


Toward Normalization of the Tumor Microenvironment for Cancer Therapy

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Abstract

The tumor microenvironment (TME) is a complex ecosystem, including blood vessels, immune cells, fibroblasts, extracellular matrix, cytokines, hormones, and so on. The TME differs from the normal tissue environment (NTE) in many aspects, such as tissue architecture, chronic inflammation, level of oxygen and pH, nutritional state of the cells, as well as tissue firmness. The NTE can inhibit the growth of cancer at the early tumorigenesis phase, whereas the TME promotes the growth of cancer in general, although it may have some anticancer effects. In particular, the TME plays a crucial role in the generation and maintenance of cancer stem cells, which lie at the root of cancer growth. Therefore, normalization of the TME to the NTE may inhibit cancer growth or improve cancer therapeutic efficiency. This review focuses on the recent emerging approaches for this normalization and the action mechanisms.

Keywords

tumor microenvironment, TME, normalization of the TME, stromal cells, inflammatory cells, cancer therapy

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Introduction

It is widely accepted that human cancers are genetic diseases due to mutations.¹ However, biological evolution is also based on mutations, and cancers are actually natural products of human evolution. It is impossible to completely eradicate cancers at the cost of human evolution.² We need to learn how to control cancers, not just kill them.

The environment in which cancer cells grow is called the tumor microenvironment (TME). It is a complex ecosystem, including blood vessels, immune cells, fibroblasts, extracellular matrix (ECM), cytokines, hormones, and other factors. Cells in the TME interact with cancer cells, and they depend on each other to promote cancer progression. For example, platelet-derived growth factor (PDGF), colony-stimulating factor 1 (CSF-1), and vascular endothelial growth factor (VEGF) from cancer cells can be potent stimuli for the growth of fibroblasts, macrophages, and endothelial cells essential for tumor survival. PDGF also induces the production of insulin-like growth factor-II (IGF-II) by fibroblasts, which in turn acts as an epithelial mitogen.³ Stromal cell-derived factor 1 (SDF1) secreted by fibroblasts/cancer-associated fibroblasts, in turn, stimulates the growth of endothelial cells and cancer cells via its receptor C-X-C chemokine receptor 4 (CXCR4) on these cells.⁴

Epidermal growth factor from macrophages also stimulates the growth of epithelial cancer cells.⁵ These findings suggest that cooperative paracrine loops between cancer cells and stromal cells exist in the TME, and these stromal cells influence tumor evolution and therapeutic response.⁴

The TME differs from the normal tissue environment (NTE) in many aspects, such as tissue architecture, chronic inflammation, level of oxygen and pH, nutritional state of the cells, as well as the consistency of ECM (Table 1). The NTE generally inhibits cancer at early tumorigenesis, whereas effects of the TME on cancer are complex with a bias toward promoting the growth of cancer.⁶ So if we can revert the TME to NTE, cancer cells may be outcompeted by surrounding normal cells. For example, cancer tissue usually has a lower extracellular pH (6.7–7.1) than normal tissue (~7.4),⁷ which is adverse to normal cells and can induce apoptosis of these healthy cells, suggesting that

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Table 1. Comparison of the Normal Tissue Environment and the Tumor Microenvironment.

Feature	Normal Tissue Environment	Tumor Microenvironment
Tissue architecture	Normal	Abnormal
Chronic inflammation	No	Yes
Macrophage	Normal macrophage	Tumor-associated macrophage
Tumor immunity	Immunosupportive	Immunosuppressive
Platelet	Inactivated	Activated
Level of oxygen	Normoxia	Hypoxia
pH	~7.4	~6.7-7.1
Fibroblast	Normal fibroblast	Carcinoma-associated fibroblast
Extracellular matrix	Homeostasis	Remodeled and fibrosis
Nutritional state	Normal	Lack of nutrition
Blood vessel	Mature and well organization with pericytes	Immature and poor organization, pericyte-poor

cancer cells have developed mechanisms to be resistant to the acid-mediated apoptosis. If the TME is normalized, cancer cells might be inhibited or outcompeted. It is worth mentioning that so-called normalized TME is relative. Cancers are more common in old people than young people. Many factors are involved in this phenomenon. One of these factors is that the stability of the tissue environment in old people is lower than that in young people. This may be caused by decreased overall levels of DNA methylation with age.⁸ DNA hypomethylation has been found in many types of human cancer because global genomic hypomethylation increases genomic instability, which is a basic character of cancer. When people grow old, the altered tissue environment makes mutant cells easily survivable and proliferative, whereas the tissue environment in young people may restrain the growth of mutant cells or kill them. We may reverse the TME to NTE, but we cannot reverse our age. This is why it is impossible to completely eradicate cancers.

Traditional cancer therapies initially focus only on cancer cells. Despite advances in many kinds of cancer in the past several decades, the overall benefits from traditional cancer therapies are limited so far.⁹ There are many reasons for this, and intratumor heterogeneity is the major one. It is well known that cancers consist of different subclones with differential sensitivity to therapies. Although most therapy-sensitive cells are killed, rare therapy-resistant cells will become dominant later. In addition, therapy may also induce new resistant subclones. For example, parts of breast and prostate cancers are initially sensitive to endocrine therapy; however, they inevitably develop to endocrine-resistant cancers after treatment, suggesting that novel mutations play an important role in the development of acquired endocrine resistance.¹⁰ Even for molecular target therapies, cancer cells might also adapt to the drug environment by activating alternative pathways,¹¹ suggesting that treatments can induce genetic and epigenetic changes in cancer cells to influence cancer evolution. Given these factors, we

need to rethink the anticancer strategies besides killing cancer cells and also utilize the TME as a therapeutic target for cancer therapy. Normalization of the TME may change the pro-tumor microenvironment to anti-tumor microenvironment, block growth signals for cancer cells, and enhance cancer therapeutic efficiency.

The Tumor Microenvironment

The TME differs from the NTE in many aspects. For example, in normal tissue, the relationship between epithelial cells and stromal cells is to inhibit each other (Figure 1). When the local environment is altered, it induces genetic and epigenetic changes in epithelial and stromal cells.¹² Stromal cells gradually lose this inhibitory effect and begin to stimulate the growth of transformed epithelial cells. Macrophages and epithelial cells have a similar relationship. Normal macrophages play an important role in immune surveillance in our body. When epithelial cells are transformed, they educate these macrophages to become tumor-associated macrophages (TAMs) via epigenetics.¹³ TAMs lose the function of immune surveillance and begin to promote the growth of epithelial tumor cells.¹⁴ So actually, transformed epithelial cells are coevolved with stromal cells in the TME during cancer evolution. Since the TME is critical for the fate of cancer cells, normalization of the TME is a promising direction for cancer therapy (Figure 1).

Changes of the microenvironment can induce abnormal differentiation of tissue stem cells. For example, the continual inflammatory microenvironment in inflammatory bowel disease is thought to be responsible for the development of colitis-associated colorectal carcinoma.¹⁵ Inflammation and tissue damage in inflammatory bowel disease cause the local tissue architecture changes and alter the physiological microenvironment and produce an unfavorable microenvironment for the intestinal stem cell differentiation. The inflammatory microenvironment also increases the chances of stem cell mutations and facilitates

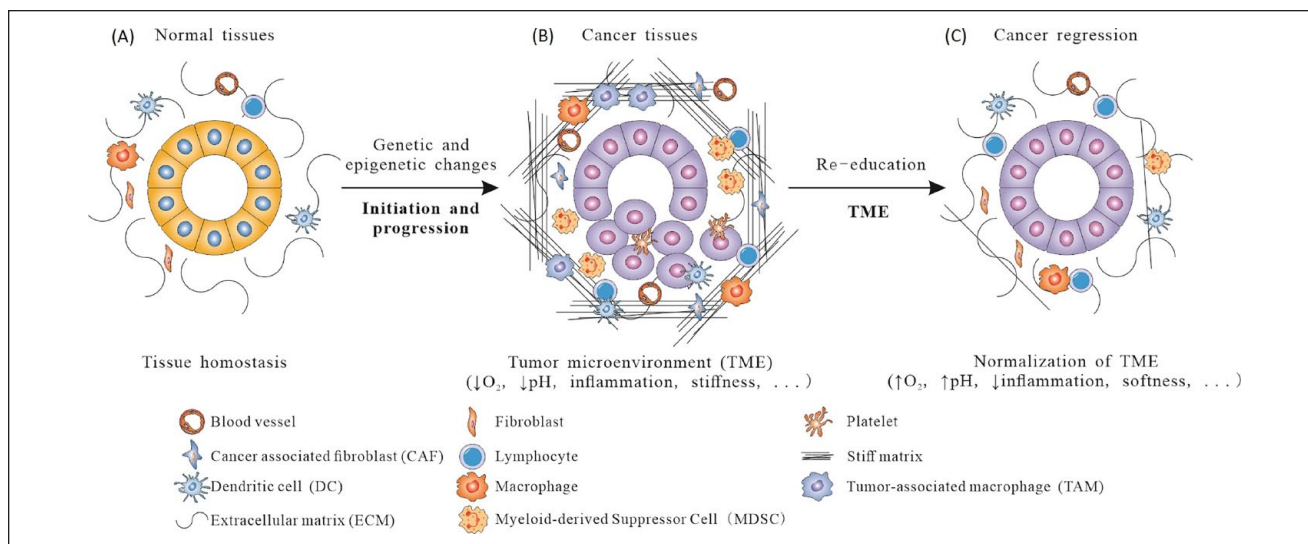


Figure 1. Normalization of the tumor microenvironment (TME) for cancer therapy. (A) There are order tissue architecture in normal tissues. Epithelial and stromal cells are mutually inhibited via negative feedback signals in the normal tissue environment (NTE). (B) The TME differs from the NTE in many aspects, such as loss of tissue architecture, low levels of oxygen and pH, existing infiltration of chronic inflammatory cells, extracellular matrix remodeling, and so on. The interactions between cancer and stromal cells are complex. Generally, stromal cells in the TME promote the growth of cancer cells although they may have some anticancer effects. (C) Normalization of the TME may inhibit cancer growth or promote cancer regression or enhance cancer therapeutic efficiency.

the mutant stem cell survival and proliferation in the local lesions. Another example is cirrhosis. Cirrhosis, an end-stage process of various liver lesions, is a precancerous lesion of hepatocellular carcinoma (HCC). Cirrhosis results in the loss of architecture of normal liver tissue, and these structural changes are unfavorable for stem cell differentiation.¹⁶ Sometimes, although the metaplastic cells induced by inflammation are differentiated cells, they are in the wrong place and have increased cancer risk. For example, Barrett's esophagus easily develops adenocarcinoma via intestinal metaplasia.¹⁷

Normalizing the Tumor Microenvironment

Given the key role of TME in cancer progression, many strategies have been developed for the normalization of the TME (Table 2).

Anti-Inflammation for Normalization of the TME

It is well known that cancer-associated chronic inflammation is a common feature of cancer tissues,⁵ and its formation is a complex process that involves intricate interactions between environmental factors and cancer tissues themselves. Infiltrated inflammatory cells in cancer tissues are mainly chronic inflammatory cells, such as macrophages, lymphocytes, myeloid-derived suppressor cells (MDSCs), and so on. The initial goal of the inflammatory response is

to eliminate foreign invaders or damaged tissues. However, the composition and function of inflammatory cells are usually changed in the TME, and generally, the chronic inflammation in cancer tends to generate an immunosuppressive TME and contribute to tumor-immune escape.⁶ For example, macrophages are polarized into M1 and M2 (see the following section), and neutrophils are polarized into N1 and N2 in response to different stimulants. M1 and N1 types are considered to exert anticancer effects via cytotoxicity and immune rejection, while M2, similar to TAMs, and N2, similar to tumor-associated neutrophils, are considered to promote cancer growth via degradation of ECM, angiogenesis, and immunosuppression.^{18,19} In the TME, macrophages and neutrophils tend to be polarized into M2 and N2, respectively. Therefore, anti-inflammation can improve the TME and aid cancer therapy.

Supporting the critical roles of inflammation in cancer, abnormal expression of inflammatory mediators (chemokines and cytokines), and enzymes regulating their synthesis in cancer tissues, like cyclooxygenase-2 (COX-2), are reported.^{20,21} Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the rate-limiting enzyme COX in the synthesis of prostaglandins and thromboxane, which are important inflammatory mediators. In mammalian cells, COX exists in 2 distinct isozymes: constitutive COX-1 and inducible COX-2. Increasing evidence shows that aspirin and other NSAIDs are effective on the prevention and treatment of many cancers, including colorectal cancer (CRC).²²⁻²⁴ The mechanisms involved for most NSAIDs are both

Table 2. Current Approaches for Normalizing the Tumor Microenvironment.

Approach	Target	Agent/Method
Anti-inflammation	Cyclooxygenase	Aspirin, NSAIDs ^{22-24,27}
	Platelet	Aspirin ²⁵
	cGAS	Aspirin ²⁶
	Chemokines and their receptors	Plerixafor, ^{29,30} maraviroc ^{31,32}
	Myeloid-derived suppressor cell	NSAIDs, ^{34,35} sunitinib ³⁷
Immune checkpoint blockade	PD1	Pembrolizumab, nivolumab ⁴⁰
	CTLA-4	Ipilimumab ⁴⁰
Targeting TAM	Depletion or repolarization	RG7155, ^{45,48} BLZ945, ^{46,48} PLX3397, ^{47,48} trabectedin, ^{50,51} low dose of radiation ⁹²
Stromal normalization	Carcinoma-associated fibroblast	Vitamin D, ⁵⁵⁻⁵⁷ vitamin A, ⁵⁸⁻⁶⁰ JQ1, ⁶⁹ I-BET151, ⁷⁰ pirfenidone ⁷²
	TGF- β /TGF- β receptor	SB431542, ⁶¹ LY2157299 ⁶²
Tumor vessel normalization	Hedgehog (Hh) signaling pathway	Hh antagonists, ⁶⁴ AZD8542, ⁶⁵ Saridegib ⁶⁶
	VEGF/VEGFR	Bevacizumab, ^{77,78} cediranib ^{79,80}
	Pericyte and others	LIGHT/TNFSF14 ^{84,85}
	PHD2	Genetic, ^{86,87} DMOG, and GSK360A ⁸⁸
Based on pH-centric therapy	Na ⁺ /H ⁺ exchanger I (NHE1)	Sinomenine, ⁹⁰ chloroquine, ⁹¹ low dose of radiation ⁹²
	Carbonic anhydrase IX (CA IX)	Amiloride, ^{98,99} cariporide ^{100,101}
	Monocarboxylate transporter	Acetazolamide, ¹⁰³ girentuximab (cG250) ¹⁰⁴ CHC, ¹⁰⁶ AZD3965, ^{105,108,109} AR-C155858 ¹⁰⁷

Abbreviations: CTLA-4, cytotoxic T lymphocyte-associated protein 4; cGAS, cyclic GMP-AMP synthase; DMOG, dimethylallyl glycine; NSAIDs, nonsteroidal anti-inflammatory drugs; PD1, programmed cell death protein 1; PHD2, prolyl hydroxylase domain protein 2; TAM, tumor-associated macrophage; TGF- β , transforming growth factor- β ; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

COX-dependent and COX-independent.^{22,23} Aspirin may also improve the TME via antiplatelet effect.²⁵ Activated platelets in the TME contribute to cancer growth and metastasis by releasing pro-inflammatory cytokines and other chemical mediators. Aspirin blocks thromboxane A2 synthesis via COX-1 inhibition and results in suppression of platelet aggregation, which in turn can balance the proangiogenic and antiangiogenic factors released from platelets and contribute to tumor vessel normalization (see the following section). Aspirin was recently found to inhibit cyclic GMP-AMP synthase (cGAS) activation via cGAS acetylation.²⁶ It is known that the damage-associated molecular pattern released by dead and dying cells excites inflammatory responses via damage-associated molecular pattern sensors, such as Toll-like receptors and cGAS-stimulator of interferon genes (STING) on innate immune cells. Therefore, more studies are needed to demonstrate whether aspirin inhibits inflammation in the TME via cGAS acetylation.

Besides anti-inflammation, some NSAIDs can also inhibit cancer cell growth or induce apoptosis *in vivo*. For example, sulindac, celecoxib, and aspirin have been shown to cause regression of colorectal polyps and prevent recurrence of colorectal polyps.²⁴ Celecoxib, a selective COX-2 inhibitor, inhibited colon cancer cells *in vitro* and 7,12-dimethyl benz(a)anthracene (DMBA)-induced tumors in rat model *in vivo*.²⁷ An interesting observation is that celecoxib can also suppress cancer through COX-2-independent mechanisms.

For example, celecoxib suppresses human colon cancer HT-29 cells primarily via downregulating leukotriene B₄ production.²⁸

Chemokines are a family of small molecular proteins that act primarily as chemoattractants for leukocytes to the inflammatory sites. Chemokines exert their activities by binding to specific G-protein-coupled receptors, such as CXCR4 (the receptor for the chemokine SDF1) and CCR5, on target cells. Plerixafor is a specific CXCR4 antagonist approved by the US Food and Drug Administration (FDA) for non-Hodgkin lymphoma and multiple myeloma treatment. As a CXCR4 antagonist, plerixafor prevents the infiltration of TAMs into the tumor tissues. Liu et al found that co-delivery of VEGF siRNA (small interfering RNA) and AMD3100 (plerixafor) improved the TME, leading to delayed tumor progression.²⁹ Mao et al found that the implanted biliary stents for alleviating pain and obstructive jaundice could cause local inflammation and increased levels of serum SDF-1, a ligand for CXCR4. AMD3100 significantly reduced local inflammation and inhibited cancer cell growth, resulting in improved survival of the tumor-bearing mice.³⁰ This inhibitory effect was also related to the prevention of the infiltration of TAMs into the tumor sites.

Maraviroc, a CCR5 antagonist, has been approved by the FDA for the treatment of patients infected with the human immunodeficiency virus-1 (HIV-1). The CCR5 ligand is the chemokine CCL5, which can recruit TAMs. Frankenberger

et al found that maraviroc significantly reduces TAM infiltration and tumor growth in xenotransplant mouse tumor models, suggesting that maraviroc might be used to normalize the TME.³¹ A recent study showed that maraviroc reshaped macrophage repolarization toward an antitumor functional state in patient-derived tumor models. These antitumoral effects of maraviroc were confirmed in a phase I trial in patients with liver metastases of advanced refractory CRC. In these patients, CCR5 blockade mitigated tumor-promoting inflammatory microenvironment and led to clinical therapeutic responses.³²

MDSCs are a heterogeneous population of immature myeloid cells that are formed during pathological conditions such as inflammation and cancer. Cytokines released by, for example, the tumor promote the proliferation of immature myeloid cells and block their differentiation, which then results in the accumulation of MDSC.³³ The NSAID indomethacin showed different effects on MDSCs in tumor-associated and tumor-free microenvironments. Indomethacin inhibited MDSC activation in tumor-bearing mice and tumor cells in vitro as well. However, in tumor-free mice, indomethacin enhanced MDSCs activation and amplified their protumor activity.³⁴ As prostaglandin E₂ induces expansion of MDSCs, aspirin or celecoxib also suppress gliomagenesis by inhibiting MDSCs development and accumulation in the TME.³⁵

In addition, MDSCs express chemokine receptor CX3CR1. Its ligand CCL26 has high expression in HCC cancer cells due to hypoxia-inducible factor (HIF) activation. Knockdown or blockade of CCL26 or blockade of CX3CR1 by neutralizing antibody substantially suppressed MDSC recruitment and tumor growth.³⁶ Sunitinib, a tyrosine kinase inhibitor approved for metastatic renal cell carcinoma (RCC), has been shown to improve outcomes in the tumor-bearing mice by partly reducing MDSCs.³⁷ However, this effect of sunitinib on MDSC depends on signal transducer and activator of transcription 3 (STAT3); and it has no effect on STAT3-negative MDSCs.³⁸ So, it may influence its usage in the clinic.

Many studies showed that exercise may be a promising adjunctive strategy for cancer patients.³⁹ The anticancer effects of exercise are multifaceted, such as increasing blood perfusion and immune function, improving tumor metabolism, and enhancing muscle-to-cancer cross-talk. Exercise can regulate the inflammation-immune axis in cancer via decreasing chronic inflammation and increasing anticancer immunity. Aerobic exercise is convenient, safe, and has no side effects, and can be integrated as a component of cancer control.

Immune Checkpoint Blockade

Cancer immunotherapy has recently received much attention again due to the success of immune checkpoint blockade

(ICB) in treating melanoma.⁴⁰ Differing from traditional cancer immunotherapy, ICB treats cancers via activating T-cells rather than using traditionally monoclonal antibodies (mAbs) against cancer cells. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD1) are immune checkpoint proteins on the T-cell surface.⁴⁰ These inhibitory receptors can be bound by ligands on cancer cells and other cells in the TME to lead to T-cell inactivation. Disruption of this mechanism by immune checkpoint inhibitors will reactivate T-cell-mediated cancer cell death. At present, the FDA has approved several mAbs against PD1 or CTLA-4 in clinical usage. For example, ipilimumab is a CTLA-4 inhibitor for melanoma and RCC. Pembrolizumab and nivolumab are PD1 inhibitors for melanoma, non-small cell lung cancer, RCC, Hodgkin lymphoma, head and neck squamous cell carcinoma, HCC, and gastric and gastroesophageal carcinoma.⁴⁰ Although ICB therapy is successful in some cancers, the response rate to ICB therapy remains 20% to 30% depending on cancer type.^{41,42} The mechanisms of the limited response to ICB therapy remain to be further investigated. The TME might influence the response of cancer to ICB therapy. Several studies and clinical trials show that normalization of the TME by antiangiogenics might increase the effectiveness of immunotherapy and diminish the risk of immune-related adverse effect (see the following sections).⁴²

TAM-Targeted Therapies

Macrophages in tissues are heterogeneous and plastic cells. In response to microenvironmental changes, macrophages can be polarized into 2 types: M1 and M2. M1-type macrophages release proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin 1 (IL-1), and IL-12, enhance T-cell functions, and are involved in antitumor immunity, whereas M2-type macrophages release anti-inflammatory cytokines, such as IL-10, transforming growth factor- β (TGF- β), and arginase, suppress T-cell functions, and are involved in protumor growth.¹⁹ However, macrophage polarization in the TME is a broad phenotypic spectrum, and this classification represents a simplistic description of the macrophage heterogeneous population. Efforts are currently being made to develop treatments targeting M2 cells or reprogramming M2 to M1 cells. CSF-1, a major regulator of macrophages, often has high levels in tumors and is an indicator of poor prognosis of cancer patients.⁴³ A study also showed that CSF-1 receptor (CSF-1R) expression in stromal macrophages, but not cancer cells themselves, is associated with worse prognosis in classical Hodgkin lymphoma.⁴⁴ Administration of emactuzumab (RG7155), a mAb against CSF-1R, to patients led to striking reduction of CSF-1R⁺CD163⁺ macrophages and increase of T-cells in tumor tissues, which resulted in

clinical benefit for patients with diffuse-type giant cell tumor.⁴⁵ Furthermore, CSF-1R kinase inhibitor BLZ945 blocked tumor progression and improved survival in mice with glioblastoma multiforme (GBM) via reducing M2 macrophage polarization.⁴⁶ Pexidartinib (PLX3397), another tyrosine kinase inhibitor of CSF-1R, also improves the antitumor efficacy of adoptive cell transfer immunotherapy with chicken ovalbumin₂₅₇₋₂₆₄ peptide-activated OT-1 splenocytes or gp100₂₅₋₃₃ peptide-activated pmel-1 splenocytes in melanoma through skewing of MHC-II^{low} to MHC-II^{high} macrophages and increasing antitumor T-cell activity.⁴⁷ Emactuzumab, BLZ945, and pexidartinib are currently under phase I to III trials.⁴⁸ However, the long-term effects of these agents on clinical outcomes are unknown. Quail et al recently reported that acquired resistance to CSF-1R inhibitor BLZ945 eventually emerges within a subset of mice. Resistance was found to be driven by phosphoinositide 3-kinase (PI3K) hyperactivation in recurrent GBM, driven by macrophage-derived IGF-1 and tumor cell IGF-1 receptor (IGF-1R). Combining IGF-1R or PI3K blockade with CSF-1R inhibition in recurrent tumors significantly prolonged overall survival.⁴⁹

Trabectedin, a synthetic anticancer agent originally isolated from the Caribbean tunicate *Ecteinascidia turbinata*, has been approved for the treatment of patients with soft tissue sarcoma. The anticancer mechanism of trabectedin differs from other anticancer drugs and involves interference with cancer cells as well as the TME.⁵⁰ In the TME, it selectively targets TAMs and monocytes via the activation of caspase 8 through TNF-related apoptosis-inducing ligand (TRAIL) receptors other than lymphocytes and neutrophils. Trabectedin significantly reduced inflammatory mediators and growth factors produced by TAMs and tumor cells. The anti-inflammatory and antiangiogenic effects of trabectedin were confirmed in vivo.⁵¹

Stromal Normalization

Normal fibroblasts can inhibit proliferation and motility of tumor cells when cocultured in vitro.⁵² This inhibitory effect is both contact and soluble factor dependent.⁵² In a 3-dimensional (3D) context, normal mammary fibroblasts induced reversion of the malignant phenotype of primary breast carcinoma cells. The reversion was confirmed by the baso-apical polarity axis and established polarity markers.⁵³

Cancer tissues are often found harder than surrounding normal tissues in consistency. This is because cancers have more stroma than normal tissues, and the stroma is remodeled due to changes of their components. For example, fibrosis (desmoplasia) is often found in pancreatic, breast, and prostatic cancers. Fibrosis contributes to chemoresistance by hindering chemical drugs from penetrating cancer tissues.

Cancer fibrosis is associated with carcinoma-associated fibroblasts (CAFs). CAFs consist of various fibroblasts of distinct cellular origins that become activated during carcinogenesis and express specific biomarkers such as α -smooth muscle actin (α -SMA), fibroblast activation protein- α (FAP α), and vimentin. CAFs have different functions from normal fibroblasts and promote cancer progression by secreting growth factors, cytokines, and ECM remodeling.⁵⁴ So, if we can reprogram CAFs to normalized fibroblasts or toward a less active state, it may improve the TME and make cancer tissues become soft.

Several approaches have been developed for this purpose. For example, calcipotriol, the active form of vitamin D, reduced fibrosis and inflammation and increased the sensitivity to the anticancer agent gemcitabine via vitamin D receptor (VDR)-mediated stromal reprogramming in pancreatic cancer.⁵⁵ $1\alpha,25$ -dihydroxyvitamin D₃ (1,25D₃), the active form of vitamin D₃, has distinct effects on normal mammary-associated fibroblasts from CAFs. After 1,25D₃ treatment, functional analysis revealed that genes associated with proliferation (NRG1, WNT5A, and PDGFC) were downregulated and genes involved in immune modulation (NFKBIA, TREM-1) were upregulated in CAFs from breast cancer patients, consistent with the antitumor activities of 1,25D₃ in breast cancer. Whereas a distinct subset of genes involved in anti-apoptosis, detoxification, antibacterial defense system, and protection against oxidative stress were induced by 1,25D₃ in normal mammary-associated fibroblasts from the same patients, which may limit carcinogenesis.⁵⁶ Ding et al also found that VDR knockout mice spontaneously developed hepatic fibrosis, suggesting VDR ligands as a potential therapy for liver fibrosis.⁵⁷

Similar to vitamin D, vitamin A derivatives are also found to reprogram stromal cells and exert anti-pancreatic cancer effects via reducing fibrosis and improving the TME.⁵⁸⁻⁶⁰ Retinoic acid (RA) was found to inhibit the proliferation and migration of pancreatic cancer cells via the downregulation of pancreatic stellate cells (PSCs) activation.⁵⁸ Quiescent PSCs store retinol, and this function is lost on activation in the microenvironmental changes. The activated PSCs are similar to CAFs in morphology and function and cause the desmoplastic reaction in pancreatic cancer. Another study showed that RA inhibited the migration of tumor cells via CAFs inhibition.⁵⁹ Both studies suggest that the TME plays an important role in promoting tumor migration. Han et al recently designed a TME-responsive nanosystem and utilized it to co-deliver all-trans RA and siRNA targeting heat shock protein 47, a collagen-specific molecular chaperone, to reeducate PSCs. They found that this system induced PSCs quiescence and inhibited ECM hyperplasia, thereby promoting drug delivery to pancreatic tumors and significantly enhancing the efficacy of chemotherapy.⁶⁰

The role of TGF- β signaling in tissue fibrosis is well known. Using a microfluidic chip, Hsu et al analyzed the paracrine loop between lung cancer cells and fibroblasts. They found that TGF- β was a major component in this paracrine loop and considered as a key factor that activated fibroblasts to myofibroblasts, which increased the migration speeds of cancer cells. This paracrine loop could be interrupted by a TGF- β receptor inhibitor SB431542 on fibroblasts.⁶¹ Calon et al also found that all poor-prognosis CRC subtypes shared a gene program induced by TGF- β in tumor stromal cells. LY2157299, another TGF- β inhibitor, was able to block the cross-talk between cancer cells and the microenvironment and therefore reduce the metastatic potential of CRC in vivo.⁶²

Hedgehog (Hh) signaling plays a crucial role in embryonic development, tissue regeneration, and organogenesis. Aberrant Hh signaling has been found in nevoid basal cell carcinoma syndrome, medulloblastoma, rhabdomyosarcoma, pancreatic cancer, prostate cancer, hematological malignancies, and so on. Studies revealed that overactivated Hh signaling leads to fibrogenesis in many types of tissues.⁶³ Therefore, Hh signaling is also a potential therapeutic target. Yauch et al found that Hh inhibition in the mouse stroma resulted in growth inhibition in xenograft tumor models,⁶⁴ suggesting that targeting Hh signaling in stromal cells may contribute to normalization of the TME in some cancers. Hwang et al reported that pancreatic CAFs (human PSCs) expressed high levels of the Hh signaling molecule smoothened (SMO) receptor and low levels of Hh ligands, whereas cancer cells showed the converse expression pattern in an orthotopic model of pancreatic cancer. Hh antagonist AZD8542 treatment inhibited tumor growth only when human PSCs were present, indicating a paracrine signaling mechanism dependent on stroma.⁶⁵ In order to improve drug perfusion in mouse models of pancreatic adenocarcinoma, Olive et al used gemcitabine combined with IPI-926 (sari-degib), a drug that reduces tumor-associated stromal tissue by inhibition of Hh signaling. They found that the combination therapy produced a transient increase in intratumoral vascular density and intratumoral concentration of gemcitabine, leading to transient stabilization of disease.⁶⁶

Bromodomain and extra-terminal (BET) proteins are epigenetic regulators and consist of bromodomain-containing protein 2 (BRD2), BRD3, BRD4, and BRDT. BET proteins bind to acetylated chromatin to recruit other regulatory complexes to influence gene expression. Some members of BET proteins, for example, BRD4 and BRD2, have emerged as novel targets for cancer therapy as their expression was upregulated in a number of hematological and solid tumors.^{67,68} Bromodomain inhibitors are also the direction for stromal normalization. As epigenetic regulators, they can turn the TME from protumoral to antitumoral. Several bromodomain inhibitors, such as JQ1 and I-BET151, are developed in clinical development. JQ1, an inhibitor of

BRD4, has been shown to reduce carbon tetrachloride-induced liver fibrosis in mouse models.⁶⁹ As liver fibrosis is a precursor of HCC, JQ1 may be used to improve the TME in fibrosis. I-BET151 may also be used to normalize the TME as it can suppress the expression of inflammatory genes and matrix-degrading enzymes in rheumatoid arthritis synovial fibroblasts.⁷⁰ However, BRD2 and BRD4 are expressed in nearly all cells of the body, so BET inhibitors may be toxic to normal tissues, which can be addressed by tumor-specific delivery systems in the future.

Recently, 2 drugs, pirfenidone and nintedanib, have been approved for the treatment of idiopathic pulmonary fibrosis.⁷¹ Pirfenidone has antifibrotic and anti-inflammatory activities via multiple targets such as TGF- β , TNF, IL-10, while nintedanib is an angiokinase inhibitor via VEGF receptor (VEGFR), fibroblast growth factor receptor (FGFR), and PDGF receptor (PDGFR). As activated fibroblasts in inflammatory conditions have similar characteristics as CAFs, they may be used as adjunct anticancer drugs. Mediavilla-Varela et al showed that the combination of low doses of cisplatin and pirfenidone could kill tumor cells and CAFs to decrease tumor progression, suggesting this combination approach may produce a clinical benefit greater than chemotherapy alone.⁷² However, stromal cells display conflicting effects on cancer cells as both anti- and pro-cancer effects are reported. This means that targeting CAFs still needs caution. For example, Özdemir et al showed that depletion of CAFs in a mouse model of pancreatic cancer accelerated cancer progression with reduced survival.⁷³

Tumor Vessel Normalization

Antiangiogenic therapies have been widely studied in oncology. Although some cancer patients may have some short-term benefits from these therapies, long-term benefits are still in question. Some studies pointed out that VEGF inhibitors might also promote tumor invasion, metastasis, and treatment resistance,⁷⁴ as they can induce tumor hypoxia and acidic microenvironment.

In contrast to normal vessels, tumor vessels are immature, irregular, tortuous, and lack a basement membrane and pericytes, which play key roles in vessel maturation and stabilization.⁷⁵ Therefore, strategies for tumor vessel normalization, from pruning abnormal vessels to becoming closer to the structures and functions of normal vessels, are emerging as complementary therapy methods for cancer.⁷⁵ The morphology of the “normalized” vessels is less tortuous and more uniform with pericyte coverage. The functions of the “normalized” vessels can alleviate vascular permeability, edema, and hypoxia; they can also reprogram the TME from immunosuppressive to immunosupportive as well as improve the delivery and efficacy of chemotherapy and immunotherapy. Hypoxia, a basic characteristic in the TME of solid cancers, is an important driver of MDSC

recruitment in the TME³⁶ and is also a major inducer of the hypermethylation of tumor suppressor genes via reducing the activity of oxygen-dependent ten-eleven translocation (TET) enzymes while restoration of tumor oxygenation abrogates this effect,⁷⁶ suggesting increase of oxygenation in the TME can inhibit cancer development. Cancer fibrosis is also associated with hypoxia in the TME. Tumor vessel normalization may improve hypoxia and reduce fibrosis.

At present, several approaches are under clinical trials for tumor vessel normalization.

Antiangiogenic Agents. Low-dose VEGF/VEGFR inhibitors may improve anticancer responses and patient survival via vessel normalization and inhibition of new vessel growth^{77,78} because high-dose inhibitors can cause excessive pruning of the vessels to result in the reduction of perfusion. Bevacizumab, an antihuman VEGF antibody, has been successfully used in several human cancers, such as rectal and breast cancers. Preclinical and clinical studies indicated that bevacizumab could induce vessel normalization.⁷⁷ This, however, does not mean that bevacizumab can improve therapeutic efficiency in cancer patients. For example, when combined with other anticancer mAbs, bevacizumab did not improve therapeutic efficiency as vessel normalization might decrease the uptake of large molecular mAbs. In contrast, bevacizumab-induced vessel normalization could improve the therapeutic efficiency of chemotherapy via reducing interstitial fluid pressure and increasing drug uptake.⁷⁷ Cediranib, an oral pan-VEGFR kinase inhibitor, prolonged the survival of patients with GBM via vascular normalization with elevated blood perfusion.⁷⁹ Furthermore, it improved tumor oxygenation in GBM via increasing blood perfusion when combined with chemoradiation and improved the overall patient survival.⁸⁰ Since VEGF is a negative regulator of pericyte function and vessel maturation,⁸¹ VEGF/VEGFR inhibitors are promising drugs for tumor vessel normalization.

The angiopoietins (Angs) and their receptor Tie2 axis is an alternative regulator of tumor angiogenesis. Ang1 is considered as a stabilizer of angiogenesis, whereas Ang2 acts as a destabilizer of angiogenesis via competitive inhibiting Ang1.⁸² Park et al found that combination of Tie2 activation and Ang2 inhibition induced tumor vascular normalization, leading to enhanced blood perfusion and chemotherapeutic drug delivery, markedly lessened lactate acidosis, and reduced tumor growth and metastasis,⁸² suggesting that tumor vascular normalization can improve the TME and is a promising adjuvant antitumor approach.

LIGHT/TNFSF14. LIGHT, also called tumor necrosis factor superfamily 14 (TNFSF14), is a member of the TNF family and has been shown to reduce tumor growth by inducing an antitumor immune response.⁸³ Intratumoral delivery of LIGHT can stimulate perivascular macrophages to secrete

TGF- β , which in turn affects the pericyte contractile and vessel integrity. Vessel integrity is not only dependent on endothelial cells, but it also requires pericyte coverage of the vascular sprout for stabilization of vascular walls. Mechanistically, intratumoral LIGHT induces pericyte differentiation and normalization via Rho kinase signaling.⁸⁴

He et al recently also reported that injection of fused LIGHT with the tumor vessel-homing peptide CGKRK intravenously into murine orthotopic glioblastoma models could normalize the tumor vasculature by inducing pericyte contractility and increasing endothelial barrier integrity. They also found that this treatment increased the immune response via high endothelial venule induction, and reducing vasogenic edema in murine tumors.⁸⁵

Prolyl Hydroxylase 2. Hypoxia induces expression of HIF- α . Endothelial cells bear the oxygen-sensitive enzyme prolyl hydroxylase domain protein 2 (PHD2), which targets HIF- α for degradation. Recently, PHD2 has received a lot of attention. For example, heterozygous deficiency of *PHD2* restores tumor oxygenation and inhibits metastasis via endothelial normalization.⁸⁶ Reduced activity of PHD2 in endothelial cells normalizes tumor vessels and increases perfusion and the delivery of chemotherapeutics to the tumor, suggesting that PHD2 inhibitors are potential drugs for cancer therapy.⁸⁷ Except endothelial cells, loss of *PHD2* in CAFs decreased tumor stiffness and metastasis via reverting CAF activation.⁸⁸ Several PHD inhibitors, such as dimethyloxyallyl glycine and GSK360A, are already used in clinical studies.⁸⁸

Miscellaneous. Besides T-cell activation, a recent study showed the ICB therapy improved vessel normalization since type 1 T helper (T_H1) cells play a crucial role in vessel normalization.⁸⁹ Mutual regulation of T lymphocytes and vessel normalization is positive, that is, infiltrated lymphocytes, especially T_H1 cells, mediate vessel normalization via improving the TME and vessel normalization, in turn, improves the microenvironment for T lymphocyte activity.

Zhang et al reported that 100 mg/kg sinomenine hydrochloride resulted in suppressed mammary tumor growth and metastasis via partial vascular normalization.⁹⁰ Sinomenine is an alkaloid extracted from the Chinese medicinal plant, *Sinomenium acutum*, which has been utilized to treat rheumatism in China for over 2000 years. However, 200 mg/kg sinomenine hydrochloride did not exhibit similar inhibitory effect on tumor progression due to the immunosuppressive microenvironment caused by excessive vessel pruning, granulocyte-CSF upregulation, and granulocyte macrophage-CSF downregulation, suggesting that a suitable dose of vascular inhibitor is important for successful therapies.⁹⁰

Chloroquine, a lysosomal inhibitor, was shown to reduce tumor growth and improve the tumor milieu via

normalizing tumor vessel structure and function and increasing perfusion. Chloroquine vessel normalization activity mainly relied on alterations of endosomal Notch1 trafficking and signaling and vascular endothelial cell cadherin function in endothelial cells.⁹¹

Radiotherapy not only kills cancer cells but also changes the TME that will result in therapeutic success or failure. For example, local low-dose γ irradiation (2 Gy) reprogrammed TAM toward the M1 phenotype, promoted normalization of aberrant vasculature, T-cell-mediated tumor rejection, and prolonged survival in xenotransplant mouse tumor models. It was indicated by a reduction in the CD31⁺ vessel area, average vessel size, and hemorrhagic lesions, as well as by an increase of the vessel circularity index in tumors.⁹² Also, pigment epithelium-derived factor (PEDF) enhances tumor response to radiation through vasculature normalization in allografted lung cancer in mice.⁹³ PEDF is a 50 kDa glycoprotein belonging to the serpin protease inhibitor family and has multiple functions, such as neurotrophic, neuroprotective, anti-inflammation, antitumor, and antiangiogenesis activities.

pH-Based Anticancer Therapy

One hallmark of solid cancer is the acidic microenvironment, which is caused by multiple factors, such as hypoxia, alterations of oncogenes, and tumor suppressors, increased glycolysis, defective vessel system, and other factors. This acidic TME influences cancer cell behavior, such as proliferation, the evasion of apoptosis, immune escape, invasion and metastasis, maintaining cancer stem cells, metabolic adaptation, and chemotherapeutic response.⁷ Improving the acidic TME is considered a potential adjuvant option to increase therapy sensitivity and overcome therapy resistance.^{7,94}

Several enzymes in the plasma membrane regulate pH gradients, such as Na⁺/H⁺ exchangers (NHEs), carbonic anhydrases (CAs), monocarboxylate transporters (MCTs), and vacuolar H⁺-ATPase, and so on. Their expressions are usually upregulated in human cancers⁹⁵⁻⁹⁷ resulting in increased intracellular pH (pH_i) and decreased extracellular pH (pH_e), which influence the biological behaviors of cancer cells.⁷

NHE1, a prototype of NHEs, has been widely studied for its role of H⁺ excretion and usually has higher expression in tumor cells.⁹⁷ Among NHE1 inhibitors, amiloride family members are widely studied. Initially used as diuretics in the clinic, they are recently used in research for cancer therapy. Amith et al reported that the combination of paclitaxel and amiloride analog HMA (5-[N,N-hexamethylene]-amiloride) was significantly more effective than either paclitaxel or HMA alone in triple-negative breast cancer cells. Furthermore, the NHE1-knockout triple-negative breast cancer MDA-MB-231 cells had markedly lower rates of migration and invasion in vitro. In vivo xenograft tumor

growth in female athymic nude mice was also dramatically decreased compared with parental cells.⁹⁸ Besides inhibiting NHE1, amiloride family members also inhibit the urokinase plasminogen activation system, which might enhance anticancer and anti-metastasis effects of amiloride and its analogs.⁹⁹ Cariporide (HOE-642), another NHE1 inhibitor, is also found to have some anticancer effects. Cong et al¹⁰⁰ found that NHE1 expressed in primary human glioma cells (GC), glioma xenografts, and glioblastoma, but not in human neural stem cells or astrocytes. GC treated with the anticancer agent temozolomide was associated with elevated NHE1 expression, which enhanced the resistance of GC to temozolomide-mediated toxicity. Application of cariporide suppressed the migration and invasion of human GC and augmented temozolomide-induced apoptosis. These effects on GC may be associated with the decreased pH_i state induced by cariporide, which has been reported as one major mechanism of cariporide's inhibitory effect on human tongue squamous cell carcinoma Tca8113 cells.¹⁰¹ This inhibitory effect was also related to the downregulation of matrix metalloproteinase 9 (MMP-9), an acid-activated enzyme for degradation of ECM, such as collagen and fibronectin. However, in a phase III myocardial protection trial, cariporide showed an unanticipated higher mortality rate due to cerebrovascular events.¹⁰² Therefore, cariporide might not be safe for use in treatment of glioblastoma.

Carbonic anhydrases catalyze the reversible conversion of CO₂ + H₂O to HCO₃⁻ + H⁺. Among all 15 human α -CAs, CA IX has the highest catalytic activity. Since CA IX is induced by hypoxia and upregulated in several human cancers, such as clear cell RCC, intrahepatic cholangiocarcinomas,⁹⁶ it is considered a potential target for cancer treatment. Acetazolamide (ATZ), a CA IX inhibitor, is an oral diuretic drug. ATZ alone or combined with temozolomide inhibited GBM. The combination therapy was particularly effective against brain tumor stem cells when ATZ was incorporated into a nanocarrier in 3D spheroid models.¹⁰³ Girentuximab (cG250), a mAb against CA IX, is currently being evaluated for RCC (NCT01826877). Zatovicova et al found that girentuximab treatment was effective against xenografts induced by human colorectal carcinoma HT-29 cells, which display high expression of CA IX even under normoxia.¹⁰⁴ In addition, the current clinically used tyrosine kinase inhibitor imatinib, nilotinib, and the COX2 inhibitor celecoxib also have inhibitory effects on CA IX.

Dysregulated glycolysis is a common feature of tumors and also a major cause of the acidic microenvironment due to production of the terminal product lactate. Export of lactate is executed by MCTs, which also mediate the bidirectional transport of other monocarboxylates, like pyruvate and ketone bodies, to cross the plasma membrane with cotransported protons (H⁺).¹⁰⁵ MCTs belong to the SLC16 gene family and are composed of 14 isoforms, of which only MCT1 to 4 have been biochemically characterized.

Upregulation of MCT1 and MCT4 has been reported in several human cancers.⁹⁵ In cancers, MCT1 is preferentially expressed on the cell membrane of oxidative cancer cells where it facilitates the uptake of lactate and protons, whereas MCT4 is preferentially expressed on the cell membrane of hypoxic cancer and stroma cells where it facilitates the export of lactate and protons.¹⁰⁵

Now, several MCTs inhibitors are developed, such as α -cyano-4-OH-cinnamate (CHC), AR-C155858, and AZD3965. CHC, an inhibitor of MCT1/2 and MCT4, can block lactate secretion and decrease pH_i levels in multiple myeloma cells when combined with metformin, an oral hypoglycemic agent, which further lowered pH_i levels and enhanced cytotoxicity.¹⁰⁶ AR-C155858, a first generation MCT1/2 inhibitor, was able to suppress lactate efflux and glycolysis in Ras-transformed fibroblasts and inhibited tumor growth, whereas ectopic expression of MCT4 conferred these cells with resistance to AR-C155858 and re-established tumorigenicity,¹⁰⁷ suggesting that hypoxia-induced MCT4 is an important anticancer target.

AZD3965, a second generation MCT1 inhibitor, was synthesized on the basis of AR-C155858 by AstraZeneca. Bola et al showed that AZD3965 inhibited bidirectional lactate transport and increased intracellular lactate while decreasing the amount of extracellular lactate in small cell lung cancer (SCLC) cells and gastric cancer cells. The reduction of extracellular lactate might improve the acidic TME. Combination of AZD3965 with radiation to treat SCLC xenografts exhibited a significantly greater therapeutic effect than the use of either modality alone.¹⁰⁵ Currently, AZD3965 is tested in a phase I clinical trial (NCT01791595). However, the sensitivity of cells to AZD3965 depends on whether MCT4 is expressed. Polanski et al found that AZD3965 sensitivity varied in SCLC cells in vitro and SCLC cells with MCT4 expression were resistant to AZD3965.¹⁰⁸ In another study, Hong et al also showed that MCT4 expression portends resistance to AZD3965,¹⁰⁹ suggesting that MCT4 can be used as an alternative pathway in MCT1 blockade.

Conclusion

Cancer development is a complex biological process. It is known from a variety of experimental systems that cancer cells are not autonomous in most cases, and their growth is dependent on the local microenvironment. So, changes in the local microenvironment may change the fate of cancer cells. If we may reeducate the TME to the NTE, it may induce cancer inhibition or regression, or enhance cancer therapeutic efficacy.

The TME is gradually initiated and established by both cancer and stromal cells. The interactions between cancer and stromal cells promote cancer evolution via cell-cell contact or soluble small molecules in the TME. In this

process, cancer cells alter some properties of stromal cells via these interactions. The altered stromal cells, in turn, influence the behavior of cancer cells in similar ways. The major altered stromal cells in the TME are infiltrating inflammatory cells, immune cells, fibroblasts, and endothelial cells, all of which might serve as targets for normalizing the TME. Soluble small molecules also play important roles in the communication between cancer cells and stromal cells via a paracrine loop, and their levels are often dysregulated in the TME. Interrupting this loop may contribute to normalization of the TME.

It is worth mentioning that different cancers have a different TME, so the approaches for normalizing the TME should be different. Many approaches are currently being studied and developed to normalize the TME for cancer therapy. Although cancer cells are dominant cells in cancer biology, there is no doubt targeting both cancer and stromal cells would be more efficacious than targeting cancer cells alone.

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