10 Tumor Microenvironment and Nitric Oxide: Concepts and Mechanisms

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Abstract

The cancer tissue exists not as a single entity, but as a combination of different cellular phenotypes which, taken together, dramatically contribute to the entirety of their ecosystem, collectively termed as the tumor microenvironment (TME). The TME is composed of both immune and nonimmune cell types, stromal components, and vasculature—all of which cooperate to promote cancer progression. Not all immune cells, however, are immune-suppressive; some of them can promote the immune microenvironment to fight the invading and uncontrollably dividing cell populations at the initial stages of tumor growth. Yet, many of these processes and cellular phenotypes fall short, and the immune ecosystem more often than not ends up stabilizing in favor of the "resistant" resident cells that begin clonal expansion and may progress to metastatic forms. Stromal components, making up the extracellular matrix and basement membrane, are also not the most innocuous: CAFs embedded throughout secrete proteases that allow the onset of one of the most invasive processes—angiogenesis—through destruction of the ECM and the basement membrane. Vasculature formation, because of angiogenesis, is the largest invader of the TME and the reason metastasis happens. Vasculature is so sporadic and omnipresent in the TME that most drug therapies are mainly focused on stopping this uncontrollable process. As the tumor continues to grow, different processes are constantly supplying it with the ingredients favorable for tumor progression and eventual metastasis. For example, angiogenesis promotes blood vessel formation that will allow the bona fide escape

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1277,
https://doi.org/10.1007/978-3-030-50224-9_10

of tumor cells to take place. Another process like hypoxia will present itself in several forms throughout the tumor (mild or acute, cycling or permanent), starting mechanisms such as epithelial to mesenchymal transitions (EMT) of resident cells and inadvertently placing the cells in such a stressful condition that production of ROS and DNA damage is unavoidable. DNA damage can induce mutagenicity while allowing resistant cells to survive. This is where drugs and treatments can subsequently suffer in effectiveness. Finally, another molecule has just surfaced as being a very important player in the TME: nitric oxide. Often overlooked and equated with ROS and initially assigned in the category of pathogenic molecules, nitric oxide can definitely do some damage by causing metabolic reprogramming and promotion of immunosuppressive phenotypes at low concentrations. However, its actions seem to be extremely dose-dependent, and this issue has become a hot target of current treatment goals. Shockingly, nitric oxide, although omnipresent in the TME, can have a positive effect on targeting the TME broadly. Thus, while the TME is a myriad of cellular phenotypes and a combination of different tumor-promoting processes, each process is interconnected into one whole: the tumor microenvironment.

Keywords

Tumor microenvironment (TME) · Cancer · Immune surveillance · Angiogenesis · Angiogenic switch · Sprouting angiogenesis · Cancer metabolism · Hypoxia · Nitric oxide · Cancer-associated fibroblasts (CAFs) · Tumor-associated macrophages (TAMs) · Innate and adaptive immunity · Stromal cells · Immunosuppression · Immune elimination/equilibrium/escape · Immunotherapy · Treatment resistance

10.1 Tumor Microenvironment

Long gone is the idea that a tumor is simply a combination of cancer cells that are involved in uncontrolled clonal expansion; instead, there has been a shift to a more revolutionary idea that a tumor is a combination of heterogeneous populations of cells: tumor cells, immune cells and nonimmune cells, stromal components, and vasculature. Together, these create an ecosystem—a cancerous organ-like structure that exists and grows on its own [1–3]. For this reason, the development of current drug therapies has evolved from inhibiting one or many of the specific components that reside in the tumor microenvironment (TME) to the more concrete approach of targeting the tumors broadly [4]. Tumorigenesis is initiated when oncogenic activation disrupts normal gene expression patterns, thereby interrupting normal tissue homeostasis and initiating a secretion of cytokines and growth factors that recruit stromal cells and vascular components [5, 6]. These cells include cancer-associated fibroblasts (CAFs), endothelial cells (ECs), adipocytes, pericytes, and immune cells such as macrophages, monocytes, lymphocytes, and dendritic cells (DCs) that become trapped in the extracellular matrix and are affected by its changing biophysical parameters [7–10]. Thus, the TME is not a static process of resident cell populations but a dynamic and ever-evolving ecosystem that is crucial for the initiation, progression, and metastasis of cancer. To reach significant growth and expansion and establish metastatic niches, the tumor microenvironment involves several important processes that contribute to tumor progression: angiogenesis, hypoxia, endothelial to mesenchymal transition (EMT), macrophage infiltration, and regulatory effects of secreted factors such as reactive oxygen species (ROS) or nitric oxide (NO) (Fig. 10.1) [11–13].

10.1.1 Composition of the Tumor Microenvironment

Cells in the TME are heterogeneous in origin and nature and can come from the bone marrow, blood vessels, or the stroma [14]. The cellular plasticity seen in these cells is mediated by EMT, loss of E-cadherin function, and loss of apical-basal polarity [15]. These cells provide the foundation for the TME.

Stromal Components The stroma is a network of the extracellular matrix (ECM) supported by the basement membrane, which is lined with endothelial cells [16]. The ECM scaffolding is composed of collagen, fibronectin, proteoglycans, and laminins, all of which are intricately interwoven and well organized. The interesting thing about the ECM in tumor tissues is that it has an extremely abnormal morphology—it often exhibits aberrant patterns of fibril deposition, which lead to invasion of the surrounding tissue. Furthermore, the stroma plays a critical role in angiogenesis as it is intertwined with a busy network of blood vessels. As far as cell types residing in the stroma are concerned, these include cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs), and tumor-associated macrophages (TAMs). CAFs are known to enhance angiogenesis, tumorigenesis, and metastasis, as well as promote drug resistance. Angiogenesis is usually triggered by CAFs' ability to secrete matrix metalloproteinases (MMPs) and other enzymes that destroy the ECM as well as factors that upregulate expression of the vascular endothelial growth factor (VEGF), which stimulates angiogenesis [17]. On the other hand, MSCs residing in the TME attempt to repair the injured cells by transferring mitochondria via nanotubules but can also differentiate into CAFs, which further promote angiogenesis and metastasis [18]. Thus, while MSCs mean well, in the context of the TME, these cells may actually promote cancer survival and progression. Finally, macrophages are recruited to the TME via signaling molecules and cytokines to fight the rapidly growing ecosystem; however, they can become polarized and converted to TAMs, the M2 phenotype, which actually plays a significant role in cancer progression [19]. Thus, the stroma of the TME is a supportive network that plays an important role in establishing tumor integrity, all the while promoting its subsequent growth and expansion.

Immune Surveillance The main role of the mammalian immune system is to find, tag, and eliminate a pathological invader in order to protect the organism against infectious agents and eliminate damaged cells [20]. However, unlike in normal tissue, cancerous tissue is marked by persistent immunological cell populations that not only expand but also diversify due to malignant processes such as fibrosis, angiogenesis, and neoplasia [21, 22]. Three stages of immune involvement in cancer have been proposed: elimination, equilibrium, and escape [23]. In the first stage, the immune system tags uncontrollably growing cell populations and is particularly efficient at destroying and eliminating them. However, in the equilibrium stage the immune system is not as efficient at fighting the ever-growing malignant cells, giving them sufficient time to adapt to the new immune microenvironment and differentiate into other cell types by undergoing EMT. This allows the establishment of a cancer niche that is full of immune-resistant cells, which will inadvertently develop into a solid tumor. Finally, the involvement of the immune system has been well documented at the escape stage, where it reduces anticancer proteins and other surveillance mechanisms, allowing tumor cells to escape their original niche, migrating to distant metastatic sites. In this sense, the immune system evolves from a mechanism that fights cancer invasion to a mechanism that becomes completely entrapped by the tumor ecosystem and thus promotes cancer progression.

The tumor ecosystem contains cells of both adaptive and innate immunity, both of which play a role in tumor establishment and progression, modulation of angiogenesis, and subsequent immune escape. Adaptive immune cells include T lymphocytes and B cells, while innate immune cells include dendritic cells, natural killer cells, monocytes and macrophages, neutrophils, mast

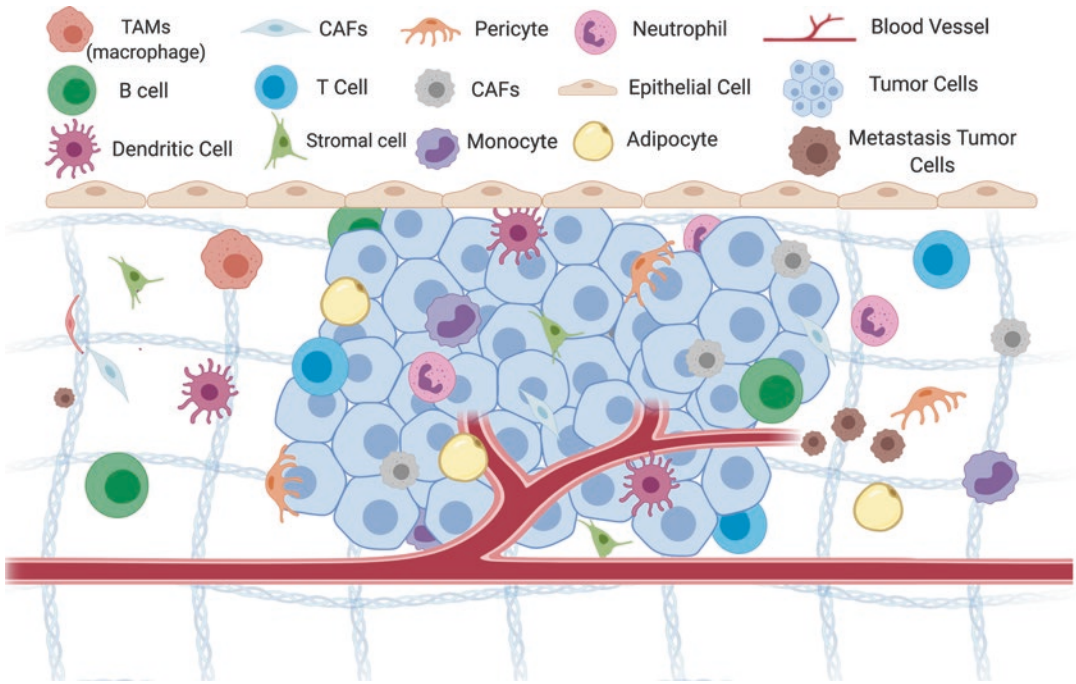

Fig. 10.1 Composition of the tumor microenvironment. TME is a combination of different cellular phenotypes, all of which dramatically contribute to the entirety of their ecosystem. The TME is composed of both immune (T and B cells, dendritic cells, monocytes, TAMs) and nonimmune cells types (CAFs, epithelial cells, etc.), stromal components, and vasculature—all of which exist in unison to allow cancer progression to take place. Not all immune cells, however, are immunosuppressive; some of them can promote the immune microenvironment to fight the invading and uncontrollably dividing cell populations at initial stages of tumor growth. However, many of these processes and cellular phenotypes fall short, and the immune ecosystem more often than not ends up stabilizing in favor of the "resistant" resident cells that begin clonal expansion and may progress to metastatic forms. Stromal components, constituting the extracellular matrix and basement membrane, contain potentially hazardous CAFs, which secrete proteases that initiate angiogenesis through destruction of the extracellular matrix (ECM) and the basement membrane. Vasculature is the biggest invader of the tumor microenvironment, and the reason metastasis occurs. The fusion of the immune and nonimmune cells, stromal components, and vasculature creates a favorable microenvironment for the progression of cancer. TAMs: tumor-associated macrophages; CAFs: cancer-associated fibroblasts

cells, and eosinophils [24]. T cells in the TME can be CD4+ (helper T cells) or CD8+ (cytotoxic T cells), which secrete IFN-gamma, TNF-alpha, and IL-17 that mediate adaptive immune responses and exhibit antitumor effects. T-cell infiltration has been shown to be associated with a positive outcome in cancer patients; however, tumors have evolved to display dominant inhibitory mechanisms that work against proliferation of T effector cells. Currently, a hot target of immunotherapy approaches are immune checkpoint blockade inhibitors, such as cytotoxic T-lymphocyte antigen-4 (CTLA4), programmed cell-death-1 (PD1) and its ligand, PDL1. B-cells, which can be divided into immature B cells, plasma cells, or memory cells, express different immunoglobulins on their surface for antigen recognition, and these phenotypes can vary depending on the stage of tumor as well as tumor type, such as IgM, IgD, IgA, or IgG [22]. In addition, dendritic cells (DCs) express inflammatory cytokines IL-12, IL-23, and IL-1 that promote IFN-gamma CD4+ T-cell responses [25]. Natural killer (NK) cells express HLA class-I receptors, which can recognize and eliminate malignant cells [26]. There are also immunosuppressive cell

types in the TME, which includes the T-regulatory cell population (Treg), myeloid-derived suppressor cells, and M2 macrophages [27–29].

Other Cells In addition to stromal and immune components, there are multiple other cell types residing in the TME that contribute to tumor growth. For instance, endothelial cells continue to grow and divide uncontrollably during cancer progression, which express the VEGF receptor on the cell surface, which allows them to continually stimulate angiogenesis. Platelets within the TME can be an additional source of VEGF and both pro- and antiangiogenic proteins that are usually carried in alpha-granules of platelets [30]. Pericytes are another important cell type that maintains the integrity of blood vessels, but which begin to loosen their attachment upon activation of angiogenesis via signaling molecules such as PDGF, TGF-beta, angiopoietin, and Notch [31]. Loss of pericyte attachment leads to higher permeability of blood vessels and increased metastatic spread of tumor cells.

Vasculature Just like normal tissue, malignant tissue develops a network of blood and lymphatic vessels to supply the necessary oxygen, remove waste and carbon dioxide, and provide a route for immune surveillance [32]. However, unlike in normal tissues, these vascular networks often contain leaky capillaries. During angiogenesis, the vasculature becomes even more complicated. An ever-hypoxic state of the TME initiates aberrant blood vessel formation that allows tumor cells to escape the low-oxygen setting and disseminate to distant sites, where nutrient and oxygen levels are not yet depleted. Hypoxia triggers the release of hypoxia-inducible factor 1 (HIF-1), leading to upregulation of genes such as VEGF and PDGF, which stimulate angiogenic factors [33]. Formation of new blood vessels begins with degradation of the basement membrane around the tumor and disruption of the EC monolayer, followed by tube formation and EC invasion into the surrounding tissue [34]. Pericyte recruitment then stabilizes the newly formed blood vessels, providing structural support and allowing for the necessary crosstalk between ECs that further stimulates VEGF production [35].

10.2 Angiogenesis

10.2.1 Angiogenic Switch

In the absence of new vasculature, tumor growth is restricted with a well-maintained balance between proliferation and apoptosis [36]. An angiogenic switch occurs when this homeostasis—the balance between proangiogenic and angiogenic pathways—skews in one direction over the other. This loss of angiogenic homeostasis may occur for several reasons, but evidence from many studies points to genetic and epigenetic remodeling as being the main contributors to such a switch. The angiogenic switch is correlated with both loss of tumor suppressor genes, such as p53, and upregulation of oncogenes, such as Myc, which increases production of VEGF by 10-fold [37–39]. Regardless of the reasons this happens, angiogenic switch starts a cascade of processes that make it much more likely and favorable for cancer to progress.

Hypoxia is a well-known inducer of angiogenic switch, as it forces a very rapid metabolic reprogramming, skewing this well-maintained angiogenic homeostasis [40]. In the absence of oxygen, cells go into a crisis mode, trying to get nutrients and oxygen from nearby tissues. Tumors are no exception to this—they are highly hypoxic structures with abnormal vascular networks that are constantly trying to survive. Hypoxia shifts the cellular metabolism in a way that the extracellular space becomes more acidic and glucose metabolism along with lactic acid production become upregulated, which subsequently lowers the pH in the TME [41]. Such a pH decrease is correlated with rigorous EMT, cell dissemination, and eventual metastasis [42–45]. An acidic environment is an important contributor to an increase in angiogenic factors through upregulated expression of VEGF [46]. Hypoxia triggers additional processes, ranging from mobilization of bone-marrow-derived precursor cells to

immune activation [47]. Hypoxia is also one of the main contributors to induce expression of VEGF, MMPs, and angiopoietin-like 4 (ANGPTL4)—all of which are promoters of angiogenic pathways [48, 49].

10.2.2 Mechanisms That Drive Angiogenesis

Pathological vessel proliferation is one hallmark of cancer progression [50]. In normal tissue, blood vessels appear as ordered tubular networks that facilitate the transport of gases, nutrients, and cells around the body, and are carriers of different trophic signals, all of which are necessary for normal organ homeostasis [51]. They are categorized into veins, arteries, and capillaries, and comprise a thin monolayer of epithelial cells on the luminal side, the basement membrane on the outside covered with pericyte, and vascular smooth muscle cells. Two processes are required for the maintenance of vascular networks, namely, vasculogenesis and sprouting angiogenesis, both of which are essential mechanisms for cancer progression. Vasculogenesis is the de novo formation of new blood vessels, while sprouting angiogenesis is the formation of new vessels from a pre-existing network of capillaries.

Sprouting angiogenesis is the first process in blood vessel formation, which involves an intricate interplay between the ECM, stromal cells, and soluble factors [52]. During sprouting angiogenesis, endothelial cells begin to loosen their contact with pericytes, which are the stabilizing cells surrounding blood vessels, whose function it is to maintain the vessels' integrity and quiescent state. Once endothelial cells have been destabilized, they undergo EMT, where they acquire a highly migratory and invasive personality. This process is accompanied by the basement membrane destabilization and ECM degradation, much needed for angiogenesis to proceed by allowing the formation of an immature blood vessel [53]. Vessel maturation occurs when a process known as mesenchymal to endothelial transition (the reverse of EMT) occurs, which restores endothelial cells to their quiescent state, followed by the synthesis of a new basement membrane [54]. Initiation of an angiogenic sprout is controlled by VEGF and the Notch signaling pathway [55]. The growing end of the sprout is known as the "tip cells," which respond to VEGF signaling by extending filopodia that sense their environment and recruit stromal cells for stabilization and support. Endothelial cells that are located in the stalk portion of the angiogenic sprout are known as the "stalk cells," which undergo the same process but sprout sideways, contributing to extensive branching—most often in response to VEGF-A signaling [56].

Vasculogenesis begins with the mobilization of endothelial progenitor cells (EPCs), which get recruited in response to chemokines, cytokines, and growth factors released by both tumor and stromal cells [57]. In hypoxic conditions, expression of HIF is seen to activate VEGF, PDGF, C-X-C chemokine receptor Type 4 (CXCR4), and stromal-derived factor-1 (SDF-1), which are important for EPC proliferation [57, 58]. In response to VEGF and PDGF particularly, EPC mobilization occurs through the release of matrix metalloprotease 9 (MMP9), which activates the Kit ligand, a stem-cell migratory cytokine that allows EPC mobilization to take place [59]. Besides its role in primary tumor growth, vasculogenesis has also been implicated in the dissemination of cells and eventual metastasis via soluble factors such as SDF-1, which recruit EPCs to distant sites [60]. The interaction of SDF-1 on EPCs and the CXCR4 receptor on tumor cells establishes the development of a pre-metastatic niche.

10.2.3 Metastasis Due to Angiogenesis

Unfortunately, angiogenesis is the main contributor to cancer progression from a primary tumor ecosystem to a metastatic tumor ecosystem, where cells disseminate and invade the surrounding tissue. As already discussed, VEGF is the main inducer of multiple processes that make metastasis much more likely—it upregulates pro-

tease production that degrades the basement membrane and secretes factors that weaken endothelial-tumor cell interactions, a necessary process for metastasis [61]. The pericyte lining the blood vessels also loosens their attachments to endothelial cells on the luminal side of the vessel, leading to a decrease in endothelial cell survival and creation of a leaky environment through the intercellular gaps that allow tumor cells to escape and travel to disseminated sites [62–64].

10.2.4 Blocking Vessels in the TME

Since angiogenesis is such an important part of neoplasms, modern therapies have focused on finding a suitable therapy that targets this process. There has even been marginal success in the treatment of several tumor types with such drugs as Sutent and Avastin against kidney and colorectal cancer [50, 65–67]. However, modern approaches still rely on standard chemotherapy, which seems to fall short due to its low selectivity of cancer cells and its high toxicity to normal cells [68]. While drug delivery to tumors is inefficient because of highly abnormal vasculature, as already discussed, multiple targets are being developed to inhibit or induce regression of neoplastic blood vessels [69].

Direct vessel signaling inhibition. EPC mobilization and seeding are the absolute requirement necessary to start angiogenesis, which occurs via targeting of tyrosine kinase (TK) receptors by angiogenic growth factors such as VEGF [70, 71]. Therefore, approaches that inhibit TK receptors or their ligands are being investigated as an antiangiogenic therapy approach, including antibodies, soluble factors, and small-molecule inhibitors [71–73]. Examples of TK inhibitors (TKIs) include Sorafinib, which downregulates Raf signaling along with VEGFR-2 and PDGFR-beta [71], and Sunitinib, a TKI for both VEGFR-2 and PDGFR-beta and a potent inhibitor of c-kit [72].

Vascular environment inhibition. Another approach is to inhibit the vascular environment of the TME, and since angiogenesis begins with EPC recruitment and establishment of EPC metastatic niches, this process may also target pharmacologically. For instance, as the SDF-1/CXCR4 signaling axis is the main regulator of EPC mobilization and homing, antibodies against CXCR4 might be a plausible target [60].

Vessel normalization. Another promising type of treatment is actually the opposite of the two aforementioned therapeutic approaches—a desire to stabilize vascular networks [74]. As already mentioned, in contrast to normal vasculature in nonmalignant tissue, which is efficient and follows predictable patterns, the vasculature of a tumor is in a state of extreme disarray, characterized by aberrant, disorganized, and dilated morphologies. This decreases pericyte association, elevates chances for hypoxia, increases permeability to escaping tumor cells, and lowers perfusion. One of the main issues of chemotherapeutic drugs and immune therapies is that they cannot reach the target area because of this faulty vasculature [75]. Thus, drugs have developed to stabilize the vascular networks, with the goal of improving pericyte recruitment and tightening cell-to-cell junctions in a process known as vascular normalization [76]. Such drugs include bevacizumab (Avastin) and trebananib, which have shown favorable clinical outcomes when used in combination with chemotherapy in breast and ovarian cancer patients [77–79].

10.3 Hypoxia

10.3.1 Role of Hypoxia in the TME

Hypoxia is at the forefront of cancer growth and progression [80]. Because of oncogene activation, initial cell proliferation is so aggressive that there are not enough available nutrients and oxygen in the environment to supply the cells, and so the environment becomes hypoxic as those resources quickly deplete [81]. This lack of nutrient and oxygen supply triggers a cascade of changes in the TME that increases production of angiogenic factors and revascularization events [82]. However, as already mentioned, vascular structures in the tumor environment are not perfectly ordered; instead, they are chaotic and

sporadic with constant angiogenic mechanisms triggered in response to hypoxic episodes, which lead to vascular leakiness and nonlaminar blood flow [83, 84]. Because tumors are heterogeneous structures with dynamic fluctuations in blood flow, within a single tumor ecosystem there may exist regions of both mild hypoxia and acute hypoxia; those fluctuations in blood flow can lead to cycling hypoxia, which can vary from hours to days. Two frequencies of cycling may be detected: higher-frequency cycling usually results from small alterations in red blood cell perfusion, while lower-frequency cycling results from large-scale remodeling of the vascular network and angiogenesis [85]. Short-term hypoxia activates autophagy as well as apoptotic and metabolic adaptation of cells to survive in adverse conditions [86, 87] and production of reactive oxygen species (ROS), which contributes to tumor survival and growth [88, 89]. Acute hypoxia induces metastasis and is associated with aggressive tumor phenotypes [90]. Long-term hypoxia contributes to long-term cellular and genetic changes, such as DNA breaks, higher DNA replication errors, genetic instability, and mutagenesis [91–93]. Regardless, neither chronic nor acute hypoxia is good news for a growing tumor—these sporadic events at irregular intervals usually present with adverse clinical manifestations.

10.3.2 Hypoxia in Blood Vessel Formation

Hypoxia induces overexpression of transcription factors such as HIF-1-alpha and HIF-2-alpha (Fig. 10.2), which target blood vessel formation and metastasis, and play a role in resistance to treatment [94]. Abnormal angiogenesis ensues in response to the pathological condition in which, because of rapid cell proliferation, nutrients and oxygen are used up by the rapid cell increase [95, 96]. The hypoxic state allows the production of proangiogenic factors, thus skewing the intricate balance that maintains the normal angiogenic equilibrium, resulting in rapid vessel formation. These disordered vessels lack structure, organization, and proper pericyte contacts, which make them leaky and more susceptible to metastatic spread. Thus, angiogenesis results from a cell's attempt to relieve the hypoxic state, thus inducing the formation of more blood vessels to relieve the oxygen demands, but inadvertently restarting the vicious cycle [51]. However, the cycle continues as soon as another need to improve hypoxia arises. There are some antiangiogenic drug therapies being developed that target highly malignant and invasive cancer types, including bevacizumab, an anti-VEGF monoclonal antibody approved for colorectal cancer and other solid tumor types [97].

10.3.3 Hypoxia in Metastasis

A bona fide metastatic process results from hypoxia-induced angiogenesis, where the cells end up escaping the highly hypoxic conditions via the newly formed blood vessels to relieve oxygen demands and survive [51]. However, as the result of sporadic growth, the new vasculature is so fragile, highly permeable, and heterogeneous that it permits the massive relocation and delivery of tumor cells to distant organs via circulation. Levels of tumor oxygenation and overexpression of HIF-alpha has been shown to correlate with highly metastatic and aggressive tumors and the poor overall survival of patients [98]. It may not come as a surprise, therefore, that previous hypoxic cells can also keep their ability to metastasize at a higher rate than cells only cultured in normoxic conditions, as was shown by an orthotopic mouse model, where lymph node metastasis seemed to increase due to acute hypoxia followed by normoxia [99]. Mechanistically, hypoxia seems to trigger an invasive and migratory phenotype of cells by inducing EMT [100, 101]. On the genetic regulatory level, genes responsible for maintaining an epithelial phenotype are reduced (E-cad, beta-catenin) [102], while mesenchymal-like gene expression is stimulated (N-cad, vimentin, SMA, CXCR4) [103, 104]. Though the bona fide master regulator of the physiological EMT is TGF-beta, it is increased in response to hypoxia, activating

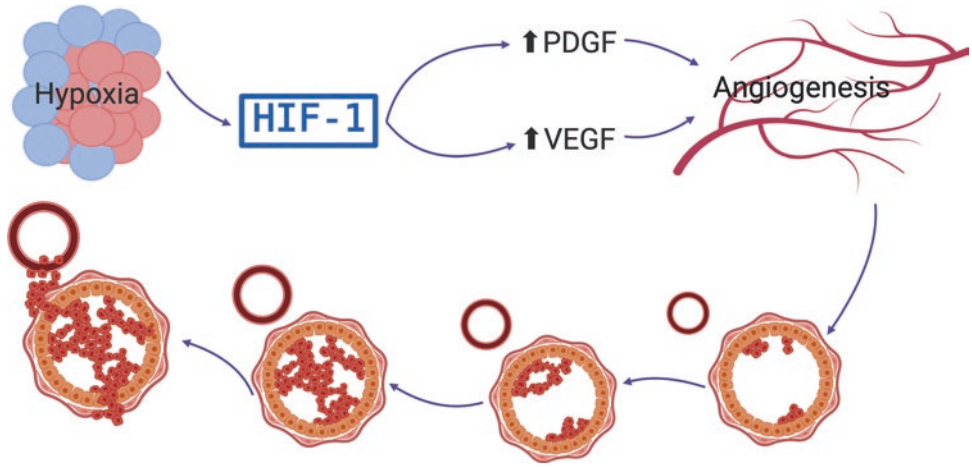

Fig. 10.2 Role of hypoxia in the TME. Hypoxia can present itself in several forms throughout the tumor—mild or acute, cycling or permanent—initiating mechanisms such as epithelial to mesenchymal transitions (EMT) of resident cells as well as inadvertently placing the cells in such a stressful condition that production of ROS and DNA damage is unavoidable. The reason DNA damage to mild forms of hypoxia (or cycling hypoxia) is so dangerous is because it can induce mutagenicity while allowing resistant cells to survive. This is where drugs and treatments can subsequently suffer in effectiveness. Hypoxia causes release of inducible factor 1 (HIF-1) that upregulates vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) that stimulates angiogenesis. Angiogenesis degrades the basement membrane, disturbs the endothelial cell (EC) monolayer, and results in an invasion into the surrounding tissue

downstream transcription factors (TFs) such as Snail, Smad, Slug, and Twist with the inhibition of E-cadherin expression [105], thus inducing massive EMT. The development of resistance to radio- and chemotherapy has been linked to faulty EMT processes that are regulated via Snail and Slug [105]. HIF inhibition has been viewed as a major promising therapeutic approach, especially for metastatic and solid tumor types, and there has been marginal success with several drugs that have undergone phase I and II clinical trials [106, 107]. Thus, there are multiple correlations between metastasis, development of drug resistance, and hypoxia-induced EMT changes that take place in terminal cancers.

10.3.4 Hypoxia in Radiation and Drug Resistance

Resistance to treatment-induced apoptosis from radio- or chemotherapy is one of the biggest obstacles in cancer treatment [108]. Often, this occurs because residual cells that are resistant to treatment are left over and multiply, contributing to a clonal expansion of treatment-resistant cells that can quickly lead to tumor recurrence and metastasis [109]. Hypoxia can also cause resistance of cancer cells to treatment, often leading to various physiological states that allow cells to survive via a variety of mechanisms such as cell cycle arrest (quiescence), a state of reduced proliferation that protects cells from external stress, inhibition of apoptosis, senescence, autophagy, and increased mitochondrial activity [110–112]. In normoxic conditions, an abundance of oxygen supply causes oxygen to react with free radicals generated by ionizing radiation during treatment in a process known as "oxygen fixation," which leads to irreversible DNA damage and profound cell death [113]. However, when oxygen supplies are low, there is a slow generation of free radicals that would otherwise contribute to DNA damage, allowing cells to adapt and survive. These "left-over" cell populations after treatment are dangerous because they can come back at full force. An additional disadvantage is that radio- or chemotherapy often targets the bulk of rapidly proliferating cells. Hypoxic cells are difficult to target because they are usually quiescent, low-

proliferating, have stem-cell-like properties, and live in the most hypoxic (innermost) regions [105, 112]. The least sensitive cell cycle phases to ionizing radiation are G1 and the end of S phase, while the most sensitive are G2 and M, when DNA repair mechanisms are most susceptible [114]. Since these facts about hypoxia-induced treatment resistance have surfaced, researchers have turned to attempting to block HIF-1 with inhibitors to stimulate the cells to respond to treatment in the same way that normoxic cells do. For instance, the HIF-1 inhibitor (YC-1) was tested in tumor-bearing mice and found to cause radiation-induced vessel damage, while HIF1-alpha inhibitor (PX-478) re-sensitized squamous and pancreatic cancer cells, cultured in a hypoxic environment, to radiation therapy [115, 116].

10.4 Nitric Oxide

10.4.1 Nitric Oxide in the Tumor Microenvironment

Nitric oxide (NO) is an intriguing molecule that has resurfaced in the recent decade after much debate as to its pathogenicity. NO, however, is also a known inducer of apoptosis and may play a therapeutic role in cancer rather than just a pathological one [11]. Thus, NO has a dual role as both a physiological and a pathophysiological molecule. NO is a product of a metabolic reaction that converts L-arginine to L-citrulline using nitric oxide synthase (NOS), and can exist in several forms depending on the origin of its production: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [12]. NO by itself is reactive and thus has been implicated to play a significant role in activating multiple signaling pathways. It is regulated by intracellular calcium concentrations (nNOS and eNOS), but it can also be brought about with no calcium present (iNOS) by the upregulation of factors such as endotoxins, inflammatory cytokines, hypoxia, and oxidative stress [12, 117]. Overexpression of different NOS isoforms has been linked to many solid tumors [13]. The most striking feature of NO is that it can exhibit a dose-dependency, so that at high concentrations it acts as the source of nitrosative and oxidative stress, causing DNA damage and mitochondrial dysfunction along with upregulating apoptosis, while at low concentrations it decreases apoptosis and promotes angiogenesis, thus displaying tumoricidal roles (Fig. 10.3) [11, 118]. However, because of its obvious antitumor effects, NO has been gaining popularity in anticancer treatments. For instance, as already discussed, resistance to chemo- and radiotherapy is a main issue in metastatic forms of cancer, but NO has been shown to sensitize cells to subsequent treatment, thus providing a combinatorial therapy approach to cancer treatment [119].

Besides promoting many of the TME essential processes (angiogenesis, metabolism, apoptosis), NO might also play an important role in reprogramming the immune component of the TME.

10.4.2 NO in Immunosuppression

NO can play the role of an immunosuppressive messenger in the TME. One of the main immune cell populations that NO targets is T-cell-mediated antihumoral responses by mediating several mechanisms. In one study, it was shown that NO-derived peroxinitrite inhibits T-cell proliferation, a mechanism which consequently induces apoptosis of T cells [120]. NO can also interfere with T-cell humoral recognition by inhibiting migration of T cells into the TME. One explanation for this interesting observation could be that high concentrations of NO in the TME induces S-nitrosylation of CCL2, a chemoattractant chemokine, which abolishes the tumor's ability to attract CD8+ T cells into the tumor core [121]. In addition, there is another population of cells regulated and attracted by CCL2—myeloid-derived suppressor cells (MDSCs), which produce NO and thus further restrict T-cell migration into the tumor by downregulating E-selectin [122]. INOS was also shown to promote recruitment of T-regulatory cells (Tregs), an immunosuppressive cell type, by modulating IL-12 expression [123]. Additional studies have pointed

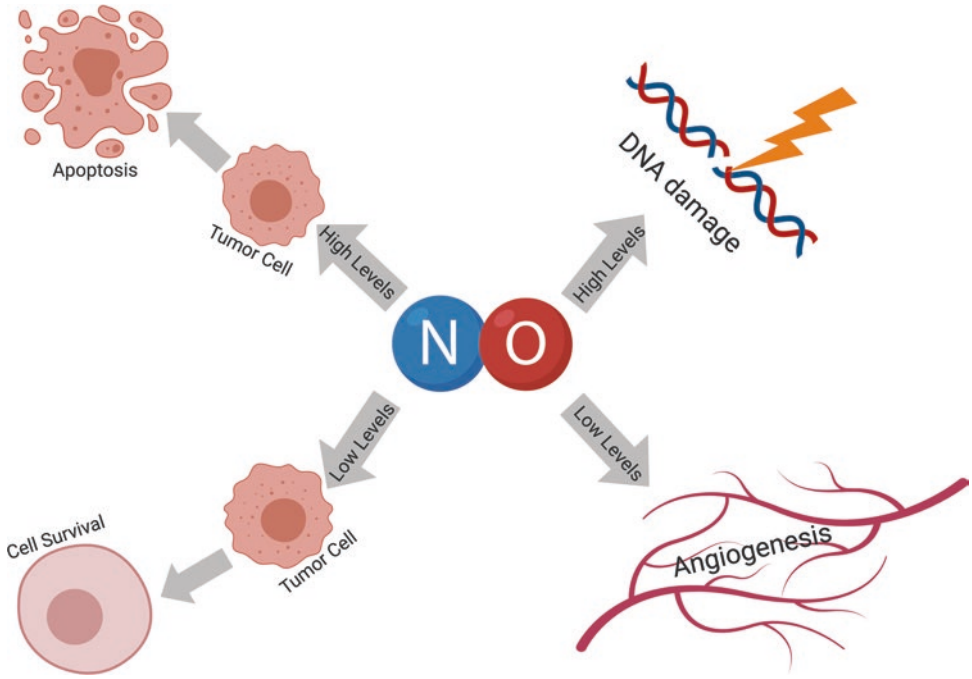

Fig. 10.3 Role of nitric oxide in the TME. Nitric oxide (NO), previously grouped with ROS in the pathogenic molecule category, was recently found to be an important molecule in the TME. Though NO has been found to induce damage by causing metabolic reprogramming and the promotion of immunosuppressive phenotypes, it has also been found to have a positive effect on targeting the TME broadly. Furthermore, it has been found to be exceptionally dose-dependent with regard to both its negative and positive effects on the TME. NO at low doses decreases apoptosis along with promoting angiogenesis, while at high doses it causes DNA damage and increases apoptosis

to other mechanisms of NO-mediated tumor immunosuppression, such as inhibiting antigen presentation from dendritic cells to CD4+ helper T cells [124] and directly impairing natural killer (NK) cell functions [125]. It may also play a role in immune-activation processes, as NO was shown to be released by activated macrophages in the TME, thus inducing their cytotoxic antitumor activity [126].

10.4.3 NO in Evasion of the Immune Response by Cancer Stem Cells

Considering all of this, there is a large body of evidence that points to NO also exerting other immunosuppressive functions on the TME by regulating the "stemness" of cancer cells. Tumors seem to be so good at evading immune system recognition because of a subset of cells in the TME termed "cancer stem cells," and this has led scientists to refer to cancer as a "stem cell disease" [127]. The "cancer stem cell model" states that there is a subpopulation of cancer stem cells (CSCs) at the initiation stages of tumor growth, which display pluripotent and renewing properties. These properties allow the initial tumor seeding events to take place and eventual propagation and metastasis, which are responsible for the bulk of failures of many conventional therapies and poor cancer survival rates [128, 129]. The effect of stem cell signaling on the TME seems to be driven by the active WNT/beta-catenin signaling pathway and a complete absence of T-cell gene expression signature in human melanoma [130]. In addition, CSCs do not exhibit tumor antigen expression and show a

defective MHC-antigen presentation pathway and downregulation of MHC class I molecules [131]. CSCs can also recruit cells that further promote immunosuppressive functions, supporting the CSC phenotype and stabilizing their niche in the TME [132].

NO metabolism contributes to the maintenance of "stemness" that is characteristic of CSCs. As was shown in glioblastoma, eNOS activates the Notch signaling pathway, which promotes the CSC phenotype [133]. CSCs also promote expression of the iNOS isoform, which cranks up the synthesis of NO [134]. The maintenance of the CSC phenotype by NO signaling is demonstrated in several cancer types, including breast [135], colorectal [136], lung [137], and liver [138] cancers. NO produced by immunosuppressor cells in the TME may contribute to the plasticity of cancer cells themselves that allows them to gain and maintain a stem cell phenotype [139].

10.4.4 Metabolic Reprogramming by NO in the TME

Tumors adapt rapidly to stress conditions by rewiring their metabolic pathways. Shockingly, most energy in the TME is generally derived from aerobic glycolysis, which is not as efficient at producing ATP but is a fast process that can generate some energy to be used immediately. Unfortunately, the downside is that aerobic glycolysis quickly builds up lactic acid in the extracellular space, lowering the pH [140]. The acidic microenvironment induces expression of VEGF that, besides increasing angiogenesis, also leads to polarization of the M2 macrophage phenotype [141]. At early stages of tumor growth, TAMs maintain a proinflammatory and antitumorigenic phenotype, the M1 state, while at later stages of metastasis and tumor progression M1 differentiates into the M2 phenotype, which displays a protumoral phenotype and contributes to immunosuppression [142]. In high-grade tumors, TAMs are mostly the M2 phenotype, which also produces NO and has endogenous mechanisms that protect tumor cells from chemotherapy [143]. Since hypoxia induces the upregulation of enzymes involved in glycolysis and the inhibition of mitochondrial function, in this sense, NO-induced hypoxia contributes to the "Warburg effect" (aerobic glycolysis metabolism observed in cancer) [144]. NO has also been shown to prevent differentiation of M1 macrophages into the M2 phenotype by abolishing mitochondrial respiration and reducing their plasticity [145].

Acknowledgments We would like to thank The UM writing center, specifically Valerie A. Gramling, for proofreading the draft. Images were created with BioRender.com.

Conflict of Interest None: See disclosures.

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