Hypoxia-Induced Resistance to Chemotherapy in Cancer

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Abstract

A major barrier to the successful management of cancer is the development of resistance to therapy. Chemotherapy resistance can either be an intrinsic property of malignant cells developed prior to therapy, or acquired following exposure to anti-cancer drugs. Given the impact of drug resistance to the overall poor survival of cancer patients, there is an urgent need to better understand the molecular pathways regulating this malignant phenotype. In this chapter we describe some of the molecular pathways that contribute to drug resistance in cancer, the role of a microenvironment deficient in oxygen (hypoxia) in malignant progression, and how hypoxia can be a significant factor in the development of drug resistance. We conclude by proposing potential therapeutic approaches that take advantage of a hypoxic microenvironment to chemosensitize therapy-resistant tumours.

Keywords

Hypoxia · Drug resistance · Chemotherapy · Tumor microenvironment · HIF-1 · Metastasis · Autophagy · Nitric oxide · Glyceryl trinitrate · PD-1/PD-L1

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9.1 Drug Resistance in Cancer

9.1.1 Intrinsic Drug Resistance

The development of drug resistance in cancer is complex and multifactorial; moreover, several mechanisms of drug resistance appear to be clinically relevant. Some of these mechanisms operate at the single cell level and include the overexpression of drug efflux proteins such as the multidrug resistance protein (MRP1; and related MRP2 and MRP3), as well as the P-glycoprotein (P-gp) efflux pump [2]; increased levels of detoxification and DNA repair enzymes such as glutathione-S-transferase (GST) and 06-alkylguanine DNA alkyltransferase [3, 4]; and mechanisms interfering with drug-induced apoptosis [1, 5]. Alternatively, drug resistance can occur at the multicellular level, where the tumour architecture plays an important role. In this case, cells can acquire resistance to several classes of drugs (multidrug resistance or MDR) via multiple mechanisms [6–9].

Cancer cell 'stemness' has emerged as a major contributor to drug resistance and recurrence. Cancer stem cells (CSCs) are capable of limited differentiation, self-renewal, and tumourigenicity, and exhibit enhanced proliferation [10] and survival [11] in response to chemotherapy. Importantly, resistance of these tumour initiating cells to both chemotherapy and radiation therapy [12] results in selective enrichment of heteroge-

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neous subpopulations [12, 13]. Acquisition of drug resistance in CSCs can arise as a result of multiple cell intrinsic mechanisms (Reviewed by Abdullah and Chow [14]). Most critically though, drug resistance in CSCs is dependent on maintenance of pluripotency, which contributes to tumour heterogeneity – a feature of MDR. Recent work has additionally highlighted the importance of autophagy in conservation of pluripotency [15] such that inhibition of autophagy sensitizes tumour cells to chemotherapy and thus represents a potential strategy to overcome drug resistance [16].

9.1.2 Role of the Tumour Microenvironment in Drug Resistance

Until relatively recently, tumour cell intrinsic pathways were the focus of mechanistic drug resistance studies. Accumulating evidence now shows that the tumour microenvironment (TME) plays a pivotal role in facilitating acquired drug resistance. Contributing to the TME is a network of fibroblasts, immune cells, host microbes, lymphatics and vasculature [17]. These cellular constituents reside within a complex stromal scaffolding made up of extracellular matrix (ECM) proteins often within an environment deficient in oxygen [18]. The TME contributes to drug resistance in a multifactorial manner. In the following paragraphs we describe some of the mechanisms by which elements of the TME can mediate drug resistance.

(a) Biomechanical and biophysical properties

Tissue architecture, including cellular organization, polarity [19] and deposition and composition of the ECM [20] can regulate apoptotic responses to chemotherapy. Relative to normal tissue architecture, the ECM of solid tumours is often rigid. The biomechanical properties of this stiffened ECM regulate and direct malignant cellular behaviours [21] including migration and invasion [22], dormancy, proliferation and chemosensitivity [20]. Increased tumour stiffness is predictive of neoadjuvant chemotherapy response in breast cancer [23] and is linked to chemoresistance in pancreatic cancer [24, 25]. High interstitial pressure resulting from a rigid ECM in solid tumours can also lead to drug resistance by preventing the transport of chemotherapeutic agents away from blood vessels (Reviewed by Munson and Shieh [26]). Poor delivery of molecules resulting from high interstitial pressure and inadequate blood perfusion can lead to a hypoxic environment and a deficit in nutrients such as glucose. Glucose deprivation was shown to induce resistance to doxorubicin and etoposide in Chinese hamster ovary cells, as well as human colon and ovarian cancer cell lines [27–29]. Similarly, changes in the pH of the tumour microenvironment resulting from increased anaerobic respiration and decreased removal of toxins cause alterations in cell membrane permeability, which in turn can limit cellular uptake of chemotherapeutic agents [30, 31].

Biomechanical properties of the ECM can also be altered by neoplastic progression. For example, tumour cell expression of the intracellular protein tyrosine kinase focal adhesion kinase (FAK) regulates local tissue fibrosis and promotes an immunosuppressive TME associated with therapy resistance in pancreatic ductal adenocarcinoma [25]. Indeed, stromal depletion has been utilized as an approach to enhance delivery of chemotherapeutic agents to desmoplastic tumours and has been successful at improving survival in mouse studies [32, 33].

(b) Host microbiome

The potential role of the microbiome in the modulation of therapy responses in cancer is an area of investigation that has received a great deal of attention in recent years [34], but is still a relatively new concept. It is becoming evident that

gaining an understanding of pharmacomicrobiomics, the study of interactions between host microbes and drugs, is important to implementing effective cancer treatments [35]. While much of the work currently under way focuses on promoting a favourable intestinal microbiome for successful immune therapy [36], there is also evidence that intratumoural bacteria metabolize chemotherapeutic agents and thus contribute to chemoresistance [37].

(c) Immune microenvironment

Tumours are described as wounds that do not heal [38] and tumour-promoting inflammation is now widely accepted as a hallmark of cancer [39]. The tumour immune microenvironment (TiME) is dynamic and consists of innate and adaptive immune cells as well as humoral factors that are largely immunosuppressive [40]. Immune cells within the tumour microenvironment are functionally distinct from their counterpart immune cells of the adjacent normal stroma and are often described as being pro-tumourigenic. These tumour promoting immune cells within the TME negatively influence responses to radiotherapy and chemotherapy (Reviewed by Medler et al. [41]), in part by preventing tumour cell apoptosis [42]. Myeloid cells, including tumour associated macrophages (TAMs), neutrophils and myeloid-derived suppressor cells (MDSCs) are well-studied immune cells contributing to chemoresistance (Reviewed by Cotechini et al. [43]). However, cells of the adaptive immune system, including B cells and $CD4^+$ T cells, also contribute to chemoresistance and radioresistance in part by regulating the mobility and antitumour functions of cytotoxic $CD8^+$ T cells (CTL) [44–46].

An important signalling axis regulating immune cell activity and, in particular, $CD8^+$ cytotoxic T cell responses, is the immune checkpoint Programmed Death Receptor 1 (PD-1)/Programmed Death Ligand 1 (PD-L1). PD-1 is a monomeric transmembrane receptor present on activated T cells, B cells, dendritic cells, NK cells and monocytes. Binding of PD-1 to its cognate ligands, PD-L1 or PD-L2, renders T cells hyporesponsive to antigen stimulation and manifests as inhibition of proliferation and dampened effector (cytotoxic) functions [47–49]. PD-L1 is expressed by many different cell types, including epithelial cells, B cells, T cells, monocytes and antigen presenting cells [50]. Importantly, tumour cells from various cancers including breast, colorectal, ovarian, bladder and lung cancers, as well as glioblastomas, lymphomas, melanomas and leukemias express PD-L1, and expression of this immune checkpoint is predictive of poor clinical prognosis [51–59].

There is recent evidence from our work [60] and that of others [61], that the PD-1/PD-L1 signalling axis is bi-directional and that reverse signalling endows tumour cells with enhanced resistance to conventional anti-cancer drugs. *In vitro* work from Azuma and colleagues revealed that PD-L1 overexpressing mouse mastocytoma (B7-H1/P815) cells are resistant to $PD\text{-}1^+$ CTL-mediated killing as well as to Fas and drug-mediated apoptosis [61]. Using *in vitro* and *in vivo* approaches, we recently discovered that PD-1/PD-L1 signalling endows human and mouse prostate and breast cancer cells with resistance to conventional chemotherapeutic agents likely via signalling through PI3K-AKT-mTOR and MEK-ERK pathways [60]. Importantly, our group has also shown that hypoxia induces PD-L1 expression in murine and human tumour cells leading to immune escape [158]. Hypoxia is a characteristic of solid tumours and occurs as a result of an imbalance between oxygen consumption and oxygen availability [62]. While a reduction in the local amount of oxygen can be initially detrimental to rapidly-proliferating cells, tumour cells adapt to hypoxia by activating oxygen sensitive transcription factors (described below). Tumour cells co-opt physiological adaptations to hypoxia in order to evade immune destruction and survive radiotherapy and chemotherapy.

9.2 Hypoxia and Malignant Progression

9.2.1 HIF-1: A Mediator of Hypoxia-Induced Malignant Phenotypes

The most well characterised transcription factor responsible for many cellular adaptations to hypoxia is the hypoxia-inducible transcription factor (HIF), a dimeric protein consisting of a constitutively active subunit (HIF-1β) as well as an oxygen-sensitive subunit (HIF-1α) [63]. Under well-oxygenated conditions, HIF-1α is unstable and rapidly degraded. HIF-1α is hydroxylated by the oxygen-dependent enzyme prolyl hydroxylase domain 2 (PHD2) and interacts with the von Hippel-Lindau tumour suppressor protein (pVHL). This interaction leads to the recruitment of E3 ubiquitin ligase that mediates the polyubiquitination of HIF-1α, which ultimately leads to the proteasomal degradation of HIF-1α [64, 65]. HIF-1α is also hydroxylated by factor inhibiting HIF-1 (FIH-1), which prevents binding of its coactivator p300/CBP and inhibits transcriptional activity [66, 67]. Due to the oxygen requirement for PHD2 activity, hypoxia prevents the hydroxylation of HIF-1α, thereby allowing it to bind to HIF-1β and mediate the transcription of hypoxia-inducible genes [63].

Many HIF-1 gene targets encode proteins involved in promoting tumour growth and malignant phenotypes such as angiogenesis, glucose metabolism, ECM remodelling, epithelial-to-mesenchymal transition, cell survival, and proliferation [68]. Glucose transporter 1 (*glut-1*) is a HIF-1 target gene and is involved in regulation of glucose uptake [69]. HIF-1 regulates angiogenesis by activating various genes, most notably vascular endothelial growth factor (*VEGF*), a master regulator of neo-vessel formation [70], as well as genes that mediate endothelial cell and pericyte proliferation, migration, adhesion, and maturation, vascular permeability and vasoactivity [71]. Despite activation of angiogenesis in response to hypoxia, blood vessels within the TME are tortuous and leaky and do not function in a normal capacity [72]. Hypoxia is also a central regulator of lymphatic vessel formation or lymphangiogenesis [73]. In addition to the presence of lymphatic vessels being associated with lymphogenous spread of disease, recent work examining lymphatic vessel density (LVD) in human melanoma revealed a positive correlation between LVD and the presence of immunosuppressive factors within the TME and tumour-draining lymph nodes [74]. Taken together, hypoxia enables tumour growth by promoting the classical hallmarks of cancer [39, 75].

9.2.2 Hypoxia and Radioresistance

As early as the 1950s, radiobiologists were aware that hypoxia within solid tumours reduces the efficacy of radiation therapy [76]. Gray and colleagues discovered that tumour cells were three times more sensitive to radiation under normoxic conditions compared to those in anoxia [76, 77]. Successful radiotherapy depends on the presence of relatively high levels of oxygen required for the generation of free radicals that cause irreversible DNA damage, and hence tumour cell death [78]. To overcome hypoxia-induced radio-resistance, studies have focused on developing therapeutics that function to increase oxygen delivery via improving blood flow, mimicking oxygen or targeting and destroying hypoxic cells [79]. Studies combining fractionated radiotherapy with oxygen mimetics such as 2-nitroimidazoles, or use of cytotoxic agents that specifically target hypoxic cells have shown increased tumour cell killing during radiotherapy [80]. However, despite decades of strong evidence revealing that modification of hypoxia is clinically efficacious in radiotherapy, it has yet to become a standard of care [81]. Similarly, it was discovered that many chemotherapeutic agents (*e.g.* carmustine and alkylating agents) display reduced cytotoxicity toward hypoxic tumour cells, as these drugs also require oxygen for maximal activity [82]. These early observations led to the development of novel chemotherapeutic bioreductive agents which are cytotoxically active only under limited levels of oxygen [82].

9.2.3 Hypoxia and Cancer Metastasis

Studies have demonstrated that hypoxia within the tumour mass is an independent marker of a poor prognosis for patients with various types of cancers such as carcinoma of the cervix [83], soft tissue sarcoma [84], carcinoma of the head and neck [85], cutaneous melanoma [86] and prostatic adenocarcinoma [87, 88]. In some of the above studies, disease-free survival for patients with tumours having median pO_2 values of less than 10 mmHg was found to be significantly lower than for patients with tumours having higher pO_2 values. Moreover, clinical studies now have provided evidence that low tumour oxygen levels are associated with increased tumour growth and metastasis [83, 84] and with biochemical relapse and recurrence of prostate cancer following radiotherapy [89].

Local tumour hypoxia is a serious impediment to the successful treatment of cancer in part as a result of hypoxia-mediated acquisition of malignant phenotypes that promote the spread of tumour cells. Experimental evidence in support of hypoxia having a direct stimulatory effect on metastasis was initially provided by the work of Hill and co-workers using various cell lines [90–93]. Their earlier studies demonstrated that exposure of mouse fibrosarcoma cells to hypoxia induces DNA over-replication and selects for tumour cell variants with increased metastatic potential [92]. More recently, we and others showed that hypoxia (both *in vitro* and *in vivo*) rapidly and transiently increases the invasiveness and metastatic potential of various tumour cell lines [90, 91, 93–99]. Our studies linked the hypoxia-mediated invasive ability of tumour cells to elevated expression of metastasis-associated molecules such as the urokinase plasminogen activator receptor (uPAR: a cell surface glycoprotein necessary for tumour cell invasion through the extracellular matrix) and the plasminogen activator inhibitor 1 as well as with decreased expression of tissue inhibitor of metalloproteinases 1 [95, 100, 101]. In support of these observations, Rofstad et al. reported that hypoxia promotes lymph node metastasis in human melanoma xenografts by up-regulating uPAR expression [102].

The above-mentioned hypoxia-associated tumour intrinsic mechanisms contributing to metastasis are often described as ‘seed factors’ [103] in relation to Stephen Paget’s ‘Seed and Soil’ hypothesis [104]. It is becoming evident that the metastatic niche is an important factor to consider when discussing metastasis and metastatic potential since this fertile soil contributes to the metastatic microenvironment (MME). Similar to the TME, the MME is a hypoxic, immunosuppressive milieu consisting of dysregulated cellular and acellular components. Metastatic tumour cells often exhibit organotropism with respect to dissemination to secondary sites. For example, breast cancer frequently metastasizes to the lungs. There is evidence, using human breast cancer cells and metastatic murine models, that HIF-1 orchestrates metastatic programs driving lung-specific metastasis through various mechanisms [105]. In general, the MME favours seeding and outgrowth of disseminated metastatic tumour cells and thus contributes to malignancy. However, it is also important to consider the role of the pre-metastatic niche. This unique microenvironment is established prior to the dissemination of tumour cells and is primed by transformed cells within the primary neoplasm to enable colonization of metastatic cells. Indeed, recent work has shown that factors secreted by hypoxic tumour cells support the establishment of an immunosuppressive pre-metastatic niche [106, 107].

9.2.4 Role of Hypoxia in Autophagy

Autophagy – a cell-intrinsic process of ‘self-eating’ that maintains cellular homeostasis – is regulated by numerous stimuli and pathways, one of which is hypoxia [108]. Both HIF-1-dependent and HIF-1-independent mechanisms are known to control this process [109]. HIF-1-independent

pathways tend to be activated in more severe hypoxic conditions, and work in concert with other cell stressors such as metabolic stress and nutrient starvation [109]. One important HIF-1α-independent pathway of hypoxia-induced autophagy is the unfolded protein response (UPR). Rouschop et al. demonstrated that hypoxia activates transcription of microtubule-associated protein 1 light chain 3β (*MAP1LC3B)* and autophagy-related gene 5 (*ATG5)* in multiple tumour cell lines via the UPR [110]. *MAP1LC3B*, encoding microtubule-associated protein light chain 3 (LC3B), and *ATG5* are important for autophagosome formation and thus play important roles in autophagic processes. Similar results were shown by Rzymski et al., who reported that autophagy-related gene 4 acts on *MAP1LC3B* transcription by directly binding to a cyclic AMP (cAMP) response element binding site in the promoter [111]. Both studies indicate that the increased transcriptional activation of *MAP1LC3B* leads to a replenishment of the LC3B pool, thus prolonging autophagy and allowing cells to survive through extended periods of hypoxia [110, 111]. There is also evidence that 5'-AMP-activated protein kinase (AMPK) regulates hypoxia-induced autophagy. In a study of androgen-dependent prostate cancer, it was discovered that hypoxia and androgen deprivation lead to activation of autophagy through AMPK and a mechanism partially mediated by Beclin-1 [112]. Similarly, Papandreou et al., showed that hypoxia increases autophagy in tumour cells via activation of AMPK, in a manner independent of HIF-1 and its target genes [113]. Recent studies have also shown an emerging role for micro-RNAs (miR) in regulating hypoxia-induced autophagy. For example, expression of miR-96, which can either promote or inhibit autophagy, is increased in response to hypoxia in prostate cancer cells [114].

Activation of autophagic processes provides a survival advantage to cancer cells subjected to hypoxic stress. HIF-1-dependent mechanisms of hypoxia-induced autophagy are thought to require the expression of Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) as well as a similar protein, BNIP3L (also known as Nix) [115, 116]. It has been proposed that these molecules lead to autophagy by releasing Beclin-1 (a key mediator of autophagy) from Bcl-2/Beclin-1 or Bcl-XL/Beclin-1 complexes [117]. Tracy et al. showed that hypoxia-induced autophagy was dependent on HIF-1-mediated activation of BNIP3 [118]. HIF-1 has also been shown to induce autophagy via activation of miR-210, leading to a downregulation of Bcl-2 [119]. HIF-1 also stimulates autophagy in hypoxia via the p27-E2F1 pathway in esophageal cancer cells [120]. It is important to note that there is some controversy regarding whether hypoxia-induced autophagy is a cell survival or a cell death-inducing mechanism [121]. However, in the context of hypoxia-induced drug resistance in cancer cells, autophagy acts as a survival mechanism.

9.3 Mechanisms of Hypoxia-Induced Drug Resistance

As stated previously, many chemotherapeutic agents exhibit reduced cytotoxicity toward hypoxic tumour cells as such drugs often require oxygen for maximal activity [82]. Regardless of the oxygen requirement for anti-cancer drug activity, studies have also revealed that pre-incubation of certain human and non-human tumour cell lines under hypoxia alters their phenotype such that they transiently increase their resistance to drugs such as etoposide, and doxorubicin [4, 122–127]. Some explanations suggested for this form of resistance have included the upregulated expression of glucose- and oxygen-regulated proteins, DNA over-replication, cell cycle arrest, altered cellular metabolism, increased drug efflux pumps and greater genetic instability [82].

Various hypoxia-inducible genes with well-established roles in resistance to anticancer agents have been identified. For example, functional HIF-1 target hypoxia response elements (HREs) have been identified in genes encoding the multidrug resistance 1 protein (MDR1/ABCB1) and breast cancer resistance protein

(Bcrp/ABCG2), which are members of the ATP-binding cassette (ABC) transporter family that confer resistance through active efflux of a wide range of anti-cancer agents [128, 129]. As observed for most HRE-containing genes, increased HIF-1 binding activates the MDR1 or Bcrp gene promoters, resulting in increased expression of these drug transporters under hypoxic conditions [130].

Chemotherapeutic agents can trigger tumour cell death through the induction of pro-apoptotic pathways. However, it is important to recognise that tumour cells that have undergone drug-induced DNA damage can also be eliminated via other forms of programmed cell death such as autophagy, mitotic catastrophe, and necrosis [131]. Moreover, certain anti-cancer drugs are known to induce senescence in tumour cells [132–137], and drug-induced senescence and mitotic catastrophe may, in fact, be more prominent than apoptosis [132, 136]. Senescence is characterized by an irreversible arrest of the cell cycle and can be induced by various stresses including telomere dysfunction, oxidative damage, DNA damage, and aberrant expression of oncogenic proteins such as Ras [138]. Senescence is categorized as either replicative senescence, a physiological process triggered to limit the life span of non-malignant cells, or accelerated senescence, associated with a rapid onset of terminal proliferation arrest in response to cell damage such as drug- or radiation-induced DNA damage [138]. A study from our laboratory revealed that hypoxia-induced resistance to anti-cancer drugs is associated with decreased tumour cell senescence and that it requires HIF-1 activity [139]. While there is evidence that hypoxia can inhibit replicative senescence by increasing telomerase activity [140–143], it is doubtful that increased telomerase activity accounts for the hypoxia-mediated resistance to drug-induced senescence. It is rather likely that a lack of drug-induced senescence in hypoxic tumour cells is indirectly a result of hypoxia-triggered inhibition of DNA damage, as evidenced by another study from our group [144]. In that study we demonstrated that hypoxia prevents etoposide-induced DNA damage in cancer cells through a still to be characterised mechanism involving HIF-1α [144].

As mentioned previously, hypoxia is an important driver of autophagy. Although autophagy has been shown to have both pro-apoptotic and pro-survival roles in tumour cells, there is a link between hypoxia-induced autophagy and drug resistance [121]. As a protective response against chemotherapy- and radiotherapy-induced apoptosis, tumour cells undergo autophagic processes that degrade damaged cellular components [145]. For example, in response to cisplatin, glioma cell lines stimulate protective autophagic responses via up-regulation of AMPK and subsequent down-regulation of mammalian target of rapamycin (mTOR) [146]. It was subsequently revealed that hypoxia amplifies cisplatin-induced autophagy in a HIF-1-dependent manner and that inhibiting a crucial autophagy mediator, ATG5, restored sensitivity to cisplatin in lung cancer cells [147]. Similar results were observed in non-small cell lung cancer cells, where inhibition of LC3B restored cisplatin sensitivity under hypoxic conditions [145]. Hepatocellular carcinoma cells cultured in hypoxia also exhibited increased resistance to cisplatin, epirubicin, gemcitabine and mitomycin via hypoxia-mediated autophagic processes [148]. Furthermore, bladder cancer cells exposed to gemcitabine exhibit increased autophagy, which is augmented by hypoxia in a manner dependent on the HIF-1α/BNIP3/Beclin-1 signaling pathway [149]. The anti-cancer effects of other drugs, including paclitaxel, were also shown to be decreased under hypoxic conditions in a manner dependent on HIF-1 and autophagy [150, 151]. A study by Notte et al. showed that taxol induces the UPR in hypoxic breast cancer cells, and that upregulation of ATF4 leads to hypoxia-induced autophagy, as well as increased resistance to taxol [152].

While hypoxia-induced autophagy mediates resistance to conventional chemotherapeutic agents, there is also evidence that hypoxia-induced autophagy mediates resistance to anti-angiogenic agents in a HIF-1α/AMPK dependent manner [153], as well as resistance to ionizing

radiation via the HIF-1α/miR-210/Bcl-2 pathway [119, 154].

In addition to the tumour-cell intrinsic mechanisms of hypoxia-mediated drug resistance described above, there are also tumour-cell extrinsic factors contributing to therapy resistance. The recent successes of immune therapy combined with the recognition that tumour-associated inflammation is a potentiator of malignant progression have led to recent exploration of links between hypoxia, the TiME and drug resistance. For example, hypoxic niches within the TME harbour CSCs [155], and thus contribute to cellular heterogeneity and drug resistance. In addition, hypoxia-induced release of macrophage chemoattractants results in recruitment of TAMs to the TME. These myeloid cells subsequently release factors that promote tumour cell survival and amplify resistance to therapy [156]. In addition, chemotherapy- and radiotherapy-induced immunogenic cell death, which is characterized by antigen-specific immune responses against dead-cell antigens, is hindered by the presence of TAMs [157]. Cytotoxic $CD8^+$ T cell responses against tumour antigens are dampened by checkpoint molecules expressed on immune cells and tumour cells within the TME [41]. Our group discovered that hypoxia is an important driver of PD-L1 expression in various human and mouse tumour cells. We found that HIF-1α-induced expression of PD-L1 results in resistance to CTL-mediated target cell lysis thus enabling immune escape [158]. Work by Noman and colleagues revealed that HIF-1α binds directly to the HRE in the proximal promoter of the PD-L1 gene and results in increased PD-L1 expression on various immune cells including macrophages, MDSCs and dendritic cells [159]. In addition, our more recent work describes a novel mechanism by which reverse signalling of PD-1/PD-L1 confers chemoresistance to tumour cells [60]. Thus, hypoxia within the TME drives an immunosuppressive phenotype, limits cytotoxicity, and promotes chemoresistance, which altogether potentiate malignancy and promote metastasis.

9.4 Therapeutic Opportunities

9.4.1 Nitric Oxide Mimetic Agents

There is evidence that cellular adaptations to hypoxia, such as the acquisition of malignant properties by tumour cells, are in part a consequence of a hypoxia-mediated inhibition of the nitric oxide/cyclic guanosine monophosphate (cGMP) signalling pathway [96, 97, 160–165]. The generation of nitric oxide results from the conversion of L-arginine into L-citrulline [166–168] (Fig. 9.1). This reaction is catalyzed by the enzyme nitric oxide synthase (NOS), of which there are three known isoforms: NOS-1, -2, and -3. Production of nitric oxide depends on the availability of several co-factors and co-substrates, including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), tetrahydrobiopterin, and oxygen [166, 169–171]. Consequently, the process of endogenous nitric oxide production is complex. Moreover, in the absence of oxygen, as it is the case in solid tumours, endogenous production of nitric oxide is limited [170, 171]. Under well-oxygenated conditions, nitric oxide generated by NOS binds to and activates soluble guanylyl cyclase (sGC), which in turn catalyzes the conversion of guanosine triphosphate (GTP) into cGMP. The latter is a known regulator of ion channel conductance, glycogenolysis, and apoptosis. It also causes smooth muscle relaxation and vasodilation. An important function of cGMP is the activation of protein kinase G (PKG), a serine/threonine-specific kinase that phosphorylates a number of biologically important targets.

Limited availability of cGMP under hypoxia leads to decreased activation of PKG and reduced protein phosphorylation, an important aspect of cellular adaptations to hypoxia. Our research has revealed that pharmacological inhibition of NOS, soluble guanylyl cyclase, or PKG in well oxygenated tumour cells results in the acquisition of phenotypes similar to those induced by hypoxia, such as increased invasive and metastatic ability [96, 97], as well as drug resistance [162, 172].

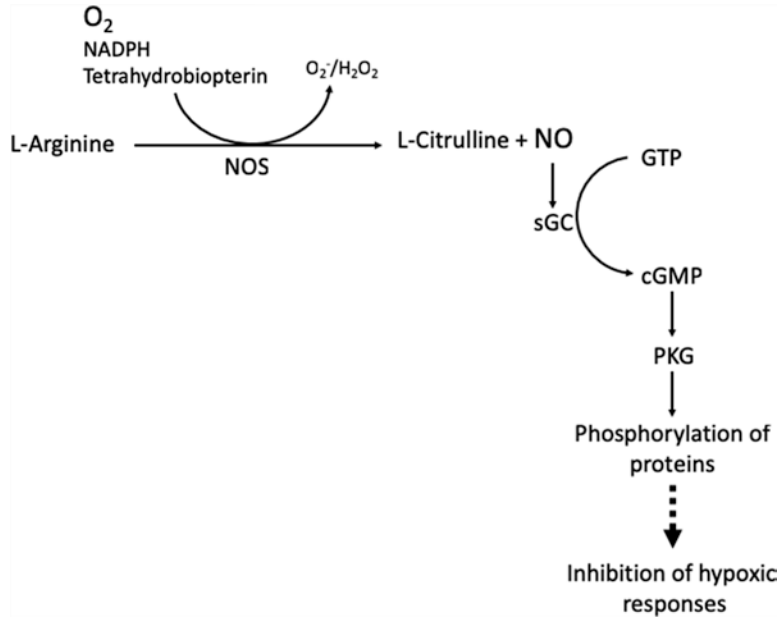

Fig. 9.1 Generation of nitric oxide (NO) and the NO/cGMP signalling pathway. NO is produced during the conversion of L-arginine into L-citrulline in an oxygen-dependent reaction catalyzed by nitric oxide synthase (NOS). Nitric oxide activates soluble guanylyl cyclase (sGC), which in turn leads to the activation of protein kinase G (PKG) and the phosphorylation of target proteins. Inhibition of this pathway due to hypoxia leads to adaptive responses

Furthermore, pharmacological activation of soluble guanylyl cyclase with various nitric oxide mimetic agents, such as glyceryl trinitrate (GTN; nitroglycerin), diethylenetriamine nitric oxide adduct (DETA-NO) and sodium nitroprusside, blocks the acquisition of malignant properties in tumour cells exposed to hypoxia [96, 97, 158, 162, 172]. A similar inhibition of hypoxia-induced acquisition of malignant phenotypes is achieved by direct activation of PKG using the non-hydrolysable cGMP analogue, 8-bromo-cGMP [96, 97, 172].

There is evidence that nitric oxide signalling interferes with tumour cell adaptations to hypoxia by inhibiting HIF-1α accumulation [173–175]. While high concentrations (>1 μM) of nitric oxide are capable of stabilizing HIF-1α during normoxic conditions, low concentrations of nitric oxide (<400 nM) have been reported to facilitate HIF-1α degradation thereby impairing HIF-1 signalling [176]. There is also evidence that under mildly hypoxic conditions inhibition of mitochondrial respiration by nitric oxide leads to a redistribution in intracellular oxygen and activation of the PHD enzymes responsible for HIF-1α degradation [174].

Thus, it appears that tumour cell adaptations to hypoxia are tightly regulated by nitric oxide and HIF-1 activity. These observations have led to the design of studies aimed at determining whether nitric oxide mimetic agents can delay disease progression or chemosensitize tumours in the clinical setting. We completed a phase II trial on prostate cancer patients with biochemical recurrence showing that continuous transdermal delivery of low doses (0.03 mg/h) of GTN may be effective at delaying disease progression [177]. This finding revealed that activation of nitric oxide signalling may have cancer inhibitory properties independent of potential chemosensitizing effects. Yasuda *et al.* reported improved response rates to vinorelbine plus cisplatin therapy in lung cancer patients treated with GTN for angina pectoris compared with patients without angina who did not use GTN [178]. This observation prompted subsequent studies to determine therapeutic benefits associated with clinical use of nitric oxide mimetics as adjuvants to chemotherapy. A Phase II trial involving patients with previously untreated stage IIIB/IV non-small-cell lung cancer revealed that, compared with patients treated with a placebo trans-

dermal patch, transdermal delivery of GTN combined with vinorelbine and cisplatin was associated with significantly increased response rate and median time to progression [178]. A follow-up study revealed a lower incidence of cells immunoreactive for HIF-1α, P-gp, and vascular endothelial growth factor (VEGF), in lung adenocarcinomas from GTN treated patients relative to tumours from non-treated patients [179].

9.4.2 Checkpoint Inhibitors

Interfering with the PD-1/PD-L1 signalling axis using monoclonal antibodies has shown promising and unprecedented results for many types of cancers [180]. At the time of writing, there were two US Food and Drug Administration-approved anti-PD-1 therapies (Nivolumab and Pembrolizumab) and three anti-PD-L1 therapies (Atezolizumab, Durvalumab and Avelumab) for treatment of patients with melanoma, non-small cell lung cancer, metastatic urothelial bladder cancer, renal cell carcinoma, Hodgkin's lymphoma, advanced gastroesophageal cancer, metastatic colorectal cancer, hepatocellular carcinoma and Merkle cell carcinoma [181]. It is important to note, however, that most of these checkpoint inhibitors have not yet been approved for use as first-line therapy and, as such, patients will have received, or will concurrently be receiving, standard-of-care chemotherapy and radiotherapy. In addition, despite the successes of immune therapy, only a fraction of patients has shown durable responses. Therefore, targeting additional mechanisms of drug resistance may be important for achieving higher response rates in individuals receiving checkpoint blockade therapy. One such approach could involve simultaneous inhibition of HIF-1α in combination with PD-L1/PD-1 blockade. The findings from our own work and those of others discussed above call upon additional studies to elucidate the mechanism(s) behind the hypoxia-driven PD-L1 expression and its significance in cancer development. It is important to note that PD-L1 expression is known to be driven by several oncogenic pathways [50] of which hypoxia is an important regulator.

9.5 Conclusion

Here we have outlined tumour-cell intrinsic and extrinsic (microenvironmental) mechanisms by which hypoxia contributes to malignancy and drug resistance. It is clear that tumour hypoxia is an impediment to the successful management of cancer. An important challenge in developing successful therapeutic options to mitigate hypoxia-induced acquisition of malignant phenotypes is to identify therapies that selectively target hypoxic tumour cells and/or other cells in the tumour microenvironment that contribute to the acquisition of malignant phenotypes. Furthermore, identifying patients likely to respond to treatment and mechanisms of hypoxia-induced drug resistance is critical. Important also is the need to identify and to better understand what role, if any, hypoxia might have in patients that fail to respond to therapy. Thus, it is evident that more basic research is required to determine mechanisms by which hypoxia is associated with development of resistance to therapy. While relying on basic, pre-clinical research to inform and guide drug development and clinical trials is important, it is also worth noting that adopting a bedside-to-bench approach is an invaluable translational opportunity and will be beneficial in the design of strategies to overcome drug resistance.

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