Mechanisms of hypoxia-mediated immune escape in cancer

Ivraym B. Barsoum1,2, Madhuri Koti1, D. Robert Siemens1,3, and Charles H. Graham1,3,4

Departments of 1Biomedical and Molecular Sciences, 2Pathology and molecular Medicine, and
3Urology

4Corresponding author: grahamc@queensu.ca

Running title: Hypoxia induces immune escape

Disclosure of Potential Conflicts of Interest: CH Graham has ownership interest in and serves
as a consultant to Nometics Inc. The other authors have no conflicts to disclose.

Précis: We discuss the contribution of hypoxia to cancer immune escape and provide evidence
for a novel role of cGMP signalling in hypoxia-induced immune escape, thereby supporting a
potential immunotherapeutic value of cGMP mimetic agents.
Abstract

An important aspect of malignant progression is the acquired ability of tumor cells to avoid recognition and destruction by the immune system (immune escape). Clinical cancer progression is also associated with the development of tumor hypoxia, which is mechanistically linked to the acquisition of malignant phenotypes in cancer cells. Despite the well-established role of hypoxia in tumor cell invasion and metastasis, and resistance to therapy, relatively few studies have examined the contribution of hypoxia to cancer immune escape. Accumulating evidence reveals that hypoxia can impair anti-cancer immunity by altering the function of innate and adaptive immune cells and/or by increasing the intrinsic resistance of tumor cells to the cytolytic activity of immune effectors. Here, we discuss certain aspects of the contribution of hypoxia to tumor immune escape and provide evidence for a novel role of cyclic guanosine monophosphate (cGMP) signalling in the regulation of hypoxia-induced immune escape. Thus, we propose that activation of cGMP signalling in cancer cells may have important immunotherapeutic applications.
Introduction

Hypoxia, a characteristic of many solid cancers, develops from an imbalance between oxygen consumption and oxygen supply. While hypoxia is an important driver of tumor invasion and metastasis as well as resistance to therapy (1), there is limited knowledge on the contribution of hypoxia to tumor cell escape from destruction by innate and adaptive immune effector mechanisms.

Immune escape in cancer is a multifaceted process resulting from the suppression of immune effector mechanisms and/or the acquisition of intrinsic tumor cell resistance to the cytotoxic activity of immune effectors. Hypoxia can influence these aspects of immune escape by modifying the intrinsic properties of tumor cells and of the stromal compartment. Here we review some of the mechanisms by which hypoxia contributes to immune escape in cancer. Furthermore, we propose that activation of cyclic guanosine monophosphate (cGMP) signalling in cancer cells, via administration of low doses of nitric oxide (NO) mimetic drugs, may be a novel therapeutic approach to interfere with certain mechanisms of immune escape.

Hypoxia-induced release of immunosuppressive molecules by tumor cells

Upon exposure to hypoxia, tumor cells release a variety of immunosuppressive molecules. For example, in the severely hypoxic tumor microenvironment dying cells release ATP that is metabolized to adenosine by CD73 and CD39 (2). Soluble adenosine in the extracellular matrix binds specific receptors on T cells to increase their intracellular levels of cAMP, which in turn suppresses T cell functions (3). Tumor-derived cytokines released under hypoxic conditions, such as IL-10 and TGF-β, induce the differentiation of tumor-associated macrophages (TAMs).
into M2 macrophages with immune-suppressive activities (4). TGF-β released by tumor cells also inhibits T cell proliferation and effector function, promotes the generation of regulatory T cells (T_{regs}), and blocks the expression of receptors required for the cytotoxic function of natural killer (NK) cells (5). In addition, TGF-β negatively regulates the antigen presentation function of dendritic cells (DCs), resulting in the inhibition of T cell function and differentiation (5).

Interestingly, emerging evidence links hypoxia-induced angiogenesis with immune tolerance (6, 7). Hypoxia drives angiogenesis within the tumor microenvironment by inducing the secretion of vascular endothelial growth factor (VEGF) and other pro-angiogenic molecules by tumor cells. Tumor-derived VEGF suppresses the maturation of DCs and blocks the presentation of tumor associated antigens to helper T cells, thereby promoting immune escape (6). Moreover, in response to tumor-derived VEGF, DCs increase their expression of the Programmed Death Ligand 1 (PD-L1 or B7-H1), a negative regulator of T cell function (7). VEGF promotes the accumulation of myeloid derived suppressor cells (MDSCs) in tumor tissues and secondary lymphoid organs (6). MDSCs are potent suppressors of anti-cancer T cell responses and also contribute to tumor progression by releasing factors that promote angiogenesis and metastasis (for a review on MDSCs see reference (8)). Consequently, VEGF is a potential target for immune therapy. In support of this, anti-VEGF therapy was shown to be associated with an increase in activated DCs and T cell function in cancer patients (9). However, targeting VEGF as an immunotherapeutic approach may lead to tumor hypoxia via inhibition of angiogenesis, thereby resulting in the activation of other hypoxia-induced immune escape pathways.
Tumor cells can secrete proteins such as CC-chemokine ligand 22 (CCL22) and various chemokines that inhibit effector T cell responses and promote the generation and recruitment of immunosuppressive T\textsubscript{regs} (10). In an ovarian cancer model, hypoxia was shown to promote the recruitment of T\textsubscript{regs} via increased tumor cell expression of CCL28 (11). T\textsubscript{regs} in turn can also secrete VEGF, thereby contributing to the VEGF pool in the tumor microenvironment that contributes to immune tolerance (11).

Tumor cells can also produce galectin-1 and galectin-3 to induce apoptosis of activated lymphocytes (12, 13). In melanoma patients, there was a strong correlation between expression of galectin-3 and apoptosis of tumor infiltrating lymphocytes (TILs) (12). In Wilms tumors and Schwannomas, galectin-3 was shown to co-localize with hypoxia-inducible factor 1\textalpha (HIF-1\textalpha) (14), a heterodimeric transcriptional regulator of adaptations to hypoxia, consisting of an oxygen-regulated HIF-1\textalpha subunit and a constitutively expressed HIF-1\textbeta subunit. Additionally, it was reported that galectin-1 expression is transcriptionally regulated by HIF-1 in colorectal cancers (15) and head and neck squamous cell carcinomas (16).

Hypoxia was also shown to induce immunosuppression by up-regulating COX-2 expression in tumor cells; and HIF-1-mediated up-regulation of COX-2 increased colorectal tumor cell survival and VEGF production (17). COX-2 is a pro-inflammatory enzyme that converts arachidonic acid into prostaglandin E\textsubscript{2} (PGE\textsubscript{2}). The latter causes immunosuppression by increasing adenosine/cAMP signalling in effector T cells (18). PGE\textsubscript{2} secreted from tumor cells can also inhibit anti-tumor immunity by inhibiting the maturation of DCs (19). Also, PGE\textsubscript{2} enhances the suppressive activity of T\textsubscript{regs} and supports the differentiation of T\textsubscript{regs} (20). Finally, PGE\textsubscript{2} can
stimulate the immunosuppressive functions of MDSC by binding to EP-4 receptors on these cells (21). Hypoxia-induced immune suppression via COX-2 can explain why chronic administration of indomethacin, a COX-2 inhibitor, in the drinking water of mice led to significant reduction in the growth rate and metastasis of mammary tumors as well as restoration of splenic NK cell activity (22). A recent study revealed that use of NSAIDs reduced recurrence of breast cancer in overweight and obese women (23).

The above studies indicate that the secretion of immunosuppressive molecules by tumor cells under conditions of hypoxia provides a survival advantage, and therefore support the concept that hypoxia represents a strong selection pressure driving immune escape.

**Direct effects of hypoxia on immune effectors**

Hypoxia can also directly impair anti-tumor immune responses. For example, hypoxia in the tumor microenvironment can induce the release of VEGF by M2 macrophages (24). Furthermore, TAMs suppress T cell function in a manner dependent on HIF-1 (25), and TAMs in hypoxic regions of tumors exhibit increased expression of M2 promoting molecules, such as TGF-β (26). Hypoxia inhibits the *in vitro* cytolytic activity of other immune effectors such as the NK cell-mediated killing of hepatocellular carcinoma cells and multiple myeloma cells (27, 28).

Hypoxia was shown to decrease T cell survival (29), and incubation of naive T cells under hypoxia decreases their secretion of the trophic cytokine IL-2 in a HIF-1-dependent manner (30). CD4+ and CD8+ T cells derived from HIF-1α deficient mice exhibit increased proliferation, produce higher levels of interferon γ, and display increased anti-tumor responses (31). HIF-1 was also shown to mediate T<sub>reg</sub> differentiation via increased expression of FoxP3 (32). Increased
numbers of Tregs in the tumor stroma have been associated with poor survival of patients with various cancers (33, 34).

Another mechanism of tumor cell immune escape involves binding of the cytotoxic T lymphocyte antigen-4 (CTLA-4, an immune checkpoint regulator) to its natural receptors, CD80 (B7.1) and CD86 (B7.2). Interestingly, hypoxia was shown to increase the expression of CD86 by bone marrow-derived mouse DCs in a HIF-1 independent manner (35). Studies revealed that CTLA-4 blockade attenuates the growth of several mouse tumors (36), reduces tumor-infiltrating Tregs, and promotes effector T cell function in humans (37).

It is important to note that not all of the reported effects of hypoxia on T cells are detrimental to their function. Hypoxia was reported to up-regulate CD137, a member of the TNF receptor family that is known for its co-stimulatory activity on T cells (38). Expression of CD137 on activated mouse T cells was shown to be stimulated by hypoxia (39), and tumors from HIF-1α-deficient mice exhibited undetectable CD137+ TILs (39). In tumor growth assays, hypoxia induced the activation of T-cells via the up-regulation of surface CD137 in a HIF-1-dependent manner, which in turn resulted in improved immune response and slower tumor growth (39).

**Hypoxia induces immune tolerance via regulation of immune checkpoints**

In addition to decreasing the cytolytic potential of immune effectors, hypoxia increases the intrinsic resistance of tumor cells to immune-mediated killing. One strategy that tumor cells employ to avoid immune detection and destruction is to alter their expression of cell surface immune checkpoint regulators. For example, tumor cells may shed stress-induced MHC class I chain-related proteins A and B (MICA/B) from their surface to avoid interaction with NKG2D
receptors on NK cells, γδ T cells, and CD8+ αβ T cells (40) thereby escaping cytolysis (41). We have shown that exposure of tumor cells to hypoxia leads to the shedding of surface MICA, which in turn results in increased resistance to lysis by innate immune effectors (42). Importantly, we also showed that the hypoxia-induced release of MICA and resistance of tumor cells to lysis required HIF-1-mediated expression of the metalloproteinase ADAM 10 in the tumor cells (43) (Figure 1).

As discussed earlier, there is also evidence that tumor cells can suppress cytotoxic T lymphocyte (CTL) function through the interaction of inhibitory co-stimulatory molecules with their ligands. Certain members of the B7 family of co-stimulatory molecules expressed on the surface of tumor cells provide signals that suppress CTL responses. For example, binding of PD-L1 with PD-1 or with CD80 (B7.1) on activated CTLs leads to suppression of immune responses via mechanisms that include induction of apoptosis and anergy (non-responsiveness to antigen) in the T cells (44). Recent clinical studies revealed that therapy with blocking anti-PD-1 antibody (Nivolumab) produced objective responses in patients with non-small-cell lung cancer, melanoma, or renal-cell cancer (45). Also, reinduction therapy with anti-PD-1 antibody for late tumor recurrence showed durable remissions in patients with colorectal cancer, renal cell cancer, and melanoma (46). In another study, concurrent therapy with anti-CTLA-4 antibody (Ipilimumab) and Nivolumab resulted in tumor regression in a substantial proportion of patients with unresectable, stage III or IV melanoma (47). We recently provided evidence that, when exposed to hypoxia, human and mouse cancer cells increased their expression of PD-L1 and acquired resistance to CTL-mediated lysis in a manner dependent on HIF-1α (48) (Figure 1).
Furthermore, the hypoxia-induced expression of PD-L1 in tumor cells led to increased apoptosis of co-cultured CTLs as well as Jurkat T cells (48).

Additionally, hypoxia may induce immune escape in cancer cells via epigenetic mechanisms. For example, tumor cells can up-regulate miR-210 in lung cancer and melanoma (49). In turn, miR-210 was shown to block the susceptibility of tumor cells to lysis by antigen-specific CTLs. This effect was mediated via increased expression of protein tyrosine phosphatase, non-receptor type 1 (PTPN1), homeobox A1 (HOXA1), and tumor protein p53 inducible protein 11 (TP53I11) (49). Further studies are required to elucidate the mechanisms employed by these molecules to suppress CTL activity.

Exposure of tumor cells to hypoxia also resulted in resistance to autologous CTL-mediated lysis in a manner dependent on the signal transducer and activator of transcription (STAT) 3 (50). STAT3 modulates the cross-talk between tumor and immune cells (51). A small molecule inhibitor of STAT3, WP1066, was reported to reverse immune tolerance in malignant glioma patients (52). Another STAT3 inhibitor, sunitinib, reduced the immunosuppressive phenotype of renal cell carcinomas (53) and reversed MDSC-mediated immune suppression via increased recruitment of CD4^+CD8^+ cytotoxic T cells (54).

Regulation of immune tolerance via hypoxia-induced autophagy

Cancer cells often rely on autophagy as a mechanism of survival under conditions of stress including hypoxia, nutrient starvation, growth factor withdrawal, and chemotherapy (55, 56). However, the mechanisms by which autophagy enables survival of normal or malignant cells are not well known.
Hypoxia-induced autophagy is partly dependent on the HIF-1/BNIP3-BNIP3L-Beclin1 axis (57), and partly on HIF-1/ platelet-derived growth factor receptor signalling (58). Through the activating transcription factor 4 and C/EBP homologous protein (CHOP), hypoxia increases the expression of microtubule-associated protein 1 light chain 3 (LC3) and autophagy protein 5 (ATG5) involved in formation and maturation of autophagosomes (59).

Hypoxia-induced autophagy is known to promote tumor cell survival via several mechanisms, including the removal of damaged mitochondria that produce cytotoxic reactive oxygen species (57) and the degradation of harmful protein aggregates (59). Activation of autophagy in cancer cells during hypoxia or exposure to other microenvironmental stressors may also lead to inhibition of death signals such as those triggered by CTLs (60). Furthermore, stress-induced release of the molecular pattern molecule HMGB1 induces cytoprotective autophagy and leads to recruitment of T\(_{reg}\) (60).

Autophagy can also promote activation of anti-cancer immunity. For example, autophagy has been shown to be crucial for proliferation of immune cells as well as for their effector functions such as antigen presentation and T cell-mediated tumor cell cytotoxicity (61). In T cells, autophagy is activated upon TCR engagement in both CD4\(^+\) and CD8\(^+\) T cell subtypes (62). The knockdown of the essential autophagy-related genes ATG5 or ATG7 during TCR stimulation leads to a significant decrease in cellular proliferation demonstrating the importance of autophagy during T cell activation (62, 63). Furthermore, culture of DCs under low oxygen results in the stabilization of HIF-1\(\alpha\), which initiates BNIP3 expression and promotes survival of
mature DCs, possibly due to induction of autophagy (64). Hypoxia-induced autophagy in antigen presenting cells infiltrating a tumor can occur via toll-like receptor (TLR) signalling (65).

Together, the above findings indicate a dual role for autophagy in cancer immune escape. Therefore, immunotherapeutic strategies designed to target autophagy will need to consider its impact on the immune system.

**Nitric oxide/cGMP-mediated inhibition of hypoxia-induced immune escape**

Our research over the last 15 years has revealed that classical NO signalling involving cGMP production functions as an O$_2$-sensing mechanism playing a key role in tumor cell adaptations to hypoxia (42, 43, 48, 66-69). Based on our findings, we postulated that an important aspect of the mechanism by which cancer cells adapt to hypoxia involves inhibition of endogenous NO/cGMP signalling. This concept is supported by results showing that NO mimetics (e.g. glycercy trinitrate (GTN), DETA/NO) at very low concentrations (nM – pM) known to solely activate sGC inhibit malignant adaptations to hypoxia, i.e. increased invasiveness, metastatic ability and drug resistance (66-69). Moreover, since NO production is dependent on O$_2$ availability, endogenous NO generation is severely limited in cells exposed to hypoxia (70, 71). This is despite the fact that hypoxia was shown to increase the expression of inducible nitric oxide synthase (iNOS) in the same cells (RAW 264.7 macrophages) (71). We previously reported that cGMP levels are decreased in MDA-MB-231 breast tumor cells incubated for six hours in 0.5% O$_2$ (68). This observation is consistent with the more recent findings of Hickok et al., who reported decreased sGC activation in a murine macrophage line incubated under 5% O$_2$ (71). It is likely that our observed effects of NO/cGMP signalling on hypoxia-induced malignant
phenotypes are at least partly mediated via inhibition of HIF-1 transcriptional activity. This conclusion is based on evidence that NO mimetics, including the cGMP analogue 8-bromo-cGMP, as shown by us (43), inhibit the accumulation of HIF-1α in cells exposed to hypoxia (43, 72). Our research has also revealed that NO mimetics interfere with the HIF-1-mediated up-regulation of ADAM10 expression involved in the shedding of MICA from the tumor cell surface and resistance to immune mediated lysis (43) (Figure 1). In that same study, treatment of mice with GTN attenuated the growth of transplanted prostate tumors via a mechanism dependent on innate immune effectors. More recently, we demonstrated that low concentrations of GTN interfere with hypoxia-induced escape from T cell-mediated immunity in tumor cells by preventing the HIF-1-dependent expression of PD-L1 (48) (Figure 1). Together our studies indicate that activation of NO/cGMP signalling may have important applications in the prevention and/or treatment of cancer.

Conclusions

While there is evidence that hypoxia can activate certain components of pathways involved in anti-tumor immunity, most studies indicate that hypoxia is a major contributor to cancer immune escape. Hypoxia-induced tumor cell escape from innate and adaptive immunity is likely a consequence of multiple mechanisms operating in a complementary, and sometimes redundant, manner. Thus, targeting individual mechanisms of hypoxia-induced immune escape will likely prove to be ineffective as a therapeutic strategy. However, it is clear that several mechanisms of such immune escape rely on the transcriptional activity of HIF-1. This raises the possibility that interference with hypoxia response pathways involving HIF-1 activity may be a fruitful immunotherapeutic approach. Interference with such pathways could be achieved
through the use of molecules that directly inhibit HIF-1 activity or block HIF-1α accumulation in hypoxia. Our studies on the inhibitory effect of NO/cGMP signalling on HIF-1α accumulation and malignant adaptations to hypoxia, including tumor cell escape from innate and adaptive immunity, support the therapeutic potential of NO mimetic agents. In this review, we highlighted some key mechanisms of hypoxia-mediated immune escape. However, since tumor cell avoidance of immune destruction is multi-faceted, it is likely that hypoxia influences escape mechanisms not described herein. The role of the hypoxic tumor microenvironment on other key aspects of cancer immune surveillance, such as antigen presentation, additional immune checkpoints and effector mechanisms of tumor cell destruction, warrants investigation.

Acknowledgements

Our studies on the role of hypoxia in cancer immune escape are funded by a grant from the Canadian Institutes of Health Research awarded to CHG and DRS (grant number MOP 79267).
References


Figure Legend

Figure 1. Proposed mechanisms of hypoxia-induced tumour cell escape from innate and adaptive immunity. Hypoxia increases the accumulation of HIF-1α in tumour cells, which in turn leads to higher levels of ADAM 10 and PD-L1 on the surface of tumour cells. ADAM 10 cleaves MICA from the cell surface to limit binding to NKG2D receptors on NK cells, leading to escape from innate immunity, whereas interaction of PD-L1 with PD-1 or CD80 on activated CTLs causes apoptosis in the CTLs and escape from adaptive immunity. NO/cGMP signalling is proposed to block the effect of hypoxia on ADAM 10 and PD-L1 up-regulation by inhibiting HIF-1α accumulation.
Hypoxia

Apoptosis

CTL

NO/cGMP

NK cell

PD-L1

Tumour cell

HIF-1

MICA

ADAM 10

PD-L1

Immune escape

Immune escape

= PD-L1

= PD-1 or CD80

= MICA

= NKG2D
Mechanisms of hypoxia-mediated immune escape in cancer


Cancer Res  Published OnlineFirst October 24, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-2598

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.