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The hypoxia-adenosine link during intestinal inflammation

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Abstract

Intestinal inflammation is a key element in inflammatory bowel disease (IBD) and is related to a combination of factors, including genetics, mucosal barrier dysfunction, bacteria translocation, deleterious host-microbe interactions, and dysregulated immune responses. Over the past decade, it has been appreciated that these inflammatory lesions are associated with profound tissue hypoxia. Interestingly, an endogenous adaptive response under the control of hypoxia signaling is enhancement in adenosine signaling, which impacts these different endpoints, including promoting barrier function and encouraging anti-inflammatory activity. In this review, we discuss the hypoxia-adenosine link in IBD, intestinal ischemia/reperfusion injury, and colon cancer. Additionally, we provide a summary of clinical implications of hypoxia and adenosine signaling in intestinal inflammation and disease.

Keywords

Adenosine; Adenosine Receptors; Hypoxia; HIF; Intestinal Inflammation; Inflammatory Bowel Disease; Ischemia/Reperfusion Injury; Colon Cancer

Introduction

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is a chronic disorder of the intestinal tract characterized by intestinal inflammation and epithelial injury. The exact set of causes of IBD remains unclear. However, significant evidence indicates dysfunction of the mucosal immune system plays an important role in the pathogenesis of IBD. Strong evidence also implicates genetic susceptibility, deleterious host-microbe interactions, mucosal barrier dysfunction, and environmental factors (1, 2). There is also now significant evidence these inflamed lesions become profoundly hypoxic. Inflamed tissues experience significant changes in tissue metabolism. Oxygen supply and other metabolic factors are limited to tissues due to vascular occlusion, damaged blood supply, and/or compression of the tissue (3, 4). The metabolic demand of inflammatory cells also is a burden. For example, activated neutrophils consume significant amounts of oxygen, so much so that they imprint on the tissue environment, making it hypoxic (5). Over the past decade, it has been appreciated that inflammatory-hypoxia (tissue inflammation leading to tissue hypoxia (3)) increases extracellular adenosine/adenosine signaling and serves as an

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essential endogenous anti-inflammatory pathway that protects tissues on multiple levels (6, 7). Though hypoxia is an inflammatory stimulus, there are examples of low oxygen promoting tissue protection (8–15). In this review, we discuss the hypoxia-adenosine link, particularly hypoxia-inducible factors (HIFs) in regulating adenosine pathway genes. We also discuss the hypoxia-adenosine link in IBD, intestinal ischemia/reperfusion injury, and colon cancer and conclude with a summary of clinical implications of these pathways in intestinal inflammation and disease.

Adenosine Signaling during Intestinal Inflammation

Extracellular adenosine and adenosine signaling has shown to be an essential endogenous anti-inflammatory pathway in a number of conditions and diseases, including acute lung injury (16–21), myocardial injury (14, 22–25), intestinal ischemia/reperfusion injury (26–28), and IBD (11, 29–33). During inflammation, adenosine triphosphate (ATP) is released from stressed, apoptotic and/or necrotic cells, and bacteria (34, 35). ATP is converted to adenosine by cell surface ectonucleotidases, ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5′nucleotidase (CD73). CD39 generates adenosine diphosphate (ADP) and monophosphate (AMP) from ATP, and CD73 generates adenosine from AMP. Adenosine signals through membrane-spanning adenosine receptors, A1R, A2AR, A2BR, and A3R and is terminated by adenosine re-entering the cell through equilibrative or concentrative nucleoside transporters (ENTs, CNTs) or through activity of adenosine deaminase (ADA) (34–36). A2BR is the predominant adenosine receptor expressed on intestinal epithelial cells (32, 33, 37), whereas A2AR is expressed by most immune cells (34).

Mucosal Epithelial Cells

Epithelial cells play an important role in IBD pathogenesis. The breakdown of their barrier encourages bacterial translocation as well as promotes the release of pro-inflammatory cytokines, exciting tissue-damaging immune cells. The role of adenosine signaling in protecting tissue barriers was appreciated three decades ago, beginning with outstanding studies on polymorphonuclear leukocyte (PMN; neutrophil)-derived secretagogue (38–40). PMNs were found to release purine nucleotides (AMP (40) and ATP (41)) during transmigration. Subsequently, ATP/AMP is converted to adenosine by endothelial/epithelial cell CD39 or CD39 family members and CD73 and tissue barriers resealed by A2BR (39, 40). Adenosine signaling induces barrier protection by inducing actin polymerization (42) and through changes in the actin cytoskeleton, involving vasodilator-stimulated phosphoprotein (VASP) (43, 44). Indeed, a significant phenotype of mice deficient for adenosine pathway genes is barrier dysfunction. For example, wild-type mice gavaged with CD73 inhibitor, APCP experience severe intestinal epithelial barrier permeability in hypoxia (12). Similar, CD73 (13) and A2BR (8) deficient mice exposed to low oxygen suffer massive barrier breakdown and inflammatory cell accumulation in several tissues. As well, CD39 (29) and CD73 (30) deficient mice and mice with global or tissue-specific deletion of A2BR (32, 33) suffer more severe disease during experimental colitis and is associated with the breakdown of the intestinal epithelial barrier. A2BR on intestinal epithelial cells also regulates inflammatory cytokines, limiting immune cell infiltration. For example, cytokine

interleukin-10 (IL-10) levels are reduced in inflamed mucosa from A2BR deficient mice (33). The anti-inflammatory role of IL-10 is significant in intestinal inflammation; IL-10 deficient mice develop spontaneous colitis (45). Extracellular adenosine/A2BR also reduces intestinal epithelial cell expression of pro-inflammatory cytokines interleukin-8 (IL-8) (46), interferon-αA, interleukin-1β, and tumor necrosis factor-α (TNF-α) (47). Studies using bone-marrow chimeras show A2BR activity on non-immune cells is essential in suppressing inflammatory cytokine and chemokine production during sepsis (48). A2BR activity on intestinal epithelial cells also promotes electrogenic chloride secretion, which flushes the lumen of toxins and pathogens (secretory diarrhea) (37, 38) and is another level of protection during intestinal inflammation. Loose stools are a clinical concern with IBD. Accordingly, there is some concern of severe diarrhea being a side effect with A2BR agonists.

Innate Immune Cells

Besides epithelial barrier breakdown, dysfunction of the mucosal immune system is significant in the pathogenesis of IBD. Several lines of evidence support A2AR on immune cells promotes profound endogenous anti-inflammatory responses (49, 50) Accordingly, interests in harnessing the immunosuppressive actions of extracellular adenosine/adenosine signaling (e.g., graft versus host disease and IBD) as well as to inhibit this response (e.g., tumors) for therapeutic benefit have been a recent topic of interest. A2AR activity on neutrophils inhibits neutrophil adhesion to and killing of endothelial/epithelial cells as well as modulates the production and release of pro-inflammatory cytokines (e.g., TNF-α) (51), chemokines (52), and prostaglandins (e.g., PGE2) (53, 54). A2AR activity on neutrophils, en route to sites of inflammation, is tissue protective by inhibiting the release of toxic oxygen species (55, 56). A2AR activity on macrophages limits their production of pro-inflammatory cytokines (e.g., interleukin-12 (IL-12) and TNF-α) and promotes their release of anti-inflammatory cytokine, IL-10 (57–61). Similar, A2AR on mature dendritic cells shifts their cytokine profile, reducing IL-12, IL-6, and interferon-α (IFN-α) (pro-inflammatory) and increasing IL-10 (62–64). A2BR activity on macrophages increases IL-10 (65) and also dampens TNF-α (59); dampening TNF-α appears operational only when not masked by A2AR (59). A2AR and A2BR activity on macrophages can also promote alternative macrophage activation, which may be important in IBD (66–68). In IBD, anti-inflammatory factor, netrin-1 interacts with A2BR, which dampens the infiltration of neutrophils into the intestinal mucosa (11, 31, 69). Adenosine receptors, A1R and A3R are less described, but show to support pro-inflammatory events (e.g., chemotaxis) leading up to A2AR and A2BR activity (56). Impaired innate immunity is implicated particularly in Crohn’s disease (70).

Adaptive Immune Cells

T cells are particularly sensitive to the immunosuppression/immunomodulatory activity of A2AR. During inflammation, A2AR is up-regulated on effector T cells. Subsequent activation of A2AR on these cells inhibits their proliferation, expansion, cytotoxic activity, and cytokine production (71–73). A2AR activity decreases both T helper 1 (Th1) and 2 (Th2) development and effector function (74). A2AR activity on these effector T cells also enhances the formation of Tregs and their expression of programmed cell death protein 1 (PD-1) and CTLA-4, negative regulators of inflammation (75). Naïve T cells express CD73

and is downregulated upon activation and differentiation, whereas Tregs express both CD39 and CD73 (76). Studies by Simon Robson have shown an essential regulatory loop, whereby extracellular adenosine generated by Tregs activates A2AR on effector T cells, suppressing effector T cell activity (77). Extracellular adenosine also activates A2AR on Tregs, promoting their expansion and immunoregulatory activity (self-reinforcing loop) (78). Accordingly, A2AR deficiency on Tregs reduces their immunosuppressive efficacy (79). A2AR agonists prevent colitis induced by pathogenic T cells in the absence of Tregs (80), whereas adoptive transfer studies show Tregs from wild-type mice fail to prevent colitis induced by pathogenic T cells from A2AR deficient mice (80). A2AR on myeloid cells is also required for controlling intestinal inflammation, as cotransfer of Tregs and pathogenic T cells from wild-type mice into rag/A2AR double deficient mice still leads to colitis (80). Interestingly, Th17 cells function similar to Tregs to suppress pro-inflammatory responses as well as show effector Th17 features, such as producing interleukin-17 and low levels of A2AR. CD39 is involved in the transition of Th17 cells into suppressor-like Th17 cells (81, 82). Many studies are directed at understanding the immunopathogenesis of IBD and relatedness of adenosine signaling. These studies will provide greatly to new ideas for treating intestinal inflammation.

Hypoxia Signaling

Inflamed lesions are often severely hypoxic. Tissues have evolved essential mechanisms to detect and adapt to low oxygen. Prolyl hydroxylases are essential to sensing oxygen and hypoxia inducible factors (HIFs) to initiating adaptive responses. HIFs are αβ-heterodimeric transcription factors that interact with hundreds of genes that encode glycolytic enzymes, inhibit mitochondrial respiration, regulate apoptosis, modulate inflammation, and promote angiogenesis (83). Oxygen-dependent prolyl hydroxylases (PHDs) and von Hippel-Lindau (VHL), an E3 ubiquitin ligase, control the stability of HIFs (84–88). In normoxia, HIF-1α and HIF-2α are hydroxylated at proline residues by PHDs, promoting their ubiquitination by VHL and proteosomal degradation (89). Mice deficient in PHDs or VHL show features of increased HIF activity, including increased vascularization and high levels of erythropoiesis (90–94). Factor inhibiting HIF (FIH), an oxygen-dependent asparaginyl hydroxylase, also controls HIFs through hydroxylation of asparagyl residues of α-subunits, which blocks the association of HIFs with transcriptional coactivators (95, 96). In hypoxia, PHDs and FIH activity is inhibited by the low availability of oxygen. HIF-α subunits combine with HIF-1β and subsequently bind to HIF-responsive elements (HREs) in gene promoters. HIF-1α and HIF-2α share a few, but largely have distinct target genes (97, 98). Recently, HIF-2α has been indicated as having a non-transcriptional role, indicating that much more remains to be discovered regarding HIF activity. HIFs also can be stabilized by inflammatory factors, such as bacterial component lipopolysaccharide (LPS), involving Toll-like receptor-4 (99), as well as bacterial release of iron-binding siderophopres, which stabilize HIFs by forming chelated complexes with iron ions, inhibiting PHDs (100). While PHDs require oxygen as a cofactor, iron is also necessary. PHDs are not limited to targeting HIFs. For example, studies by Cormac Taylor have shown that IKKβ (subunit of IκB kinase complex, member of the NF-κβ pathway) contains a conserved prolyl hydroxylation site similar to HIF-1α, and that hypoxia increases IKKβ stability through inhibition of IKKβ hydroxylation by PHD1,

directing NF-κB disassociation from its inhibitor, thus promoting NF-κB to induce pro-inflammatory gene expression (101). NF-κβ can directly regulate HIF-1α and, in turn, NF-κβ can directly regulate HIF-1α (102). Moreover, studies involving macrophages demonstrate TNF-α stabilizes HIF-1α in normoxia (103). Recent studies involving Type 1 regulatory cells (Tr1), aryl hydrocarbon receptor, and CD39 provide additional insight into hypoxia signaling in integrating immunological, metabolic, and environmental signals to regulate immune responses (104). Taken together, HIF signaling in epithelial and immune cells is essential to tissue adaptation to low oxygen.

The Hypoxia-Adenosine Link during Intestinal Inflammation

The normal intestinal mucosa exists in a state of hypoxia (physiological hypoxia) (6, 105, 106) and has shown to be particularly resistant to low oxygen (105), a feature important to priming the tissue for oxygen changes and adaptation. Accordingly, HIFs are found stabilized in normal gut mucosa and are elevated in IBD patients (107). While searching for barrier protective pathways, Sean Colgan recognized CD73 and CD39 were increased several fold in hypoxic intestinal mucosa and that CD73 is a target of HIF-1α (12). Extensions of these studies identified that Sp1 increases CD39 expression in hypoxia (14). Sp1 is strongly implicated in targeting hypoxia adaptive genes, such as vascular endothelial growth factor (VEGF) (108). In contrast, HIFs repress ENT1 (9) and ENT2 (109) expression and reduce the conversion of adenosine to AMP by adenosine kinase (AK) (110, 111). AK is repressed by HIF-1α (111). ENT1 and ENT2 are bidirectional adenosine transporters, controlling adenosine efflux or intracellular uptake according to the concentration gradient (112). Studies involving radio-labeled adenosine showed extracellular adenosine uptake slows in hypoxia and that both ENT1 and ENT2 expression significantly decreases in intestinal epithelial models. ENT1 and ENT2 promoters also contain HREs and are targets of HIF-1α (9, 109). Consistent is that intestinal epithelial-targeted HIF-1α deficient mice show increased expression of ENTs (9, 109). Moreover, ENT inhibitor, dipyridamole promotes intestinal epithelial barrier function and attenuates mucosal inflammation in murine models of hypoxia (109). Collectively, ENT1 and ENT2 down-regulation by HIFs are significant in raising the levels of extracellular adenosine/adenosine signaling during hypoxia. Indeed, dipyridamole tissue protection is reduced in A2BR deficient mice (18). A2BR appears to have a select role in barrier protection in hypoxia (8). Intestinal epithelial models show A2BR is a HIF-1α target (113). HIF-2α appears to target A2AR (114). Netrin-1 (which interacts with A2BR) is also targeted by HIF-1α (11). The tissue environment also promotes HIF-mediated adenosine signaling. For example, low oxygen or tissue inflammation greatly favors the release of extracellular ATP/ADP (3, 35). As well, colitis studies show that transmigrating neutrophils consume local oxygen, making the tissue even more hypoxic (5). Inflammation in general also induces tissue hypoxia. To summarize, hypoxia/HIFs increase adenosine pathway gene expression that support an increase in extracellular adenosine/adenosine signaling and at the same time decreases genes that reduce extracellular adenosine levels/adenosine signaling (Figure 1).

In recent years ample evidence has indicated that hypoxia/HIFs and extracellular adenosine/adenosine signaling play a prominent role in modulating immune cells during inflammation. Indeed, pharmacological and genetic studies strongly indicate that no other mechanism/

pathway can compensate for the immune modulating/immunosuppression actions of extracellular adenosine (49, 50, 115). As well, HIF-1α importantly regulates the metabolic switch of immune cells from aerobic energy to glycolysis and multiple facets of myeloid and T cell biology, including development, proliferation, survival, and cytokine production (116). As discussed, hypoxia/HIFs increase extracellular adenosine levels/adenosine signaling, whereby activation of A2AR and/or A2BR on many immune cells is a potent "off" signal to the cells. In addition to promoting inflammation, hypoxia can promote immunosuppression (116). Many studies support a potential link between hypoxia and adenosine in immune cells during inflammation. Hypoxia/HIFs impair T cell receptor (TCR)-mediated activation and reduce proliferation, IFN-γ production, and cytotoxicity. Similar, A2AR and A2BR activity inhibits T cell TCR-mediated activation, proliferation, and cytokine (e.g., IFN-γ) production. Both are considered to act in concert to mediate these effects (117, 118). Notably, studies also indicated adenosine receptor-independent mechanisms (118). The exact mechanisms are yet to be clearly defined. HIF-1α additionally plays a role in influencing Treg differentiation and proliferation and also may coordinate with extracellular adenosine/adenosine signaling to mediate this response (77, 119–121). Metabolic control of Tr1cells is balanced through coordination of hypoxia/HIF-1α target genes and ATP/adenosine metabolism (104). Moreover, HIF-1α-induced netrin-1 interacts with A2BR on PMNs, attenuating their transepithelial migration and limiting tissue damage. Recently, HIF-1α was shown to up-regulate A2BR on alternatively activated macrophages in chronic inflammatory models (122). Suffice to say, there is great excitement for the possible benefit of therapeutic agents targeting hypoxia/adenosine signaling in regulating immune responses in number of health conditions and disease.

Examples for Hypoxia-Adenosine Link in Intestinal Inflammation and Disease

Above, we have summarized adenosine signaling in protecting the intestinal epithelial barrier and its potent anti-inflammatory responses. Additionally, we discussed hypoxia signaling and the link between adenosine signaling. Below, we discuss the hypoxia-adenosine link in IBD, intestinal ischemia/reperfusion injury, and colon cancer (Figure 2).

Inflammatory Bowel Disease

IBD is characterized by excessive inflammation and profound hypoxia. Several genetic and pharmacological studies support that hypoxia signaling is protective during IBD (6). For example, mice with deletion of HIF-1α in intestinal epithelial cells are more susceptible to intestinal inflammation and have more severe disease during experimental colitis. Mice experience significant weight loss, colonic shortening, and suffer extensive increases in barrier permeability (105). In as much, mice with intestinal epithelial cell-specific deletion of VHL (105) or deletion of PHD1 (123) are protected during experimental colitis. These mice benefit from much improved intestinal barrier function and reduced inflammation (105, 123). Notably, increased HIF stabilization associates with increased expression of HIF-1α barrier protective genes, including CD73, in VHL deficient mice (105). Several studies show mucosal HIF-1α stabilization as having therapeutic promise for IBD. Stabilization of HIFs in immune cells is also protective. Loss- and gain-of-function studies show HIF-1α induces

Treg differentiation, whereby Tregs deficient for HIF-1α fail to limit inflammation in models of T cell-mediated colitis (121). HIF-1α deficiency in dendritic cells also results in the failure to dampen intestinal inflammation, resulting in the increase of pro-inflammatory cytokines and diminished numbers of Tregs (124). HIF-2α appears to have a different role from HIF-1α in IBD; HIF-2α promotes the severity of colitis in mice (125). HIF-2α promotes pro-inflammatory responses (125) and barrier dysfunction (126).

Significant tissue hypoxia during IBD has been shown by 2-nitoimidazole compounds (6, 105, 106). As mentioned, CD73, A2AR (114), and A2BR (113) are targets of HIFs; CD39 is a gene target of hypoxia-induced Sp1. Both *cd39*$^{-/-}$ (29) and *cd73*$^{-/-}$ (30) mice experience severe disease during experimental colitis, including significant weight loss, colonic shortening, enhanced immune cell infiltration, and increased barrier permeability. Wild-type mice treated with APCP experience the same devastation (30), whereas wild-type mice receiving apyrase, a soluble factor with enzyme activity identical to CD39, experience significant protection (29). Notably, CD39 polymorphisms are associated with IBD in humans (29), which provides additional support of the importance of this pathway in IBD. Several studies support that adenosine signaling by A2BR and A2AR provides protection during IBD. Indeed, mice with global or tissue-specific deletion of A2BR experience increased severity of colitis (32). Similar, wild-type mice treated with A2BR antagonist, PSB1115 experience increased weight loss, colonic shortening, and leukocyte infiltration. A2A receptor agonist, ATL146e decreases both leukocyte infiltration and the production of inflammatory cytokines by T effector cells in IBD models (127). As mentioned, netrin-1 is directly induced by HIF-1α and interacts with A2BR, dampening neutrophil trafficking (11, 31). Accordingly, netrin-1 deficient mice also suffer significant disease severity during experimental colitis. Taken together, these studies support that HIF-mediated extracellular adenosine/adenosine signaling is protective in IBD. Of note, other studies suggest that A2BR deletion increases the severity of colitis (128–130). Reasons for these differences are unclear, possibly related to differences in experimental models and design.

As well, A2AR is essential to regulating adaptive immunity in IBD (34). A2AR agonists in the absence of Tregs prevent colitis by pathogenic T cells, whereby A2AR deficient mice suffer severe disease even with transfer of wild-type Tregs (80). These studies show that A2AR expression on both CD45RB^{high} and CD45RB^{low} cells are important to controlling T cell-mediated colitis by suppressing pro-inflammatory cytokine expression while sparing anti-inflammatory activity mediated by IL-10 and transforming growth factor-β (TGF-β) (80). Adoptive transfer studies show that A2AR activity on lymphoid and nonlymphoid cells are additionally important for suppressing immune responses in colitis (131). Taken together, the ability of HIF-1α to increase extracellular adenosine/adenosine signaling in both immune and mucosal cells is essential in protecting tissues during IBD.

Intestinal Ischemia/Reperfusion Injury

Intestinal ischemia is a life-threatening condition associated with thrombosis, hypotension, necrotizing enterocolitis, bowel transplantation, trauma, and chronic inflammation. Extracellular adenosine/adenosine signaling has long been linked to ischemia protection in tissues (22, 132–134). Mounting evidence suggests a protective role of adenosine signaling

in intestinal ischemia/reperfusion (I/R) injury. For example, A2BR expression is increased in intestinal mucosal scrapings following I/R in mice (26), whereby A2BR deficient mice suffer more profound intestinal I/R injury (26). Similar, A2BR antagonism enhances intestinal inflammation and injury during I/R in wild-type mice (26), whereas A2BR agonist treatment protects from intestinal injury, inflammation, and barrier breakdown (26). Adenosine treatment also attenuates intestinal I/R injury (27, 28). In general, CD73 and A2BR deficient mice experience more severe tissue injury with I/R (22, 26), and ischemia results in a robust increase in HIFs (15). Notably, studies of heart ischemia show HIF-1α-mediated cardioprotection is dependent on CD73 and A2BR signaling. Here, wild-type mice receive significant cardioprotective benefit from HIF activator, dimethyloxalylglycine (DMOG) treatment, whereas DMOG treatment in CD73 and A2BR deficient mice provides no protection against ischemia injury (15). As well, Sp1-mediated increase of CD39 provides significant barrier protection during tissue ischemia (14, 135). Though additional studies are needed, these studies provide consideration of a link between hypoxia and adenosine in intestinal I/R injury. Therapeutic agents targeting hypoxia or adenosine signaling are widely available. Future laboratory studies will hopefully examine the potential of these agents in intestinal I/R injury.

Colon Cancer

Colitis-associated cancer (CSC) is a primary example of the induction of cancer by chronic inflammation. Ulcerative colitis patients carry an 18% life-time risk of developing colon cancer (136). CSC and sporadic colon cancer have different development pathways, however share similar inflammatory pathways. Inflammation in sporadic colon cancer can develop by oncogenes inducing inflammatory transcriptomes (137, 138) as well as through microbiota-mediated mechanisms (139). In general, all solid tumors eventually outpace the supply of oxygen which in turn promotes inflammation. Similar to IBD (125), HIF-1α and HIF-2α may have different roles. For example, HIF-1α stabilization is associated with poor prognosis, whereas HIF-2α shows no prognostic value and is inversely associated with high tumor grade and HIF-1α (colon tumors, n=731) (140). Additionally, xenograft studies show HIF-1α deficiency inhibits colon tumor growth, whereas HIF-2α deficiency stimulates tumor growth (141). Moreover, inhibiting HIF-1α dimerization with acriflavine halts the progression of CSC in immunocompetent mice (142). Interestingly, HIF-1α overexpression does not increase tumorigenesis in sporadic colon and CSC cancer models and does not result in spontaneous tumor formation in mice (143). In contrast, intestinal epithelial disruption of Vhl shows to increase tumor progression, which is HIF-2α dependent (144). Suffice to say, there is much excitement surrounding the possible clinical benefit of targeting HIFs. However, at the same time, much remains to be understood.

Extracellular adenosine/adenosine signaling is also associated with many hallmarks of cancer, particularly immunosuppression. Both tumor promoting inflammation and antitumor immunity co-exist in tumors. The immunosuppressive actions of adenosine in cancer have been demonstrated in CD39, CD73, and A2AR deficient mice and by pharmacological and genetic studies in immune competent and immunodeficient mice (145–149). Recently, studies show co-inhibition of CD73 and A2AR is superior in improving antitumor immune responses (150). A2BR also is immunosuppressive. Tumors grow considerably slower in

A2BR deficient mice and wild-type mice treated with A2BR antagonists (ATL-801, PSB1115) (151–153). Here, immune cell immunity is necessary for the antitumor effect, as A2BR antagonist treatment is not effective in nude mice (153) and T cell-deficient animals (152). Reduced tumor growth by ATL-801 or PSB1115 increases T cell and reduces Treg and myeloid-derived suppressor cell (MDSC) infiltration. A2BR is found up-regulated in colon tumors and cell lines and appears essential for colon cancer growth (154). A3R is up-regulated in colon cancer, however is largely shown to inhibit cancer growth (155, 156). Interestingly, treatment of colon cancer cells with caffeine (pan-adenosine receptor antagonist) inhibits A3R-stimulated HIF-1α stabilization (157). Studies in melanoma also show adenosine increases HIF-1α stabilization in a dose-dependent and time-dependent manner exclusively by A3R (158). IB-MECA (A3R agonist) also inhibits the growth of melanoma tumors in syngeneic models (159) and lung metastases models (160, 161). It is not clear if A3R-mediated increase in HIF-1α promotes tumor progression or supports the tumor suppressive actions of A3R.

Recent studies show whole body exposure to hyperoxic atmosphere (60% oxygen) reduces tumor growth (162, 163). Notably, adenosine, CD39, CD73, A2AR, and A2BR are reduced in these tumors and enhanced antitumor immune activity is seen. Accordingly, hyperoxia may have therapeutic benefit in cancer. These studies also strongly support the presence of the hypoxia-adenosine link in tumors. Indeed, another recent study has demonstrated that HIFs directly induce ectonucleoside triphosphate diphosphohydrolase 2 (ENTPD2), a family member of CD39, to prevent MDSC differentiation in hepatocellular cancer (164). Taken together, there is a real opportunity to target hypoxia and adenosine signaling in CSC/colon cancer. However, more studies are necessary for understanding these pathways in colon tumors.

Clinical Implications

Targeting HIFs have proven to have therapeutic benefit for patients with kidney disease (165). Many more studies are ongoing for a number of conditions and diseases (7). DMOG, FG-4497, and TRC160334, all pan-PHD inhibitors, are profoundly protective in models of colitis (166–168). A concern is that in contrast to HIF-1α (143), chronic activation of HIF-2α results in robust spontaneous intestinal inflammation (125), barrier dysfunction (126, 169), and tumorigenesis (144). Therefore, optimal therapies activating HIFs in IBD may be agents that specifically increase HIF-1α. PHD inhibitor, AKB-4924 may be promising. AKB-4924 robustly activates HIF-1α with only modest HIF-2α activation (170). AKB-4924 is currently being developed for use in IBD (NCT02914262). Similar, lower dosages of DMOG are capable of inducing HIF-1α with minimal effects on HIF-2α. Interestingly, HIF-2α–induced inflammation can be reduced with nonsteroidal anti-inflammatory drug (NSAID), nimesulide (125). Thus, combining PHD inhibitors with NSAIDs may have therapeutic potential for IBD patients. Additional concerns of PHD inhibitors are that they are non-specific for PHD isoforms (PHD1, PHD2, and PHD3 (84, 171)) and that many tissues express PHDs. Here, oral administration of PHD inhibitors, including AKB-4924 may minimize side effects. In a head-to-head comparison, though oral delivery of AKB-4924 reduced colon epithelial HIF-1α stabilization compared to intraperitoneal injection, oral AKB-4924 was sufficient enough to induce HIF gene targets

and reduce disease severity in colitis models (172). Oral delivery reduced HIF stabilization and HIF target gene expression in extraintestinal tissues (172). Accordingly, with AKB-4924 there may be a trade-off between potency and toxicity. Roxadustat (FG-4592), another oral-available PHD inhibitor, is currently in clinical trials for kidney disease (7). Studies also support the therapeutic benefit of targeting adenosine signaling in IBD; both A2AR (127) and A2BR (32) agonists provide significant benefit in colitis models. Given that many tissues express adenosine receptors, strategies for local delivery of A2AR and A2BR agonists may be worthwhile to assess. Studies also suggest that autologous Treg–based therapies, involving the enrichment of patient's endogenous CD39+ Tregs and reconditioning the cells with cytokines, may lead to better control of inflammation in IBD patients (173). The role of A3R in IBD is controversial (174, 175), however A3R agonists may have clinical benefit (176). Patients with intestinal I/R injury may also benefit from therapeutic strategies that increase HIF stabilization and adenosine signaling (26) (Figure 3).

In contrast, inactivation of hypoxia/adenosine pathways may decrease immunosuppression activity in tumors in addition to reducing metastasis and resistance to therapy (75). Hypoxia/adenosine signaling can potentially be targeted at different steps in CSC/colon cancer (75), such as improving tissue oxygenation either by HIF-1α inhibitors (177) or hyperoxic atmosphere (162, 163); reducing extracellular adenosine by inhibiting CD73 (146–149); or modulating adenosine signaling using agonists and antagonists (145) (Figure 3). Given the complexity of anti-tumor immunity, the combination of immune therapies may have the most promise for patients. Preclinical studies show anti-hypoxia/adenosine therapy in combination with immune checkpoint inhibitors, such as anti-CTLA-4 and anti-PD-1 provides great benefit (147, 178, 179). Combination therapies are currently in early phase clinical trials (7). Much hope awaits the completion of these studies.

Conclusions

The understanding of the link between hypoxia and adenosine and their potential as therapeutic targets has advanced considerably over the years. It is now appreciated that inflammatory lesions are profoundly hypoxic and that the enhancement of adenosine signaling by HIFs is an essential endogenous adaptive response. A significant challenge that remains is the movement of agents targeting these pathways into clinical practice. A focus on advancing cell or tissue-specific delivery of agents as well as defining challenges that have hampered the success of prior clinical studies of adenosine signaling agents will be important (180). Additionally, we must continue to gain new insights into these pathways. These efforts will certainly provide additional support in moving these pathways closer to being treatments and may make all the difference.

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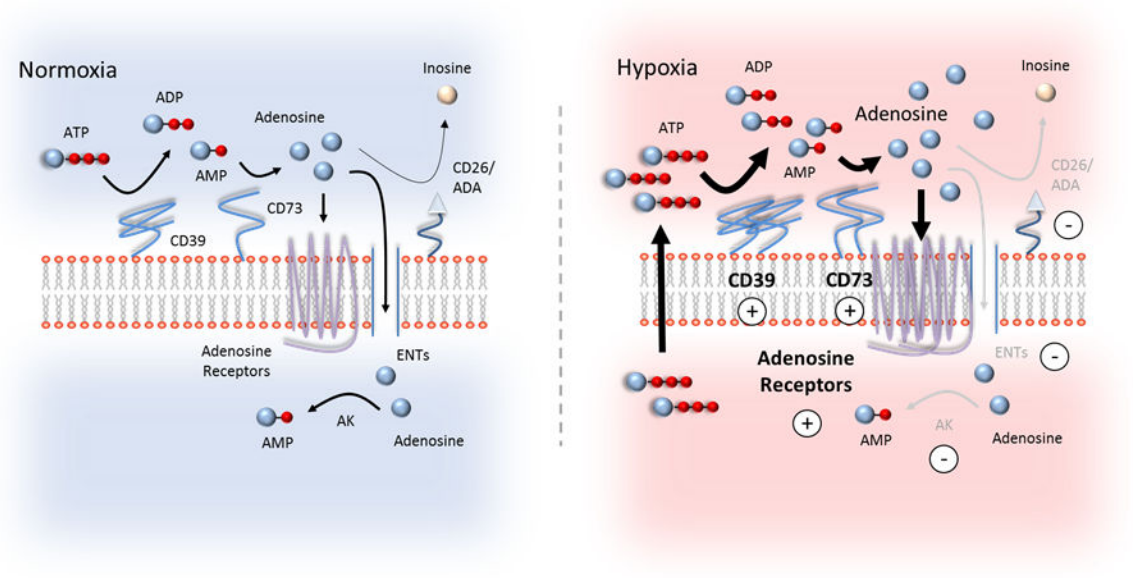

Figure 1.

Increased extracellular adenosine and adenosine signaling by hypoxia. In normoxia, the concentration of adenine nucleosides (ATP, ADP, and AMP) at the cell surface is low. Extracellular ATP is converted to adenosine by two phosphohydrolysis reactions by cell surface nucleotidases, CD39 and CD73. CD39 converts ATP/ADP to adenosine and CD73 converts AMP to adenosine. Adenosine can activate adenosine receptors (A1R, A2AR, A2BR, A3R); be transported into the cell via equilibrative nucleoside transports (ENTs); or be converted to inosine at the cell surface by CD26-bound adenosine deaminase (ADA). In normoxia, adenosine is primarily distributed between high affinity adenosine receptors (e.g., A1R, A2AR, and A3R) and ENTs. In hypoxia, adenosine signaling is enhanced. Hypoxia increases the release of extracellular ATP/ADP via lytic and non-lytic pathways. Additionally, HIFs up-regulate (indicated by positive circles) adenosine metabolizing and signaling genes, including CD73 and adenosine receptors (e.g., A2AR and A2BR) while down-regulating (indicated by negative circles) genes that dampen adenosine signaling, including ADA, ENTs, and adenosine kinase (AK). CD39 is up-regulated in hypoxia by Sp1. In the gut, CD39 is largely restricted to immune cells and vascular endothelium. ATP/ADP metabolism on epithelial cells may involve ectonucleoside triphosphate diphosphohydrolase 7 (ENTPD7), a CD39 family member, and alkaline phosphatases. Together, these events increase extracellular adenosine and adenosine signaling, which dampens tissue inflammation and protects tissue barriers.

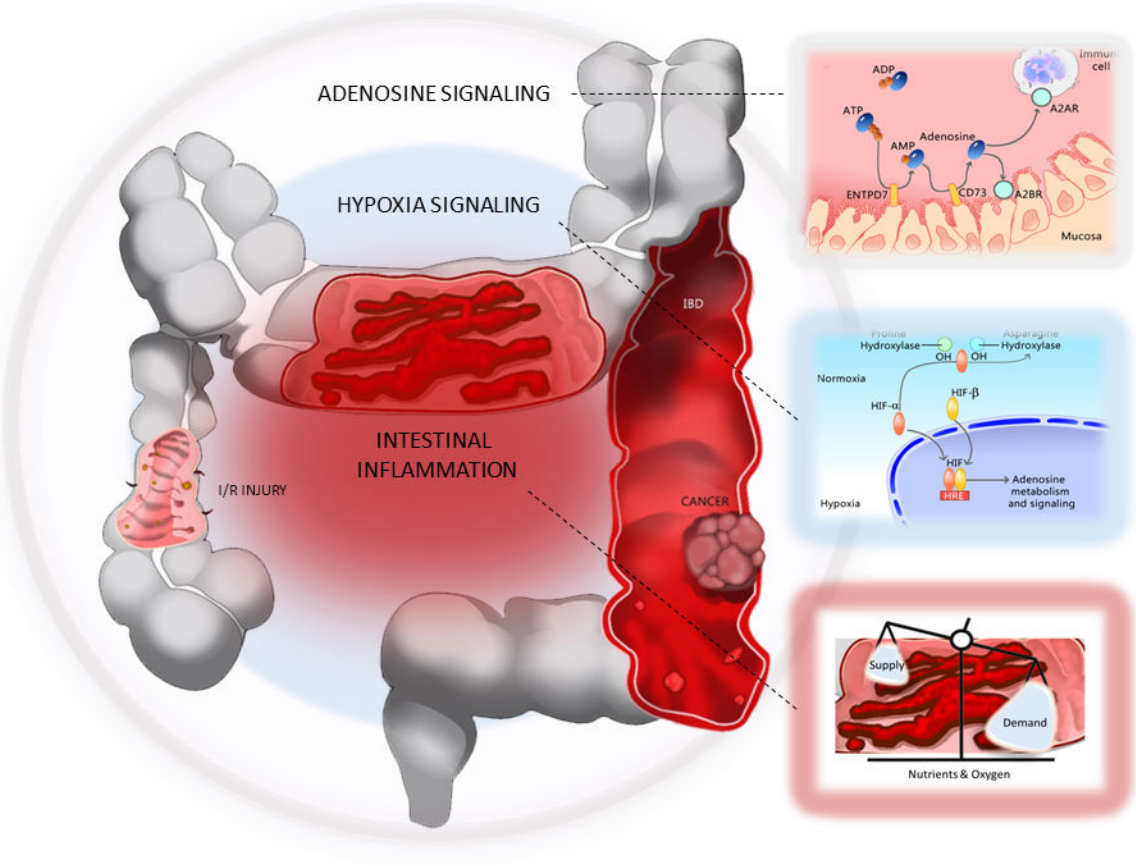

Figure 2.

Summary: the hypoxia-adenosine link during intestinal inflammation and disease. Inflamed tissues often become severely hypoxic, whereas tissue hypoxia can lead to tissue inflammation. HIFs are stabilized in these conditions, binding hypoxia-response elements (HREs) in target genes. Adenosine signaling pathway genes are direct gene targets of hypoxia/HIFs. In general, HIF-1α-mediated increase of adenosine signaling is tissue protective (e.g., IBD and intestinal I/R injury), whereas in cancer, HIFs may target extracellular adenosine/adenosine signaling genes to promote tumorigenesis. Boxes: Lower right: Inflammation alters tissue metabolism, reducing the supply of nutrients and oxygen to tissues. In turn, inflamed tissues become profoundly hypoxic. Middle right: Hypoxia signaling. Both proline hydroxylases (PHDs) and asparaginyl hydroxylase factor inhibiting HIF (FIH; indicated as asparagine hydroxylase) serve an important role as oxygen sensors, controlling the activity of HIFs. In normoxia, HIFs are rapidly targeted for degradation by proline hydroxylase (PHDs) and von Hippel-Lindau (VHL; not shown), an E3 ubiquitin ligase. In hypoxia, HIF-α is stabilized. Oxygen is an essential co-factor of PHDs and asparagine hydroxylase. HIFs (α and β subunits) translocate to the nucleus and dimerize, binding to HREs of target genes. Top right: Adenosine signaling. Multiple adenosine pathway genes are targets of hypoxia/HIFs. The increased release of ATP/ADP to the cell surface and hypoxia/HIF-mediated regulation of extracellular adenosine pathway genes (see Figure 1. for details) together enhance adenosine signaling.

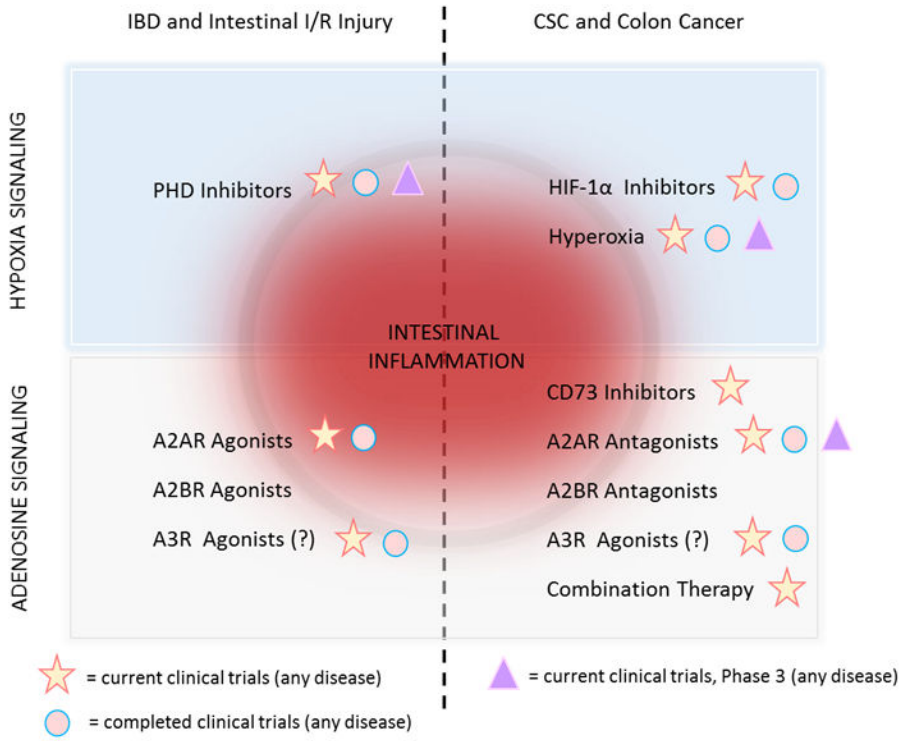

Figure 3.

Targeting hypoxia and adenosine signaling in intestinal inflammation and disease. Increasing HIF-1α by PHD inhibitors or increasing adenosine signaling by adenosine receptor agonists (e.g., A2AR, A2BR, and possibly A3R) may provide therapeutic benefit for IBD and intestinal I/R injury patients. In contrast, inhibiting HIF-1α (e.g., HIF inhibitors, hyperoxia) or inhibiting extracellular adenosine (e.g., CD73 inhibitors)/adenosine signaling (e.g., A2AR, A2BR antagonists) may prove beneficial for patients with CSC or sporadic colon cancer. A2BR agonists and antagonists are currently used only in basic science laboratories. PHD inhibitors, Vadadustat (AKB-6548), Daprodustat (GSK1278863), and Roxadustat (FG-4592) are in clinical trials for anemia associated with chronic kidney disease (7). HIF-1α inhibitors and hyperoxia are in clinical trials of various cancers and respiratory conditions/diseases, respectively. A2AR agonist, Regadenoson is in clinical trials for sickle cell anemia (NCT01788631). A2AR antagonists, PBF-509 (NCT02403193) and CPI-444 (NCT02655822) or CD73 inhibitor, MEDI9447 (NCT02503774) are in clinical trials in solid tumors as anticancer therapies to boost immune responses against tumor cells. Completed clinical trials for A2AR antagonists include Parkinson's disease (e.g., NCT01691924, NCT02111330). A3R agonist, CF-102 may reduce tumor burden and improve survival in hepatocellular cancer (NCT02128958).