Hypoxia, Therapeutic Resistance, and Sphingosine 1-Phosphate

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Abstract
Hypoxia, defined as a poor oxygenation, has been long recognized as a hallmark of solid tumors and a negative prognostic factor for response to therapeutics and survival of patients. Cancer cells have evolved biochemical mechanisms that allow them to react and adapt to hypoxia. At the cellular level, this adaptation is under the control of two related transcription factors, HIF-1 and HIF-2 (hypoxia-inducible factor), that respond rapidly to decreased oxygen levels to activate the expression of a broad range of genes promoting neoangiogenesis, glycolysis, metastasis, increased tumor growth, and resistance to treatments.

Recent studies have identified the sphingosine kinase 1/sphingosine 1-phosphate (SphK1/S1P) signaling pathway—which elicits various cellular processes including cell proliferation, cell survival, or angiogenesis—as a new regulator of HIF-1 or HIF-2 activity. In this review, we will focus on how the inhibition/neutralization of the SphK1/S1P signaling could be exploited for cancer therapy.
1. THE HYPOXIA-INDUCIBLE FACTORS FAMILY: REGULATION, ROLE IN CANCER AND THERAPEUTIC IMPLICATION

Hypoxia, or low oxygen tension, occurs in a variety of physiological conditions including embryonic development, as well as pathological conditions such as chronic inflammation, neurodegenerative disorders, ischemic cardiovascular disease, and cancer (Semenza, 2011). There is ample evidence that hypoxia is a feature of many solid human tumors as the aberrant cell proliferation rate is associated with a disequilibrium between oxygen supply and consumption (Harris, 2002). Because of their rapid proliferation, cancer cells become located too far from functional blood vessels for adequate diffusion of oxygen and nutrients. As a consequence, the establishment of a neovasculature is critical to the growth of the tumor (Hanahan & Folkman, 1996). However, this neoangiogenesis displays abnormalities—with irregular and leaky vessels, disordered network structure, and impaired transport characteristics—resulting in spatial and temporal inadequacies in delivery of oxygen leading to highly heterogeneous tissue oxygenation and areas of hypoxia (Jain, 2005; Liao & Johnson, 2007).

The many effects of hypoxia on cancer biology include not only promotion of progression and metastasis (Lu & Kang, 2010; Sullivan & Graham, 2007) but also resistance to radiotherapy or chemotherapy (Cosse & Michiels, 2008; Rohwer & Cramer, 2011; Tredan, Galmarini, Patel, & Tannock, 2007). The presence or not of oxygen influences the efficacy of ionizing radiation, a phenomenon known as the “oxygen effect,” and hypoxic fraction of human cancers is resistant due to reduced generation of oxygen radicals and failure to induce DNA breaks (Bristow & Hill, 2008; Moeller, Richardson, & Dewhirst, 2007; Wilson & Hay, 2011).

For chemotherapeutics, because of the leakiness of intratumoral vasculature, the delivery of drugs via blood flow is inefficient (Jain, 2005). In addition, the contribution of hypoxia to drug resistance involves increased drug efflux through the upregulation of the multidrug resistance 1 (MDR1) gene encoding the P-gp membrane-resident glycoprotein (Comerford et al., 2002; Ding et al., 2010; Wartenberg et al., 2003), or the multidrug-associated protein 1 (MRP1) (Chen et al., 2009; Liu et al., 2008; Zhu et al., 2005), both belonging to a family of ATP-binding cassette (ABC) transporters. By acting as drug efflux pumps, MDR1 and MRP1 can lower the intracellular concentration of chemotherapeutic drugs (Gottesman, Fojo, & Bates, 2002).
The mechanisms of resistance of hypoxic cells to chemotherapeutics also include cell cycle arrest, inhibition of apoptosis, inhibition of DNA damage, and likely induction of autophagy (Daskalow et al., 2010; Rohwer & Cramer, 2011; Sullivan & Graham, 2009; Wilson & Hay, 2011; Wirthner, Wrann, Balamurugan, Wenger, & Stiehl, 2008).

Reflecting these major roles in tumor biology and therapy, there is compelling evidence that hypoxia can compromise clinical outcomes (Vaupel & Mayer, 2007). Numerous studies using the Eppendorf pO2 probe have shown a connection between hypoxia and disease progression in a wide array of human tumors including carcinomas of the cervix, prostate, the head and neck, and soft tissue sarcomas (Milosevic, Fyles, Hedley, & Hill, 2004; Vaupel, 2004). The tumor oxygen status is currently considered as a prognostic marker that impacts on malignant progression and outcome of tumor therapy (Milosevic et al., 2012).

Hypoxia should result in the death of cancer and normal cells but cancer cells can adapt to this hostile environment by undergoing genetic changes, which allow them to survive and even proliferate. One way that cancer cells respond to reduced oxygen levels is through the hypoxia-inducible factors (HIFs), a family of transcription factors identified as important regulators of the cellular response to hypoxia (Majmundar, Wong, & Simon, 2010; Semenza, 2012). HIFs consist of a stable oxygen-insensitive HIF-1β subunit (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) and an oxygen-labile HIF-α subunit. Three HIF-α homologues have been identified: HIF-1α (Wang, Jiang, Rue, & Semenza, 1995), HIF-2α (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian, McKnight, & Russell, 1997), and HIF-3α (Makino, Kanopka, Wilson, Tanaka, & Poellinger, 2002). HIF-1α and HIF-2α are by far the best characterized α-subunits as little is known about the function of HIF-3α on tumor progression in hypoxic conditions (Heikkila, Pasanen, Kivirikko, & Myllyharju, 2011), although it may function as an inhibitor of HIF-1α, being involved in feedback regulation as its expression is transcriptionally regulated by HIF-1α (Makino et al., 2007).

Whereas HIF-1α is expressed ubiquitously in all cells, HIF-2α and HIF-3α have a limited tissue expression. For example, HIF-2α is particularly abundant in endothelial cells, from which it was first described (Ema et al., 1997; Tian et al., 1997). HIF-2α is also detected in highly vascularized organs or hypoxic tissues including kidney epithelial cells, cardiac myocytes, bone marrow macrophages, or pancreatic parenchymal cells (Talks et al., 2000; Wiesener et al., 2003).
Under normoxic conditions, the HIF-α subunit is hydroxylated on either one or two proline residues found in an oxygen-dependent domain (ODD), which controls the protein stability (Ivan et al., 2001; Jaakkola et al., 2001). This reaction is mediated by members of the oxygen-dependent specific prolyl-4-hydroxylase domain (PHD) family (Bruick & McKnight, 2001; Ivan et al., 2001; Jaakkola et al., 2001). The hydroxylation of HIF-α subunit is required for its recognition by the von Hippel–Lindau tumor suppressor gene product (pVHL) of the E3 ubiquitin ligase complex (Iwai et al., 1999; Lisztwan, Imbert, Wirbelauer, Gstaiger, & Krek, 1999), leading to its polyubiquitination and proteasomal degradation (Cockman et al., 2000; Ohh et al., 2000; Tanimoto, Makino, Pereira, & Poellinger, 2000).

Under hypoxia or in cells lacking functional pVHL (renal cell carcinomas and other tumors regrouped in the VHL syndrome), the HIF-α subunit remains unhydroxylated, and therefore accumulates and then translocates to the nucleus where it dimerizes with HIF-1β, and associates with coactivators such as CBP (cAMP Response Element-Binding Protein-binding protein)/p300 (Arany et al., 1996). The HIF-1 or HIF-2 heterodimer binds to hypoxia-response elements (HREs) located in the promoter region of its multiple target genes (Fig. 5.1): angiogenesis-promoting factors, glucose transporters, enzymes of glycolytic pathway, proteins involved in extracellular matrix remodeling, cell proliferation, and epithelial to mesenchymal transition (Hickey & Simon, 2006; Piatir & Harris, 2006; Semenza, 2010; Thiery, Acloque, Huang, & Nieto, 2009). It also includes those that are directly involved in therapeutic resistance such as the MDR1 (Comerford et al., 2002; Ding et al., 2010; Wartenberg et al., 2003) or MRP1 (Chen et al., 2009; Liu et al., 2008; Zhu et al., 2005).

A wealth of reports based on immunohistochemical studies of human tumor sections indicate that HIF-1α and/or HIF-2α are overexpressed in the majority of human cancers and these elevated levels correlate with cancer-related death (Semenza, 2010) (Keith, Johnson, & Simon, 2012). Despite their extensive sequence similarity and coexpression in many cell types, HIF-1α and HIF-2α play nonoverlapping roles in tumor progression and sometimes opposing activities (Keith et al., 2012). Differences exist in their targets, with HIF-1α activating glucose metabolism genes whereas HIF-2α preferentially promotes activation of genes regulating angiogenesis (VEGF) or proliferation (TGF-α, cyclin D1) (Hu, Wang, Chodosh, Keith, & Simon, 2003; Raval et al., 2005; Wang, Davis, Haque, Huang, & Yarchoan, 2005). Recent evidence also indicates that HIF-1α and HIF-2α can exert distinct, often opposite, direct or indirect interaction with oncoproteins and tumor suppressors such as c-myc, p53, or mTOR (An et al., 1998; Brugarolasa et al., 2004; Gordan, Bertout, Hu, Diehl, & Simon, 2007). The distinct roles of HIF-1α and HIF-2α in promoting tumor growth
have been mainly defined in von Hippel–Lindau disease-associated clear cell renal carcinoma (ccRCC) (Kaelin, 2008), which can produce either HIF-1α or HIF-2α alone, and where the role for HIF-2α as a driver of a more aggressive disease has been established (Gordan et al., 2008).

2. WHAT DOES SPHINGOSINE 1-PHOSPHATE SIGNALING HAVE TO DO WITH HYPOXIA?

Sphingosine 1-phosphate (S1P) is a bioactive phospholipid regulating pleiotropic activities in cancer biology such as proliferation, survival, migration, inflammation, or angiogenesis (Pitson, 2011; Pyne & Pyne, 2011;
S1P is generated from sphingosine, the backbone component of all sphingolipids and a proapoptotic sphingolipid (Cuvillier, 2002; Cuvillier, Edsall, & Spiegel, 2000; Cuvillier et al., 2001), in a reaction catalyzed by two sphingosine kinase isoforms, SphK1 and SphK2 (Fig. 5.2). The balance between the levels of S1P and its metabolic precursors ceramide and sphingosine has been regarded as a switch that could determine whether a cell proliferates or dies (Cuvillier et al., 1996). S1P can be secreted even if the mechanism of extracellular transport is not clear and is present at high nanomolar concentration in the circulation (Pappu et al., 2007). Extracellularly, S1P signals as a ligand of five high-affinity G-coupled receptors, named S1P1–5 (Rosen, Gonzalez-Cabrera, Sanna, & Brown, 2009). The receptors differ in their tissue distribution and the specific effect of S1P is driven by the predominance of the S1P receptor subtypes expressed (Rosen et al., 2009). Intracellular functions exist with recent studies suggesting that S1P could regulate histone

Figure 5.2 The ceramide/sphingosine 1-phosphate biostat. Ceramide, a proapoptotic sphingolipid, can be produced by de novo synthesis or degradation of membrane sphingomyelin in response to stress-associated stimuli (death receptors, chemotherapeutics, ionizing radiations, hormone deprivation, etc.). Sphingosine, the metabolite of ceramide, which can be easily converted into ceramide, has been associated with apoptosis. Conversely, sphingosine 1-phosphate, produced by the stimulation of sphingosine kinase activity by cell growth-associated stimuli, acts as an antiapoptotic, prosurvival, proinflammatory, proangiogenic sphingolipid. Sphingosine 1-phosphate exhibits both intracellular effects beginning to be elucidated, and extracellular effects (autocrine and paracrine) through the binding to five dedicated G protein-coupled receptors (S1P1 to S1P5).
acetylation in the nucleus, linking S1P to epigenetic regulation of gene expression (Hait et al., 2009). S1P was also reported to act as a cofactor for the ubiquitin ligase activity of tumor necrosis factor receptor-associated factor 2 necessary for the activation of NF-κB (nuclear factor-kappa B) (Alvarez et al., 2010). Although SphK1 and SphK2 differ in size, they share a high degree of sequence similarity (Liu et al., 2000). SphK1 and SphK2 have different tissue distribution and subcellular localization, suggesting distinct physiological roles (Pitson, 2011; Spiegel & Milstien, 2007). SphK1 is activated by various stimuli, among which growth and survival factors are prominent, thus primarily promoting cell survival and proliferation (Strub, Maceyka, Hait, Milstien, & Spiegel, 2010). On the contrary, the role of SphK2 appears by far more complex generally displaying a proapoptotic role but antiapoptotic effects have also been reported (Strub et al., 2010). The subcellular localization of both SphK1 and SphK2 is likely to be critical in determining their function (Strub et al., 2010).

Importantly, the agonist-induced S1P production as well as its downstream effects can be impeded by inhibition of the SphK1 gene expression or enzymatic activity illustrating that SphK1 plays a crucial role in the observed effects played by S1P. A wide array of studies support the convincing role of SphK1 in the promotion of oncogenesis in addition to being a cellular target for many anticancer treatments (Cuvillier, 2007). SphK1 expression has been found upregulated in a number of solid tumors, and high SphK1 expression has been correlated with a significant decrease in survival rate in patients with several forms of cancer (Cuvillier et al., 2010; Pyne & Pyne, 2010). On the other hand, anticancer regimens (chemotherapeutics, radiation therapy, hormonotherapy, chemopreventive agents) have been shown to downregulate SphK1 activity and decrease S1P levels in various cancer cell and animal models (Bonhoure et al., 2008, 2006; Brizuela et al., 2010; Dayon et al., 2009; Li et al., 2007; Nava et al., 2000; Pchejetski et al., 2005; Taha et al., 2004), suggesting that SphK1 could act as a “sensor” to anticancer therapies (Pchejetski et al., 2005). A number of preclinical studies have shown that pharmacological inhibition of SphK1 could be efficacious in decreasing tumor size (French et al., 2003, 2006; Kapitonov et al., 2009; Paugh et al., 2008) or sensitize to chemotherapy or radiotherapy (Pchejetski et al., 2010, 2008), whereas its enforced expression can accelerate tumor proliferation (Nava, Hobson, Murthy, Milstien, & Spiegel, 2002; Pchejetski et al., 2005).

Accumulating evidence has linked SphK1/S1P signaling with adaptation of noncancer cells to hypoxia notably in cardiomyocytes (Karliner, Honbo,
Summers, Gray, & Goetzl, 2001; Tao, Zhang, Vessey, Honbo, & Karliner, 2007; Zhang et al., 2007). Hypoxia also stimulates the proliferation of smooth muscle cells in a S1P-dependent manner (Ahmad, Long, Pyne, & Pyne, 2006; Yun & Kester, 2002). Of note, S1P could act as a nonhypoxic activator of HIF-1α independently of the level of oxygen, by a mechanism requiring a S1P2 receptor-dependent signaling in endothelial cells (Michaud, Robitaille, Gratton, & Richard, 2009). In line with data showing that S1P2 activation in endothelial cells increases vascular permeability (Sanchez et al., 2007), the S1P2 receptor was also shown to be induced in hypoxia–triggered pathological angiogenesis of the mouse retina characterized by evident abnormal vascular growth (vessels were tortuous and dilated) (Skoura et al., 2007). Interestingly, neovascularization was suppressed in the S1P2−/− animals subjected to ischemia–driven retinopathy, suggesting that S1P2 is crucial for endothelial cell responses to hypoxia (Skoura et al., 2007).

Despite the crucial role of hypoxia in solid tumors, the link between SphK1/S1P signaling and adaptation to hypoxic conditions has recently emerged in cancer cells. In a study conducted with five distinct tumor cell models (glioblastoma, prostate, breast, lung, renal cell carcinoma), SphK1 activity was shown to be rapidly stimulated under hypoxic conditions suggesting a posttranslational effect (Ader, Brizuela, Bouquerel, Malavaud, & Cuvillier, 2008). Hypoxia-induced SphK1 activation was dependent on reactive oxygen species (ROS) production as the ROS scavenger N-acetyl cysteine could prevent both SphK1 stimulation and HIF-1α accumulation (Fig. 5.3). Numerous studies have reported that exposing cells or tissues to hypoxia increases oxidative stress, although the mechanism(s) by which reduced oxygen concentration augments the production of ROS is not known (Poyton, Ball, & Castello, 2009). Moreover, the addition of exogenous pro-oxidants such as hydrogen peroxide has been shown to be sufficient to trigger HIF-1α accumulation under normoxia (Bell et al., 2007; Simon, 2006). Mitochondria, as the primary oxygen-consuming organelles in cells, have been proposed to be the source of ROS under hypoxia (Chandel et al., 1998, 2000; Guzy et al., 2005; Klimova & Chandel, 2008). The neutralization of ROS by antioxidants, the inhibition of the mitochondrial electron transport chain, or the use of cells depleted in mitochondria were able to abolish the hypoxic induction of HIF-1α (Bell et al., 2007; Chandel et al., 1998).

A large body of evidence suggests that stability of the HIF-α protein subunit is regulated by hydroxylation of two proline residues located within the oxygen-dependent degradation domain (ODD) via the PHD enzymes
ROS can not only directly modulate HIF-1α level through direct inhibition of prolyl hydroxylases or their cofactors (Kaelin & Ratcliffe, 2008) but also indirectly by activating signaling cascades upstream of HIF-1α such as mitogen-activated protein kinases (Du et al., 2011; Mylonis et al., 2006; Richard, Berra, Gothie, Roux, & Pouyssegur, 1999) or the phosphatidylinositol 3-kinase (PI3K)/Akt (Du et al., 2011; Flugel, Gorlach, Michiels, & Kietzmann, 2007; Gao et al., 2002; Koshikawa, Hayashi, Nakagawara, & Takenaga, 2009; Liu et al., 2006; Mylonis et al., 2006; Zhou et al., 2007). PI3K/Akt has several downstream targets (Galanis et al., 2008) including the glycogen synthase kinase 3-β (GSK3β), which has been involved in the destabilization of HIF-1α (Chen, Mazure, Cooper, & Giaccia, 2001; Flugel et al., 2007; Mottet et al., 2003; Skuli et al., 2006; Sodhi, Montaner, Miyazaki, & Gutkind, 2001). Akt inactivates the
phosphorylation of GSK3β in the ODD and therefore promotes HIF-1α accumulation (Sodhi et al., 2001). Interestingly, the SphK1-mediated accumulation of HIF-1α levels under hypoxia was found to be Akt/GSK3β pathway dependent (Ader et al., 2008).

How does S1P produced upon SphK1 stimulation activate the Akt/GSK3β signaling? The main hypothesis is that S1P could act through the S1P receptors (Fig. 5.3), as the PI3K/Akt signaling can be activated by Gt-coupling of all subtypes of S1P receptors (Lappano & Maggiolini, 2011). Activation of the PI3K/Akt pathway leading to GSK3β phosphorylation by addition of exogenous S1P has been reported in endothelial cells (Gonzalez, Kou, & Michel, 2006). Work conducted in our lab establishes that neutralizing exogenous S1P by an antibody-based approach (Visentin et al., 2006; Wojciak et al., 2009) controls HIF-1α expression under hypoxia in various cancer cell lineages (I. Ader & O. Cuvillier, unpublished data).

The inhibition (pharmacological inhibition or RNA interference) of SphK1 activity prevents activation of the Akt/GSK3β signaling, accumulation of HIF-1α, and its transcriptional activity in all human cancer cell lineages (Ader et al., 2008). As the seminal paper by Wang et al., the main regulatory mechanism of HIF-1α accumulation under hypoxia is its pVHL-mediated proteasomal degradation (Wang et al., 1995) although alternative mechanisms have been described such as the HAF (hypoxia-associated factor) pathway (Koh, Darnay, & Powis, 2008). The degradation of HIF-1α induced by inhibition of SphK1 is mediated by the proteasome through a pVHL-dependent mechanism as shown by inhibition of the proteasome by the MG132 compound or using pVHL-deficient and reconstituted pVHL cell models (Ader et al., 2008). The ROS/SphK1/Akt/GSK3β signaling cascade activated by hypoxic conditions was recently confirmed by Kim and coworkers in prostate cancer cells (Cho et al., 2011).

Obeid and coworkers suggested a relationship between the SphK1/S1P pathway and HIF-2α in U87 glioblastoma cells (Anelli, Gault, Cheng, & Obeid, 2008). Chemically induced hypoxia by cobalt chloride (CoCl2) or 0.5% oxygen conditions triggered the accumulation of HIF-1α and HIF-2α, the increase in SphK1 mRNA (but not SphK2) and protein, and secretion of S1P (Fig. 5.3). Under CoCl2, the silencing of HIF-2α by RNA interference increased HIF-1α content, abolished the induction of SphK1, and decreased extracellular S1P content. Conversely, HIF-1α downregulation resulted in slight increased HIF-2α and SphK1 expression. With regard to the regulatory mechanisms, it was suggested that both HIF-1...
and HIF-2 transcription factors could compete for binding to putative HREs located in the promoter region SphK1 under normoxia, and HIF-2 association increasing and HIF-1 decreasing under hypoxia (Anelli et al., 2008). Although in apparent contradiction with our data where SphK1 activity was found to be an upstream regulator of HIF-1α (Ader et al., 2008; Cho et al., 2011), it should be noted that the influence of SphK1 activity on HIF-1α protein levels was not evaluated in this study. Hypoxia mimetics such as CoCl2 used for the most part in this study should not be considered equivalent to bona fide hypoxia, and results should be interpreted with caution. Nevertheless, it cannot be ruled out that SphK1 activity might first regulate HIF-1α (and potentially HIF-2α) activity, which in turn could transcriptionally regulate(s) the proangiogenic and prosurvival SphK1/S1P pathway (Fig. 5.3). More work is needed to elucidate whether (i) SphK1 can be a target gene of HIF-2α as proposed by Obeid and coworkers (Anelli et al., 2008) and (ii) SphK1 activity can regulate HIF-2α, as it does for HIF-1α (Ader et al., 2008), particularly in a relevant model such as the von Hippel–Lindau–associated ccRCC. Recent studies conducted in our lab indicate that SphK1 signaling controls HIF-2α expression under hypoxia (P. Bouquerel & O. Cuvillier, unpublished data).

With regard to the SphK2 isoform, contradictory data have been reported for its potential role in mediating adaptation to hypoxia. In cerebral microvascular endothelial cells (in which SphK2 is the most prominent isoform) of Swiss-Webster ND4 mice, SphK2 was found activated (but not SphK1) during hypoxic preconditioning (Wacker, Park, & Gidday, 2009), whereas SphK2 activity and mRNA expression were unchanged in hypoxic EA.hy 926 human endothelial cells (Schwalm et al., 2008). In cancer cells, a single study has reported the upregulation of SphK2 under hypoxia in A549 lung cells, associated with a release of S1P able to protect against chemotherapeutics by a mechanism relying potentially on S1P1/3 signaling (inhibitory effect of the VPC23019 pharmacological antagonist), yet the relationship between SphK2 and HIF-1α or HIF-2α was not examined (Schnitzer, Weigert, Zhou, & Brune, 2009). In hypoxic U87 glioblastoma cells, whereas SphK1 expression was augmented, SphK2 expression was unaltered (Anelli et al., 2008) or downregulated (Zhang et al., 2012). Further studies are obviously required to elucidate whether SphK2 represents or not a mediator of hypoxia.

It is important to point out that (when examined) the stimulation of SphK1 or SphK2 was associated with the release of S1P from the hypoxic tumor cells (Anelli et al., 2008; Schnitzer et al., 2009). These findings suggest
that S1P released from hypoxic tumors could exhibit an autocrine activity (Fig. 5.3) on cancer cells including (i) a potential feed-forward amplification loop of the HIF1/2 signaling (yet to be proven) or (ii) the induction of tumor cells survival in response to anticancer therapies as shown in hypoxic A549 cells where the conditioned medium could protect these cells to etoposide by a mechanism suggesting the involvement of S1P1/3 signaling (Schnitzer et al., 2009). A paracrine proangiogenic effect of S1P (Fig. 5.4) released from hypoxic tumor cells can effect endothelial cells. HUVECs have been shown to lose their ability to form tubes and migrate in the presence of hypoxic U87-conditioned medium if pretreated with VPC23019, the antagonist of S1P1 and S1P3, or silenced by siRNA (Anelli et al., 2008). Another potential paracrine effect of S1P can interfere with cells from innate immunity such as macrophages (Fig. 5.4). Hypoxic microenvironment is well known to favorize the infiltration of macrophages (Yotnda, Wu, & Swanson, 2010), which once present in the tumor are identified as

Figure 5.4 The distinctive biological effects of S1P on endothelial cells. This schematic depicts the contrasting effects of S1P on endothelial cells depending on the S1P receptor isoform activated. Like the VEGF, S1P can trigger vascular permeability or weakening of VE–cadherin junctions through S1P2 signaling, which results in loss of association of endothelial cells. Hypoxia can also upregulate the expression of S1P2 receptor subtype, which is associated with HIF-1α activation and release of the vascular permeability factor VEGF. Conversely, S1P can promote endothelial cell integrity via S1P1 signaling (see text for details).
tumor-associated macrophages (TAMs). The high number of macrophages correlates with tumor progression and unfavorable clinical prognosis in many cancer types (Lewis & Pollard, 2006). Hypoxic TAMs switch from immune to protumor cells, being polarized toward the M2 phenotype (Yotnda et al., 2010). In conjunction with inhibition of antitumor response, they play a critical role in promoting tumor progression through stimulation of angiogenesis and extracellular matrix changes (Condeelis & Pollard, 2006; Mantovani, Schioppa, Porta, Allavena, & Sica, 2006). It is now well established that S1P can trigger the polarization of macrophages toward the M2 phenotype when added to the cells (Hughes et al., 2008) or when being present in the supernatant of apoptotic tumor cells (Weigert et al., 2007; Weis, Weigert, von Knethen, & Brune, 2009). A recent work from Brüne and coworkers has shown that S1P by binding to S1P1 receptors expressed on macrophages provokes their activation by increasing HIF-1α expression and activity, which could amplify tumor angiogenesis (Herr et al., 2009).

### 3. SPHINGOSINE 1-PHOSPHATE SIGNALING AS A TARGET FOR ANTIHYPOXIC STRATEGY?

Given the critical role of HIF-1 and HIF-2 in the regulation of hypoxia, it is clear that decreasing their activity should represent a valid strategy to control tumor hypoxia and its molecular consequences: increased potential for invasion, angiogenesis, metastasis, and patient mortality. The search for specific HIF inhibitors is not easy as transcription factors are conventionally considered complex if not impractical targets for the discovery of small molecule inhibitors (Melillo, 2007). A growing number of chemical compounds have been shown to inhibit HIF activity through various mechanisms including decreased HIF-1α synthesis, increased degradation, decreased interaction with the HIF-1β subunit, decreased DNA-binding capabilities or decreased transcriptional activity (Rodriguez-Jimenez & Moreno-Manzano, 2012; Semenza, 2012). Signal transduction pathways involved in HIF-1 or HIF-2 stabilization occurring during hypoxic stress can also be targeted to inhibit HIF-1 activity. This has been nicely illustrated with the inhibition of the PI3K/Akt signaling, which downregulates HIF-1α (and VEGF expression) and sensitizes to ionizing radiations (Pore et al., 2006).

Because the SphK1/S1P pathway appears to act as a key regulator of HIF-1 activity, we hypothesize that its inhibition should represent
an unconventional and valid strategy to control tumor hypoxia and its consequences, notably the formation of an aberrant tumor vasculature due to unleashed angiogenesis. For instance, in vitro studies have shown that SphK1-mediated inhibition of HIF-1 blocks the release of the vascular permeability factor VEGF and the glucose transporter GLUT-1 from hypoxic tumor cells (Ader et al., 2008; Cho et al., 2011). Anti-SphK1 strategies also decrease S1P secretion from hypoxic tumor cells hence impeding the potential prosurvival effect of S1P on hypoxic tumor cells (Schnitzer et al., 2009), or interfering within the tumor microenvironment by inhibiting the effects of S1P on tube formation and migration of endothelial cells (Anelli et al., 2008) or the activation of TAMs (Herr et al., 2009). Similarly, strategies aimed at (i) neutralizing circulating S1P by anti-S1P antibody to prevent its binding to its receptors (Visentin et al., 2006) or (ii) targeting a specific S1P receptor such as S1P1 or S1P2, the roles of which have been suggested in hypoxic conditions, could be employed. For instance, S1P1 could mediate the effect of S1P on tumor cell survival (Schnitzer et al., 2009) and on tube formation and migration of endothelial cells (Anelli et al., 2008), and its therapeutic targeting seems to be beneficial to prevent tumor growth and angiogenesis in animal models (Chae, Paik, Furneaux, & Hla, 2004; LaMontagne et al., 2006).

Similarly to anti-PI3K strategies that downregulate VEGF production (Fokas et al., 2012; Pore et al., 2006) or to direct anti-VEGF directed strategies (Winkler et al., 2004), one can envision that inhibiting SphK1/S1P signaling might also increase tumor oxygenation according to the concept of “vascular normalization” (Jain, 2005), and increase the efficacy of irradiation and chemotherapy. As aforementioned, the overproduction of VEGF and other proangiogenic factors (such as S1P) leads to the formation of an abnormal tumor vasculature, continuously remodeled and characterized by a marked increase in vessel leakiness, consequence of a decrease in interendothelial junctions, and loss of coverage by perivascular stromal cells, namely, pericytes, known to stabilize blood vessels (Goel et al., 2011). By improving connections between adjacent endothelial cells and increasing pericyte coverage, strategies designed to “normalize” vessels can improve delivery and efficacy of cytotoxic therapies in preclinical and clinical settings (Goel et al., 2011).

Mature endothelial cells are connected by adherens junctions including vascular endothelial (VE)–cadherin (Dejana, Tournier-Lasserve, & Weinstein, 2009), that foster vessel stabilization (Fig. 5.4). Downstream signaling from VEGF–VEGFR2 coupling promotes the weakening of VE–cadherin junctions, and thus a loosening of association of endothelial cells (Dejana et al., 2009).
2009). As a consequence, endothelial cells within hypoxic tumors are poorly connected, with less VE–cadherin junctions (Hashizume et al., 2000). Interestingly, despite the fact that S1P has originally been shown to promote endothelial cell integrity (Garcia et al., 2001; Lee et al., 1999) through S1P₁ signaling, it has been reported that S1P could increase vascular permeability (Sanchez et al., 2007) similar to VEGF, the canonical vascular permeability factor (Fig. 5.4). The mechanism of action involved the activation of the S1P₂ subtype S1P receptor and required the signal transduction of the Rho–ROCK–PTEN signaling. This observation was extended to the in vivo model of vascular permeability in the rat lung, in which the S1P₂ antagonist JTE013 significantly inhibited H₂O₂–induced permeability in the rat lung perfused model (Sanchez et al., 2007). Although S1P₁ receptor is regarded as the principal S1P receptor isoform expressed in VE cells (Michel, Mulders, Jongsma, Alewijnse, & Peters, 2007), in vivo studies have shown that S1P₂ was strongly upregulated under hypoxic stress in pathological angiogenesis of the mouse retina, establishing its essential role in pathological neovascularization (Skoura et al., 2007). One might hypothesize that the balance between S1P₁ and S1P₂ receptors in the endothelium could be altered under hypoxia, with a shift toward higher S1P₂ expression that could increase vascular permeability. With respect to S1P₂, the addition of exogenous S1P to normoxic endothelial cells also induces the activation of HIF-1 and subsequent rise in VEGF release, suggesting a potential amplification of VEGF signaling (Michaud et al., 2009). Last, S1P₂–deficient (S1P₂⁻/⁻) mice implanted with Lewis lung carcinoma or B16 melanoma cells displayed increased number of matured and functional tumor vessels, showing increased pericyte coverage (Du et al., 2010). Although it remains to demonstrate whether (or not) anti-S1P strategies might directly impact vascular permeability, it has been recently established that neutralizing exogenous S1P by using anti-S1P antibody could reduce plasma levels of VEGF in xenograft experimental models (Visentin et al., 2006).

4. CONCLUDING REMARKS

Since the discovery that HIF-1α was overexpressed in common human cancers, a large body of evidence has since decrypted many of the mechanisms and consequences of hypoxia-inducible factor overexpression during cancer progression and resistance to conventional therapeutics. Accordingly, the discovery and development of novel agents targeting hypoxia signaling pathways is an attractive area of cancer therapeutics. More studies will be needed to better understand the role of the SphK1/S1P
metabolism in regulating hypoxia-inducible factors-dependent pathways and provide opportunities for therapeutic intervention. Based on the currently available data, the inhibition/neutralization of the S1P signaling in hypoxic tumors is anticipated to interfere directly or indirectly with tumor cells, immune cells such as TAMs and the tumor vasculature. Of particular interest, interrupting the S1P signaling by reducing VEGF levels could be associated with a decrease vascular permeability resulting in normalization of tumor vessels, a prerequisite for improving delivery and efficacy of cytotoxic therapies.

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