EXPERT REVIEWS

The influence of pH and hypoxia on tumor metastasis

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Mariam Abaza and Yunus A Luqmani*

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, PO Box 24923, Safat 13110, Kuwait
**Author for correspondence:*
Tel.: +965 97608971
Fax: +965 24638689
yunus@hsc.edu.kw;
yluqmani@yahoo.com

Rapid malignant proliferation, prior to effective tumor neoangiogenesis, creates a microenvironment around solid cancers, which is predominantly hypoxic and characterized by a high interstitial fluid pressure. Presumably as an adaptive response, tumor cells favor metabolic activity with apparently inefficient energy output, and production of intermediates that promote cellular replication, preferentially through anaerobic glycolysis, a phenomenon that persists even in re-established normoxic conditions (anomalously referred to as 'aerobic glycolysis'). Extrusion of the consequently excessive accumulation of lactate and protons decreases extracellular pH, leading to a microenvironment considered conducive to promotion of tumor motility, invasion and metastasis, and one that will invariably influence response to drug treatment. This review will critically assess the evidence forming the basis of current understanding of the precise pH conditions in the extracellular tumor matrix, its regulation by cancer cells and relationship with hypoxia, its relevance to malignant progression and its exploitation for therapeutic advantage.

Keywords: aerobic glycolysis • cancer • hypoxia • metastasis • pH • Warburg effect

Metastasis is the principal cause of cancer morbidity, accounting for 90% of malignancy-related deaths from solid tumors [1]. During this process, most epithelial tumors will have an indeterminate proportion of cells in the primary lesion that acquire the ability to dissociate cell–cell adhesion, and undergo a process of modified cell configuration that allows a higher degree of mobility. Invasion through the basement membrane, frequently involving disruption of glandular structures, leads to contact with blood vessels forming around the tumor in response to angiogenic signals and results in intravasation into the circulatory system. To achieve this, such cells must first sever links with their site of origin and form new attachments to elements of the extracellular matrix (ECM) into which they move in an amoeboid fashion. They are able to do so by secreting a variety of proteinases and other matrix digesting enzymes that essentially clear a path for them. In some cases, such as breast neoplasms, the tumor cells may lose their normal asymmetric basolateral polarity and trans-differentiate into a more motile mesenchymal type cell (a process referred to as epithelial to mesenchymal transition or EMT). On the other hand, primitive neuroepithelial tumors including neuroblastoma, medulloblastoma and Ewing sarcoma do not initially seem to demonstrate asymmetric polarity as such but may do so afterward. Evidence for EMT is so far restricted to a few tissue types.

Those cells that survive the immune system finally extravasate from the blood and colonize into a secondary site [2], likely undergoing a reversal of the morphological transformation that allowed them to escape from their previous location and which now facilitates their survival as self-sustaining replicative secondary cancer colonies. Figure 1 illustrates some of the current alternative ideas. Trans-differentiated cells may regain epithelial characteristics (mesenchymal to epithelial transition) and polarity. The actual significance of changes in polarity, if any, is not clear.

The success of this hazardous journey is clearly dependent upon how well a cell interacts with the structural and dynamic components of a complex ECM [3]. It has long been apparent that the ECM, in addition to its rich content of fibrillar collagens, proteoglycans and various structural glycoproteins, also exhibits biochemical signaling capacity via communication networks extending into the cell through the action of an extensive reservoir of growth factors, enzymes and other factors that may themselves originate from cellular activity. Rapidly growing tumors that expand beyond the vascular supply

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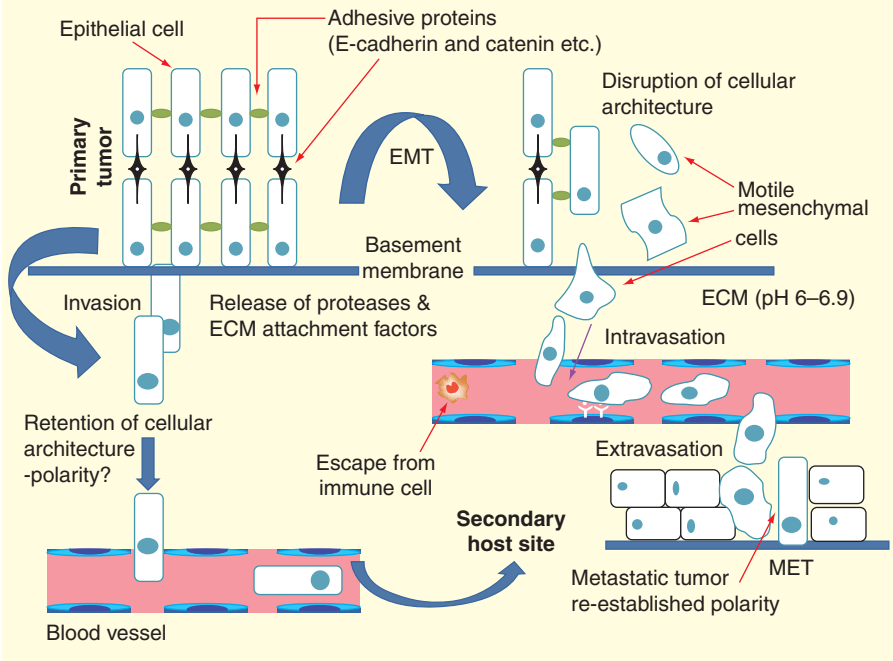

Figure 1. Cellular events leading to metastasis. During tumor progression, some cells disaggregate through loss of adhesive factors. They may either retain their features including polarity (which may or may not be subsequently altered) or trans-differentiate into more mobile mesenchymal-like cells. In either cases, they express and secrete a variety of factors allowing them to invade through the basement membrane, interact with the extracellular matrix and penetrate into growing blood vessels. Cells that escape immune destruction extravasate from the circulation into host tissues where they are thought to maintain or revert back to an epithelial type and start growing as a secondary deposit.
ECM: Extracellular matrix; EMT: Epithelial to mesenchymal transition; MET: Mesenchymal to epithelial transition.

of their tissue find themselves in an essentially hypoxic environment particularly at their core, which is eventually, if only partially, resolved by neoangiogenesis that results in raising of the interstitial fluid pressure (IFP) [4]. This with increasing extracellular acidity due to modified glucose metabolism, clearly distinguishes the tumor ECM from that which surrounds normal tissue. Tumors are able to adapt and flourish in this hostile environment by modulating expression of various genes that promote tumor growth, invasiveness, angiogenesis, metastasis and when confronted with therapeutic intervention, drug resistance. Currently, much research is focused on trying to understand how tumor cells interact with the ECM and determine how to exploit this special environment to improve the selectivity of the various treatment strategies to promote drug efficacy while minimizing undesirable side effects. One aspect of the extracellular environment that has until recently received relatively little attention is the pH, which can crucially affect both enzyme and receptor function as well as operation of ion channel transport proteins.

Tumor microenvironment

Tumor acidity

Tumor pH has been determined in animal models using non-invasive magnetic resonance spectroscopy, indicating a neutral to alkaline intracellular pH (pHi) of 7–7.4 similar to that of normal cells [5], with a more acidic extracellular pH (pHe) of 6–6.9, below the pHe around normal cells of 7.3–7.4. Several groups [5–9] have described the acidic microenvironment of tumors, which is thought to be due to the accumulation of lactic acid and protons derived from aerobic and anaerobic glycolysis and is sustained by the abnormal neovascularization of the tumor mass and absence of functional lymphatic vessels within large tumors. Interestingly, however, an *in vivo* study on rat glioma cells using spectroscopic nuclear magnetic resonance (NMR) imaging [10] showed significant spatial disparity between the intracellular sites of lactate accumulation and the pHe measured at the same and other sites. It may be that there is redistribution of protons along the cytoplasmic face of the plasma membrane to other proton transporters (discussed in later sections). This also raises the intriguing possibility of local fluctuations in pHe, which may provide insights into why some tumor cells are more prone to metastasis than others.

However, this may be, the increased capacity for transmembrane pH regulation further contributes to the lower pHe. Both intracellular neutralization of acid with bicarbonate and the pentose phosphate shunt release CO_2 from cells, contributing to lowering of the pHe. Hydrolysis of ATP in an energy-deficient environment also contributes to acidosis during hypoxia [11].

Tumor hypoxia

In a growing tumor mass, oxygen demand outstrips availability and hence, tumors exhibit a hypoxic environment. The reduction in oxygen delivery is the result of insufficient blood supply due to abnormally formed and poorly functional blood vessels found within most solid tumors, and an increased diffusion distance between blood vessels and oxygen-consuming cells [12]. Although reduced oxygen tension can be lethal for normal cells, many tumor cells adapt to this condition by activation of the transcriptional modulator HIF-1α [13]; this upregulates the expression of over 80 genes that are involved in glucose metabolism, cell survival, angiogenesis, cancer progression and invasion [14]. HIF-1α promotes angiogenesis by upregulating VEGF expression to stimulate endothelial cell proliferation to form new blood vessels [15]. In addition, it promotes cell proliferation and survival by targeting several growth factor genes such as insulin-like growth factor-2 and TGF-α [16]. Patients with a high proportion of hypoxic tumor cells in their primary

tumors have decreased disease-free and overall survival, probably due to a higher rate of metastasis, supporting the notion that hypoxia promotes a more aggressive, metastatic tumor phenotype [17].

Tumor IFP

The interstitium of a tissue is essential for molecular transport from blood vessels to cells and vice versa. As early as the 1950s, it was discovered that solid tumors have a high IFP with levels of up to 100 mmHg. It was suggested that three factors are involved in raising the IFP; the fenestrated tumor vasculature and lack of functional lymphatics, the osmotic forces that draw solutes into the tissue and the contractile characteristics of the tumor stroma. An elevated IFP can act as a barrier to efficient drug delivery due to the reduced transcapillary fluid flow into the tumor interstitium [7].

Adaptation of tumors to their microenvironment

Tumor cells can adapt and survive the stressful conditions of sustained low oxygen tension and high acidity in part by activating a process known as autophagy, in which cytoplasmic constituents are isolated from the rest of the cell within double-membrane vesicles (autophagosomes), which are then fused with lysosomes and their contents either degraded or recycled to sustain cellular metabolism [18] as illustrated in Figure 2. Autophagy can have a dual role in cancer; while it can suppress tumor growth by removing damaged proteins and organelles and limiting genomic instability [19], it can also promote the survival of cancer cells by maintaining cellular biosynthesis during nutrient deprivation and stress. Prevailing evidence indicates that the prosurvival autophagy has the predominant role in cancer and may result in therapeutic resistance and continued tumor growth [18]. Several treatment strategies exploiting autophagic dependence of cancer cells have been reported. Inhibition of the pro-survival autophagy by genetic knockdown of autophagy-related genes or pharmacological means in preclinical models was shown to be lethal to cancer cells and triggered apoptosis [20]. Moreover, the use of autophagy inhibitors in conjunction with chemotherapy can inhibit tumor growth and promote cell death more effectively than chemotherapy alone, both *in vitro* and *in vivo*. The autophagy inhibitors, chloroquine and hydroxychloroquine, have been used recently in preclinical studies and clinical cancer trials [21]. However, there is a need to develop more potent and specific inhibitors of autophagy since several of the existing drugs lack selectivity. Cellular 'cannibalism', which is increased in acidic conditions, is another feature relating to malignancy and aids in the survival of tumor cells despite nutrient deprivation through feeding off other cells, including T lymphocytes. This process is facilitated by reorganization of the actin cytoskeleton, which allows the formation of the 'cannibalistic vacuole' that has an efficient digestive machinery [22]. Suppressing the cannibalistic activity of cancerous cells by targeting its components provides a novel strategy in targeting tumors [23].

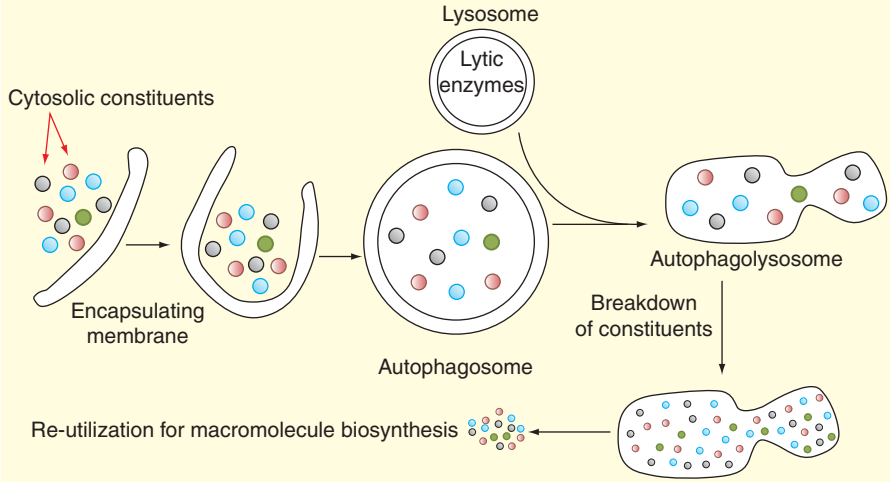

Figure 2. Scavenging and recycling of unwanted cell components through autophagy.

Major factors determining pHi/pHe in normal tissues & tumors

Warburg effect

In 1931, Otto Warburg reported that most solid tumors displayed a high rate of glucose utilization and underwent anaerobic glycolysis even in the presence of adequate oxygen supply (a phenomenon that has become commonly referred to as the 'Warburg effect' or anomalously 'aerobic glycolysis'). It is now generally accepted that tumors have considerably greater reliance on this metabolic pathway for the production of ATP than normal cells since mitochondrial oxidative phosphorylation assumes a relatively subsidiary role [24], although it nevertheless still remains a significant source of energy for the cancer cell. One of the identified mechanisms responsible for the high rate of glycolysis is overactivation of HIF-1α, which upregulates the expression of the glucose transporters GLUT1 and GLUT3 [13] and the transcription of several glycolytic enzymes [6]. There is also evidence that HIF-1α activates lactate dehydrogenase (LDH) [25], pyruvate dehydrogenase kinase-1 (PDK-1) [26] and PDK-3 [27]. Activation of PDK-1 and PDK-3 leads to the inhibition of pyruvate dehydrogenase (PDH) thereby reducing conversion of pyruvate to acetyl-coA and depriving the tricarboxylic acid (TCA) cycle of intermediates to generate reduced NAD/FAD for subsequent ATP generation through mitochondrial oxidative phosphorylation [27]. Therefore, conversion of pyruvate to lactate is enhanced. Developing drugs that target LDH isoenzymes could be a useful therapeutic approach since inhibition of this enzyme by short-

interfering RNA (siRNA) has been shown to reduce tumor growth and modify its behavior [28].

In some cancer cells, aerobic glycolysis may also be sustained by loss of tumor suppressors or activation of oncogenes particularly the immediate early response genes such as myc, jun and fos. Wang and colleagues [29] demonstrated differing roles for myc and HIF-1 in T-cell mitogenesis. A complex relationship between HIF-1α and N-myc has been described in neuroblastoma [30]. There is also considerable evidence for the involvement of miRNAs in regulation of glycolytic enzymes [31] both independently and through HIFs [32].

Another possible explanation for the tumor preference for aerobic glycolysis is its need for certain macromolecular precursors toward its inexorable aim of proliferation. These include ribose for nucleotides and glycolytic intermediates for synthesis of nonessential amino acids [9].

Mechanism of pHi control

Maintenance of pHi is crucial to prevent autodigestion of cancerous cells by the activation of lytic enzymes triggered by acidosis, which is due to the excessive lactate and proton production. Thus, there are several cell membrane transporters in tumor cells active in preventing cytosolic acidification and facilitating the maintenance of pHi within a narrow range. These regulators include vacuolar H^+-ATPases (V-ATPases), the Na^+/H^+ exchanger (NHE), bicarbonate transporters, monocarboxylate transporters (MCTs) and carbonic anhydrase (CA) [5].

V-ATPases

V-ATPases are ATP-dependent proton transporters that are ubiquitously expressed in the plasma membrane as well as in the membranes of several organelles including lysosomes, endosomes and secretory vesicles. Tumor cells with high metastatic potential appear to operate these pumps more than the other transporters, indicating the relative importance of V-ATPases in invasion and metastasis [33]. They pump protons into the extracellular space or into the lumen of organelles in order to maintain a relative neutral to alkaline pHi and an acidic pHe. V-ATPases contribute to multidrug resistance in cancer through several mechanisms; these include decreased drug internalization, neutralization of drugs either intra- or extracellularly and inhibition of apoptosis [201].

The NHE

NHE is an ion exchanger in the plasma membrane that leads to influx of Na^+ and efflux of H^+, driven by a Na^+ gradient across the cell membrane. However, when the pHi approaches a certain point, the antiport becomes inactive in spite of a large Na^+ gradient [34]. This transporter can be blocked by the diuretic drug amiloride and many of its analogs (such as the more specific and potent ethylisopropylamiloride) by competing for the Na^+ channel [35]. Several NHE inhibitors have completed Phase II/III clinical trials, but there are toxicity issues. Cariporide inhibited breast cancer cells *in vitro* but was associated with an unacceptable level of stroke observed during a Phase III clinical trial, leading to cessation of further clinical development [36]. There is a challenge to develop agents that selectively target NHE in tumors since this transporter is present in many tissues and its role in crucial physiological processes confers potential risk of side effects with the existing class of inhibitors [9].

Bicarbonate transporters

The bicarbonate transporters that aid in the regulation of pHi are the Na^+-dependent Cl^-/HCO_3^- exchanger (NDCBE), Na^+-independent Cl^-/HCO_3^- exchanger and Na^+/HCO_3^- co-transporter (NBC). The NDCBE protects from intracellular acidification by exchanging intracellular Cl^- with extracellular Na^+/HCO_3^- complex. However, the Na^+-independent Cl^-/HCO_3^- exchanger only plays a role in rare cases of intracellular alkalinization by extruding HCO_3^- out from the cells in exchange for Cl^-. All of these bicarbonate-dependent transporters are inhibited by 4,4′-diisothiocyanostilbene 2,2′-disulfonic acids [11].

MCTs

MCTs are among the most important regulators involved in pH homeostasis in cancer cells. Their isoforms 1–4 are responsible for the H^+-linked transport of monocarboxylates such as lactic acid across the plasma membrane [37]. However, within different parts of the tumor mass, there will be simultaneously both normoxic and hypoxic conditions. Cells in the latter environment produce excessive amounts of lactate, which are extruded from the cell by MCT4 and taken up by nearby cells that have a normoxic environment by MCT1. The 'normoxic' cells then utilize this lactate for conversion to intermediates that enter the TCA cycle and generate ATP through oxidative phosphorylation, in addition to their reliance on aerobic glycolysis (Figure 3). Interference with glucose/lactate utilization may be an effective therapeutic strategy for the treatment of 'hypoxic' tumors. This model emphasizes the fact that tumors cannot be considered therapeutically as a single target; even metabolically, they appear to have a diversity of bioenergetic capacity that displays a high degree of plasticity and adaptation in response to external conditions. Monotherapy with metabolic inhibitors is therefore likely to meet with limited success. The inhibition of MCT1 and MCT4 was found to reduce the lactate-fuelled respiration and render the 'normoxic' tumor cells more dependent on aerobic glycolysis for ATP generation. This strategy results in the death of 'hypoxic' tumor cells by glucose starvation [38]. MCTs can be inhibited by pyruvate and other substituted monocarboxylic acids and by bioflavonoids such as quercetin [39].

CA

To date, 13 active isoforms of CA have been described. Some of them (CA1, 2, 3, 7) are expressed in the cytoplasm, whereas others (CA4, 9, 12, 14, 15) are localized to the cell membrane. Tumor-associated forms as well as their inhibitors have been extensively discussed in a previous review [40]. Hypoxia leads to

overexpression of CA9 and CA12 in tumor cells [9]. CA9 has been reported to play an important role in hypoxic breast tumors and to be an independent prognostic indicator of distant metastases and survival [41] by allowing propagation of cancer cells in hypoxic conditions. These isoenzymes have an extracellular catalytic site and cause hydration of CO_2 molecules, which exit cells through aquaporins, to form HCO_3^- and H^+. The HCO_3^- are recycled back into the cell via the NDCBE while the H^+ get trapped in the extracellular space, contributing to its acidity [42]. Figure 4 illustrates the major ion channel membrane transporters that are involved in the maintenance of ion fluxes to regulate pH in tumor cells; potential inhibitors of these proteins are indicated.

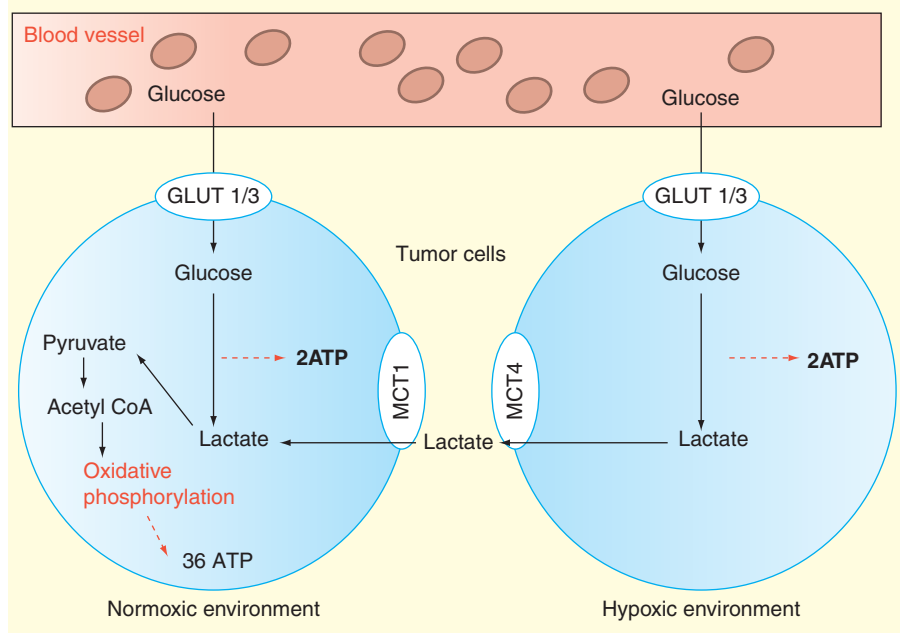

Figure 3. Glucose/lactate utilization in 'hypoxic' and 'normoxic' tumor cells.
Lactate produced and secreted by oxygen-deprived cells can be utilized by other tumor cells that have adequate oxygen, reducing their dependence on glucose uptake.
MCT: Monocarboxylate transporter.

Methods to assess the pH of tumors & their microenvironment

Key approaches that have been used to determine pHi and pHe include pH-sensitive microelectrodes, NMR and pH-sensitive cell penetrating fluorescent dyes [43–45]. To date, pH measurement approaches have been applied with some NMR nuclei such as ^1H, ^{31}P, ^{19}F and ^{13}C. One study [10] used *in vivo* ^1H spectroscopic imaging to perform spatial localization of both pH and metabolites as mentioned earlier. However, the most commonly reported and technically less demanding method for measuring pHi is with a pH-sensitive fluorescent dye, of which the most popular is 2′,7′-*bis*(carboxyethyl)-5(and-6-)-carboxyfluorescein [46]. A linear relationship between its fluorescence intensity and pHi exists in the range of pH 6.5–7.5. The fluorescence intensity can be converted to pHi units by reference to a calibration curve [39].

Role of tumor microenvironment in promoting metastasis

Effect of low pH on metastasis

The low pHe that is generated by the transporters mentioned above, of which V-ATPases appear to play the most important role in metastatic progression, enhances the secretion and activation of proteolytic enzymes that include matrix metalloproteinases, bone morphogenetic protein-1-type metalloproteinases, tissue serine proteases, adamalysin-related membrane proteases, cathepsins B, D and L and gelatinases. These enzymes cause degradation and remodeling of the ECM to facilitate cancer invasion and metastasis [2,47]. Furthermore, the activity of intracellular V-ATPases promotes the migration of secretory vesicles and lysosomes containing the proteases toward the cell surface for exocytosis [48]. The presence of hypoxia and low pHe may also enhance the expression of some angiogenic factors, such as VEGF, basic fibroblast growth factor, PDGF and IL-8, which promote neovascularization that leads to increased tumor growth and metastasis [11]. Moreover, induction of genomic instability or epigenetic regulation of gene expression can lead to metastasis of tumor cells in acidic environment. Another possible mechanism of tumor metastasis in low pHe is through the presence of lactate, which enhances the expression of hyaluronan and hyaluronidase, important components of increased invasion and metastasis [49]. It has also been reported that acidic conditions can increase formation of filopodia, which are cytoplasmic projections that aid in cell locomotion [50].

Effect of reduced oxygen tension on metastasis

Tumor hypoxia aids in malignant progression by causing inactivation of suppressor genes, activation of oncogenes and alteration in gene expression [12]. Examples of some proteins that are upregulated by hypoxia and play an essential role in invasion and metastasis are cathepsin D, matrix metalloproteinase 2, urokinase plasminogen-activated receptor, fibronectin 1, keratins 14, 18 and 19, vimentin, transforming growth factor alpha and autocrine motility factor [16]. Moreover, hypoxia promotes genomic instability through point mutations, gene amplification and chromosomal re-arrangement and results in clonal selection [12].

EMT

The E-cadherin–catenin complex plays an important role in epithelial cell–cell adhesion and in the maintenance of

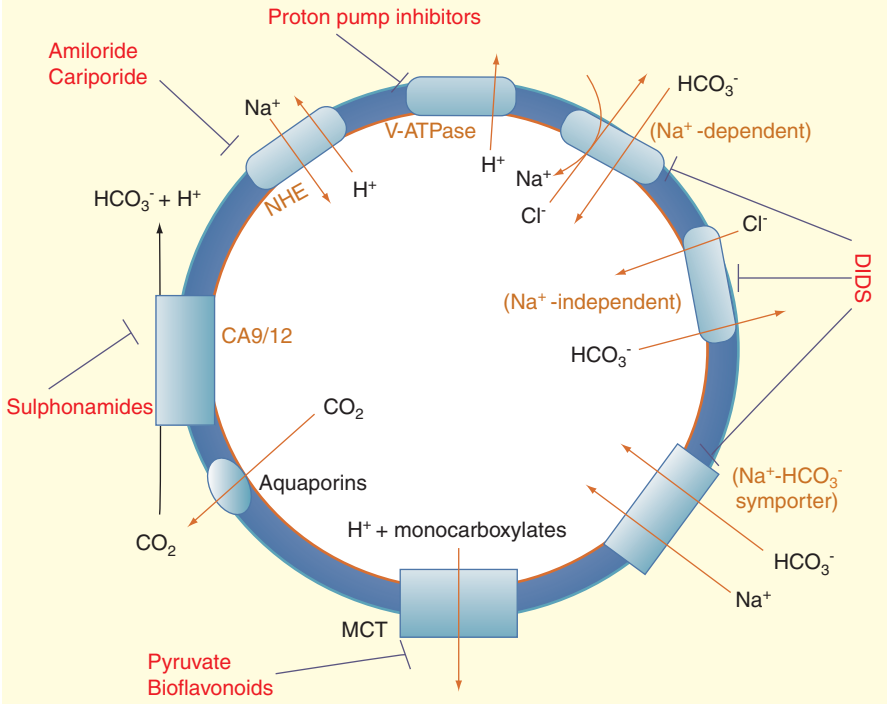

Figure 4. Major ion channel membrane transporters regulating ion fluxes to maintain pH in tumor cells and potential inhibitors.
CA: Carbonic anhydrase; DIDS: 4,4′-diisothiocyanostilbene 2,2′-disulfonic acids; MCT: Monocarboxylate transporter; NHE: Na^+/H^+ exchanger; V-ATPase: Vacuolar H^+-ATPase.

tissue architecture [51]. However, during hypoxia, activation of the lysyl oxidase–Snail pathway results in deregulation of E-cadherin expression. Inactivation of E-cadherin is an important feature of EMT, which is thought to promote tumor progression and metastasis by converting the nonmotile epithelial cells to a more motile mesenchymal type of cell. We have discussed this in more detail previously [52].

Influence of tumor microenvironment on anticancer immunity

Despite the fact that cancer cells produce proteins presented by the major histocompatibility complex-I/II and should prompt immune destruction, cancers can inhibit immune function by several mechanisms that include the presence of lactic acid, low pHe and hypoxia [8]. Hypoxia prevents the presentation of antigens to T cells and suppresses the antitumor effects of macrophages [53]. Lactic acid impairs immunity by inhibiting polymorphonuclear leukocytes, chemotaxis and respiratory activity. Additionally, it hinders proliferation and cytokine production of human cytotoxic T lymphocytes and suppresses the T-cell response to tumor-associated antigen [54]. Furthermore, the low pHe inhibits a subset of cytolytic T cells and suppresses the tumoricidal activity of natural killer cells [55]. Both lactate and acidic pHe can inhibit the production of cytokines such as tumor necrosis factor from monocytes [56]. Therefore, raising the pHe of tumors could restore immune cell function and antitumor response.

Contribution of tumor microenvironment to therapeutic resistance

Tumor acidity

The acidic microenvironment has been shown to alter tumor response to various treatment strategies such as radiotherapy, chemotherapy and hyperthermia. Radioresistance is potentiated in low pHe due to the prolonged radiation-induced G_2 arrest, which results in increased DNA damage repair [57]. On the contrary, the acidic environment can enhance cell death induced by thermal damage and photodynamic therapy [47]. The acidic pHe increases the uptake and efficacy of weakly acidic drugs such as cyclophosphamide [58], but decreases that of weakly basic drugs such as doxorubicin [59] and vinblastine [60]. This is due to the fact that drugs cross the plasma membrane in their neutral form, and since the basic drugs will get protonated in an acidic environment, this will result in their accumulation either in the acidic tumor microenvironment or in the lumen of acidic intracellular vesicles. Despite that the acidic pHe may enhance tumor response to acidic chemotherapy drugs, hyperthermia and photodynamic therapy, deliberate tumor acidification can lead to undesired tumor metastasis via the mechanisms mentioned earlier.

Tumor hypoxia

Hypoxia leads to radioresistance since radiotherapy demands oxygen in order to produce cytotoxic-free radicals that damage DNA and to sustain the damage. Hypoxia may also contribute to several types of chemotherapy resistance via multiple mechanisms. One of them is the suppression of the effect of drugs that need oxygen for optimal efficacy; these drugs include mephalan, bleomycin and etoposide [61]. Additionally, it will affect the drugs that are more active against dividing cells since extreme hypoxia will decrease the rate of cell division causing cell cycle arrest [62]. Hypoxia also results in the selection of apoptosis-resistant cells in tumors [63].

Manipulation of tumor pH as a potential therapeutic strategy

Since both pHi and pHe are altered during malignancy in order to promote cancer progression and survival, manipulating those pH levels may have considerable potential in cancer therapy. Some of these strategies include alkalinization of the external environment, administration of V-ATPase inhibitors and rapid intracellular acidification. Other strategies that have been adopted entail acidification of the extracellular space, but the disadvantage of this has already been mentioned [64].

Systemic buffering to increase pHe

Systemic buffering achieved by oral administration of sodium bicarbonate, trisodium citrate or a low-to-moderate protein diet but high in potassium-rich fruits, vegetables and juices selectively raises the pHe of tumors without affecting their pHi or the pH of blood or normal tissues [64]. This suggests that there could be a physiological buffer that prevents the alteration of pH in these tissues. Although alkalinization therapy had no effect on the growth rate of primary tumors when it was tested on nude mice implanted with a human breast cancer, there was a significant reduction in the size and number of metastasis. Moreover, the administration of sodium bicarbonate reduced the colonization of lymph nodes but with no influence on the circulating tumor cells. This observation suggests that alkalinization therapy suppresses metastasis by inhibiting 'extravasation' and colonization while not influencing 'intravasation' [2].

V-ATPases inhibitors

In view of the contribution of V-ATPases in metastasis, several approaches are being developed to target them as a new strategy against cancer. One of the procedures is to use siRNA to silence the expression of the V-ATPase c subunit gene (ATP6L) [65]. Although bafilomycins, which are inhibitors of V-ATPases, can induce apoptosis, they are tissue nonselective and result in unacceptable toxicity [66]. The similarity between V-ATPases and the gastric proton pump (H^+/K^+ ATPase) has prompted the use of proton pump inhibitors (PPIs), such as omeprazole and esomeprazole, for inhibiting V-ATPases. PPIs work by binding irreversibly to V-ATPases and inhibiting proton translocation; this results in lowering of the pHi while elevating the pHe. Since PPIs are prodrugs that need acidic conditions for their activation, their use will be associated with minimal toxicity due to their selectivity to the acidic tumor microenvironment despite that V-ATPases are ubiquitously expressed [67]. PPIs will lead to tumor self-digestion via the acidification of the cytosol, which leads to the activation of proteases and other lytic enzymes. Moreover, achieving a low pHi enhances the killing efficacy of hyperthermia [68] and the apoptotic response to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [69]. PPIs and other inhibitors of V-ATPases also suppress tumors from proliferating by reducing their cannibalism and blocking their survival. It has been shown that pretreatment with PPIs both *in vivo* and *in vitro* leads to chemosensitization of tumor cells by reversion of the pH gradients, overcoming tumor resistance to basic drugs [70].

Intra- & extracellular hyperacidification therapy

Acute intracellular acidification can kill cancer cells either directly by inducing apoptosis or by increasing their sensitivity to other cytotoxic agents whose activity is greater at acidic pH. Furthermore, low pHi will enhance the lethal effect of hyperthermia and TRAIL as mentioned earlier. Hyperacidification of the intracellular compartment can be achieved by the combination of high-dose intravenous glucose infusion, PPI and dinitrophenol. Hyperglycemia will boost tumor glycolysis resulting in the production of lactic acid and protons that will be retained intracellularly due to the effect of PPI. Dinitrophenol can further promote glycolysis by blocking mitochondrial ATP production, which can also be inhibited by meta-iodobenzylguanidine [64]. However, one limitation of this strategy is that tumors have several mechanisms to expel protons to the extracellular space. Therefore, it is not warranted to maintain intracellular acidification by just blocking V-ATPases. Acutely maximizing extracellular acidity is another therapeutic approach. This strategy allows tumor-selective delivery of drugs from pH-sensitive nanoparticles, which can be specifically designed to breakdown or fuse with cell membranes under acidic conditions [71]. Extracellular hyperacidification can be accomplished in the same way as intracellular acidification, but without the use of PPIs to allow the transfer of protons into the extracellular space.

Other new approaches for cancer therapy

Potential strategies to manipulate glycolysis

In recent years, there has been renewed interest in targeting tumor glycolysis as a therapeutic approach. Inhibition of the glycolytic pathway would deprive cancer cells with mitochondrial defects of ATP. This would block cell cycle progression and lead to apoptosis. A strategy that is currently under investigation in clinical trials is the inhibition of key glycolytic enzymes such as hexokinase (HK), phosphofructokinase and pyruvate kinase [72]. Lonidamine, a HK inhibitor, is already being used in some chemotherapy protocols [73]. Additionally, 2-deoxyglucose, a nonmetabolized analog of glucose [74] and 3-bromopyruvate are examples of HKII inhibitors [75] that can initiate cell death by inhibition of glycolysis and ATP deprivation. Moreover, the glycolytic enzymes can be inhibited indirectly via suppression of HIF-1α. Topotecan inhibits HIF-1α translation by a DNA damage-independent mechanism [76] and is now approved by the U.S. Food and Drugs Administration as a second-line therapy for small cell lung cancer and ovarian neoplasms [6].

Another approach is to enhance oxidative phosphorylation in order to decrease cellular dependence on *aerobic glycolysis*, increase pHe and induce cell apoptosis by the production of reactive oxygen species (ROS). For example, dichloroacetate (DCA) is currently in three ongoing Phase I/II clinical trials for patients with recurrent and/or metastatic solid tumors, glioblastoma multiforme and malignant gliomas [6]. This drug exerts its effect through two pathways, mitochondrial and plasmalemmal as illustrated in Figure 5. Stimulation of the mitochondrial pathway results from inhibition of PDK. This will activate PDH leading to a shift of metabolism from pyruvate to lactate in the glycolytic cascade toward the production of acetyl-CoA, which enters the TCA cycle and drives oxidative phosphorylation [77]. This leads to a reduction in the mitochondrial membrane potential and reopens the mitochondrial transition pores resulting in the release of proapoptotic factors such as cytochrome c and AIF, which will upregulate caspases and

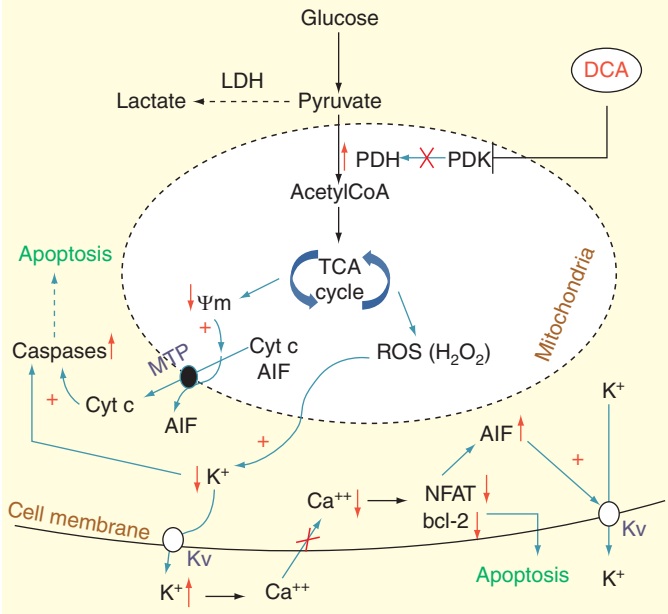

Figure 5. Effect of DCA in promoting oxidative phosphorylation in tumor cells. DCA inhibits PDK leading to enhanced PDH activity (and thereby indirectly reducing lactate production), promoting conversion of pyruvate to acetyl-CoA for entry into the TCA cycle. This decreases Ψm leading to increased release of Cyt c/AIF through MTP, and increases ROS production that leads to extrusion of K^+ to the extracellular space. High extracellular K^+ blocks Ca^{++} uptake leading to low intracellular Ca^{++} and decreased NFAT/bcl-2 activity, promoting apoptosis. Decreased NFAT relieves its transcriptional repression of AIF whose increased expression promotes upregulation of voltage gated K^+ channels resulting in exit of K^+ from the cell. Increased Cyt c and lower intracellular K^+ activates caspase-mediated apoptosis.
DCA: Dichloroacetate; LDH: Lactate dehydrogenase; Kv: Voltage-gated K^+ channels; Ψm: Mitochondrial membrane potential; MTP: Mitochondrial transition pores; NFAT: Nuclear factor of activated T cells; PDH: Pyruvate dehydrogenase; PDK: Pyruvate dehydrogenase kinase; ROS: Reactive oxygen species; TCA: Tricarboxylic acid.

promote K^+ efflux from the cell. Apoptosis will also be induced by the release of ROS as a by-product of oxidative phosphorylation [78]. Furthermore, it has been proposed that DCA causes cell apoptosis via the mitochondrial pathway by upregulating the expression of the proapoptotic protein p53 upregulated modulator of apoptosis [79]. The plasmalemmal pathway involves the activation of voltage-gated K^+ (Kv) channels in the plasma membrane by ROS. The lowered intracellular K^+, due to efflux from the cell, leads to activation of caspases that play an essential role in cell apoptosis. Moreover, the expulsion of K^+ results in membrane hyperpolarization and closure of Ca^{++} channels. The decrease in Ca^{++} entry inhibits the Ca^{++}-dependent transcriptional modulator nuclear factor of activated T cells (NFAT) suppressing the transcription of antiapoptotic bcl-2 [78]. Although DCA selectively targets tumor cells and is reported to be relatively safe, it is not effective against all cancer cells, and further studies have to be conducted to determine its selectivity [77].

HIF-1α inhibitors

Considering the diverse influence on energy metabolism and membrane transporters of HIF-1α in the response of tumor cells to hypoxia (as illustrated in Figure 6), it has obvious attractions as a therapeutic target. Several strategies to inhibit its activity include suppression of mRNA expression, protein translation and DNA binding and transcriptional activity and also by increasing its degradation [80]. HIF-1α expression increases in response to activation of growth factor receptors [81]. Gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, is effective in suppressing HIF-1α induction and VEGF release from some tumors [82]. Inhibiting HIF-1α mRNA expression can be achieved by aminoflavone and targeting it with antisense oligonucleotides. Although not HIF-1α specific, the following drugs have been used; doxorubicin and daunorubicin to block its binding to the hypoxia response elements, bortezomib to inhibit its transcriptional activity and HSP90 inhibitors and microtubule targeting agents such as 17 B 2-methoxyestradiol to induce its degradation [80]. On the other hand, PX-478 is a selective HIF-1α translation inhibitor and has demonstrable activity in several xenograft models [83]. It is currently in Phase I clinical trials for advanced solid tumors and lymphomas [6]. Acriflavine and digoxin are two more recent HIF-1α inhibitors that reduce the hypoxia-induced expression of lysyl oxidase and lysyl oxidase-like proteins which both play a role in metastasis [53].

CA inhibitors

Overexpression of CA9 in tumor cells has been targeted with isotopically labeled antibodies, prodrug activation systems and vaccines to stimulate T-cell responses [84]. Selective sulphonamides that target CA9 were shown to be effective in reducing the growth of primary tumor and metastasis in a mouse model of breast cancer [9]; other specific CA9 inhibitors are likely candidates for clinical trials [85]. Several sulfamate compounds have been successful in reducing breast metastatic tumors generated in mice [86]. Both mouse and human preclinical models show function requirement for CA9 [41] indicating its potential as a therapeutic target. It is uncertain whether blocking the extracellular enzyme alone is sufficient or whether there may be additional synergistic benefit in including intracellular CA inhibitors.

Deacetylation inhibitors

Hypoxia stimulates the expression of histone deacetylases. These are a class of enzymes that remove acetyl groups from an ε-*N*-acetyl lysine amino acid on a histone, allowing the histones to wrap the DNA more tightly. Inhibition of these enzymes will result in tumor cell death by interfering with the regulation of HIF-1α, including its transcriptional activity and protein degradation [87]. The first clinical antitumor agent of this class is suberoylanilide hydroxamic acid, which has been shown to be beneficial in treating breast cancer [53], with *N*-hydroxy7-(2-naphthylthio) heptanomide being another inhibitor of histone deacetylases with antitumor activity both *in vitro* and *in vivo* [88].

Tumor suppressor p53

The tumor suppressor p53 protein binds directly to the oxygen-dependent degradation domain of HIF-1α leading to its degradation by the proteasomal pathway [89]. A large proportion of epithelial cancers produce a dysfunctional mutant p53 protein making this an attractive target. The pharmacological p53 reactivator strategies that are currently being explored in cancer treatment include nutlin-3, p53 reactivation and induction of massive apoptosis (PRIMA-1) and reactivation of p53 and induction of tumor cell apoptosis [53]. The latter has been shown to inhibit tumor growth and induce p53-dependent apoptosis *in vivo* [90], whereas PRIMA-1 inhibits human breast cancer cells, both *in vitro* and *in vivo* [91]. Nutlin-3 restores the p53 suppression pathway by antagonizing murine double minute 2, also known as E3 ubiquitin-protein ligase, which causes degradation of wild-type p53 [92].

Exploiting the tumor microenvironment for delivery of anti-cancer drugs

Since most of the side effects experienced during the usage of conventional chemotherapeutic agents are due to their nonspecific systemic distribution, development of drug delivery systems able to selectively target cancer cells would be beneficial. Examples of such are the pH low insertion peptides nanotechnology (as mentioned earlier) and the use of acridine orange, which selectively accumulates within tumor tissues due to reversed pH gradients. This then gets activated within cancers by photodynamic and radiation therapy, exerting a very selective cytotoxic effect [93]. Another approach is the development of prodrugs that only become activated to cytotoxic metabolites at low oxygen tension. In this respect, tirapazamine, an hypoxia-activated cytotoxin, has advanced the furthest in a clinical setting and has been shown to reduce metastasis when given as neoadjuvant to radiation therapy in mice [94]. Alkylaminoanthraquinone N-oxide is a hypoxia-activated cytotoxin with preclinical activity against primary tumors [95]. Another emerging agent, 3,5-dinitrobenzamide-2-mustard PR 104, with potential activity against both primary and metastatic tumor cells [1] has completed a Phase I clinical trial [96].

Angiogenesis & IFP

As mentioned earlier, VEGF released by tumors encourages neovascularization, but the resultant blood vessels are generally malformed. Furthermore, VEGF is not only responsible for the formation of new blood vessels, but it also results in a leaky vasculature (hence, it is also known as vascular permeability factor) [97]. This leakage will result in an elevated IFP impairing efficient drug delivery. In such cases, inhibition of VEGF can result in some normalization of vessels enhancing oxygenation, chemotherapeutic drug penetration and sensitization of cancer cells to radiotherapy [53]. Bevacizumab, a monoclonal antibody that targets VEGF, can be used to decrease the elevated IFP through vascular normalization [98] and was shown to increase responsiveness to chemotherapy and delay tumor progression. However, it did not change overall survival rates [99]. Long-term

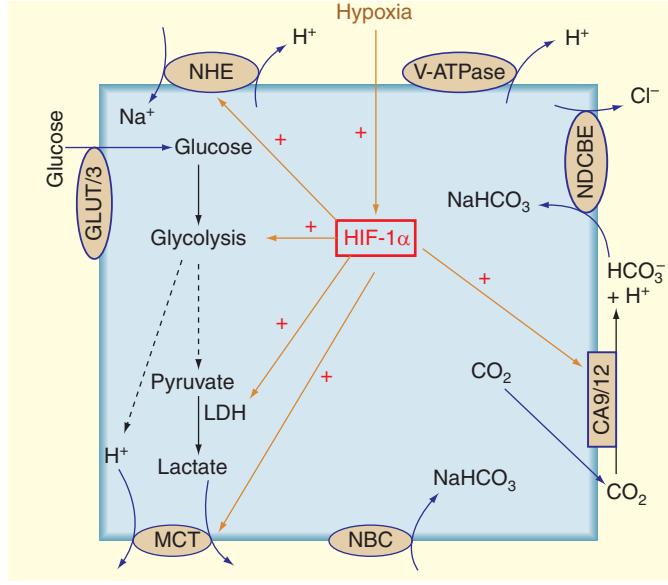

Figure 6. Cellular effects of HIF-1α initiated by hypoxia in tumor cells, regulating energy metabolism and ion transport.

CA: Carbonic anhydrase; HIF-1α: Hypoxia-induced factor-1 alpha; LDH: Lactate dehydrogenase; MCT: Monocarboxylate transporter; NBC: Na^+/HCO_3^- co-transporter; NDCBE: Na^+-dependent Cl^-/HCO_3^- exchanger; NHE: Na^+/H^+ exchanger; V-ATPase: Vacuolar H^+-ATPase.

effects of bevacizumab treatment actually suggest an induction of tumor hypoxia, presumably as VEGF is no longer available for endothelial growth, which may lead to resistance [53].

Targeting stromal fibroblast–ECM interaction has proved successful in preclinical models. *In vitro* studies suggest that PDGF is involved in modulating stromal contraction. The PDGF receptor inhibitor, Imatinib, decreases IFP [100] and, in combination with epothilone B, taxol, 5-fluorouracil and radiolabeled antibodies, improves drug delivery and efficacy [101–103] as illustrated in Figure 7. However, this effect was achieved only when the PDGF inhibitors were administered prior to chemotherapy and not when given simultaneously [7].

Expert commentary

Rapid proliferation, in the absence of adequate blood supply, creates a microenvironment around solid cancers, which is predominantly hypoxic and characterized by a high IFP. Promotion of neoangiogenesis is a peculiar feature of cancers, that through an albeit malformed vasculature can re-establish a normoxic environment, allowing them to adapt and survive under these conditions by various mechanisms, including oncogenic activation of glycolysis either independently or through the activation of hypoxia induced factor-1 alpha, miRNA control of glycolytic enzymes, autophagy and cannibalism for efficient recycling of cellular components. Indeed, tumors even appear to take advantage of a mixed hypoxic/normoxic environment for efficient utilization of energy yielding substrates between different parts of the tumor, demonstrating their remarkable ability to exploit

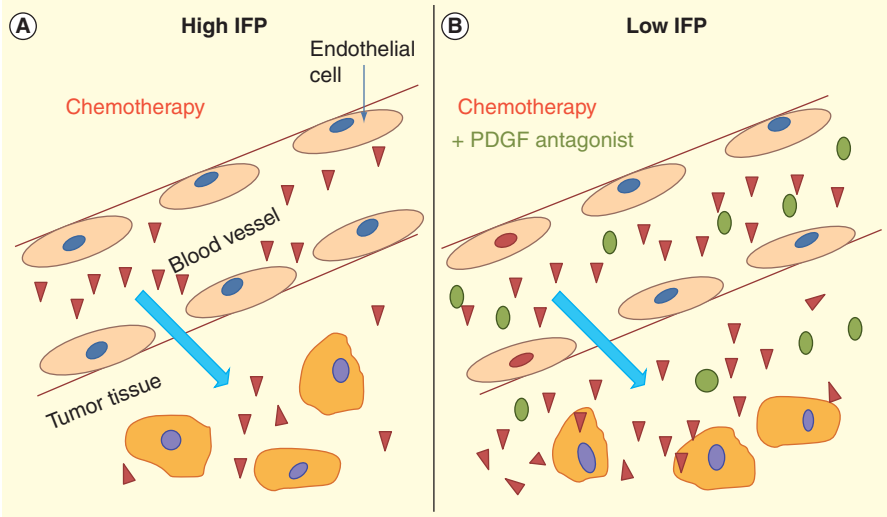

Figure 7. Effect of interstitial fluid pressure on drug uptake into tumor cells from the circulation. Under conditions of high IFP, drug uptake is inefficient; however, this can be enhanced by the simultaneous administration of PDGF that lowers the IFP. Oval represents PDGF antagonist; inverted triangle represents chemotherapy drug.
IFP: Interstitial fluid pressure.

changing environmental conditions commensurate with growth and increasing heterogeneity of the tumor mass.

It has also long been recognized that cancers, unlike normal cells, derive metabolic energy and intermediates geared toward cellular replication, preferentially from anaerobic glycolysis even in re-established normoxic conditions (anomalously referred to as 'aerobic glycolysis') with reduced dependence upon ATP production through oxidative phosphorylation. The consequent excessive accumulation of lactate and protons necessitates efficient redistribution of these and other molecules from and within the cell in order to maintain an appropriate pHi. The consensus view from pH measurements in tissues, using microelectrodes, pH-sensitive penetrant dyes and NMR, are that the tumor microenvironment is generally more acidic than that around normal tissues. As a cautionary note, however, it should be said that there is insufficient information in the literature regarding the reliability or accuracy of these methods. There is also assumption of uniformity of the extracellular space on which at least one study has cast doubt. There is a paucity of studies examining the effect of pH on *in vitro* model systems. Notwithstanding this, it is the prevailing view from animal studies that a low pH around tumors stimulates their progression and metastasis. Recent clinical data would appear to support this conclusion as ingested alkalinizing buffers have been reported to lead to a reduction in metastasis, presumably through neutralization of tumor acidity. Several key regulators of pH have been identified that include V-ATPases, the NHE, bicarbonate transporters, MCTs and the CA enzyme, whose activity is considered to be responsible for generating extracellular acidity.

Better understanding of the precise pH conditions prevalent in the extracellular tumor matrix would be helpful to the design of targeted therapies that avoid harmful effects to surrounding normal tissues. Two general types of therapeutic approaches are being investigated to exploit the tumor microenvironment to either develop drug delivery systems that are effective under acidic conditions to either selectively target cancer cells or maximize uptake, or by reversing tumor pH to retard metastasis. Several studies have also suggested the possibility of targeting the aforementioned pH-dependent transporters. Additionally, there is resurgent interest in strategies aimed at diverting tumor metabolism away from overproduction of lactate by interfering with earlier events of glycolysis; in a short therapeutic window, normal cells may be able to sustain themselves without glucose through lipid metabolism, and the brain could be protected if the drug is unable to penetrate the blood brain barrier.

As a final note, not mentioned in the general discussion due to the preliminary nature of the findings [104], we have data showing that an alkaline environment (as opposed to an acidic one) increases the invasive capacity of siRNA-mediated estrogen receptor (ER) silenced breast cancer cells while having no effect on the parental ER expressing cells. Although apparently at variance with the consensus view of external acidity promoting metastasis, this may be a peculiar adaptation of endocrine-resistant cells, which requires further investigation.

Five-year view

Despite considerable improvements in the design of cytotoxic drugs resulting in reduced side effects, the efficacy of chemotherapy of cancer remains a major challenge. Several alternative strategies that include antihormonal therapies for endocrine tumors, and various biologic approaches utilizing monoclonal antibodies against growth factor receptors or small molecule inhibitors of signaling pathways or recruitment of immune cells, all have advantages in terms of specificity. In recent years, the realization that tumor cells may also be indirectly targeted through nutritional deprivation has led to the development of antiangiogenic drugs. Now there is resurgent interest in the mechanics of tumor cell dispersion and the composition and role of the ECM in metastasis. This is largely focusing on immune-associated molecules and identification of proteolytic enzymes and ECM attachment factors. Less attention has been paid to the influence of pH particularly within the ECM, but it is likely that this will change, particularly as a result of increasing studies identifying possible targets in the glycolytic pathway to divert tumor metabolism away from lactate production with a view to normalizing extracellular pH. Enzymes are critically dependent on pH, regulated or generated through function of ion channels. Therefore, a more precise

understanding of how these channels operate and how their activity can be modified may provide another indirect means of limiting tumor progression; possibly introduction of ATPase inhibitors. Changing the pH could limit the activity of participant enzymes and we may see clinical trials with alkalinizing therapies geared toward this aim. Another avenue would be to modify existing cytotoxic agents such that their activation (of prodrugs) and/or uptake is enhanced in the particular prevailing pH environment around tumor cells. In this respect, the application of nanotechnology for drug delivery will certainly have good potential for exploiting the pH difference between normal and tumor cell matrix. This will need development of better methods of pH measurement. Our own recent data on the effects of pH on an endocrine-resistant cellular model of breast cancer suggest that it can have quite dramatic effects on cell behavior *in vitro,* without corresponding effect on either normal or hormone-sensitive cells. Although the current consensus favors the idea of an acidic extracellular pH surrounding tumors, generated in large measure by extrusion of lactate, further direct studies are needed to determine whether this is indeed the case. pH-modifying strategies could be useful in combination with existing therapies. The increasing interest in, and application, of proteomics, will add to our knowledge of the role of channel proteins in cancer cell metabolism and their involvement in metastasis.

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Key issues

- Rapid proliferation, without adequate blood supply, creates a microenvironment around solid cancers that is predominantly hypoxic and characterized by a high IFP.
- Cancers, unlike normal cells, derive metabolic energy preferentially from anaerobic glycolysis, even under a normoxic environment (Warburg phenomenon); however, reduced dependence on oxidative phosphorylation may only be a feature of some not all cells within a heterogeneous tumor.
- The excessive accumulation of lactate and protons derived from glycolysis necessitates efficient redistribution of these and other molecules from and within the cell in order to maintain an appropriate intracellular pH.
- Several key regulators of this process include V-ATPases, the NHE, bicarbonate transporters, MCTs and CA, whose activity is considered to be responsible for generating extracellular acidity.
- Tumor cells are able to adapt and survive under their hostile microenvironment in part by oncogenic activation of glycolytic enzymes, upregulating the expression of HIF-1α and by activating autophagy and cannibalism for efficient recycling of degraded cytoplasmic constituents, reducing dependence on vascular supply of external substrates.
- The peculiar features of the tumor microenvironment are key factors in promoting tumor progression, invasion and metastasis and of course response to therapeutic intervention.
- Reversal or manipulation of pH may be a strategy to modify the extracellular environment such that it impedes the movement of cancer cells into it and thereby blocks access to the vascular system.
- Manipulation of extracellular and/or intracellular pH of tumors could facilitate drug delivery by tailoring molecular structures for maximal uptake under prevailing ionic conditions.
- Several studies have suggested the potential advantages of targeting selected pH-dependent channel transporters as a new strategy in cancer treatment as these can influence the ability of cells to metastasize. Particular targets may be those channels involved in the movement of protons and lactate across the plasma membrane.

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