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pH Control Mechanisms of Tumor Survival and Growth

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A distinguishing phenotype of solid tumors is the presence of an alkaline cellular feature despite the surrounding acidic microenvironment. This phenotypic characteristic of tumors, originally described by Otto Warburg, arises due to alterations in metabolism of solid tumors. Hypoxic regions of solid tumors develop due to poor vascularization and in turn regulate the expression of numerous genes via the transcription factor HIF-1. Ultimately, the tumor microenvironment directs the development of tumor cells adapted to survive in an acidic surrounding where normal cells perish. The provision of unique pH characteristics in tumor cells provides a defining trait that has led to the pursuit of treatments that target metabolism, hypoxia, and pH-related mechanisms to selectively kill cancer cells. Numerous studies over the past decade involving the cancer-specific carbonic anhydrase IX have re-kindled an interest in pH disruption-based therapies. Although an acidification of the intracellular compartment is established as a means to induce normal cell death, the defining role of acid–base disturbances in tumor physiology and survival remains unclear. The aim of this review is to summarize recent data relating to the specific role of pH regulation in tumor cell survival. We focus on membrane transport and enzyme studies in an attempt to elucidate their respective functions regarding tumor cell pH regulation. These data are discussed in the context of future directions for the field of tumor cell acid–base-related research. J. Cell. Physiol.

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Despite numerous advancements in the treatments for various types of cancers, cancer remains a leading cause of death worldwide (Mathers et al., 2009). Thus, it is imperative to exhaust any new potential avenues of improvement in available therapies to patients. The recent surge in publications related to carbonic anhydrase IX (CA IX) has resulted in a renewed interest for the importance of extracellular (pH_e) and intracellular pH (pH_i) regulation in tumor cell survival. Rapidly growing tumors require a complementary vasculature to provide oxygen and nutrients required for survival (Fraisl et al., 2008). However, solid tumors typically develop faster than the blood supply, resulting in a hypoxic microenvironment within the tumor mass. As a result, increased metabolic acids (carbonic and lactic acids) are produced and excreted resulting in acidification of the microenvironment (pH_e) of the tumor (Gatenby and Gillies, 2004; Cardone et al., 2005; Pouyssegur et al., 2006; Brahimi-Horn and Pouysségur, 2007; Brahimi-Horn et al., 2007; Chiche et al., 2009). This poses a stress on the cells in that the extracellular acidification can ultimately lead to a decrease in pH_i. However, pH_i must be maintained within a narrow range to prevent disruption of basic cell functions including membrane permeability, enzyme activity, cellular metabolism, ATP maintenance, cell proliferation, and apoptotic mechanisms among others (Roos and Boron, 1981; Pouyssegur et al., 1984, 1985; Brooks et al., 2005). A variety of pH measurement techniques have demonstrated that cancer cells maintain a more alkaline intracellular pH_i compared to other non-cancerous cells despite their acidic surroundings (for an extensive review of pH measurements please refer to Gillies et al., 2002). This property is even more apparent in more aggressive tumors and the reversal of the pH gradient in tumor cells (high pH_i vs. low pH_e) compared to normal cells is one of the main defining characteristics of tumor cells (Cardone et al., 2005). Consequently the current consensus in the literature is that cancer cells possess efficient membrane transport machinery that extrudes H^+ and imports HCO_3^- to maintain an elevated pH_i. The overall pH_i regulating mechanism may include the Na^+/H^+ exchanger 1 (NHE1), Cl^-/HCO_3^- exchangers (CBEs), Na^+/HCO_3^- cotransporters (NBCs), H^+/lactate cotransporters (monocarboxylate transporters, MCTs), and CA II, IX, and XII working in a co-ordinated fashion (Cardone et al., 2005; Pouyssegur et al., 2006; Brahimi-Horn and Pouysségur, 2007; Brahimi-Horn et al., 2007; Swietach et al., 2007; Chiche et al., 2009, 2010a). It has been proposed that tumor cells develop an enhanced acid resistance to survive in the microenvironment where normal cells will die (Gatenby and Gillies, 2004; Gatenby et al., 2006; Fang et al., 2008). Consequently, it is hypothesized that targeting the pH_i regulating capability of tumor cells would be an effective way to kill cancer cells (Pouyssegur et al., 2006; Swietach et al., 2007; Gatenby and Gillies, 2008; Supuran, 2008; Chiche et al., 2009, 2010a; Harguindey et al., 2009; Huber et al., 2010). However, knowledge of the complete mechanism of pH_i regulation in tumor cell survival and growth remains unknown. Due to the compensatory nature of pH_i regulating systems, it is imperative that the complete pH_i regulating mechanism be understood to ensure proper development of therapies targeting pH_i disruption to cause tumor regression. The aim of this review is to discuss the recent literature relating to the control of tumor cell survival and growth by pH alterations. This review will emphasize studies relating to membrane transport control of pH_i regulation in tumor cells and their consequences for survival.

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The role of pH_i regulation in tumor cell survival was spearheaded in the 1980s. Reversal of NHE activity to induce cell H^+-suicide was used to select for NHE-deficient fibroblast mutants and demonstrated the essential requirement for pH_i regulation and cell survival (Pouyssegur et al., 1984). Following this, studies on pH_i regulation and tumor cell survival utilized the ionophore nigericin to demonstrate cellular toxicity due to acidification of pH_i (Rotin et al., 1987). Cell toxicity was realized with nigericin at pH_e values below 6.5 (Rotin et al., 1987; Luo and Tannock, 1994). However, in combination with the potent NHE inhibitor HMA, cell killing was noticed at pH_e below 6.8, which is in the range found in solid tumors (Luo and Tannock, 1994). These results were also observed in tumor xenografts in mice, but only when delivered in combination with the drug hydralazine to reduce tumor blood flow (Luo and Tannock, 1994). Similar results were observed with the more general NHE inhibitor amiloride (Newell et al., 1992) and the general bicarbonate transport inhibitor DIDS (Boyer and Tannock, 1992; Yamagata and Tannock, 1996). Interestingly, a recent study on melanoma cells reported substantial cell mortality with acid-acclimation alone in the absence of nigericin or inhibitors of pH regulating proteins (Moellering et al., 2008). However, not all cells were killed in this study and the acidic conditions selected for a more invasive phenotype (Moellering et al., 2008). Therefore, the proof of principle is well established that cellular acidification can ultimately cause tumor cell death and regression (Pouyssegur et al., 2001). Despite this, the literature remains incomplete in terms of experiments demonstrating gene disruption for an effective induction of acidosis to cause tumor cell death. The majority of data from the previous years demonstrate a reduction in cell proliferation as opposed to direct killing by intracellular acidification. It is possible that the reduction in cell proliferation, and absence of cell killing is related to an inability to knockout the pH_i regulating mechanism effectively thus far. From the earliest studies on pH_i regulation and tumor growth, cellular toxicity due to acidic pH_i was only observed in the presence of nigericin, inhibitors of pH_i regulation enhanced cellular toxicity but they did not cause toxicity individually (Rotin et al., 1987; Boyer and Tannock, 1992; Newell et al., 1992). Therefore, it appears that tumor cells are capable of regulating pH_i even with the inhibition of pH_i regulating proteins. Consequently, without inhibiting the entire pH regulating mechanism (or disrupting the most critical components of the mechanism), cell survival will continue. These are important underlying points to consider in the future development of pH-targeted cancer therapies (Huber et al., 2010).

Tumor Intracelluar pH (pH_i) Regulating Proteins

Carbonic anhydrase (CA)

CA IX. CA is an essential enzyme throughout the body catalyzing the conversion of CO_2 and H_2O to H^+ and HCO_3^-. There are 15 different isoforms of CA expressed in humans with unique tissue distribution (Supuran, 2008). CAs are further categorized based on their distribution as cytosolic (CA I, II, III, VII, and XIII), mitochondrial (CA VA and VB), membrane bound (CA IV, IX, XII, and XIV), and the secreted form found in saliva (CA VI). A hallmark feature of solid tumors is the high expression of CA IX (Wykoff et al., 2000; Supuran, 2008; Chiche et al., 2010a,b). CA IX is a hypoxia-induced protein that is controlled by the transcription factor HIF1α (for review see Brahimi-Horn and Pouyssegur, 2009). In addition, CA IX is rapidly becoming a diagnostic marker for poor patient prognosis in a variety of cancers (see Kaluz et al., 2009; Chiche et al., 2010b for an extensive summary). Located at the cell membrane with an extracellular orientation, CA IX activity contributes to extracellular acidification of the tumor microenvironment (Svastova et al., 2004; Chiche et al., 2009; Li et al., 2009; Swietach et al., 2009). CA IX was repeatedly described to not only participate in the extracellular acidification, but also to regulate the intracellular pH. However, no clear demonstration established a link between CA IX expression and a permissive pH_i. Moreover, CA IX was recently reported not to have an impact directly on pH_i in isolated cells (Swietach et al., 2008). However, it was demonstrated to be important in forming a uniform distribution of pH_i within cultured spheroids of bladder carcinoma cells (Swietach et al., 2008). Our lab then demonstrated that the role of CA IX in pH_i regulation in individual cells is observed when experiments are performed under nominally HCO_3^--free conditions (Chiche et al., 2009). These experimental conditions are more physiological in terms of the tumor microenvironment and demonstrate the essential nature of CA IX in regulating pH_i in vivo. It is also important to note that the effect of ectopic expression of CA IX on pH_i regulation was observed in the presence or absence of the key pH_i regulating protein NHE1 (Chiche et al., 2009). Monitoring pH_i in three dimensional tumor spheroids then suggested that CA IX alkalinizes pH_i by facilitating CO_2 extrusion from the cells as opposed to an enhancement of membrane H^+ extrusion (Swietach et al., 2009). This study well illustrates the importance of CO_2 movement in regulation of both pH_i and pH_e and the dependence on CA IX to maintain the diffusion gradients necessary. The function of this proposed mechanism depends on the presence of a continuous HCO_3^- import (Swietach et al., 2009) which remains unknown and will be discussed below. It was further demonstrated that the proteoglycan domain of CA IX enables the hydration of CO_2 to occur at low pH levels found in the tumor microenvironment (Innocenti et al., 2009). This same study proposes that the PG domain of CA IX can act as an intrinsic buffer due to the large number of COOH side groups in this region although this will require further research to clarify (Innocenti et al., 2009).

The above studies and others have implicated CA IX as an attractive target for tumor therapy with respect to pH disruption. Although this concept has been proposed on numerous occasions, our laboratory was the first to definitively show that knockdown of CA IX impairs tumor growth *in vivo* (Chiche et al., 2009). However, to achieve a more substantial decrease in tumor growth (85% vs. 40%) combined knockdown of CA XII was also required indicating a complementary function of CAs. Indeed, CA IX knockdown was not sufficient to abolish the membrane–CA activity due to a subsequent upregulation of CAXII (at both mRNA and protein levels). It was only when CA IX and CA XII were silenced that we reduced extracellular acidification and disturbed pH_i-regulating capacities of the cells when exposed to an acidic and bicarbonate-free environment. Supuran's group has pioneered the development of specific CA IX inhibitors to target tumor growth (for review see Supuran, 2008, 2010). In vitro studies report the use of aromatic sulfonamides in contrast to the non-specific CAs inhibitor (acetazolamide), resulting in decreased pH_i, decreased cell proliferation and induction of a ceramide-mediated apoptosis in HeLa and 786-O cells (Cianchi et al., 2010) but not in non-CAIX-expressing cells 786-O+pVHL. However, in vivo studies of these compounds are lacking to assess the potential of sulfonamide derivatives on tumor growth reduction. Proof of concept that CA inhibitors can actually cause effects on tumor growth in vivo was recently published where mice with renal cell carcinoma (RCC) xenografts treated for 1 month with CA inhibitors demonstrated a retardation of tumor growth (Ahlskog et al., 2009). The potential of CA IX inhibitors to control tumor growth via the manipulation of pH_i and pH_e will be an intense area of study in the coming years.

Other efforts to target tumor survival with respect to CA IX function are focusing on the regulation of CA IX, an area that will benefit from the recent publication of the crystal structure of the catalytic site (Alterio et al., 2009). Removal of the short intracellular (IC) tail results in CA IX localization to switch from the membrane to the cytoplasm (Hulikova et al., 2009). Mutations of basic amino acid residues within the IC tail do not alter surface expression; however, it abolishes the pH acidification of the extracellular space (Hulikova et al., 2009). These mutations also decrease inhibitor binding (FITC-sulfonamide) and reduce shedding of the CA IX ectodomains and CA IX-mediated cell dissociation (Hulikova et al., 2009). Furthermore, CA IX may play another role in signaling apart from pH regulation that contributes to cell adhesion (Svastová et al., 2003) and tumor progression. CA IX induction caused down regulation of the intracellular isoform CA II (Swietach et al., 2008) indicating that CA IX expression can interact with signaling pathways to control the expression of other genes and proteins. We have also observed this with the upregulation of CA XII in response to CA IX knockdown (Chiche et al., 2009). Undoubtedly, the biochemical characterization of CA IX and its interactions with other proteins will be an important area of future research to understand its role in tumor survival.

CA XII. CA XII is another extracellular facing CA that was originally identified due to overexpression in renal cancer cells (Ivanov et al., 1998; Türeci et al., 1998) with subsequent studies demonstrating high expression in breast cancer (Ivanov et al., 2001) and other tumor types (for review see Chiche et al., 2010a). CA XII is also a hypoxia inducible CA (Wykoff et al., 2000; Ivanov et al., 2001; Chiche et al., 2009); however, it is not as robustly regulated by hypoxia as CA IX and its HIF response element (HRE) was not identified. The regulation of CA XII expression is linked to the estrogen receptor α in breast cancer (Barnett et al., 2008). In contrast to CA IX, CA XII lacks the proteoglycan-like domain.

Our laboratory has demonstrated a role for CA XII in the regulation of pH_i and has proposed that it acts as a complementary mechanism to CA IX activity to maintain acid–base regulation in the tumor microenvironment (Chiche et al., 2009). CA XII contributed to a decrease in tumor growth when combined with CA IX silencing as mentioned above (Chiche et al., 2009) while a single knockdown of CA XII did not reduce tumor growth rate. Recently in the breast cancer cell line MDA-MB-231, CA XII knockdown reduced cellular migration in vitro along with tumor growth and metastasis in vivo although no information was provided regarding pH_i regulation (Hsieh et al., 2010). It is interesting to note that another study reported that CA XII is not expressed in MDA-MB-231 cells (Li et al., 2009). This contradiction in the literature requires clarification for the role of CA XII in breast cancer.

CA XII has also been shown to be a marker for good prognosis in invasive breast cancer patients (Watson et al., 2003) and a marker of poor prognosis in astrocytomas (Haapasalo et al., 2008). It is interesting to note that the CA XII in astrocytomas is an alternatively spliced shorter variant with eleven fewer amino acids than CA XII in other tissues raising the possibility that this CA XII functions in a different manner (Haapasalo et al., 2008). These data seem to contradict the experimental data discussed above. In fact this contradiction is only on the surface. If CA IX and CA XII share almost identical pH-regulatory functions, their expression in tumors is strikingly different. Indeed a recent study of a large cohort of patients has revealed that CA XII is a marker for good prognosis in resectable non-small cell lung cancers (NSCLC, Ilie et al., 2010a) whereas expression of CA IX in this cohort was associated with a very poor prognosis (Ilie et al., 2010b). Due to these opposing prognoses, an examination on the same tumor section for the level of both CA IX and CA XII should be performed to help clarify how CA IX expression could be contributing to the prognosis found with CA XII overexpression. The interesting point is that the 19% subset of NSCLC that overexpress CA XII is associated with low grade and well-differentiated features, a characteristic that contrasts with the subset of tumors expressing strongly CA IX. We believe that the expression of CA IX, but not of the more promiscuous CA XII, gives a selective advantage for tumor survival that occurs only when the tumor has reached an advanced grade of dedifferentiation. Before this stage hypoxia cannot "turn-on" the normally repressed *ca9* gene. Regardless, the differing reports in the literature require that the role of CA XII in both tumor survival and pH regulation are clarified to ensure proper therapeutic development targeting extracellular CA isoforms.

CA II. Reports of intracellular CA II function related to tumor biology are scarce due to weak expression in many tumors. With regards to tumor survival, CAII expression was described to not be an independent marker of poor prognosis in medulloblastomas (Nordfors et al., 2010). However, a recent study indicates that CA II is a marker for gastrointestinal stromal tumors and strong staining of CA II indicated significantly better survival rates (Parkkila et al., 2010). This article showed weak staining of CA IX; however, which could potentially be the cause of a good prognosis. The authors speculate that CA II could function to maintain alkaline pH_i and acidic pH_e as per the function of CAIX (Parkkila et al., 2010); however, no mechanism is provided for how this could occur when comparing *intra* versus *extra*cellular CA isoforms. Furthermore, the action of CA IX is shown to enhance tumor survival and be a marker of poor prognosis which would contradict their results for CA II and better survival if CA II performed the role of CA IX.

Although expression of CA II in epithelial tumors is low, there are reports for certain amount of expression in other cancers such as brain (Parkkila et al., 1995). The importance of CA II in tumor survival may be overlooked; however, as it is commonly expressed in normal cells and it is also one of the most efficient enzymes ever described in nature (Supuran, 2008). Therefore, it is possible that CA II is never detected as a prognostic marker in tumors as even low expression could be sufficient to enhance tumor growth and survival by contributing to pH dynamics. Conversely the role of CA II could be at the level of the tumor vasculature where expression in the vessel endothelium is high compared to normal endothelia in a variety of cancers (Yoshiura et al., 2005).

Knockout of CA IX leads to increase in CA II and vice versa in the stomach (Pan et al., 2006). This indicates a strong linkage between CA IX and CA II expression and function. Perhaps the linkage is at the level of pH regulation. This is very important to consider in therapeutic development as the inhibition of CA IX could be in some way compensated for by the increase in CA II. The fact that CA IX is extracellular facing and CA II is located in the cytoplasm is puzzling in terms of considering one CA compensating for the other. Perhaps it is the interaction of both isoforms with another protein that ultimately forms the pH-regulating complex. The interaction of CA II with the overall pH_i regulating mechanisms in tumors requires further consideration.

Aquaporins (AQPs)

Approximately 12 years ago, it was reported for the first time that CO_2 transport across the cell membrane is enhanced by AQPs acting as CO_2 channels in the membrane (Nakhoul et al., 1998). This contradicted the prevailing assumption that CO_2 passes through the membrane via simple diffusion. Further studies have indicated that CO_2 movement depends on AQPs to increase membrane permeability in some tissues (Endeward et al., 2006; Musa-Aziz et al., 2009). This hypothesis remains controversial as it has been argued that the contribution of AQP

to CO_2 movement is likely not relevant to physiological conditions (Missner et al., 2008). Regardless of the controversy, the movement of CO_2 has a large impact on pH both in the cellular and extracellular environment. CO_2 is suggested to provide a major contribution to the acidification of the tumor microenvironment as demonstrated in experiments performed in the absence of glycolysis and lactic acid production (Newell et al., 1993; Yamagata et al., 1998; Helmlinger et al., 2002). Therefore, it is reasonable to predict that AQP expression could play a role in the pH balance of solid tumors via the facilitation of CO_2 movement. Circumstantial evidence for this hypothesis stems from reports that the expression of AQP1 (Echevarría et al., 2007), AQP4, and AQP5 (Ding et al., 2009) can be induced by hypoxia indicating that AQP expression could be manipulated in hypoxic regions of solid tumors as well. AQP4 expression is also related to increases in VEGF expression in neuronal tissue (Kaur et al., 2006; Rite et al., 2008) which is an important factor in tumor development. AQP1 knockout was demonstrated to significantly delay tumor growth (Saadoun et al., 2005); however, the role of AQP in tumor development was attributed to angiogenesis and cell migration. Nothing is known yet about the role of aquaporins in tumor pH regulation and the possible interaction with the membrane-associated CAs. If and how aquaporins contribute to CO_2 movement in solid tumors and the subsequent impact on pH regulation with relation to tumor growth and survival remains a potential avenue of future research.

Membrane Transporters

Na^+/H^+ exchanger (NHE)

The NHE isoform 1 (NHE1) is one of the most important proteins regulating cellular pH and is expressed in almost every cell type of the body (Wakabayashi et al., 1997; Counillon and Pouyssegur, 2000). Activation of NHE1 by all growth factors (Counillon and Pouyssegur, 2000) is a key player in tumor cell transformation (for review see Cardone et al., 2005). The importance of NHE1 in tumor growth is firmly established as early studies found that mutated cells lacking NHE1 activity failed to grow tumors or demonstrated a drastic reduction in growth when implanted into nude mice (Lagarde et al., 1988; Rotin et al., 1989). Fibroblasts mutated to prevent lactic acid production or be deficient in cellular respiration (therefore producing excessive lactate) in combination with NHE1 knockout also demonstrated the importance of NHE1 and lactic acid in the ability of tumors to form in vivo (Pouyssegur et al., 2001 and for a more extensive discussion of these experiments the reader is referred to Chiche et al., 2010a). As mentioned in the introduction, a variety of studies using pharmacological inhibition of NHE1 have been shown to effectively reduce tumor growth (Rotin et al., 1987; Newell et al., 1992; Luo and Tannock, 1994). NHE1 inhibitors have also been shown to decrease pH_i and induce apoptosis in leukemic cells with 90% cells mortality being achieved after 5 h of inhibitor incubation in vitro (Rich et al., 2009). Cellular toxicity due to NHE1 inhibition was reported in breast cancer cell lines as well (Reshkin et al., 2003).

NHE1 activity also interacts with drug treatment and the pharmaceutical paclitaxel's induction of apoptosis was linked to inhibition of NHE1 via the kinases PKA and p38 in human breast cancer cell lines (Reshkin et al., 2003). Paclitaxel application was also associated with the acidification of pH_i (Reshkin et al., 2003). Although the role of NHE1 contribution to apoptosis was attributed to the signaling transduction mechanism in this study, it is possible that the contribution lies in the reduction of pH_i. This article was recently followed up by a study showing that the inhibition of NHE1 is mediated by PPARγ activation to cause inhibition of tumor growth in vitro and in vivo (Kumar et al., 2009). Another recent article has revealed a role for neurotensin in the activation of NHE1 and the progression of pancreatic cancer (Olszewski et al., 2010).

Furthermore, NHE1 expression and activity can be enhanced by hypoxia (Rios et al., 2005) and a HRE was proposed to exist in the promoter region of the gene for HIF-1 binding (Shimoda et al., 2006) although a full characterization of the HRE has not yet been presented. High levels of NHE1 were also found in the central hypoxic regions of ductal carcinomas in situ (Gatenby et al., 2007). However, the same effects of hypoxia on NHE1 expression have not been presented for human cancer cell lines and therefore the relationship to HIF-1 control in tumor development remains unknown. This potential relationship of NHE1 activity to hypoxia provides further relevance for the role of NHE1 in the tumor microenvironment. It is clear that NHE1 plays an essential role in pH_i dynamics and tumor growth; however, individual targeting of NHE1 is not necessarily sufficient for tumor regression and its therapeutic benefit will require continued exploration in combination with other cellular targets.

HCO_3^- transporters (BTs)

HCO_3^- import is proposed to be an essential component of maintaining elevated pH_i in tumor cells to ensure growth and survival. HCO_3^- transporting (BTs) proteins include Cl^-/HCO_3^- exchangers (SLC4A1-SLC4A3), Na^+/HCO_3^- co-transporters (electrogenic SLC4A4 and A5; electroneutral SLC4A7 and A10), Na^+-dependent Cl^-/HCO_3^- exchangers (SLC4A8), and promiscuous anion exchangers known to transport HCO_3^- (SLC26A3, A4, and A6). (For recent reviews see Alper, 2009; Boron et al., 2009). The exact role that HCO_3^- transporters play in tumor survival remains unknown.

Anion exchangers (AEs)

A member of the AE family has been proposed as the transporter responsible for HCO_3^- uptake in the tumor microenvironment (Pouyssegur et al., 2006; Harguindey et al., 2009). To date there is limited evidence for the role of AEs in tumor survival. AE1 (commonly known as band 3 from erythrocytes) was reported to have high expression in gastric and colon cancers; however, it did not traffic to the membrane indicating that it is a non-functional form of the protein (Shen et al., 2007). The authors proposed that AE1 in the cytoplasm interfered with the normal pH_i regulating capability of AE2 in these cells and reported an elevation of resting pH_i when AE1 was present (Shen et al., 2007). The authors' main conclusion using AE1 forced expression and siRNA was that AE1 expression correlated with p16 increase in the cytoplasm and therefore alters cell-cycle regulation (Shen et al., 2007). Consequently, no emphasis in the study was placed on understanding the potential impact of elevated pH_i and the role it could play in tumor cell survival. The question remains if non-functional AE1 expression can interfere with other AEs to disrupt HCO_3^- extrusion from the cell and contribute to the maintenance of an elevated pH_i. A follow-up study on the clinicopathological parameters of gastric cancer indicated that AE1 expression was a marker of poor prognosis (Xu et al., 2009). Therefore, the regulation of AE1 continues to be studied and its function is related to the micro RNA miR-24 (Wu et al., 2010); however, the direct linkage to pH homeostasis remains unknown.

AE2, which is expressed in many normal tissues, was shown to be regulated at the mRNA level by the tumor suppressor VHL in RCC cells (Karumanchi et al., 2001). However, the effect on pH_i regulation was unclear and has not been further investigated. Analysis of gastric cancer tissue via immunohistochemistry demonstrated a downregulation of AE2 compared to normal tissue (Yang et al., 2008). A correlation between AE2 downregulation and pH homeostasis was

proposed; however, no evidence for this mechanism has been presented to date. DIDS, a general BT inhibitor, induced apoptosis in hepatocellular carcinoma cells (HCCs) (HA22T) that were reported to overexpress AE2 (Liu et al., 2008). Since DIDS is a general inhibitor of all of the BTs, it is difficult to conclude that it is only targeting AE2 in this study. Knockdown of AE2 in these cells also induced apoptosis (Hwang et al., 2009) strengthening the results found with DIDS. These studies provide early indications for AE2 importance in at least certain hepatocellular cancers; however, more extensive study is needed to its relation to pH homeostasis, and if it is relevant to the survival of other solid tumors. It is interesting to note that in mouse fibroblasts lacking AE2, resting pH_i was unable to be regulated to wild-type levels suggesting that AE2 plays an integral pH_i regulating role in certain cells (Mardones et al., 2008).

HCO_3^- can also be exchanged for Cl^- via members of the promiscuous transporting SLC26 gene family (Ohana et al., 2009). To date there are no reports of any SLC26 isoforms being involved in tumor pH regulation and survival. Conceptually a member of the AE or SLC26 family would not appear as a prime candidate for HCO_3^- uptake in tumor cells to achieve cellular alkalinization and cell survival. This is simply because it would require that the transporter operate to secrete Cl^- and absorb HCO_3^- which is the opposite direction of its endogenous function. Indeed AEs can be bi-directional transporters based on ion gradients but the low pH tumor microenvironment would provide also a low HCO_3^- concentration making it unlikely that the AE would change its direction of transport.

Na^+-dependent Cl^-/HCO_3^- exchangers (NDCBE)

The NDCBE (encoded by the SLC4A8 gene) was implicated along with NHE1 as a component of the pH_i regulating system in tumor cells (Rotin et al., 1987; Newell and Tannock, 1989; Boyer and Tannock, 1992; Yamagata and Tannock, 1996). The conclusions of these studies were based on the use of the broad-range HCO_3^- transport inhibitor DIDS so it was difficult to isolate the exact HCO_3^- transport mechanism from these studies. Furthermore, DIDS was toxic at higher concentrations during in vivo studies preventing its use as an effective therapeutic agent (Yamagata and Tannock, 1996). A novel NDCBE inhibitor was developed by the Aventis Pharmaceutical Company, Frankfurt, Germany and applied to human and murine breast cancer cells (Wong et al., 2002). Although this inhibitor (S3705) affected pH_i recovery from acidosis and decreased pH_i when pH_e was lowered, there was no cellular toxicity found over a range of acidic pH values (Wong et al., 2002). Inhibition of NDCBE with S3705, and in combination with NHE inhibition did; however, reduce cellular proliferation at low pH_e (Wong et al., 2002). Inhibition of NDCBE was then assessed as a strategy to lower pH_i and enhance the cytotoxic effect of melphalan on breast cancer cells; however, the results obtained were deemed to be to small and inconsistent to have a therapeutic value (Wong et al., 2005). Finally, NDCBE inhibition was utilized on cholangiocarcinoma cells and caused a decrease in proliferation and induction of apoptosis (Di Sario et al., 2007). S3705 also inhibited the phosphorylation of various signaling molecules (ERK, AKT, and BAD) indicating that its effect was not a direct relation to pH_i disruption (Di Sario et al., 2007). No further articles have been published to date regarding NDCBE in tumor cells and the individual targeting of this transporter does not appear to be an effective means to control tumor cell survival.

Na^+/HCO_3^- co-transporters (NBCs)

The NBCs are a diverse family of BTs with both electrogenic and electroneutral isoforms providing a variety of functions throughout the body including pH regulation (Boron et al., 2009). Expression of the electrogenic NBCe1 (SLC4A4) isoform was presented in RCC cells (Yamada et al., 2003). This article could only speculate on the role of the transporter in RCC tumor pH regulation and survival, as further characterizations were not made. Since this publication, nothing further has been presented in the literature pertaining to NBCe1 and cancer. However, an NBC has been proposed in a general overall model of tumor pH regulation (Swietach et al., 2007).

A clinical study of breast cancer development analyzing tyrosine kinase substrates found that the electroneutral NBC (SLC4A7) was downregulated in 64% of the tumor samples compared to their normal matched samples (Chen et al., 2007). The authors predict that the alteration in SLC4A7 expression contributes to the acidity of the microenvironment and tumor development; however, no data is provided. In addition, the SLC4A7 gene has been reported as a possible causative gene in a genome wide association study of breast cancer susceptibility (Ahmed et al., 2009). Very recently, a follow-up study on the role of SLC4A7 in tumor pH dynamics and survival has been presented for MCF-7 breast cancer cells (Lauritzen et al., 2010). This study revealed that SLC4A7 contributes equally to pH_i regulation with NHE1 in these cells (Lauritzen et al., 2010). The truncated tyrosine kinase ErbB2 receptor (ΔNErbB2) that is common in breast cancer was found to upregulate SLC4A7 mRNA and protein expression levels while NHE1 expression was unaffected (Lauritzen et al., 2010). Interestingly, although SLC4A7 and NHE1 contributed equally to pH_i homeostasis, only NHE1 (and not SLC4A7) inhibition and knockdown had an effect on the application of the therapeutic cisplatin to induce cell death. This indicates that despite a commonality in pH_i regulating function, NHE1 and SLC4A7 play different roles in cellular function. A linkage between proteins was also presented with NHE1 knockdown resulting in upregulation of SLC4A7 presumably to compensate for pH_i regulation (Lauritzen et al., 2010). This literature will be expanded in the future to the tumor microenvironment conditions to better understand the interactions of SLC4A7, NHE1, and pH homeostasis in tumor cell survival.

Monocarboxylate transporters (MCTs)

The MCTs are an essential component of cellular metabolism and provide an important contribution to the regulation of tumor pH_i by coupling H^+ export with monocarboxylates such as lactate and pyruvate (Halestrap and Price, 1999; Halestrap and Meredith, 2004; Cardone et al., 2005; Pouyssegur et al., 2006; Swietach et al., 2007). MCT activity appears essential for tumor survival due to the dependence of most tumors on glycolysis and subsequent production of massive amounts of lactic acid as first described by Warburg (1956). MCT expression is high in a variety of tumors and the expression of MCT4 is induced by hypoxia via a HIF-1α-dependent mechanism (Ullah et al., 2006). siRNA targeting the MCT chaperone CD147/basigin decreased the expression of MCT1 and MCT4 with a resultant decrease in glycolysis, extracellular pH, and ATP production indicating the importance of MCTs in malignant melanoma progression (Su et al., 2009). MCT4 has also been implicated in cell migration and is suggested to play the role of NHE1 in the rare cases where NHE1 is not expressed (Gallagher et al., 2009). The transport activity of both MCT1 and MCT4 is enhanced with CA II expression as demonstrated in *Xenopus* oocyte experiments (Becker et al., 2005, 2010; Becker and Deitmer, 2008). Interestingly, the enhancement of transport does not depend on the catalytic activity of CA II. It is proposed that CA II protonation/deprotonation provides a means for enhanced H^+ diffusion to or away from the MCT pore to maintain efficient import or export

of lactate (Becker et al., 2005, 2010; Becker and Deitmer, 2008). Targeting MCTs or their chaperon has attracted a lot of attention recently and experiments are supportive of a very strong potential in counteracting growth of glycolytic tumors (Schneiderhan et al., 2009). This topic will be developed at large in a coming cancer review.

H^+-ATPase

In addition to NHEs, CAs, BTs, and MCTs, vacuolar-type H^+-ATPase (VHA) is also proposed as a contributing factor to the pH dynamics in tumors. VHA was shown to be functional at the cellular membrane of tumor cells with its membrane expression possibly due to improper sorting of normal cellular components (Martinez-Zaguilan et al., 1993). Immunohistochemical analysis in breast cancer cell lines revealed a higher membrane expression of VHA in highly metastatic breast cancer cells compared to low metastatic cells (Sennoune et al., 2004). Early studies using the VHA inhibitor bafilomycin revealed a retardation of pancreatic xenograft tumor growth in vivo (Ohta et al., 1998) suggesting that VHA plays a role in tumor survival via maintenance of pH_i.

A comprehensive study of the growth inhibition of tumor cells by bafilomycin revealed an induction of HIF-1α expression, subsequent p21 induction, and cell-cycle arrest (Lim et al., 2006). Therefore, the effect of VHA inhibition by bafilomycin on tumor survival does not appear to be at the level of pH_i regulation as previously proposed. This interaction was confirmed in a follow-up study where it was demonstrated that the effect of bafilomycin on cell survival was not a result of pH_i acidification (Lim et al., 2007). Instead it was found that bafilomycin enhances the binding of the VHA subunit ATP6V0C to the n-terminus of HIF1α and alters the protein structure to prevent pVHL binding and thus results in HIF1α stabilization (Lim et al., 2007). Despite this clear demonstration that VHA inhibition by bafilomycin causes tumor suppression via a mechanism other than acidification, proton pump inhibitors (PPI) are being pursued experimentally and clinically as a method to reduce tumor growth (De Milito et al., 2010; Spugnini et al., 2010). The attractive component of PPIs is that they require acid conditions to be converted into the active form (Mullin et al., 2009) which provides the possibility for tumor-specific selection (Spugnini et al., 2010). The PPI esomaprazole (ESOM) was administered to melanoma cells and caused cell death in extremely acidic (pH 5.0) and in unbuffered media (De Milito et al., 2010). This was correlated with an induction of acidification and caspase-dependent cell death in vitro (De Milito et al., 2010). Elevation of pH_e and depression of pH_i was also observed in tumor xenografts in mice with ESOM treatment, while in vivo tumor growth was reduced by $\sim$50% (De Milito et al., 2010). Despite the fact that the reduction in tumor growth was not as effective as has been achieved in CA knockout studies (Chiche et al., 2009), ESOM treatment increased mean survival time by $\sim$20 days while higher doses of ESOM increased survival time by 80% (De Milito et al., 2010). These results need to be interpreted with some caution; however, as the different doses of ESOM had the same effect on tumor size despite a difference in survival. Therefore, we must understand further the mechanism involved in VHA inhibitors to clarify the direct role in pH regulation and tumor survival.

Acidification and Metastasis

Another key component of the pH discussion concerning tumor growth concerns the ability of tumor cells to metastasize. Hypoxia is a key contributor to metastasis by causing the induction of various genes that contribute to tumor cell invasion and spreading (for review see Bertout et al., 2008). Acidification in hypoxic tumors promotes metastasis as well where it is proposed that the extracellular acidification results in normal cell death and extracellular matrix degradation to allow for the advancing acid-adapted tumor cells to proliferate (Gatenby and Vincent, 2003; Gatenby and Gillies, 2004; Gatenby et al., 2006). Supporting evidence for the acid-mediated invasion hypothesis revealed an acidic gradient in the peritumoral region of tumor xenograft models (Gatenby et al., 2006). This study also found evidence for normal cell toxicity and extracellular matrix degradation in the acidic regions (Gatenby et al., 2006). The mechanism of individual tumor cell migration is a multi-step process and each component is also dependent on pH_i and pH_e (reviewed by Stock and Schwab, 2009). Additional evidence for the role of acidosis in metastasis was shown when pretreatment of melanoma cells in vitro with acidic pH_e promoted the development of metastases in vivo by upregulating the expression of various proteolytic enzymes (Rofstad et al., 2006). A follow-up study suggested that acidosis selects for a stable invasive phenotype that is not reversible upon re-acclimation to physiological pH (Moellering et al., 2008). Thus, it appears that the acidic pH of the tumor microenvironment plays a key role in the invasive tumor phenotype for human cancer cells.

NHE1 and metastasis

NHE1 is known to be a key player in tumor cell motility and metastasis (for reviews see Cardone et al., 2005; Stock and Schwab, 2009). In fibroblasts, NHE1 mutants were made that lacked cytoskeletal anchoring but retained ion transport capability and vice versa (Denker and Barber, 2002). These mutants revealed that the cytoskeletal anchoring of NHE1 is required for focal adhesion assembly and the ion transport component of NHE1 is required for de-adhesion with both events being key regulators of cell migration (Denker and Barber, 2002). An upregulation of NHE enhanced the invasive capabilities of breast cancer cells (Reshkin et al., 2000). Melanoma migration was then demonstrated to be reliant on pH_e and NHE1 as well (Stock et al., 2005). Cell adhesion was strongest in acidic conditions and weakened in basic pH_e with a mechanism that involved the integrin $\alpha2\beta1$ and NHE1 (Stock et al., 2005). Recently, siRNA targeting NHE1 and amiloride were used to demonstrate a reduction of cell invasion in a HCC line (Yang et al., 2010b). siRNA of NHE1 had only a minor effect on cell proliferation in these cells (Yang et al., 2010b). The NHE1 inhibitor EIPA also reduced invasion and motility in a different HCC line under in vitro hypoxia conditions that was associated with a reduction in pH_i and increase of pH_e (Yang et al., 2010a). NHE involvement in metastasis has been linked to the regulation of lysosme (the major storehouse of cellular proteases) trafficking to the cell membrane via pH alterations (Steffan et al., 2009). However, shRNA knockdown of NHE1 (by $>$90%) did not inhibit the acidic pH_e induction of lysosomal trafficking and the mechanism of NHE involvement continues to be explored. The current consensus for the mechanism of NHE1 action in metastasis is that NHE1 localizes to the front (invading) portion of the cell to direct cellular invasion via the alkalinization of pH_i and acidification of pH_e (Cardone et al., 2005; Steffan et al., 2009; Stock and Schwab, 2009). In fact, the reliance on pH manipulations for metastasis is so great that all of the pH regulating proteins including CA IX, AE2, NBC, MCT4, and NHE1 are proposed to localize to the invading region of the cell to ensure an acidified pH_e and alkaline pH_i in this area (for review see Stock and Schwab, 2009).

VHA is also suggested to play a major role in the acidification and breakdown of the extracellular matrix, thus leading to enhanced metastasis. Knockdown of the VHA c subunit (ATP6L) by 60% using siRNA in human hepatocellular carcinomas reduced the ability of cells to recover pH_i following

acidosis (Lu et al., 2005). This was associated with decreased expression of MMP-2 and gelatinase activity, decreased growth of xenograft tumors in nude mice, and a marked decrease in the number of metastases (Lu et al., 2005). Other results from in vitro studies have suggested VHA inhibitors as important therapeutic targets for the prevention of metastases (Fais et al., 2007). However, as discussed above with regards to bafilomycin interactions with HIF1α, the mechanisms of VHA inhibitors remain poorly understood (for review see Pérez-Sayáns et al., 2009) and therefore their usefulness in the clinic is debated.

Bicarbonate and Acid Therapy

An intriguing therapeutic avenue pertaining to pH regulation and tumor growth involves the simple ingestion of $NaHCO_3$. This hypothesis was presented in a paired study where computer simulations and mathematical modeling were utilized to demonstrate the role of systemic pH buffers to reduce the acidification of the tumor microenvironment (Silva et al., 2009). The authors' mathematical model predicted an elevation of tumoral pH_e due to $NaHCO_3$ ingestion that was matched experimentally by the addition of $NaHCO_3$ to the drinking water of rats in the companion study (Robey et al., 2009; Silva et al., 2009). Ingestion of $NaHCO_3$ elevated tumor pH_e but did not have an impact on systemic pH (Robey et al., 2009). $NaHCO_3$ ingestion also did not have a significant impact on pH_i (although this should not be unexpected as pH_i regulation is efficient in tumor cells). Importantly, bicarbonate therapy resulted in a massive decrease in metastases in in vivo mouse experiments and an increase in overall survival despite there being no impact on the primary tumor growth (Robey et al., 2009). The use of bicarbonate to control metastases via alterations in tumor pH is an exciting area of future research. However, the lack of effect on the primary tumor could pose concern for its efficacy in future trials.

Contrary to HCO_3^- therapy, acid exposure is also suggested to be beneficial in the prevention of tumor growth. Physical exercise is one parameter that consistently correlates with preventing the development of numerous types of cancer (Friedenreich and Orenstein, 2002). Specifically, the risk of breast cancer is reduced by almost 50% with exercise as observed across a wide range of studies (Bernstein et al., 1994; Kruk, 2007; Suzuki et al., 2008). The beneficial effects of exercise are widespread and have been proposed to target a variety of cellular functions; however, the exact mechanism of action remains unknown. A new hypothesis suggests that the beneficial effect of exercise relates to periodic disruptions of serum pH (Smallbone et al., 2010). This suggestion relates to the progression of tumor development that requires an upregulation of glycolysis and adaptation to acidosis (Gatenby and Gillies, 2008). The concept is that exercise will cause a repeated transient decrease in the pH_e found in in situ cancers (Smallbone et al., 2010). This would then result in cell death along with a disruption in the adaptive mechanisms required for the development of the malignant cancer (Smallbone et al., 2010). The authors' preliminary mathematical modeling suggests that indeed repeated transient reductions in tumor pH_e will have a significant impact on disrupting the evolutionary development of the invasive tumor phenotype. These preliminary suggestions await confirmation but provide a potential means for the prevention of tumor growth via pH manipulation. Following the logic of this hypothesis; however, exercise would be considered to impact cancer patients in a negative way when a tumor has developed as it would drive the selection of a more aggressive phenotype. Therefore, with so many parameters changing during exercise it will be important to use caution when extending the benefit to pH-related processes.

pH Sensors and Cancer

A final area to consider in the discussion of pH control of tumor growth relates to the pH sensing mechanisms that exist in cancer cells. Conceptually, sensors of pH changes will be essential for tumor cell survival by ensuring the initiation of appropriate cell responses to the pH stress (for a general review of cellular pH sensing see Srivastava et al., 2007; Casey et al., 2010; Tresguerres et al., 2010). It is suggested; however, that for the majority of pH-sensitive proteins within the cell, pH_i changes will only act as a secondary regulator of their activity while pH_e changes can act as a true signal (Casey et al., 2010).

An important link has been demonstrated for intracellular pH sensing of p53 in a study of Brazilian children who have increased susceptibility to adrenocortical carcinoma (ACC) (DiGiammarino et al., 2001; Ribeiro et al., 2001). These children were found to harbor a mutation in the tetramerization domain of the tumor suppressor p53 (Ribeiro et al., 2001). The tetramerization domain of p53 is required for suppressing tumor activity (Stürzbecher et al., 1992). This mutation results in the tetramerization domain of p53 to become highly sensitive to pH changes which can lead to pH-dependent p53 dysfunction that is correlated with the increased risk of ACC in these Brazilian children (DiGiammarino et al., 2001). This p53 mutation resulting in a gain of pH sensing illustrates how pH_i dynamics can control tumor growth at multiple levels within the cell.

With the integral role that CA IX is suggested to play in tumor cell survival, sensing changes in extracellular CO_2 or HCO_3^- may be fundamental components of tumor cell survival. Neuronal CO_2 sensing has been elegantly demonstrated in *Drosophila* (Jones et al., 2007) but the link to mammalian non-neuronal cells and any potential implications for tumors remain unknown. Interestingly, the extracellular facing CA IV acts as the principle CO_2 "taste" receptor on the tongue (Chandrashekar et al., 2009). In the context of the tumor microenvironment this study provokes the question if CA IX/CA XII acts as a principle CO_2 sensor to initiate cellular events in tumor cells. CO_2 can also be sensed by G-protein regulated adenylyl cylases (ACs) which are a member of the class III family of ACs (Townsend et al., 2009). Soluble AC (sAC) is another member of this family that is directly stimulated by HCO_3^- to produce cAMP (Chen et al., 2000). sAC involvement in sensing pH changes was illustrated in Ae2-deficient mice fibroblasts that showed an elevated resting pH_i of 0.22 U higher than wild-type cells (Mardones et al., 2008). In these cells, knockout of Ae2 led to an increase in sAC expression and activity along with an increase in cAMP which in turn affected CREB phosphorylation (Mardones et al., 2008). This study indicates the importance of sAC in sensing changes in intracellular HCO_3^- and pH (Mardones et al., 2008). However, nothing is presented yet for the involvement of any AC members in tumor CO_2, HCO_3^-, or pH sensing and it remains to be seen if these sensors have a role in regulating tumor growth. Furthermore, direct extracellular acid sensing is possible via H^+-regulated G-protein coupled receptors (Ludwig et al., 2003) while intracellular acid sensing can occur via Pyk2 signaling (reviewed by Tresguerres et al., 2010). Additionally, acid-sensing ion channels (ASICs) are controlled by extracellular H^+ and are predominantly neuronal proteins with expression in other non-neuronal tissues as well (for review see Lingueglia, 2007). Functional expression of pH-sensing ASICs was reported in adenoid cystic carcinoma (ACC) cells (Ye et al., 2007). The authors suggested that ASICs could function in these cells to sense the external acidosis found in tumors and initiate gene transcription; however, the data is not yet sufficient to support this conclusion. ASIC1 importance in glioblastoma progression was demonstrated with the elevated expression in glioma cells compared to normal astrocytes and the knockdown of ASIC1 using shRNA inhibiting cell migration

(Kapoor et al., 2009). The role of tissue acidification was not explored in this study and the role of ASICs in other non-neuronal cancers remains to be explored as well. The pH sensors reviewed briefly here, and others, may provide insight in future studies for the control of tumor growth by pH disruptions.

Summary and Perspectives

Tumor cell metabolism studies in recent years have re-vitalized the efforts to understand the relationship of pH dynamics to tumor growth and survival. Numerous studies reviewed here provide proof of principle that alterations in pH regulation provide an effective means to limit tumor proliferation. The redundancy of pH_i regulating machinery appears to have limited the ability to realize efficient killing of tumor cells by pH_i disruptions thus far. In consideration of the link between pH regulation, tumor growth, and metastasis it is imperative that the role of various pH regulating proteins and their interactions continue to be investigated. Furthermore, if pH is an essential component of tumor cell survival, then the mechanisms that sense changes in pH will ultimately control the initiation of adaptive responses within the cell. Achieving an understanding of pH sensing and the subsequent initiation of pH regulation in tumor cells will enable the exploitation of the one defining marker of tumor cells, that of reversed pH gradients compared to normal cells, to provide effective cancer therapy.

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