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Regulation of tumor pH and the role of carbonic anhydrase 9

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Abstract The high metabolic rate required for tumor growth often leads to hypoxia in poorly-perfused regions. Hypoxia activates a complex gene expression program, mediated by hypoxia inducible factor 1 (HIF1α). One of the consequences of HIF1α activation is up-regulation of glycolysis and hence the production of lactic acid. In addition to the lactic acid-output, intracellular titration of acid with bicarbonate and the engagement of the pentose phosphate shunt release CO_2 from cells. Expression of the enzyme carbonic anhydrase 9 on the tumor cell surface catalyses the extracellular trapping of acid by hydrating cell-generated CO_2 into HCO_3^- and H^+. These mechanisms contribute towards an acidic extracellular milieu favoring tumor growth, invasion and development. The lactic acid released by tumor cells is further metabolized by the tumor stroma. Low extracellular pH may adversely affect the intracellular milieu, possibly triggering apoptosis. Therefore, primary and secondary active transporters operate in the tumor cell membrane to protect the cytosol from acidosis. We review mechanisms regulating tumor intracellular and extracellular pH, with a focus on carbonic anhydrase 9. We also review recent evidence that may suggest a role for CA9 in coordinating pH_i among cells of large, unvascularized cell-clusters.

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1 The pH of tumors

A fundamental paradigm of whole-body acid/base regulation is the maintenance of a favorable extracellular pH (pH_e) at around 7.4 [1]. In line with the Henderson-Hasselbalch equation (Fig. 1), pH_e is dependent on the concentration of CO_2 and HCO_3^-, the components of carbonic buffer. In health, pH_e is held constant through the fine tuning of plasma CO_2 partial pressure (PCO_2) and bicarbonate concentration ($[HCO_3^-]$) by the lungs and kidneys, respectively (Fig. 1). The effect of acid/base disturbances on pH_e will be minimized by an immediate reaction with carbonic buffer, followed by slower physiological adjustments that return PCO_2 and $[HCO_3^-]$ to optimal levels. A major source of acid is aerobic and anaerobic cellular respiration, which generates CO_2 and lactic acid, respectively. If allowed to accumulate inside cells, intracellular pH (pH_i) would fall to dangerously low levels. Acidic pH_i has been shown to affect cell function, growth and division [1–3], it can be clastogenic [4] and even induce apoptosis [5]. To counter this acid-load, cells have evolved multiple membrane transport mechanisms to extrude acid into the extracellular environment and maintain a favorable pH_i of ~7.2 (Fig. 1).

Since most tissues have alkaline pH_e and near-neutral pH_i, it could be postulated that this arrangement provides a selective advantage for cell growth and development. Nobel prize-winning work by Otto Warburg in the 1930s [6] showed that tumors, in contrast to normal tissue, have a very high capacity to produce lactic acid (even in the

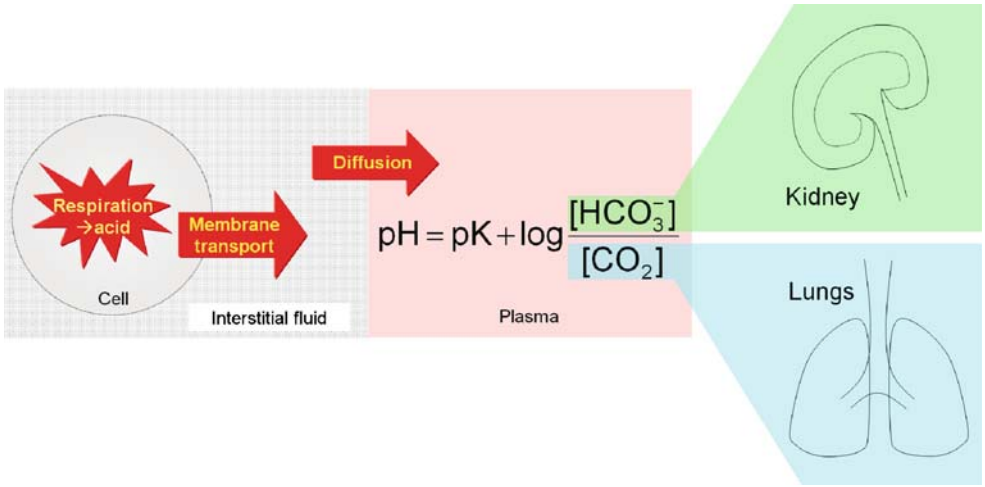

Fig. 1 Acid is the end-product of metabolism. There is therefore a tendency for intracellular pH to fall. This is prevented by driving acid-equivalents out of cells, thereby acidifying the extracellular milieu. The principal extracellular buffer is carbonic (CO_2/HCO_3^-) buffer (pK 6.1). Cell-generated acid will reduce by extracellular HCO_3^-, thus raising the $[CO_2]/[HCO_3^-]$ ratio. In cells that are near capillaries, changes in carbonic buffer will be minimized by the tight regulation of CO_2 partial pressure and $[HCO_3^-]$, performed by the lungs and kidneys, respectively. If the diffusive coupling between the immediate cell environment and blood plasma is reduced (e.g. longer diffusion distances due to inadequate vasculature), then the steady-state pH in the cell surroundings will acidify

presence of O_2) which, combined with inadequate vasculature gives rise to acidosis. The acidic nature of tumors was confirmed with electrode studies [7, 8] and for many years it was believed that both pH_i and pH_e were low. The implementation of ^{31}P nuclear magnetic resonance (NMR) technology on living tissue in the 1980s showed that tumors were, in fact, alkaline [9]. The apparently conflicting results obtained by the two methods could be reconciled by considering that ^{31}P NMR measurements are weighted towards pH_i whereas electrode measurements sample pH_e. It soon became apparent that tumor cells have low pH_e and a higher pH_i. This concept was confirmed by studies using extracellular probes for ^{31}P NMR e.g. 3-aminopropylphosphonate [10] and more recently with ^1H probes such as IEPA [11]. NMR technology was a considerable step forward as it permitted spatial pH imaging that could not be achieved with single electrode studies. Even better spatio-temporal resolution (μm^3 and seconds versus mm^3 and minutes) can be attained with fluorescent dyes such as carboxy-SNARF-1 [12, 13], selectively targeted to the intra- or extracellular compartments. The current consensus is that, in most tumors, pH_i lies between 7.0 and 7.4, similar to non-tumor cells, whereas pH_e is typically between 6.9 and 7.0 [14], although values as low as 6.0 have been reported [15]. Indeed, some *in vivo* tumors can acidify venous blood in patients [16] because of their low pH_e (Fig. 1).

Low pH_e appears to give selective advantage for tumor growth and development [17]. Extracellular acidity has been shown to support *in vitro* invasion in melanoma cells [18] and promote *in vivo* metastasis in sarcoma and melanoma tumors [19], possibly by favoring degradation of the extracellular matrix and basement membrane [20]. Reaction-diffusion models also propose a key role for low pH_e in tumor invasiveness [21]. As discussed earlier, intracellular acidosis poses a threat to cell survival, and tumor cells are no exception. Maintenance of a normal pH_i is a key cellular strategy to protect against apoptotic death [22] and permit cell proliferation [23]. An additional protective mechanism in cancer cells is a mutation in the transcription factor p53 which otherwise recruits cells into apoptosis [24].

Warburg's original model of elevated lactic acid production in tumors must be modified to explain how tumor cells maintain a high pH_i but a low pH_e. A notable and near-universal acid-extruder is the amiloride-sensitive Na^+-H^+ exchanger (NHE), a secondary active transporter, described in several tumors including malignant gliomas [25], mammary sarcoma and breast cancer [26]. Some studies (e.g. [26]) have proposed that a stilbene-blockable Na^+-dependent Cl^-/HCO_3^- exchanger (NCBE) is a more potent regulator of pH_i. NCBE does not directly transport H^+ ions, but its inward transport of HCO_3^- neutralizes intracellular H^+ ions, thus raising pH_i. In this way, NCBE mediates an H^+-equivalent efflux. In the same way, $Na^+ - HCO_3^-$ co-transport, operating in the HCO_3^- influx mode, can produce an H^+-equivalent efflux. High expression of the vacuolar (V-type) H^+-ATPase has been reported in tumor cells [27]. The expression of this type of pH regulator may be a reflection of the unreliability of the ionic composition of the extracellular milieu which drives secondary active transport. V-type ATPase may serve an anti-apoptotic role in cancer cells. Neutrophil apoptosis is secondary to cytosolic acidification, and both events can be delayed by stimulation

of the V-type ATPase. Moreover, transfection of fibroblasts with V-type ATPase [28] (or indeed NHE [29]) renders cells tumorigenic.

2 Lactic acid production and transport

Positron emission tomography for ^{18}fluorodeoxyglucose [30, 31] has confirmed that elevated lactic acid production is coupled to a near-universal increase in glucose utilization by tumors. It has been hypothesized [17] that the high glycolytic rate observed in tumors gives cells a strong survival advantage over aerobic respiration, despite the considerably lower ATP yield. A correlation has been reported between glycolytic flux and tumor behavior: the aggressive MDA-m6-231 cell line has a much greater degree of glycolysis up-regulation than the non-invasive breast cancer cell line MCF-7 [17]. In line with the so-called Pasteur effect, hypoxia is classically known to play a central role in the development of the tumor glycolytic phenotype. The combination of a high metabolic rate required to sustain cancer growth and inadequate capillary perfusion can lead to O_2 depletion in tumors. Using window chambers in animal models, oxygen electrodes have detected a fall of O_2 partial pressure to around nil at distances as short as 100 μm from vasculature [32]. This is in agreement with classical work by Krogh [33] who used a mathematical approach to derive a capillary-cell distance of 150 μm as the upper limit for adequate tissue oxygenation. Although angiogenesis is a cardinal tumor phenotype, it is believed to be preceded by raised glycolysis [34]. Even after the onset of angiogenesis, O_2 delivery may remain inadequate because the vasculature is often abnormal and heterogeneous [35].

Low O_2 up-regulates glycolysis through a signaling cascade that involves activation of hypoxia-inducible factor-1 (HIF1α). HIF1α activation was first shown to up-regulate erythropoietin transcription [36]. Currently, over 40 genes are known to be under the control of HIF1α [37, 38]. It is accepted that the Warburg effect is partly mediated by HIF1α since several gene targets code for proteins involved in glycolysis [39] (Fig. 2), and because there is a correlation between tumor growth and HIF1α over-expression or mutation [38].

Elevated glycolytic flux is achieved by increasing intracellular glucose availability through up-regulation of GLUT1/3 carriers and hexokinase activity [40] (Fig. 2). Cell metabolism switches to lactic acid production by up-regulation of lactate dehydrogenase (LDH) to increase pyruvate-to-lactate flux [40, 41] and of pyruvate dehydrogenase kinase (PDK) to block pyruvate recruitment into the Krebs cycle [42, 43] (Fig. 2). Although HIF1α activation can account for raised lactic acid output, recent studies have

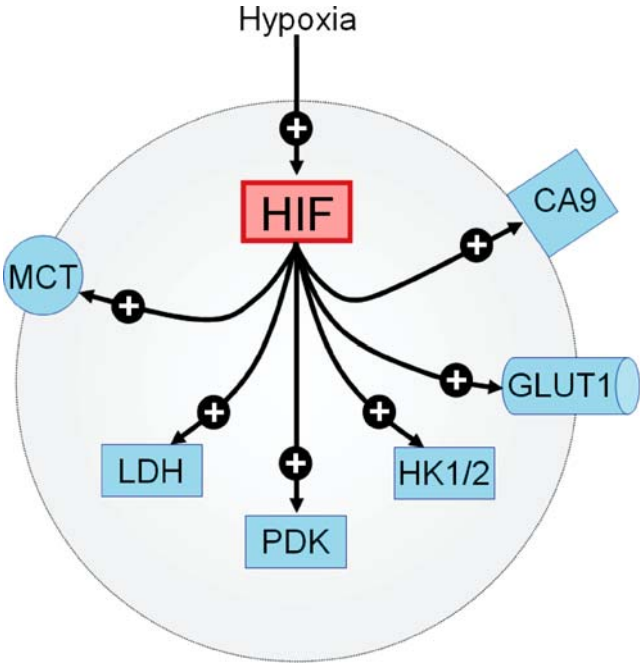

Fig. 2 Hypoxia (O_2 partial pressure of the order 1–0.1%) activates hypoxia-inducible factor (HIF1α) in cells. At present, over 40 genes are known to be regulated at the level of transcription by HIF1α activation. The diagram summarizes key HIF1α targets, related to glycolysis and pH regulation. MCT, monocarboxylate transporter; LDH, lactate dehydrogenase; PDK, pyruvate dehydrogenase kinase; HK1/2, hexokinase 1 and 2; GLUT1, glucose transporter-1; CA9, carbonic anhydrase 9

shown that glycolysis persists in tumors which show measurable periodic fluctuations in PO_2 [44]. Additional mechanisms are required to sustain aerobic glycolysis, e.g. up-regulated Akt signaling [45]. Spatio-temporal heterogeneity in oxygenation status may be a means by which tumor cells are selected for, as these cells will engage persistently in glycolysis.

The coincidence that tumors have high lactic acid output and low pH_e [46] has led to a popular belief that lactic acid (pK_a=3.9) is the source of acidosis (net $2H^+$/glucose [47]), especially under conditions of poor blood perfusion [8, 48]. The idea was reinforced by classical work that reported a reduction of pH_e following hyperglycaemia in rodent sarcomas *in vivo* [49] (although controversy surrounds the interpretation of this work [50]). The realization that pH_i is alkaline required a mechanism for lactic acid extrusion to prevent its cytosolic accumulation. The discovery of monocarboxylate transport (MCT) [51] offered a mechanism for achieving low pH_e while keeping pH_i alkaline.

Monocarboxylate transporters (MCTs) co-transport H^+ ions with monocarboxylate anions such as lactate or pyruvate [51]. There are four known transporter families (MCT1-4) which differ in their distribution [52]. MCT1 is fairly widely expressed, whereas MCT4 is more strongly expressed in glycolytic tissue such as white muscle. MCT2

is not expressed in most human tissues and MCT3 is mainly expressed in retinal pigment epithelium. Therefore MCT1 (also known as SLC16A1) and MCT4 (SLC16A3) are of particular importance to tumor cells. MCT4 has been reported to be inducible by hypoxia in bladder cancer cell lines [53] and the underlying mechanism was later linked to HIF1α [54] (Fig. 2).

3 The role of CO_2 as a source of extracellular acidity

The concept that lactic acid is responsible for generating low pH_e was challenged by experiments performed on tumors with downgraded glycolytic ability. Glycolysis-deficient Chinese hamster lung fibroblasts (lacking phosphoglucose isomerase activity), when transfected into mice, produced an acidic extracellular milieu of pH 6.7 despite negligible *in vitro* lactic acid production [55]. The cells were not, however, capable of acidifying culture media *in vitro*. Chinese hamster ovary cells with deficient lactate dehydrogenase (LDH) activity showed considerably lower glucose utilization and lactic acid output. Nonetheless, they produced an acidic extracellular environment when transplanted into mice [56] but not *in vitro*. These findings suggest that the acid responsible for low pH_e *in vivo* must be volatile i.e. CO_2. A high CO_2 partial pressure (59–84 mmHg vs 50–66 mmHg for venous blood) has indeed been measured in rodent solid tumors as early as the 1960s [57], but its importance as an acid was somehow overshadowed by lactic acid.

There are three possible sources of CO_2. Firstly, the Krebs cycle produces a molecule of CO_2 for each carbon atom. This source is probably negligible in most tumor cells because of their commitment to glycolysis, although it may occur in the tumor stroma (see Fig. 6). CO_2 can also be obtained from the pentose phosphate shunt, which has a particularly high flux during periods of demand for nucleotide synthesis, and therefore may be an appealing mechanism in tumors. A study on tumor cells with reduced phosphoglucose isomerase (PGI) activity [58] has suggested that the down-regulation of this enzyme shifts glucose metabolism from glycolysis to the pentose phosphate shunt. It is plausible that tumors where PGI activity has not been experimentally down-regulated may still show high flux through the pentose phosphate shunt as a result of glucose-6-phosphate accumulation.

Another route for CO_2 generation, independent of glycolytic or Krebs-cycle enzymes, is a titration of bicarbonate (HCO_3^-) by metabolically-generated acid (Fig. 3). HCO_3^- is the conjugate base of carbonic acid. Provided CO_2/HCO_3^- can function as an open buffer, it will ensure an ample supply of HCO_3^-, especially if pH_i is

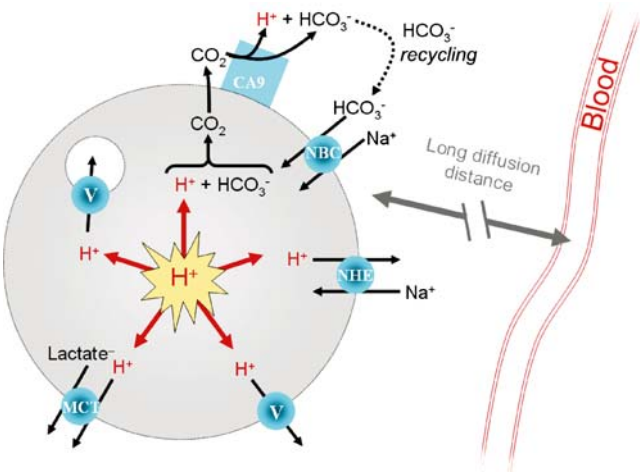

Fig. 3 Cancer cells generate considerable amounts of H^+-ions as a consequence of their high metabolic rate. These must be extruded in order to maintain an intracellular pH above 7, a prerequisite for cell growth. H^+-ions can be packaged into vesicles by V-type H^+ ATPase (V). Alternatively, they can be extruded across the surface membrane by a similar V-type H^+ ATPase (V), by Na^+-H^+ exchange (NHE) or by the monocarboxylate transporter (MCT), which co-transports H^+ with an organic anion. Intracellular H^+-ions can also titrate intracellular HCO_3^-, forming CO_2 that is freely membrane-permeant and can exit cells. H^+, carried in the form of CO_2, can be trapped extracellularly by CO_2 hydration, catalyzed by the surface tethered carbonic anhydrase 9 (CA9). Intracellular $[HCO_3^-]$ can be replenished by Na^+-driven bicarbonate co-transport processes (NBC) such as $Na^+ - HCO_3^-$ co-transport or Na^+-dependent Cl^-/HCO_3^- exchange. Thus HCO_3^- cycles at the membrane and, in doing so, deposits H^+-ions in the extracellular space. Poor extracellular perfusion, which is characteristic of tumors, implies large diffusion distances

high [1] (sustained by the mechanisms outlined in Fig. 3). In addition, HCO_3^- influx mechanisms such as $Na^+ - HCO_3^-$ co-transport or Na^+-dependent Cl^-/HCO_3^- exchange can maintain a high steady-state $[HCO_3^-]_i$ (Fig. 3). In most cells at resting pH_i, $[HCO_3^-]_i$ is typically ~12 mM. For a system open to CO_2, this is equivalent to an intracellular buffering capacity of 28 mM/pH [1] i.e. it takes 28 mM of H^+-equivalents to reduce pH by 1 unit.

For effective removal of H^+-equivalents from cells, CO_2 must be preferentially hydrated in the extracellular space. Otherwise, cell-derived CO_2 could be re-hydrated in the cytosol and short-circuit acid-extrusion. The kinetics of spontaneous CO_2 and HCO_3^- inter-conversion are slow. Provided there is a sufficient outward PCO_2 gradient, CO_2 can exit cells before being hydrated intracellularly. Acid can be trapped extracellularly when CO_2-hydration is catalyzed by the enzyme carbonic anhydrase (CA) present in the extracellular medium. Acid-trapping has been illustrated experimentally by the acidification of cell-suspensions following the addition of purified CA [59]. To date, 13 active isoforms of CA have been described [60, 61]. Some CA isoforms are expressed in the cytoplasm (e.g. CA1, 2, 3, 7) and would favor intracellular acid trapping. Several isoforms have been localized to the

surface membrane (e.g. CA4, 9, 12, 14, 15) and these are potential candidates for extracellular acid trapping. Figure 3 highlights the importance of HCO_3^- influx mechanisms in sustaining acid-excretion. H^+-ions, shuttled across the membrane in the form of CO_2, are deposited extracellularly, HCO_3^- ions, also produced from CO_2-hydration, are recycled back into the cell to ensure that continuous intracellular H^+-production does not saturate the buffering apparatus. This arrangement between a transport event (e.g. NBC) and reaction catalysis (e.g. CA9) is functionally akin to a 'HCO_3^- transport metabolon' [62].

Despite convincing evidence for CO_2 as an acid-donor responsible for acidifying tumor milieu, there is an important role for lactic acid in contributing to pH_e. The inter-relationship between lactate and carbonic anhydrase function in maintaining low pH_e was recently investigated in clinical samples [16]. In patients with gastrointestinal carcinomas undergoing surgery, arterial and venous pH, oxygen levels, CO_2 content and glucose content were measured, and HIF1α, LDH5, CA9 and GLUT1 were assayed. The study showed that LDH5 was linked to low oxygen consumption, high CA9 expression was correlated with high CO_2 production, and GLUT1 expression was related to high glucose consumption. The study showed that consumption of oxygen and glucose differed substantially between cancers, but in the majority of cases there was an inverse relationship between oxygen consumption and glucose utilization. This correlation was stronger in patients with high LDH5 and HIF1α. The combination of high CA9 and high LDH5 was associated with the lowest pH in the tumor draining veins. This study emphasizes the synergy and additive affects between these two major metabolic routes to generation of acidic pH_e. The attainment of low pH_e and a higher pH_i therefore requires special adaptations in cancer cells. A summary of these pathways is illustrated in Fig. 4.

4 Carbonic anhydrase 9 and the tumor phenotype

CA9 is a transmembrane isoform of carbonic anhydrase with an extracellular-facing catalytic site [60] characterized by the highest H^+ transfer rate known among CAs [63]. The protein was first detected in HeLa cells and named the MN protein [64]. Its expression was induced by high cellular confluence which, in retrospect, was a reflection of hypoxia. Immunofluorescent studies, with an antibody against CA9 (M75), showed it was on the extracellular cell

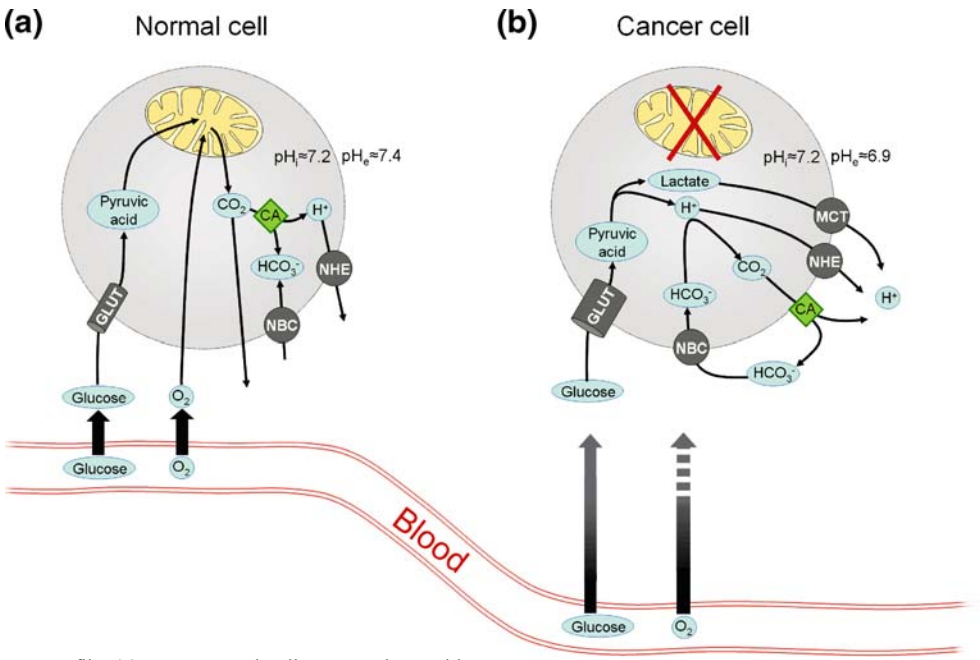

Fig. 4 The respiratory profile. (**a**) Most normal cells engage in aerobic respiration, whereby pyruvate generated from glucose is recruited into the Krebs cycle (mitochondria). This requires blood-derived O_2 and generates CO_2, an acidic molecule which is free to exit across the cell-surface. CO_2 can also be hydrated intracellularly, giving H^+ and HCO_3^-. To prevent cytosolic accumulation of acid, membrane transporters such as Na^+-H^+ exchange (NHE) can restore a favorable steady-state intracellular pH. Short cell-capillary distances underlie good diffusive coupling, thus the immediate extracellular environment of the cell has a pH close to plasma pH. (**b**) Cancer cells switch from aerobic to anaerobic respiration. Glucose is converted to lactic acid, which can either be extruded across the cell by the monocarboxylate transporter (MCT) or it can be titrated against intracellular HCO_3^- brought into cells by Na^+-driven HCO_3^- co-transport processes (NBC) such as $Na^+ - HCO_3^-$ co-transport or Na^+-dependent Cl^-/HCO_3^- exchange. Thus formed CO_2 can cross the membrane and hydrate extracellularly with the aid of carbonic anhydrase 9 (CA9). Since cell-capillary diffusion distances are greater in tumors, diffusive coupling is poor and can lead to extracellular acidosis

surface, but could also have a nuclear localization [65]. CA9 was cloned by Pastorek et al. [66] and found to be a 466 amino acid-long protein with a hydrophobic signal peptide, a proteoglycan related domain at the N-terminus, a carbonic anhydrase catalytic domain, a hydrophobic transmembrane segment and a short intracellular C-terminus. The catalytic domain shows high homology with other carbonic anhydrases including critical, zinc-binding histidine residues at the active site. The role of the C-terminal region is less clear but recent evidence has shown that it is a target for phosphorylation and may be important for signaling functions [67], dimerization and interaction with other bicarbonate transporters in a metabolon, by analogy to CA2 [62]. The role of the proteoglycan domain remains unclear but it may serve a purpose in cell adhesion [68]. This ectodomain is cleaved by a metalloprotease [69] although the significance of this *in vivo* remains to be elucidated.

CA9 is found in normal gastrointestinal cells [70] but its expression is most notable in cancer. An important link between CA9 and tumorigenesis was made when it was discovered that CA9 (and CA12, another membrane-tethered isoform) are regulated by hypoxia through HIF1α [71]. CA9 is in fact one of the most inducible hypoxic genes, an observation that may be related to the overlay between the hypoxia response element and the CA9 start of transcription [71]. Using a monoclonal antibody against CA9, extensive studies have investigated CA9 expression and its relationship to tumor behavior and prognosis. CA9 was assessed semi-quantitatively by intensity and area of tumor occupancy and found to be related to prognosis in cancers of the lung, breast, cervix and many other tumor types [72–78]. This correlation was linked to HIF1α expression and other markers of hypoxia such as LDH5 and VEGF. In some studies it was a strong, independent, prognostic factor. In breast cancer, for instance, CA9 was associated with the oestrogen receptor-negative phenotype [74] and tumors resistant to epirubicin [79].

CA9 expression has also been shown to correlate with hypoxic areas, assayed with O_2 electrodes, providing independent validation of CA9 as a marker of hypoxia [80]. However, these studies are complex in that the timescales of the measurements differed, and the biopsy versus O_2-assayed tissue areas may not have overlapped. To tackle this issue, other studies have investigated pimonidazole staining and GLUT1 expression in relation to CA9 expression [81]. These markers, in general, show a degree of overlap but correlations can be poor and may reflect multiple pathways. For example, Akt may regulate GLUT1, and delays between pimonidazole administration and surgery, and also the rate of pimonidazole metabolism, may vary considerably. The area of hypoxia may also vary through acute intermittent hypoxia [32]. These studies have highlighted the need to develop a quantitative, reproducible and reliable method for measuring hypoxia in a non-invasive way.

One way to test the suitability of CA9 as a measure of hypoxia is to investigate CA9 levels in randomized trials, and to compare these data with the outcome of a given modality of treatment that can be influenced by hypoxia. One example is a randomized trial of head and neck cancer in which patients received either continuous radiation therapy [CHART] over a 12-day period with three doses per day, versus the normal 6-week period of radiation with 5 days per week therapy. The overall outcome of the two strategies showed no significant difference. However, analyzing the subgroup of patients with primary tumors showed that those with high CA9 had a much worse prognosis than those with low CA9 [82]. There was indeed a therapeutic benefit for patients with normoxic tumors, as classified by CA9 staining. In other words, normoxic tumors did not require reoxygenation and the effect of radiation hyper-fractionation was more effective in this group. Conventional radiotherapy allows a period of reoxygenation, which is probably more important for hypoxic tumors. Another plausible explanation is that normoxic tumors have a higher growth rate and therefore would be more susceptible to hyper-fractionated radiation which impedes DNA repair.

More direct evidence has come from siRNA knockdown of CA9 expression. Cell-lines treated with siRNA against CA9 were less likely to grow to maximum density in tissue culture and showed reduced clonogenic survival both in normoxia and in hypoxia [83]. Taken together, this evidence suggests that CA9 may be useful as a stratifying factor in randomized trials of hypoxia modulation therapy, and that direct inhibition of CA9 function should be considered. However, the knockdown experiments do not prove that therapeutic benefit is due to inhibition of the carbonic anhydrase activity. There could be other reasons for the outcome, involving a role for interactions with CA9 through its internal domain or its proteoglycan domain, separately from just knocking out CA9 catalysis.

In summary, CA9 is highly regulated by hypoxia, and related to the tumor phenotype (e.g. [84, 85]). Considerable progress has been made in deciphering the molecular mechanisms of regulation. The exact physiological role of CA9, however, remains unclear.

5 Physiological role of carbonic anhydrase 9

As discussed previously, at least part of the cause of extracellular acidosis is likely to be cell-generated CO_2. The effectiveness of extracellular acid-trapping depends on the ability of CO_2 to be hydrated to HCO_3^- and H^+ outside the cell. The extracellular catalytic activity of CA9 appears

to satisfy this requirement (Figs. 3 and 4), and this has been proven, in principle, by the work of Svastova et al. [86]. In their study, MDCK epithelial cells constitutively expressing CA9 were shown to acidify culture media when exposed to periods of hypoxia. Acidification was slowed in the presence of sulphonamide inhibitors of CA. Acid generated by metabolism reacts with HCO_3^- to liberate CO_2. On exit from cells, CO_2 is catalytically hydrated by CA9 to HCO_3^- and H^+. As outlined earlier, this mechanism can only work efficiently if (1) [HCO_3^-] is replenished and (2) the catalysis of CO_2 hydration is no faster inside cells than on the surface. Replenishment can be attained with membrane transporters such as Na^+-dependent Cl^-/HCO_3^- exchange which has been characterized in mammary sarcoma (EMT6), human breast cancer (MCF-7), and Chinese hamster ovary [26]. A matched intracellular and extracellular CO_2-hydration rate is not unreasonable since most cells, with the notable exception of red blood cells [87], show a modest level of intracellular CA activity (e.g. cardiac myocytes [88], chondrocytes [89]).

Carbonic anhydrase enzymes have recently received considerable scientific interest and many novel physiological roles have been proposed. CA isoforms have been implicated in (1) facilitating transmembrane HCO_3^- transport, (2) facilitating H^+ diffusion, and (3) facilitating CO_2 diffusion. Membrane HCO_3^- transport is proposed to be augmented by CA, through the enzyme's ability to optimize HCO_3^- supply [62]. Carbonic buffer has also been shown to facilitate diffusion of intracellular H^+-ions in murine enterocytes [90] and cardiac myocytes [91]. The intracellular buffering environment has a large contribution from poorly mobile proteins which severely reduces cytosolic H^+-diffusion [92]. In the presence of CA, carbonic buffer can enhance H^+-mobility by generating H^+ from CO_2 in alkaline regions, and removing H^+ (through combination with HCO_3^-) from acidic regions. In effect, this produces an H^+-shuttle, mediated by the spatial diffusion of CO_2 and HCO_3^- in opposite directions. A similar mechanism may also enhance *extracellular* H^+-mobility. In addition to H^+-mobility, rapid equilibration between CO_2 and HCO_3^- can enhance CO_2-mobility. For example, simple CO_2 diffusion can be supplemented by a parallel flux of HCO_3^-, followed by its conversion to CO_2. Indeed, CO_2 diffusion from skeletal muscle cells to plasma is believed to be facilitated by membrane-anchored CA4 in capillary walls [93], and a similar arrangement is believed to facilitate CO_2 transfer at the lung surface [94].

As illustrated earlier (Fig. 3), the expression of CA9 may provide one component of a functional HCO_3^- transport metabolon [62, 95]. The combination of extracellular CA activity and a membrane HCO_3^- transporter such as NBC or NBCE effectively moves H^+ ions out of the cell. At present, it is not clear if this collaboration between CA9 and NBC/NCBE is merely functional, or if it requires a physical union between the two molecules i.e. whether CA9 binding to the transporter is necessary ([62, 95] cf [96]).

The potential for accelerated H^+/CO_2 mobility is relevant to tumors. As discussed earlier, tumor cells often operate outside the oxygenation limit set by Krogh cylinders, and therefore diffusion distances can be considerable. The high rate of production of metabolic acid may sometimes saturate the ability of carbonic buffer to cycle H^+-ions out of the cell. Long extracellular diffusional distances may also severely uncouple carbonic buffering between blood plasma and the tumor milieu. A situation may be envisaged in which most HCO_3^- has been titrated by intracellular H^+-ions, leaving cells poorly buffered, and eventually leading to acidosis. Since tumor cells are at varying distances from capillaries, significant pH_i and pH_e non-uniformity may occur, characterized by a core-acidosis, with consequent break-down of coordination within the developing tumor (e.g. with cells entering different stages of the cell cycle).

To salvage core cells from the adverse consequences of pH non-uniformity, diffusion of CO_2 and HCO_3^- must somehow be accelerated. In a recent study [13], we have used clusters of cells (spheroids; average diameter ~410 μm or 45 cell-lengths) of RT112 cells imaged confocally for pH_i using the fluorophore carboxy-SNARF-1. As indicated in Fig. 5, a radial non-uniformity of pH_i was observed across the cluster, with the core being more acidic than the periphery. This special non-uniformity was considerably smaller in clusters of CA9-expressing cells (Fig. 5(b); $\Delta pH = 0.10$), compared with similar clusters where CA9 was not expressed (Fig. 5(a); $\Delta pH = 0.25$). The CA inhibitor, acetazolamide, almost doubled pH_i non-uniformity in the CA9-expressing clusters. These data can be interpreted in terms of CA9 increasing the 'bandwidth' for spatial H^+/CO_2 diffusion, i.e. the capacity for spatial H^+ and CO_2-flux. This is illustrated in Fig. 5(c). To protect against low pH_i, a steep outward transmembrane PCO_2 gradient must be maintained to ensure efficient CO_2 removal from cells. CO_2 released from cells is then driven across the interstitial fluid, towards vasculature by means of a radial extracellular PCO_2 gradient. However, the diffusional CO_2-flux is low, because of the large diffusional distances from the core. The flux magnitude can be raised by rapidly converting extracellular CO_2 into HCO_3^-, thereby allowing two parallel diffusive processes (i.e. increasing the bandwidth for CO_2 flux). A certain fraction of HCO_3^- fluxes back into cells (Fig. 3; bicarbonate recycling) thus further increasing the rate of CO_2 removal from cell surroundings. This diffusion-reaction model will accelerate CO_2 removal from the intracellular milieu by sustaining a steep transmembrane CO_2 gradient. This serves to protect pH_i from acidosis at the expense of acidifying the extracellular space. Expressing CA9 can therefore increase the degree of core-periphery

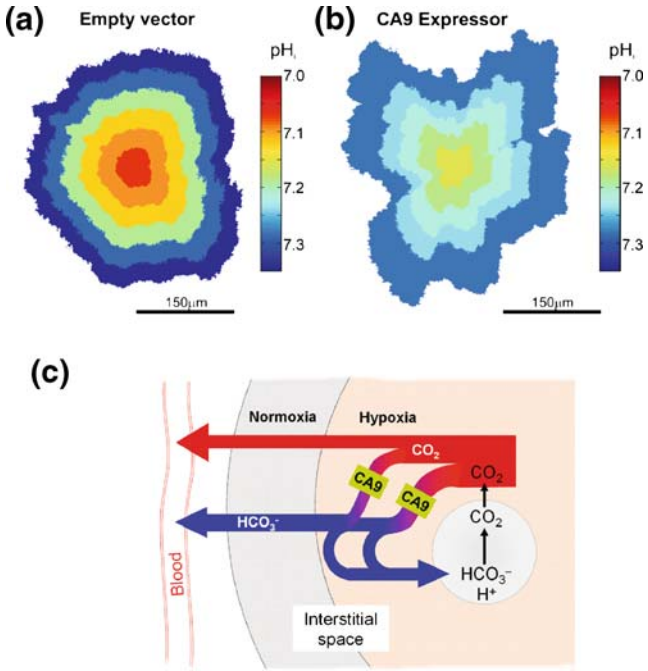

Fig. 5 (**a**) Cell cluster made of RT112 cells (diameter~31 cell-lengths) transfected with an empty vector and loaded intracellularly with the pH-sensitive fluorophore, carboxy-SNARF-1. The intracellular pH (pH_i) map obtained from confocal fluorescence recordings suggests considerable surface-to-core pH_i non-uniformity (~0.25 units), with significant core-acidosis that could trigger cell death. (**b**) Clusters of RT112 cells (diameter~35 cell-lengths) expressing surface CA9 activity have greatly reduced pH_i non-uniformity (surface to core: ~0.1 units). (**c**) Model of role of CA9 in accelerating diffusive flux of CO_2. Core cells generate CO_2 and establish an outward diffusive PCO_2 gradient across the extracellular space. In hypoxic regions where CA9 is expressed, CO_2 can be readily converted to HCO_3^- (*blue*) and H^+ (not shown; H^+-diffusion is likely to be slow due to buffering). As a result, there are two routes available for diffusion of CO_2: as CO_2 and as HCO_3^-. A certain fraction of HCO_3^- will be recycled back into cells. This arrangement increases the bandwidth for CO_2 removal and also reduces PCO_2 around cells. Consequently, the outward transmembrane PCO_2 gradient is maintained steep, protecting cytosol from acidification. The extracellular CO_2 load, also contributes to the fall of pH_e

pH_i coordination, thereby assisting tumor survival, even in poorly perfused regions.

6 Stromal metabolism of lactate

A recent study [82] of patterns of gene expression in colon cancer has suggested a key role for the tumoral stroma in metabolizing lactic acid released from cancer cells (Fig. 6), which would otherwise saturate glycolysis. In the cancer cells, hypoxia acting through HIF1α, induces expression of lactate dehydrogenase 5 (LDH5), an enzyme with high capacity to convert pyruvate to lactate. Tumor cells also show high MCT1 and GLUT1 expression. This pattern therefore suggests a high glucose uptake, catabolism into lactate and a subsequent export of lactate. In contrast, tumor-associated fibroblasts express high MCT1/2, which can capture lactate released from cancer cells. The high ratio of LDH1 to LDH5 in fibroblasts implies far less glycolytic lactate production, and the low GLUT1 expression implies reduced glucose uptake. Collectively, it appears that fibroblasts preferentially metabolize tumor-derived lactic acid, an energy-yielding substrate. The tumor-associated endothelium has a high LDH1 and low LDH5 and MCT1, suggesting that tumor cell-derived lactate is not taken up the endothelium. Instead, the endothelium metabolizes glucose aerobically. The stroma expresses very low levels of pyruvate dehydrogenase kinase 1 (PDK1) and high levels of pyruvate dehydrogenase (PDH) [97]. PDH regulates the step from pyruvate to acetyl CoA, a key link between glycolysis and the Krebs cycle. PDH is itself regulated by PDK1. Therefore, in cancer cells, the low PDH to PDK1 ratio would block the conversion of pyruvate to acetyl CoA and prevent its oxidation in the Krebs cycle, possibly as a protective measure against free radical production [42, 43]. In contrast, the high PDH activity and/or low PDK1 expression in the stroma commits pyruvate to the Krebs cycle.

Extracellular CA9 expression may play an additional role in coupling the activity of the tumor cell to its neighbors, the fibroblasts and endothelial cells. These neighboring cells, particularly the fibroblasts, will contribute additional CO_2 for CA9-catalysed hydration, reinforcing extracellular acidification of the stromal environment, thereby augmenting further tumor development (Fig. 6).

The contrasting patterns between tumor and stroma gene expression are suggestive of a special role for the stroma in lactate uptake and its metabolism (Fig. 6). The aforementioned model requires a coordinated gene expression program in both the tumor and its stroma. This may be a decisive factor for metabolic efficiency and tumor survival.

It should be noted that tumor-generated lactate could then stimulate glycolysis, via a feedback loop, through its inhibitory interaction with prolyl hydroxylases [98]. In the tumour cells, lactate is metabolized to pyruvate and even in a low O_2 environment it can be oxidized to oxoloacetate via the Krebs cycle. This molecule can bind to the 2 oxoglutarate site of HIF1α prolyl hydroxylases and block their inhibitory effect on HIF1α.

7 Therapeutic implications

High CA9 expression in hypoxic areas of tumors and relatively low expression in normal tissues, coupled with the poor prognosis and the aggressive phenotype associated with CA9, make this enzyme a candidate target for therapy. The demonstration of the survival role of CA9 in normoxic

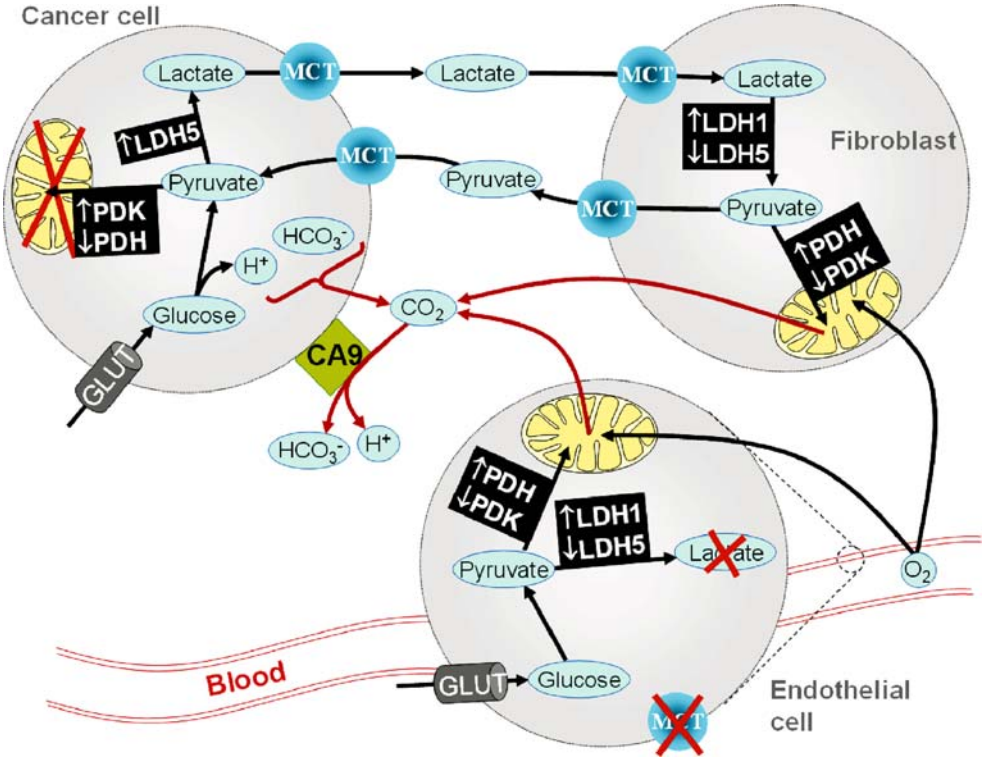

Fig. 6 Model of tumor-stroma interactions. The complementary expression patterns for enzymes (LDH lactate dehydrogenase, PDH pyruvate dehydrogenase, PDK pyruvate dehydrogenase kinase) and transporters (MCT monocarboxylate transporter, GLUT glucose transporter) suggest a key role for stroma cells in metabolizing the lactic acid load generated by respiring tumors. Lactic acid can still yield energy on oxidation thus stroma can benefit from this arrangement and the tumor environment can be protected from lactic acid overload, which could otherwise saturate glycolytic flux. Note that high expression of CA9 on tumor cells will further reduce pH_e by trapping CO_2 that fluxes from respiring stromal tissue

and hypoxic cells [83] plus a recent demonstration of its importance in maintaining pH_i uniformity [13] add further support to its development as a therapeutic target. Early studies have shown that inhibition of CA can slow tumor growth [99]. Essentially, inhibition of CA9 with inhibitors of different selectivity and membrane-permeability will need to be studied. Also the consequences of inhibiting intracellular and extracellular CA isoforms will require investigation to see whether simply blocking the extracellular enzyme alone is sufficient to obtain the maximum therapeutic effect, or whether there is synergy with intracellular carbonic anhydrases. Another therapeutic strategy, linked to the role of CA9, could target HCO_3^- influx mechanisms, which complete the cytosolic acid-removal cycle. When $Na^+ - HCO_3^-$ co-transport or Na^+-dependent Cl^-/HCO_3^- are blocked with stilbenes, the efficiency of acid-removal will be severely impaired.

Manoeuvres that raise extracellular pH may be useful, as they would affect the access of drugs that are ionized at acidic pH, allowing greater cellular uptake [100]. An example of such a compound is epirubicin, perhaps one of the most important anti-cancer drugs in terms of its widespread application for many human tumors. But it is possible that inhibition of CA9 alone may suffice to allow acidic metabolites to build up intracellularly within a tumor and inhibit proliferation.

Other approaches currently being developed against CA9 include vaccines to develop T cell responses, antibodies targeting CA9 with radioisotopes, or pro-drug activation systems targeted by antibodies. These strategies do not target the catalytic activity of CA9 but instead take advantage of selective CA9 expression in tumors. Trials will need careful patient selection so that one quantifies the degree of up-regulation of CA9 in patients to see how that relates to the response to therapy. Currently, it would not be possible to determine the percentage of cells required to express CA9 for either a worthwhile immune response or sufficient effect on pH regulation and drug uptake. Obvious candidate tumors for initial investigation would be clear cell renal cancers where mutations of *vHL* result in up-regulation of HIF1 or HIF2 and hence CA9 in all cells [101].

Lactate dehydrogenase A is also critical for maintenance of tumor function and transformation. Inhibition of LDH-A by siRNA has been shown to have dramatic effects on tumor growth and behavior [102] thus it would be interesting to consider the possibility of developing drugs that would block this target and perhaps propose a combinational therapy for inhibition of CA9 and lactate production. It would be highly attractive to be able to cause a tumor's death by its own acid metabolism. Many specific inhibitors of CA9 are being developed and it is likely that such drugs will be available for clinical trial in the near future [103].

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