Multiple effects of intracellular pH modulation in cancer cells

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Cancer cells adapt to a decreased oxygen concentration by turning to anaerobic respiration and secretion of lactic acid; thus hallmarks of solid tumors are hypoxia and the consequent upregulation of specific hypoxia inducible factors. Reversed pH is also a peculiar feature of cancer cells, being their extracellular microenvironment acid while the intracellular basic. The elucidation of the molecular determinants of tumor microenvironment as well as the identification of the crucial factors present in the extracellular matrix could be instrumental to develop new approaches for modulate cancer cells metabolism. Moreover, the manipulation of intracellular pH could influence the signaling pathways governing the survival of cancer cells.

Keywords: Amiloride; cancer; hypoxia; pH; tumor microenvironment


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Introduction

The cellular context surrounding tumor cells, generally designed as tumor microenvironment, is characterized by a number of peculiar features, including hypoxia, acid pH, increased angiogenesis, and recruitment of immune components and extracellular release of signaling molecules. In fact, tumor cells shift from oxidative phosphorylation to aerobic glycolysis to produce lactate from glucose and to ensure the high level of ATP necessary for their proliferation (Warburg effect). In turn, upregulation of glycolysis leads to increased glucose consumption and microenvironmental acidosis.

In addition, according to a model proposed by Vegran et al [¹], a ‘lactate-based dialog between cancer cells and endothelial cells’ exists. Notably, lactate represents a way for cancer cells to control the blood supply through modulation of endothelial cell phenotype. Lactate is imported by endothelial cells and stimulates the NF-κB/IL-8 pathway through ROS production, finally leading to the stimulation of cell migration and angiogenesis [¹, ²].

Within the frame of the impact of Warburg effect, also an active role for lactic acid as an immunosuppressive metabolite has been postulated; indeed, aerobic glycolysis increases lactic acid secretion in order to lower microenvironment pH and suppress T-cell function. Given that it has been shown that acidic pH in human lymphocytes can interfere with the function of immune cells [³], cancer cells are able to survive through the inhibition of anticancer immune response. This view puts the focus on lactic acid
as a predictive marker of metastasis and poor prognosis in cancer patients\cite{4,5}. The main downstream effects of lactate production on cancer cell metabolism, i.e. stimulation of proliferation, angiogenesis and metastasis, as well as impairment of the immune response, are shown in Figure 1. The regulation of oxygen and pH with respect to tumor microenvironment is below described.

Oxygen regulation. A constant supply of $O_2$ is necessary to carry on mitochondrial oxidative phosphorylation to generate ATP. Due to the increased oxygen consumption, solid tumors are often hypoxic, with oxygen pressure decreased to 1% or less. Limited oxygen supply causes cell cycle arrest, thus slowing down tumor cell proliferation; this condition could favor chemoresistance towards anticancer drugs that kill specifically proliferating cells.

The identification of the signals governing tumor response to hypoxia will help to develop innovative strategies to kill cancer cells\cite{6}. As illustrated in Figure 2, hypoxia stimulates the HIF family of hypoxia-inducible transcription factors, which regulate a cascade of pathways, RNA-protein translation and, in turn, angiogenesis, tumor growth and metastasis, aiming at bypassing the metabolic stress conditions\cite{7}. The best characterized member is HIF1; two subunits are known: HIF1-$\alpha$ is a transcription factor activating, directly or through the dimerization with HIF1-$\beta$, the transcription of genes encoding enzymes and proteins involved in oxygen homeostasis and angiogenesis\cite{8,9}. Also the isoform HIF2-$\alpha$ is involved in sensing $O_2$ availability and counteracts hypoxia by forming a heterodimer with HIF-1$\beta$\cite{8,9}.

Figure 2 shows that, under normal oxygen conditions (normoxia), HIF1-$\alpha$ associates with the tumor suppressor VHL (von Hippel-Lindau) ubiquitin ligase\cite{10}, which promotes its targeting to the proteasome to be degraded in order to keep it inactive\cite{10}. Hypoxia promotes VHL nucleolar sequestration and consequent loss of its HIF-regulating function, remaining confined to nucleoli until

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**Figure 1. Features of cancer cell microenvironment.** During cancer development, to face the requirement of oxygen and nutrients, cells shift from oxidative phosphorylation to aerobic glycolysis to produce lactate from glucose thus ensuring the intracellular high ATP level necessary for their proliferation (Warburg effect). Lactate is then carried outside the cell by MCT transporters, where it acidifies the extracellular milieu, suppressing the immune response and promoting angiogenesis and metastasis. The proton pumps and transporters (e.g. V-ATPase, NHE and CA) contribute to the acid microenvironment by maintaining this reverse pH. A strategy to counteract the acidification of extracellular milieu can be the inhibition of the transporter controlling ionic flux by specific drugs (including the amiloride derivative HMA), able to lower intracellular pH, to stimulate acid DNases and activate cell death mechanisms.
the achievement of neutral pH conditions \[11\], and leading to the cascade of HIF-mediated transcriptional events schematized in Figure 2. HIF-1α, and to a lesser extent HIF-2α, represent the principal mediators of the response to hypoxic conditions and are often overexpressed in different types of human cancers \[12,13\].

In fact, tumors expressing high levels of HIF-1 have poor prognosis and high mortality \[9,14\]. For this reason, the analysis of HIF transcriptional activity towards a number of factors implicated in tumor development and progression (including angiogenic factors, telomerase, acid-base balancers and promoters of metastasis) could help in setting a more efficient therapy against cancer, based on HIF inhibitors \[15-17\].

pH regulation. Hydrogen ion turnover is deregulated in cancer cells and tissues, which show a reversed pH situation compared to the normal counterpart, becoming the extracellular compartment acidic and the intracellular neutral or basic \[18\]. The reversal of the pH gradient across cell membranes is involved in multi-drug resistance (MDR), and possibly implicated in the resistance of cancer cells to chemotherapy. A crucial role in tumor microenvironment is played by several enzymes (schematized in Figure 1), including proton pump V-ATPases (vacuolar-type ATPases), proton transporters NHEs (Na+/H+ exchanger), MCTs (monocarboxylate transporters), CAs (carbonic anhydrases), Na+/HCO3- co-transporters and Cl-/HCO3- exchangers \[19,20\], which could alter survival, differentiation and proliferation, thus promoting tumorigenesis \[21\].

V-ATPases are multisubunit proteins localized in intracellular compartments, as well as on plasma membranes, being able to translocate protons across them. These enzymes are overexpressed in tumor cells with an MDR phenotype \[22\]. The plasma membrane NH exchangers regulate the intracellular pH through the exchange of intracellular H+ with extracellular Na+ \[23\].

MCTs are encoded by the SLC16 gene family, composed by 14 members sharing conserved sequence motifs, including transmembrane helices regions \[24\]. MCT isoforms ensure the proton-linked transport across the plasma membrane of monocarboxylate metabolites, including lactate, pyruvate and ketone bodies; each isoform has distinct properties, tissue distribution and subcellular localization \[25-27\]. MCTs are not the main actors in governing H+ transport, but they contribute to the maintenance of the acid conditions favorable to tumor cell growth by exporting lactate and regulating pH \[26\]. As reviewed by Pinheiro et al. \[26\], the analysis of the expression of MCTs in cancer tissues provided contrasting results; in fact, in some tumors the overexpression of one or more isoforms was detected, while an opposite trend was recorded for other cancers, thus rendering difficult to attribute a univocal behavior to these enzymes in cancer development. Nevertheless, different approaches leading to the inhibition of MCTs have been developed (including the use of chemicals and silencing), providing promising results with respect to tumor growth, response to therapy and prognosis \[26\].

Among the factors in charge for maintaining the acid-base balance, CAs catalyze the rapid and reversible hydration of CO2 to HCO3- and H+ \[28\]. As a consequence of convergent evolution, five distinct CA families (α, β, γ, δ and ε) were formed, without significant amino acid sequence similarity; at least 16 isoforms have been described in mammals, the active site of which contains a zinc ion, so justifying their classification as metalloenzymes \[29\]. CAs participate in the structural-functional complex named transport metabolon, formed by an array of enzymes acting in a sequential manner within the metabolic pathway, together with a family of pH regulators, including Na+/HCO3- co-transporters and Cl-/HCO3- exchangers \[29\].
The first cellular compartment involved in pH regulation is the extracellular matrix (ECM), the basic functions of which are connected to the structural support for cells and tissues; during tumorigenesis, ECM has a critical function in microenvironment traits and tumor progression. ECM is composed of a complex network of specialized molecules playing a pivotal role in migration, proliferation and metastasis [30]. Within this structure, specialized proteins are present, including fiber (e.g. collagen and elastin) and non-fiber (glycoproteins and proteoglycans) forming factors and matricellular proteins responsible for cell-matrix interactions. Of note, cells produce also proteinases, mainly matrix metalloproteinases (MMPs), called matrixins, which may degrade extracellular matrix factors and release peptides able to stimulate cell adhesion, migration, proliferation, protein synthesis and apoptosis. Two classes of small peptides (matrikines) originate from the degradation of ECM: the "natural" matrikines, responsible for the signaling in a direct way from the extracellular compartment, and the "cryptic" matrikines that expose the ligand or release it from its ECM protein after proteolytic processing. Such features confer dynamism to ECM components, thus mediating tumorigenesis.

Serine- and metallo-proteinases are extremely efficient in degrading the ECM given that they are active at neutral pH [31, 32]. At acid pH, cysteine and aspartic proteinases retain a major activity, having a predominant role in lysosomal degradation and when they are released in tumor extracellular milieu and degrade the ECM [30, 33].

Intracellular environment. The cytosolic pH is an essential physiological parameter of cells that has to be constantly monitored in order to maintain homeostasis; thus, the acid extracellular microenvironment of cancer cells, which favors the dangerous tendency to migrate and disseminate metastasis, is normally counteracted by neutral or basic intracellular pH [34]. To reverse the intracellular situation with a consequent impact on the extracellular milieu, several approaches aiming at inducing intracellular acidification have been proposed, given that many reports showed that cytosol acidification occurs in apoptotic cells, favoring DNA degradation by acid DNases (Figure 1) [35,36]. The rationale of these approaches is based on the consideration that cancer cells could be eliminated by apoptosis under conditions of intracellular acidification. In this respect, it has to be considered that cancer cell lines could be defective in acidification mechanisms; as an example, it was reported that CaCo-2 (colorectal adenocarcinoma) and MCF-7 (breast cancer) cell lines show an aberrant secretion of lysosomal hydrolases, such as (pro)cathepsin D, thus being unable to acidify their endosomal compartments [37]. Given that the acidification-deficient phenotype could be a feature of drug-resistant cancer cells, it has been postulated that cathepsin D expression level could be a suitable biomarker of tumor malignancy; however, controversial data are present in the literature (reviewed in [38,39]).

Cytosolic acidification can be obtained through the exogenous administration of H2O2 (or drugs that can induce an increase in peroxide levels), which activates the Bax-mediated intrinsic/mitochondrial apoptotic pathway [40]. In other experimental conditions, the induced acidification of cytoplasm is accompanied by an increase in the apoptotic caspase 8 activity and consequent triggering of the apoptotic extrinsic pathway [41].

Another strategy to modulate acidification has been developed by Fogarty et al. [42], who analyzed the role of V-ATPases in metastatization. Assuming that for the activity of V-ATPases the association with the transmembrane protein HRG-1 (Heme-responsive gene 1) is required, it was observed that HRG-1 and V-ATPase are co-expressed at the plasma membrane in highly invasive and migratory cancer cell lines, while in less invasive cell lines HRG-1 overexpression remains confined to the intracellular compartments. When HRG-1 expression was stably suppressed in MDA-MB-231 cells (highly invasive), acidification of the cytosol and reduction in colony forming ability were observed. Thus, HRG-1 may represent a novel target for selectively disrupting V-ATPase activity, modulate intracytosolic pH levels and affect the metastatic potential of cancer cells [42].

In a neuronal background, Hwang et al. [43] demonstrated that the increase in intracellular Ca2+ concentration by glutamate and capsaicin causes intracellular acidification and that the inhibition of NHE1 (by siRNA or chemical inhibitors) can affect the capability of cells to recover pH(i) and, ultimately, have direct consequences for neuronal excitability [43].

Recently, many groups have taken advantage of the use of the inhibitor of a known inhibitor of the Na+/H+ exchanger NHE1 amiloride. Indeed, in HCC (hepatocellular carcinoma) cells the knockdown of NHE1 expression by RNAi or amiloride significantly inhibited the invasiveness and reduced the secretion of MMP-2 [44]. So, the results obtained with use of amiloride are very promising. Several amiloride derivatives have been developed by a double substitution on 5-amino group; among the derivatives, HMA (5-(N, N-hexamethylene)amiloride) is more active inhibitor of NHEs than the lead compound [45]. In fact, HMA was reported to induce intracellular acidification in Baby Hamster Kidney (BHK) fibroblasts and stimulate acid DNase II activity. These features were accompanied by time- and dose-dependent cell survival inhibition and induction of apoptosis [46]. Thereafter, the same group extended the analysis to cancer cell lines and monitored the form of cell death induced by HMA, providing the evidence that the
drug effects are mediated by caspase-independent apoptosis \[47\]. HMA effects could be mediated by an increase in cytosolic \( \text{Ca}^{2+} \) concentration \[48\], as suggested by the observation that HMA could modulate the endoplasmic reticulum \( \text{Ca}^{2+} \) store \[49\].

We investigated the effects of HMA on colon carcinoma cells, showing that their proliferation is affected by HMA in a dose- and time-dependent manner \[50\]. Focusing on the paradigms of cell death induced by HMA in our experimental system, we observed that canonical apoptosis is initiated but not going to the end, being undetectable the final steps of this process, \textit{i.e.} PARP-1 cleavage and DNA ladder. On the contrary, the analysis of caspase-independent paradigms of cell death revealed that HMA induces the LEI/L-DNase II pathway (normally activated under acid conditions) as well as parthanatos (which is triggered by specific signaling to mitochondria) \[50\]. Remarkably, we found that HMA rapidly activates autophagy, as supported by the presence of the typical markers, \textit{e.g.} LC3 lipidation, formation of autolysosomes and activation of crucial kinases \[50,51\]. This is an intriguing observation, which add a further effect of HMA to the other above described linked to intracellular pH lowering, and points the possible antagonism between caspase-independent apoptosis and autophagy, the latter process playing opposite roles in cancer cells, \textit{i.e.} either protective or killer \[52\].

Of note, we exploited a peculiar feature of HMA to follow its intracellular localization, showing that HMA can act as a fluorescence probe \[53\]. This observation derives from the evidence obtained in human ARPE19 cells incubated for short times with HMA, which showed an intense cytoplasmic blue fluorescent signal under conventional UV-excitation conditions \[51\]. In cells incubated for longer times, the fluorescence increased, being evident in the perinuclear region and in vesicles. The possible correlation between HMA fluorescence properties and pH was investigated by monitoring spectral shape and amplitude emission; we demonstrated for the first time that pH influences the HMA fluorescence properties; this observation could suggest the use of HMA as a self-biomarker of pH alterations \[53\].

**Conclusions**

Cell populations able to resist unfavorable environmental conditions have a growth advantage, can undergo unlimited proliferation and malignant transformation, and may acquire invasion capacity (Figure 1) \[54\]. These properties, also known as the metabolic reprogramming of cancer cells \[55\], prompted many investigators to depict the molecular determinants of tumor microenvironment regulation, providing the evidence that the targeting of relevant factors could be a modern and promising approach to face tumor growth \[115,56-59\]. In fact, many trials based on the use of HIF inhibitors, thus acting on hypoxia, are going on, aiming at understanding if counteracting HIF upregulation faces tumor and/or metastasis development (clinicaltrials.gov). One trial is based on the drug AZD3965, an inhibitor of monocarboxylate transporters as an attempt to modulate the anomalous transport of chemical compounds that is responsible for pH deregulation in cancer cells (clinicaltrials.gov). However, a cautionary note is represented by the fact that tumor microenvironment shows a high degree of heterogeneity due to the presence of stroma fibroblasts, infiltrating cells and normal districts surrounding the tumor \[60\]; by consequence, the target of cancer cells could also affect the surrounding tissues.

As for the modulation of intracellular pH through the inhibition of proton pumps (Figure 2), the properties of HMA towards cancer cells justifies its consideration as a promising chemotherapeutic agent \[61\]; however, due to its multiple effects, further work is required to understand how the modulation of intracellular pH by HMA could represent a valuable tool against cancer cell proliferation and spreading without interfering with the metabolism of normal cells.

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**Conflicting interests**

The authors declare that they have no conflicting interests.

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