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# EXTRACELLULAR ACIDITY AS FAVOURING FACTOR OF TUMOR PROGRESSION AND METASTATIC DISSEMINATION

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**The bidirectional interactions between tumor cells and the so-called "host reactive stroma" play a critical role in most of the events characterizing tumor progression and distant organ colonization. This review discusses critical components of tumor environment involved in tumor cell dissemination. More specifically, it addresses some of the experimental evidences providing that acidity of tumor environment facilitates local invasiveness and metastasis formation, independently from hypoxia, with which acidity may be associated. Besides, acidity renders tumor cells resistant to radiation therapy and chemotherapeutic drugs. Therefore, this review examines the strategies for raising the low extracellular pH of tumors that might have considerable potential in cancer therapy.**
***Key Words*: extracellular acidity, invasiveness, metastatic dissemination, proton pump inhibitors.**

## CANCER AS A COMPLEX SCENARIO OF TUMOR CELLS AND AN ACTIVE STROMA

Capacity of cancer to evolve and change has been named "tumor progression" [1]. Biological characteristics that define tumor progression have been extensively described, although the underlying mechanisms remain unknown. Malignant tumor cells accumulate increasingly genetic alterations, generated by random mutational events, leading them to assume all the characteristics of invasive cells. In concert with this "genetic instability", a key role in favouring changes in tumor cells is played by local host factors [2, 3]. Among local factors, particular attention has been devoted to the interactions that tumor cells establish with various host cells that reside in or are attracted into tumor environment. The bidirectional interaction between tumor cells and host cells, is recognized as crucial for the decision whether tumor cells progress toward metastatic dissemination or remain dormant [4–8]. Indeed, tumor growth and metastasis is significantly reduced in fibroblast-deficient mice, while injection of wild-type fibroblasts into these mice can reverse this phenotype, providing a clear evidence for the involvement of fibroblasts in the emergence of metastasis [9–11]. This type of activated cells, commonly identified by the expression of α-smooth muscle actin (α-SMA) and referred as "myofibroblasts" [12], was named cancer-associated fibroblasts (CAFs) and actively participates at all stages of metastatic cascade. In addition cells of monocyte/macrophage lineage enter into the tumor mass via blood vessels throughout life span of tumors, from early-stage lesions to late-stage tumors that are invasive and metastatic, and are indicated as tumor-associated macrophages (TAMs) [13, 14]. TAMs are remarkable for the diverse activities in which they can engage on different occasions. Quiescent macrophages respond to immune or bacterial stimuli by expressing new functional activities, resulting in their capacity to recognize and destroy transformed cells. On the contrary, macrophages isolated from experimental and spontaneous tumors show a reduced level of cytotoxic activities and was proved to be relevant to tumor progression and mestastases [15, 16]. Plasticity of both CAFs and TAMs may be exploited by tumor cells to elicit distinct functions at different stages of tumor progression. It is also possible that changes expressed by these host cells during tumor development might be related to their location inside the tumor mass. Most tumors develop an environment characterized by low oxygen tension (hypoxia), elevated interstitial fluid pressure, low glucose concentration and high lactate concentration. These changes are largely caused by a combination of poor tissue perfusion due to abnormal tumor vasculature, uncontrolled proliferation and altered energy metabolism [17].

Cells require oxygen and nutrients for their survival and growth. Likewise, neoplastic cells depend on nearby capillaries for growth and once aggregates of tumor cells reach the diffusion limit for critical nutrients and oxygen, tumor cells become dormant. Indeed, some human tumors can remain dormant for a number of years at a stage where tumor cell proliferation and death are balanced. But once new blood vessel formation is initiated, the so-called angiogenic switch, tumor progression and metastasis follow [18]. The new vessel formation governed by a balance of pro- and anti-angiogenenic factors is often disturbed in tumors leading to a vasculature characterized by dilated, tortuous and incomplete vessels. The molecular mechanisms causing this abnormal vascular architecture are still debated, but the uncontrolled vascular endothelial

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*Abbreviations used*: α-SMA – α-smooth muscle actin; CA – carbonic anhydrase; CAFs – cancer-associated fibroblasts; ECM – extracellular matrix; HIF – hypoxia-inducible factor; MCTs – monocarboxylate transporters; MMPs – matrix metalloproteinases; NHEs – $Na^+/H^+$ exchangers; PAI – plasminogen activator inhibitors; TAMs – tumor-associated macrophages; uPA – urokinase-type plasminogen activator; uPAR – uPA receptor; V-ATPase – vacuolar $H^+$-ATPase; VEGF-A – vascular endothelial growth factor A.

growth factor A (VEGF-A) signalling may be a key contributor. VEGF-A is a strong mitogen and survival growth factor for vascular endothelial cells and induces mobilization and recruitment of endothelial precursor cells [19, 20]. Furthermore, VEGF-A contributes to the angiogenic phenotype by increasing the permeability of existing vessels, permitting extravasation of fibrinogen and clotting factors and resulting in a fibrin-rich stroma that supports migration of endothelial cells and formation of new vasculature. However, the uncontrolled secretion of VEGF-A results in a lower perfusion rates in tumors than in many normal tissues. Blood flow in tumors is unevenly distributed and can even reverse its direction in some vessels, therefore, regions with poor perfusion are common. These environmental features vary widely in different areas of tumors, reflecting tumor cell heterogeneity. In addition, the uncontrolled growth of tumor cells compress the intra-tumor lymphatic vessels. Consequently, there are no functional lymphatic vessels inside solid tumors, whereas functional lymphatic vessels are present only in peri-tumoral tissues [21, 22]. Both, the high permeability of tumor blood vessels and the lack of functional lymphatics are keys contributors to the development of an interstitial hypertension in neoplastic tissues [23]. As a result, the hydrostatic and colloid osmotic pressures become almost equal between intravascular and extravascular spaces, compromising the delivery of nutrients as well as therapeutic agents. Since tumor interstitial hypertension is a reflection of global pathophysiology of tumors, it may be used for diagnosis and/or prognosis. The consequent metabolic hallmark of tumor environment is hypoxia. Hypoxia characterizes the microenvironment of many solid tumors and it has been shown to affect many biological properties of tumor cells implicated in tumor progression, response to therapy, including clinical outcome of patients [24–26]. The mechanism behind these effects is related to the induction of hypoxia-inducible factor (HIF) family of transcription factors. Under conditions of acute or chronic hypoxia, HIF-1$\alpha$ is stabilized, form a heterodimer with HIF-1$\beta$, allowing this factor to bind a core sequence and increase transcription of target genes. This factor regulates many cellular processes including apoptosis, cell proliferation, angiogenesis and glucose metabolism [27, 28]. Thus, hypoxia increases genetic instability, blood vessel formation and a switch to anaerobic metabolism.

Hypoxia, elevated interstitial fluid pressure, low glucose and high lactate concentration resulting from a predominant anaerobic metabolism, are responsible of low extracellular pH (pHe) in tumor tissues. As a consequence, the second metabolic hallmark of tumor environment is tumor acidosis.

In this review, we will discuss evidence that acidity of tumor extracellular space represents a direct contributor to the process of tumor progression and that normalization of pHe could be considered a new strategy for tumor therapy.

## CONSEQUENCES OF TUMOR ACIDITY

In contrast to normal cells, which rely on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes, most cancer cells, even in the presence of sufficient oxygen to support mitochondrial respiration, use “aerobic glycolysis”, a phenomenon termed “the Warburg effect” [29, 30]. This phenomenon was first reported by Warburg in the 1920s, leading to hypothesis that cancer results from impaired mitochondrial metabolism. Although the “Warburg hypothesis” has proven incorrect, an increased conversion of glucose to lactic acid in tumor cells has been continuously demonstrated ($\frac{1}{2}$ (glucose) = lactate$^-$ + H$^+$). The clinical application of the imaging technique positron-emission tomography (PET) using the glucose analog 2-($^{18}$F)-fluoro-2-deoxy-D-glucose (FDG) tracer, demonstrated that most primary and metastatic human lesions express a high glucose uptake [31]. FDG-PET combined with computer tomography (PET/CT) has a high sensitivity and specificity for the detection of metastases of most epithelial cancers. A possible explanation for the switch to aerobic glycolysis is that proliferating tumor cells have important metabolic requirements beyond ATP, and some glucose must be diverted to macromolecular precursors such as acetyl-CoA for fatty acids, glycolytic intermediates for nonessential amino acids and ribose for nucleotides. Moreover, some tumors possess a greater capacity to pump lactic acid and protons out to the extracellular space through specific transporters, to maintain an appropriate neutral/slight alkaline intracellular pH essential for cell proliferation. The inefficient removal of protons and lactic acid from the extracellular spaces, due to the poorly perfused tumor tissue and absence of functional lymphatic vessels, creates a reversed pH gradient characterized by an acidic pHe and an alkaline intracellular pH (pHi) [32].

*In vitro* and *in vivo* studies revealed that tumor cells have pHi ranging from 7.12 to 7.56 (pHi of normal cells: 6.99-7.20), and pHe of 6.2-6.9 (pHe of normal extracellular space: 7.3-7.4). Degree of acidity in tumors tends to be associated with a poorer prognosis [33]. Indeed, tumor acidity contributes to aggressiveness of tumor cells, stimulating increased mutation rate [34]. Acute and chronic acidosis, hypoxia and reoxygenation injury all together promote DNA instability even in very small tumors leading to the selection of cells with additional genetic defects. Moreover, a minimum in pHe has been observed near tumor periphery, where tumor cells are invading normal tissues [35]. Hypoxia, also, stimulates invasiveness in tumor cells [27]. Could be expected that low extracellular pH and hypoxia always colocalize within tumor regions, instead, there is often a lack of spatial correlation among these parameters. Potential explanations of this lack of correlation could be due to the enhanced glucose uptake for glycolytic ATP generation in conditions of high oxygen tension, or to the possibility that some tumor vessels carrying hypoxic blood, are unable to deliver adequate quan-

tity of oxygen to the cells, but are able to carry away the waste products (e.g., lactic acid). Low pHe has shown to affect several steps of metastatic cascade. In some tumor cells, low pH promotes angiogenesis through VEGF [36] and IL-8 [37], however in other models of tumor cells acidosis inhibits VEGF [38]. Role of acidic pH in angiogenesis is still not completely understood. On the other hand, influence of acidity in invasiveness of tumor cells into host tissues is well demonstrated. Invasiveness is a multistep process based on extracellular matrix-degrading proteinases, such as serine and metallo-proteinases, reorganization of cytoskeleton and an integrin-mediated formation and release of focal adhesion contacts [39]. It has been reported that an acidic pHe may enhance invasion of tumor cells facilitating the redistribution of active cathepsin B, a lysosomal aspartic proteinase with acidic pH optima, to the surface of malignant cells [40, 41]. Acid-activated catepsins L also participate to amplify proteinase cascade through activation of urokinase-type plasminogen activator (uPA) [42]. The uPA system, made by uPA, two main plasminogen activator inhibitors (PAI-1, PAI-2) and uPA receptor (uPAR), is critical for tumor cell-driven degradation of extracellular matrix (ECM) in many steps of metastatic cascade [43, 44]. Activation of cathepsins D and L in an acidic tumor environment reduce perfusion of tumor regions, generating angiogenesis inhibitors such as angiostatin [45] and endostatin [46] from proteolysis of plasminogen and collagen, enhancing the chaotic vascular organization of tumors. Furthermore, acidic pH can promote the conversion of matrix metalloproteinases (MMPs) in their active forms. MMPs have long been associated with invasiveness and dissemination of tumor cells, due to their capacity to help tumor cells to cross structural barriers, including basement membranes and structural components of the ECM, such as collagen fibers [47–49]. Degradation of structural components of ECM is considered essential in tumor-induced angiogenesis. MMPs also participate in the release of cell-membrane-precursor-forms of many growth factors. The expression of MMPs in tumors is regulated in a paracrine manner by growth factors and inflammatory cytokines secreted by tumor infiltrating inflammatory cells as well as tumor cells themselves, and a continuous cross talk between tumor cells and inflammatory cells during the invasion process was demonstrated. Incubation of human and mouse melanoma cells in a low pH medium stimulate MMP expression and an increase *in vitro* invasiveness and *in vivo* metastasis formation in immunodeficient mice [50–53].

Another important component of basement membrane to be degraded by tumor cells to disseminate are the heparan sulphate chains. Toyoshima and Nakajima report that heparanase has an optimal pH of 4.2, but a significant heparanase activity persists at pH 6.0–6.5, suggesting that the acidic environment of tumors may activate the degrading properties of tumor heparanases [54].

More recently, two of the most important $H^+$ transporters, the ubiquitously expressed $Na^+/H^+$ exchanger isoform (NHE1) [55] and the plasma membrane type of vacuolar $H^+$-ATPases (V-ATPases) [56] were found to be implicated in migration of tumor cells. NHE1 influences the formation of invadopodia, structures that regulate cell motility [57]. Cell motility is driven by cycles of actin polymerization, integrin-mediated cell adhesion and acto-myosin contraction. Thus the moving tumor cells, in the absence of proteinases, make contact with collagen fibers and proceed along fibers [39]. V-ATPases are a family of ATP-dependent proton pumps particularly expressed by invasive pancreatic [58] and breast carcinomas [59], and inhibition of V-ATPases expression in hepatocarcinoma using siRNA abrogates invasion and metastatic diffusion of these tumor cells [60]. On the whole, an acidic condition may potentiate several proteinases critical for tumor cells when they detach from the primary tumor, migrate into the blood, extravasate and colonize in distant host tissues.

Moreover, extracellular lactic acid can suppress tumoricidal activity of cytotoxic lymphocytes and natural killer cells, an effect mediated by lactate/$H^+$ co-transporter that under neutral conditions remove lactic acid from leukocytes [61]. Acidification of the extracellular space may also influence radiation therapy and chemotherapy. Indeed, acidity of tumors reduce sensitivity of tumor cells to radiation therapy [62–64]. This protective effect is considered to be due to the decreased fraction of proliferating tumor cells [65] and the reduced fixation of radiation-induced DNA damage [66].

Extracellular acidity also confers a special resistance against weakly basic drugs to tumor cells. It has been reported that chronic and acute sodium bicarbonate-induced alkalosis is able to circumvent this drug resistance and enhance the anti-tumor activities of two weakly basic drugs, such as doxorubicin [67] and mitoxantrone [68]. These results suggest that induction of metabolic alkalosis using sodium bicarbonate can produce a net gain in the therapeutic index of the several chemotherapeutic agents, and open up the possibility that normalization of pHe may have a therapeutic utility.

## MANIPULATION OF TUMOR ACIDIFICATION

Since acidity of tumor environment appears to contribute to cancer aggressiveness, chemo- and radiation resistance and, even, evasion of immune reactions, measures to normalize pHe of tumors may be used in tumor therapy.

A number of researches have explored the possibility to correct the extracellular acidity of tumors.

Studies revealed that in tumors levels of $CO_2$ are higher and concentration of bicarbonate, the principal physiologic buffer used to control pH, are lower than in blood or in healthy tissues [69, 70]. Therefore, it is possible that an increased concentration of sodium bicarbonate can reduce aggressiveness of tumor

cells. Indeed, the alkalization of melanoma-bearing animals by sodium bicarbonate was found to inhibit the development of spontaneous metastases [71]. Interestingly, a similar dose of bicarbonate used in these latter experiments has been administered chronically (>1 year) in patients with renal tubular acidosis [72] and sickle cell anemia without adverse effects [73]. Computer simulation used to verify the ability of sodium bicarbonate to increase pHe of tumors *in vivo* also indicates that the normalization of tumor acidity reduces invasiveness of tumor cells without altering the pH of blood or normal tissues [74].

As an alternative strategy for correcting low pHe, several authors explored the inhibition in tumor cells of key pH regulators that maintain a neutral/alkaline intracellular pH by extruding lactate or protons [75]. pH regulators in tumor cells include extracellular forms of carbonic anhydrase (CA), $Na^+/HCO_3^-$ co-transporters, $Na^+/H^+$ exchangers (NHEs), monocarboxylate transporters (MCTs) and the vacuolar $H^+$-ATPase (V-ATPases). The raise of pHe promoted by these inhibitors is constantly associated with a decrease of intracellular pH. Acidity of pHi tends to suppress the efficiency of glycolysis, sustaining the raise of pHe [76], and may exert anti-proliferative and pro-apoptotic effects on tumor cells themselves [77–79]. Consequently, pH regulators might be considered true anticancer drugs.

Two CA isozymes, CA9 and CA12, are overexpressed in tumor cells and their activity is associated with malignancy and resistance to therapy [80]. Sulphonamide CA inhibitors that target CA9 were found effective to block the growth of primary tumor and metastases in a mouse model of breast cancer [81]. Some of these compounds are in advanced preclinical evaluation. V-ATPases, while originally identified in intracellular compartments, they have increasingly been shown to play essential roles in proton transport across the plasma membrane of a variety of cell types, including tumor cells [59, 76, 82]. The likely similarity between V-ATPase and the $H^+/K^+$ ATPase, the enzyme involved in proton secretion in gastric parietal cells, prompted the investigation of proton pump inhibitors (PPIs), such as omeprazole and esomeprazole, for inhibiting V-ATPase. When activated by acidic pHe of tumors, these drugs can inhibit V-ATPase by a covalent interaction. Both *in vitro* and *in vivo* experiences indicate that non-toxic doses of PPIs, analogous to those used for treatment of Zollinger-Ellison syndrome, exert anti-proliferative and pro-apoptotic effects on melanoma cells [83]. PPIs were also demonstrated to inhibit growth of B-cell lymphoma cells transplanted into severe combined immunodeficient mice [77]. Knockdown of V-ATPase expression by siRNA in cells isolated from a human hepatocarcinoma markedly reduced metastatic dissemination of these cells [84]. Importantly, Hashioka et al report that PPIs have anti-inflammatory effects and decrease monocytic neurotoxicity [85]. Recently, Lee et al. found that omeprazole exerts a cancer-preventive role against colitis-induced carcinogenesis, a chemopreventive action independent of gastric acid suppression [86]. Evidence that PPIs play a role in normalization of low pH and abrogate inflammation, renders these drugs suitable to target critical mechanisms involved in tumor progression. Moreover, clinical data provide that PPIs have a very low level of systemic toxicity as compared with standard chemotherapeutic agents. NHE is crucial in pH regulation and is expressed in every cell type. There are several NHE inhibitors, structurally related to amiloride and cariporide, however the diffused presence of NHE in many tissues and its role in crucial physiological processes, confers to this class of agents potential risk of side effects. Inhibitors of V-ATPase and NHE have been shown to have an additive impact on intracellular pH and on thermosensitization [87]. Therefore, it is crucial to develop agents that selectively target NHE in tumor. At the same time, potent, non-toxic selective MCT inhibitors are needed. MCTs, are overexpressed in many tumors and the isoform MCT1 regulates the entry and exit of lactate from tumor cells. The inhibition of MCT1 was found to induce a switch from lactate-fuelled respiration to glycolisis, which was accompanied by a retardation of tumor growth in a mouse model of lung carcinoma and in transplanted human colorectal carcinoma [88].

## CONCLUSION

Tumor stroma manifest some degree of plasticity, a property controlled by tumor cells themselves. Indeed, tumor cells influence host stromal elements to produce relevant effectors that act as tumor promoters. The metabolic hallmarks of this space are hypoxia and acidosis. We have elucidated how the extracellular acidity *per se,* may promote an aggressive and metastatic phenotype in tumor cells and how these findings suggest the possibility of a novel and effective therapeutic strategy based on the control of tumor acidity.

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