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Free Base Lysine Increases Survival and Reduces Metastasis in Prostate Cancer Model

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Abstract

Background: Malignant tumor cells typically metabolize glucose anaerobically to lactic acid even under normal oxygen tension, a phenomenon called aerobic glycolysis or the Warburg effect. This results in increased acid production and the acidification of the extracellular microenvironment in solid tumors. H^+ ions tend to flow along concentration gradients into peritumoral normal tissue causing extracellular matrix degradation and increased tumor cell motility thus promoting invasion and metastasis. We have shown that reducing this acidity with sodium bicarbonate buffer decreases the metastatic fitness of circulating tumor cells in prostate cancer and other cancer models. Mathematical models of the tumor-host dynamics predicted that buffers with a pka around 7 will be more effective in reducing intra- and peri-tumoral acidosis and, thus, and possibly more effective in inhibiting tumor metastasis than sodium bicarbonate which has a pKa around 6. Here we test this prediction the efficacy of free base lysine; a non-bicarbonate / non-volatile buffer with a higher pKa (~10), on prostate tumor metastases model.

Methods: Oxygen consumption and acid production rate of PC3M prostate cancer cells and normal prostate cells were determined using the Seahorse Extracellular Flux (XF-96) analyzer. *In vivo* effect of 200 mM lysine started four days prior to inoculation on inhibition of metastasis was examined in PC3M-LUC-C6 prostate cancer model using SCID mice. Metastases were followed by bioluminescence imaging.

Results: PC3M prostate cancer cells are highly acidic in comparison to a normal prostate cell line indicating that reduction of intra- and perit-tumoral acidosis should inhibit metastases formation. *In vivo* administration of 200 mM free base lysine increased survival and reduced metastasis.

Conclusion: PC3M prostate cancer cells are highly glycolytic and produce large amounts of acid when compared to normal prostate cells. Administration of non-volatile buffer decreased growth of metastases and improved survival indicating acidity plays a significant role in growth and invasion in-vivo.

Keywords: Tumor Acidosis; PC3M; Prostate Cancer; Oxygen consumption rate; Extracellular acidification rate; Buffers; Free base Lysine

Introduction

Tumor cells are highly glycolytic even in the presence of oxygen and hence produce free protons (H^+) at a higher rate than normal cells, a phenomenon known as the Warburg effect [1]. As a consequence, the microenvionment of solid tumors is acidic and significantly affects tumor growth and invasion. Low extracellular pH leads to increased release of Cathepsin B and other proteolytic enzymes that result in degradation of the extracellular matrix (ECM) [2,3]. Interestingly tumor cells are relatively resistant to acidic pHe most likely due to mutations of the p53 tumor suppressor gene or other components of the apoptotic pathway [4]. These observations have led to the acid-mediated invasion hypothesis which proposes that H^+ flow along concentrations gradients from the tumor into peritumoral normal tissue causing normal cell death and ECM degradation. Cancer cells, which are acid-adapted, are then able to invade into the damaged adjacent normal tissue. Acidic pHe has been shown to pre-dispose cancers to increased invasive and metastatic phenotypes in animal models. Exposure of tumor cells to acidic growth conditions prior to intravascular injection substantially increases their ability to metastasize [5]. Our previous studies have shown that neutralizing tumor acidity with oral sodium bicarbonate can lead to a reduction in spontaneous and experimental metastasis in different animal models including prostate [6]. The data also showed that bicarbonate inhibits extravasation and/or colonization, and not the rate of intravasation, as the level of circulating tumor cells was unchanged. Bicarbonate therapy does not alter the pH of blood and healthy tissues, which can be explained by steady-state physiological reaction-diffusion modeling [7]. These mathematical models indicated that equal and perhaps improved anti tumor effects could be obtained by non-volatile buffers with a pka closer to physiologic (i.e. pKa around 7 compared to the bicarbonate pka of 6). This was confirmed by observations that imidazoles (IEPA) is equally effective in raising the pH and reducing metastases [8]. Here we investigate the potential role of lysine, a freely available amino acid with a pKa of 10, in buffering tumors and reducing metastases. We hypothesize that lysine will alkalize the stomach, and through the alkalinization of the stomach

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lumen will increase the availability of bicarbonate in blood, in what is known as "alkaline tide".

A prostate cancer model was chosen because preliminary observation, using fluorescence microscopy in dorsal window chambers, demonstrated that prostate cancer (PC3) xenografts export acid into the surrounding normal tissue [9]. Here we investigate acid production by PC3M cells and normal prostate cells in-vitro. After demonstrating increased glycolysis and acid production we examined the effect of lysine buffer on growth of metastases in vivo.

Materials and Methods

Cell culture

Experiments were performed using PC3M cells (-Luc6 clone) obtained from Xenogen Caliber (Hopkinton, MA) and Normal Human Primary Prostate Epithelial Cells obtained form ATCC (Manassas, VA). PC3M cells were cultured using MEM/EBSS media, supplemented with 10% Fetal Bovine Serum, 1% Penicillin Streptomycin, 1% nonessential amino acids, 1% sodium pyruvate and 1% MEM vitamins. Normal prostate cells were grown in phenol red-free Prostate Epithelial Cell Basal Medium supplemented with L-Glutamine: 6 mM , Extract P: 0.4% ,Epinephrine: 1.0 µM , rh TGF-α: 0.5 ng/mL , Hydrocortisone: 100 ng/mL , rh Insulin: 5 µg/mL , Apo-transferrin: 5 µg/mL . Cells were maintained in 37°C and 5% CO_2.

Oxygen consumption and extracellular acidification measurements

Real-time basal oxygen consumption (OCR) and extracellular acidification rates (ECAR) for PC3M and normal human primary prostate epithelial cells (PCS) were determined using the Seahorse Extracellular Flux (XF-96) analyzer (Seahorse Bioscience, Chicopee, MA). The XF-96 measures the concentration of oxygen and free protons in the medium above a monolayer of cells in real-time. Cells seeded in a XF microplate were cultured for 2 hours in the presence or absence of 2 g/L D-glucose prior to OCR and ECAR measurements. Protein concentration was determined for each well using a standard BCA protein assay. OCR and ECAR values are normalized to mg/protein and are plotted as the mean +/- standard deviation.

Glycolysis stress test

Glycolysis and glycolytic capacity were determined for PC3M and PCS cells using the Seahorse Extracellular Flux (XF-96) analyzer. Cells were cultured for 2 hours in the absence of glucose. Three sequential injections of D-glucose (2 g/L), oligomycin (1 µM), and 2-Deoxyglucose (100 mM) provided extracellular acdification (ECAR) associated with glycolysis, the maxiumum glycolytic capacity, and non-glycolytic ECAR. Glycolysis was defined as ECAR following the addition of D-glucose and maximum glycolytic capacity was defined as ECAR following the addition oligomycin. ECAR following treatment with 2-Deoxyglucose is associated with non-glycolytic activity.

Animals housing and diet

All animals were maintained in accordance with IACUC standards of care in pathogen free rooms, in the USF Vivarium on site at the Moffitt Cancer Research Center. All imaging was performed within the facility. 4-6 week old male beige SCID mice (Harlan, Madison, WI) were placed in two cohorts, which were allowed to drink either tap water or 200 mM free base lysine. Lysine (free base from Sigma Aldrich, St. Louis MO) dissolved in tap water at a concentration of 200 mM was started four days prior to injection. Water consumption was recorded biweekly by weighing the water bottles. Animal weights were measured and recorded twice weekly.

Cell preparation for injections: Cells were trypsinized, then rinsed once with sterile phosphate buffered saline (PBS), prepared for injection at a concentration of 5 x 10^6 cells in 200 µL PBS. The cells were injected intravenously. Injection was confirmed by bioluminescent imaging immediately following cell injection.

Bioluminescent imaging

Animals were anesthetized with isoflurane and injected intraperitoneally with 10 µl per g body weight of sterile d-luciferin substrate prepared in PBS at 15 mg/ml. Five minutes after the mice were transferred to the thermoregulated, dark chamber of the In Vivo Imaging System (IVIS 200), a photographic image was acquired followed by overlaying of the bioluminescent image. Bioluminescent images were acquired by measuring photons emitted from luciferase-expressing cells and transmitted through the tissue. Images were analyzed using the LIVINGIMAGE V. 3.2 software.

Histology

At necropsy, ex vivo bioluminescence images were obtained of the Lung/Heart for each animal. Tissues were processed, embedded in paraffin, and 4 - 5 µm slices of the tissues were obtained. Slides were stained with hematoxylin and eosin (H&E) stain, and were graded by a pathologist (MB) for presence of tumor tissue. Histology slides were scanned using the Aperio™ (Vista, CA) ScanScope XT with a 20x/0.8NA

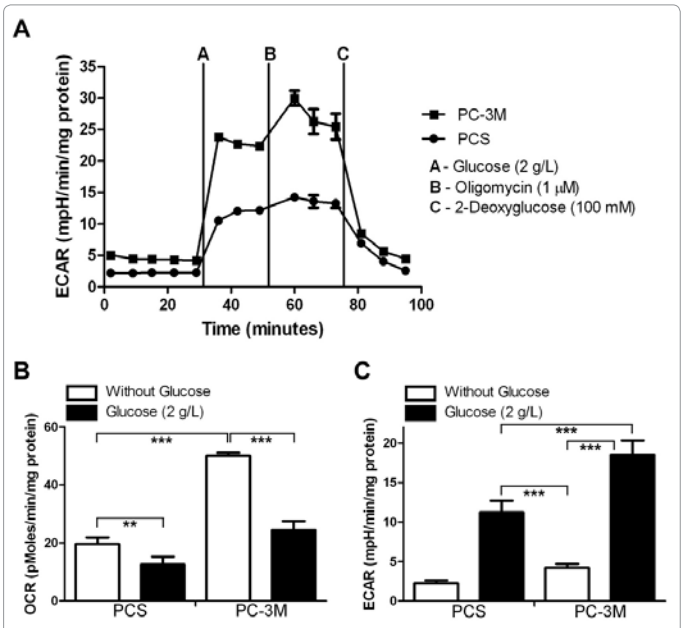

Figure 1: The glycolytic activity and maximum glycolytic capacity was determined for PC3M and PCS cells in real-time using the Seahorse extracellular flux analyzer A) A series of extracellular acidification rates (ECAR) were calculated for cells glucose starved for two hours and subsequently treated with 2 g/L D-glucose (a), 1 µM oligomycin (b), and 100 mM 2-Deoxyglucose (2-DG) (c). ECAR following the addition of glucose defines glycolysis and ECAR following oligomycin represents maximum glycolytic capacity. ECAR prior to the addition of glucose and following treatment with 2-DG represents acidification associated with non-glycolytic activity. The data represent the mean ± standard deviation. B-C) Basal oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were determined for PC3M and PCS cells either in the presence or absence of 2 g/L D-gluocose. The data represent the mean ± standard deviation. A two-tailed Student's t-test was used to calculate statistical significance: ** $p < 0.001$ and *** $p < 0.0001$.

objective lens (200x) at a rate of 2 minutes per slide via Basler tri-linear-array.

Data processing

Microsoft Excel and Graphpad Prism were used for data processing, and to calculate statistical significance.

Results

We determined the metabolic profile of PC3M and PCS cells using the XF analyzer to test the hypothesis that prostate cancer cells have acquired a glycolytic phenotype and would potentially benefit from buffer therapy (Figure 1A). Both cell lines were glucose starved for approximately 2 hours to limit extracellular acidification (ECAR) coupled to glycolysis. PC3M ECAR was approximately two fold greater than PCS cells under glucose starved conditions implying elevated acidification by cancer cells independent of glycolysis. Glyolytic ECAR was measured immediately following the addition of glucose. Acidification of the extracellular space in PC3M cultures occurred at a much greater rate than normal prostate cell cultures. Complete cellular glycolytic capacity was determined by treating cultures with oligomycin, an inhibitor of ATP synthase. Cells treated with oligomycin will reduce mitochondrial respiration and maximize glycolytic ATP production. Reserved glycolytic capacity was observed in PC3M cultures and not PCS cells further suggesting that prostate cancer cells have acquired a glycolytic phenotype to satisfy ATP energetic demands. The addition of 2-deoxyglucose (2-DG), an inhibitor of the first step of glycolysis, was used to confirm that the ECAR measured was a result of glycolytic metabolism. ECAR was restored to non-glycolytic levels in both cell lines following 2-DG treatment. Basal measurements of mitochondrial respiration (OCR) and glycolysis (ECAR) were measured in the presence and absence of glucose to study if PC3M acid production was a consequence of high glucose metabolism. ECAR increased three fold, while OCR decreased three fold in PC3M cells after administration of glucose (Figure 1B,C) indicating that acid production of PC3M cells is critically dependent on glycolysis.

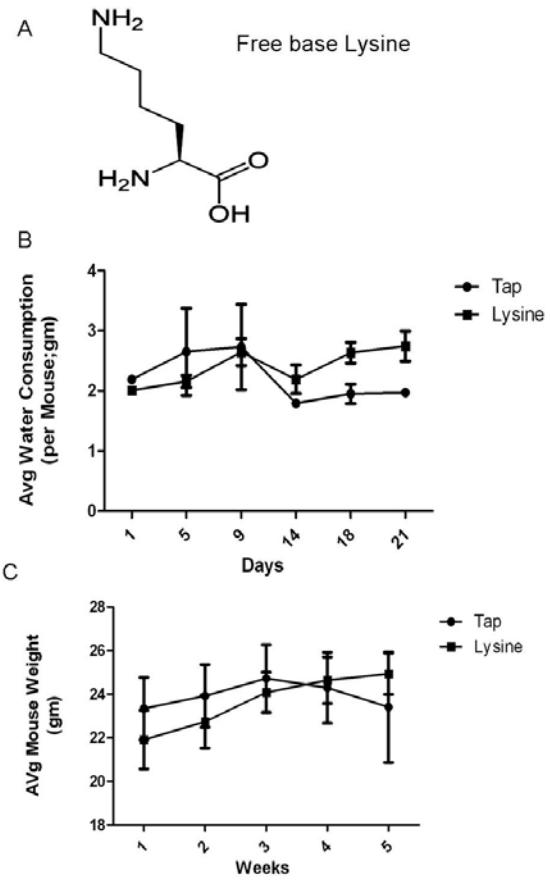

Figure 2: Average animals weight and water consumption A) Name and Chemical Structure of lysine. B) Average water consumption over time in the two cohorts (tap and lysine) indicating no difference in the amount of water consumption between the treatments. C) Average mouse weight over time between cohorts of mice drinking either tap water (Tap) or tap water buffered with 200mM free base lysine, showing no significant difference in weight gain or loss between the two treatments.

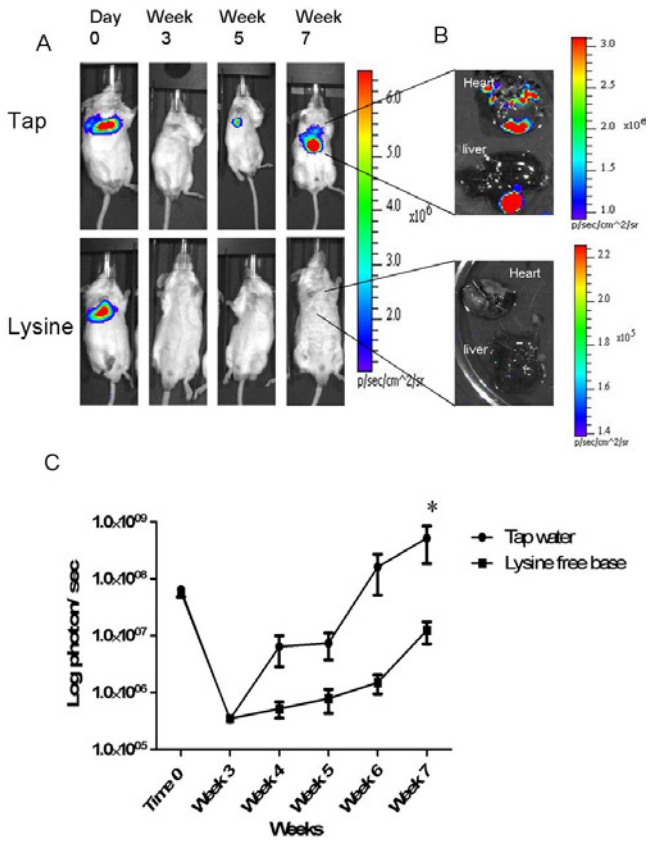

Figure 3: Effect of lysine on PC3M experimental metastasis A) Bioluminescence images of representative mice from the tap versus lysine groups at the indicated time points after venous injection of luciferin expressing PC3M cancer cells to induce experimental metastases. B) Ex-vivo Bioluminescence images of representative mice from the tap versus lysine groups at the end time point confirming the in vivo images. C) Mean tumor bioluminescence in each group after induction of experimental metastases, indicating significantly fewer metastases in the lysine cohort than in the tap ($p<0.04$) note log scale).

High acid production of cancer cells supports rapid, invasive and metastatic growth. This growth can be reduced by buffers. To examine this we used 200 mM free base lysine buffer. Animals can tolerate the lysine with no observed reduction on their weight or change in their behavior (Figure 2). Two separate experiments were conducted; the first experiment consisted of intravenous injections of PC3M cells (n=10 mice for each group) followed by treatment with or without lysine for seven weeks. The groups that were treated with lysine showed

a significant decrease in metastasis ($p< 0.04$) compared to tap water with all control animals showing metastases and only two showing metastases from the lysine group (Figure 3). The second experiment was a longitudinal survival study. Animals treated with lysine for six weeks lived significantly ($p<.002$) longer (Figure 4A) than animals on tap water. The bioluminescence imaging (Figure 4B) showed an increase in the tumor burden in all tap animals (two had to be euthanized at 5 weeks), while only two of the six animals on lysine showed any significant tumor burden (Figure 4C). Bioluminescence images from the tap water group showed signal emanating from all areas of the animal, and necropsy indicated that the tap cohort showed evidence of metastatic disease, with lymph, jaw and backbone metastases in addition to lung. Conversely, only one third of the lysine animals developed any measurable tumor burden, and these metastases were located mainly in the inguinal lymph nodes, and the lungs.

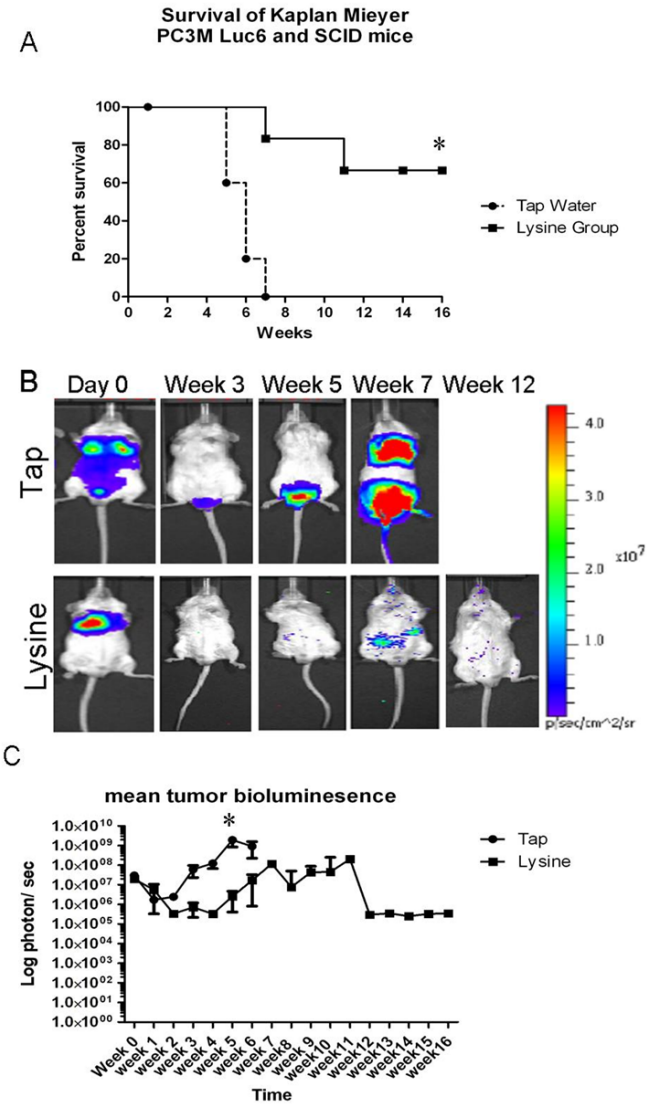

Figure 4: Effect of lysine on survival, and tumor burden A) Kaplan Meyer Survival Curve illustrating that the animals treated with lysine survived significantly ($p<.002$) longer than the tap cohort. B) Representative bioluminescence images demonstrating little tumor burden present in the lysine animal as compared to * the tap animal. C) The lifetime average tumor burden of two cohorts, showing that all tap animals had to be euthanized after 6 weeks, while only two of the six lysine animals developed measureable metastases.

($*p<.005$)

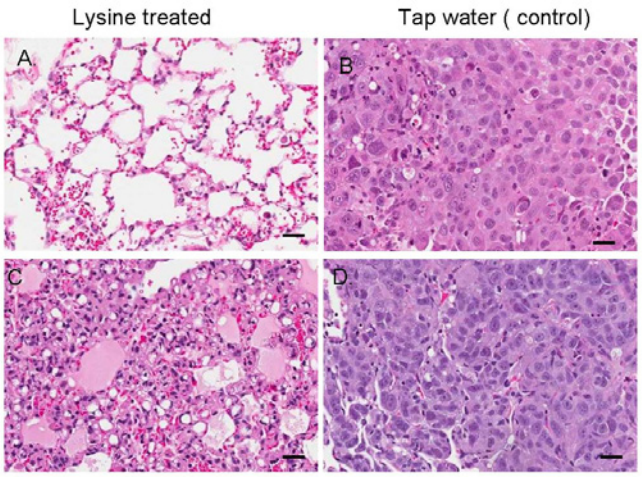

Figure 5: Histological images for treated and non treated mice. A) Representative images from mice at 7 weeks , showing a healthy lung on the lysine treated mouse B) and poorly differentiated lung on the control mouse. C-D) Representative images from the longitudinal study, showing a mouse sacrificed at 16 weeks old on lysine treated and 7 weeks on the control mouse. All images are at 200x magnification and scale bar is 100µm.

Histology of lungs collected from animals that were sacrificed at the same time (7 weeks) demonstrates that animals treated with lysine (Figure 5A) still had healthy lung tissue, where as the tap animal (Figure 5B) consisted of poorly differentiated lung tissue, indicating metastases. Moreover, histology from lung samples taken from the animals which were allowed to live until they demonstrated signs of severe discomfort show similar results; with the lysine lung appearing more normal (Figure 5C) with healthy tissue and the tap group having poorly differentiated lung tissue (Figure 5D).

Discussion

Cancers typically maintain a highly acidic microenvironment. In part this is due to regions of insufficient perfusion leading to inadequate oxygen supply (hypoxia), which requires upregulation of glycolysis to maintain ATP levels. In addition, it has been commonly observed since Warburg that many tumors use glycolytic metabolism even in the presence of normal oxygen. This is commonly described as aerobic glycolysis or the Warburg effect. Because it is less efficient in ATP production, glycolysis requires increased glucose flux resulting in increased lactic acid production and extracellular acidosis [10]. Although the glycolytic phenotype is thought to be a near-universal phenomenon in cancer cells, it has not been specifically described in PC3M. Therefore, we first investigated the metabolic state of PC3M cells in comparison to a normal prostate cell line (PCS) *in vitro*. Real time basal metabolic measurements of oxygen consumption and proton production were significantly higher in PC3M cells suggesting that prostate cancer cells are more metabolically active than normal prostate cells. In support of this observation, PC3M cells were found to have a greater glycolytic capacity in the presence of glucose and following inhibition of mitochondrial ATP production implying that PC3M cells have the ability to depend on glycolysis much more so than PCS cells to meet energetic demands.

Based on the in vitro results, we examined the potential role of a systemic buffer, lysine, in inhibiting the development of metastases

from the PC3M cell in vivo. We found, consistent with our predictions, that lysine substantially inhibits development of metastases and prolongs survival.

The precise mechanism by which lysine acts to inhibit metastases cannot be specifically answered in our study. pH plays an important role in almost all steps of metastasis [11]. Metastases are the cause of 90% of human cancer deaths [12] and metastatic disease in prostate cancer is uniformly fatal. Metastasis is a multistep process that is defined as the spread of cells from a primary tumor to a distant secondary organ or site. The metastatic process involves multiple interactions between the tumor cells and their microenvironments [13]. Tumor cells locally invade through the basement membrane into the lymphatic and blood vasculature followed by extravasation into secondary organs [14]. Numerous explanations and mechanisms could potentially contribute to the effect of buffers on tumor metastasis. Acid-mediated invasion can occur via destruction of the extracellular matrix, which is promoted by proteases and glycosidases. Metalloproteinases (MMP-2 and MMP-9) are believed to be critical for invasion and extravasation [15]. MMPs are a family of proteolytic enzymes that degrade the extracellular matrix and junctional proteins and further increase endothelial permeability [16]. Low pH up regulates angiogenic factors such as vascular endothelial growth factor (VEGEF) and interlukin 8 (IL-8) stimulating neovascularization and promoting metastasis. Thus it is possible that lysine can reverse acidosis and consequently decrease proteolytic enzyme activity and or anigiogenesis which will lead to the inhibition of extravasations and colonization of circulating tumor cells decreasing successful metastases.

The clinical application of these results will require additional investigation. A practical concern is that the amount of buffer necessary to match the dose applied to the mice is in the range of 15 to 30 grams per day. Compliance would likely be limited by the large amount and possible related GI toxicity. An alternative may focus on perturbation of proton pumps, which export protons from across the cytoplasmic membrane and acidify the intracellular compartments. These pumps play a pivotal role in the regulation of cell pH in normal cells and, to a much greater extent, in tumour cells [17]. Inhibiting these pumps can be an alternative approach to alleviate extracellular tumor acidity although this may be attenuated by systemic effects that, for example, might decrease buffering capacity and, therefore, paradoxically increase tumoral acid concentrations. Thus, this approach may be useful for tumor control [18] but it is likely that the complex tumor and systemic dynamics will require extensive experimental investigation and mathematical modeling to optimize treatment strategies.

In summary our findings suggest that manipulation of the extracellular acidosis of prostate tumor with Lysine buffer reduces metastatic disease and prolongs survival which supports the hypothesis that non-volatile buffers with pKa > 7 should be more effective in buffering extra cellular acidity. This suggests new potential therapeutic strategies in treatment of prostate cancer.

Acknowledgment

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References

1. Warburg O (1956) On the origin of cancer cells. Science 123: 309-314.
2. Montcourrier P, Mangeat PH, Valembois C, Salazar G, Sahuquet A, et al. (1994) Characterization of very acidic phagosomes in breast cancer cells and their association with invasion. J Cell Sci 107: 2381-2391.
3. Cuvier C, Jang A, Hill RP (1997) Exposure to hypoxia, glucose starvation and acidosis: effect on invasive capacity of murine tumor cells and correlation with cathepsin (L + B) secretion. Clin Exp Metastasis 15: 19-25.
4. Ohtsubo T, Wang X, Takahashi A, Ohnishi K, Saito H, et al. (1997) p53-dependent induction of WAF1 by a low-pH culture condition in human glioblastoma cells. Cancer Res 57: 3910-3913.
5. Rofstad EK, Mathiesen B, Kindem K, Galappathi K (2006) Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. Cancer Res 66: 6699-6707.
6. Robey IF, Baggett BK, Kirkpatrick ND, Roe DJ, Dosescu J, et al. (2009) Bicarbonate increases tumor pH and inhibits spontaneous metastases. Cancer Res 69: 2260-2268.
7. Silva AS, Yunes JA, Gillies RJ, Gatenby RA (2009) The potential role of systemic buffers in reducing intratumoral extracellular pH and acid-mediated invasion. Cancer Res 69: 2677-2684.
8. Ibrahim Hashim A, Cornnell HH, Coelho Ribeiro Mde L, Abrahams D, Cunningham J, et al. (2011) Reduction of metastasis using a non-volatile buffer. Clin Exp Metastasis 28: 841-849.
9. Gatenby RA, Gawlinski ET, Gmitro AF, Kaylor B, Gillies RJ (2006) Acid-mediated tumor invasion: a multidisciplinary study. Cancer Res 66: 5216-5223.
10. Vaupel P, Kallinowski F, Okunieff P (1989) Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 49: 6449-6465.
11. Hashim AI, Zhang X, Wojtkowiak JW, Martinez GV, Gillies RJ (2011) Imaging pH and metastasis. NMR in biomedicine 24: 582-591.
12. Weigelt B, Peterse JL, van 't Veer LJ (2005) Breast cancer metastasis: markers and models. Nat Rev Cancer 5: 591-602.
13. Fidler IJ (2003) The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer 3: 453-458.
14. Ruoslahti E (1996) How cancer spreads. Sci Am 275: 72-77.
15. Nguyen JH, Yamamoto S, Steers J, et al. (2006) Matrix metalloproteinase-9 contributes to brain extravasation and edema in fulminant hepatic failure mice. J Hepatol 44: 1105-1114.
16. Alexander JS, Elrod JW (2002) Extracellular matrix, junctional integrity and matrix metalloproteinase interactions in endothelial permeability regulation. J Anat 200: 561-574.
17. Martinez-Zaguilan R, Lynch RM, Martinez GM, Gillies RJ (1993) Vacuolar-type H(+)-ATPases are functionally expressed in plasma membranes of human tumor cells. The American journal of physiology 265: C1015-C1029.
18. Luciani F, Spada M, De Milito A, Molinari A, Rivoltini L, et al. (2004) Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs. J Natl Cancer Inst 96: 1702-1713.

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