The Relationship between Intracellular and Extracellular pH in Spontaneous Canine Tumors[1]

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ABSTRACT

Recently, it has been suggested that the cellular uptake of chemotherapeutic drugs may be dependent on the pH gradient between the intracellular (pH_i) and extracellular (pH_e) compartments. It has been demonstrated in murine tumor models that the extracellular environment is acidic, relative to the intracellular environment, thus favoring preferential accumulation of drugs that are weak acids into cells. However, concomitant measurements of pH_i and pH_e in spontaneous tumors have not been reported, so it is not certain how well the murine results translate to the clinical scenario. In this study, both types of measurements were performed in dogs with spontaneous malignant soft tissue tumors. On average, pH_e was more acidic than pH_i, with maintenance of a more physiologically balanced intracellular tumor environment. However, the magnitude of the gradient varied widely, and individual tumors had both positive and negative pH gradients ($pH_i - pH_e$). These data suggest that the magnitude and direction of the pH gradient may need to be measured for individual patient tumors and/or that manipulation of pH_e may be required if exploitation of the pH gradient is to be achieved for tumor-selective augmentation of intracellular drug delivery.

INTRODUCTION

It is well established that solid tumors tend to have a more acidic microenvironment than normal tissues (1, 2). The increase in hydrogen ion concentration is thought to be due to a combination of a more glycolytic phenotype, as well as reduced oxygen availability, leading to lactic acidosis from glycolysis (3). A poor and chaotic tumor vascularization leads to the inefficient washout of the acidic products and contributes further to development of the chronically acidic extracellular environment. It has been further established that the excess hydrogen ion is excreted from the cell via hydrogen ion pumps, such that the intracellular environment is maintained at a more physiologically normal pH (4, 5). This process depends on the buffering capacity of the cell and on membrane-based ion exchangers, the Na^+/H^+ antiport, and Na^+-dependent HCO_3^-/Cl^- exchange mechanism (6, 7). Thus, the extracellular environment tends to be more acidic than the intracellular environment, leading to a pH gradient (pH_{grad}) across the cell membrane.

The magnitude and the direction of pH_{grad} across the tumor cell membrane may be important for certain kinds of therapy. For example, it has been speculated that pH_{grad} may affect intracellular accumulation of weakly acidic or basic drugs, thereby affecting the efficacy of such agents (2, 4, 5). A low pH_e enhances the uptake of weakly acidic drugs (8–10) and topoisomerase I inhibitors (11). It increases the activation of bioreductive agents (12, 13) and potentiates the interaction of alkylating agents and platinum-containing drugs with DNA (14, 15). However, a low pH_e reduces the uptake of mitoxantrone (16, 17) and the cytotoxicity of weakly basic drugs, such as doxorubicin (15). It has also been shown that the probability of thermoradiotherapy response of human tumors is higher when pH_e is relatively acidic (18), as well as when pH_i is more basic (19). All of these results suggest that the magnitude and direction of the pH gradient may be important factors that can determine and predict treatment response. Although pH gradients have been measured in murine tumors, there has not been any systematic attempt to measure them in spontaneous tumors in either humans or dogs. This report presents such data on a series of 31 tumor-bearing canine patients.

MATERIALS AND METHODS

Patient Characteristics. Thirty-one privately owned dogs with spontaneous malignant soft tissue tumors were the subjects for this study (Table 1). None of the tumors had been treated previously. Tumor volumes were calculated from dimensions obtained from T_2 weighted MR[3] images. The animals were cancer patients at the College of Veterinary Medicine at North Carolina State University. The dogs were brought to Duke University from North Carolina on the day of study and returned the same day.

The protocol for measurement of pH values was approved

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[3] The abbreviations used are: MR, magnetic resonance; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; CPT, camptothecin; NTP, nucleoside triphosphate; TPT, topotecan.

Table 1 Patient characteristics (tumor-bearing dogs)

Dog ID[a]	Histology	Grade[b]	Age (yr)	Tumor volume (cm^3)	No. of positions measured[c]	pH_e mean (±SE)	pH_i mean (±SE)	pH_{grad} (gradient)	C_i/C_e[d] $pK_a = 6.0$	C_i/C_e[d] $pK_a = 8.0$
BP	FSA	Low	4.9	1067.5	16/8	6.94 (±0.03)	6.71 (±0.07)	−0.23	0.63	0.97
GC	SA	High	10.0	346.2	12/8	7.17 (±0.04)	6.99 (±0.03)	−0.18	0.68	0.96
SBe	LPS	Low	11.0	761.4	8/3	7.33 (±0.04)	7.15 (±0.02)	−0.18	0.68	0.94
BN	MCT	High	7.0	160.9	7/2	7.35 (±0.01)	7.24 (±0.02)	−0.11	0.79	0.96
JY	FSA	Low	12.5	20.2	8/1	7.22 (±0.02)	7.11	−0.11	0.80	0.97
BC	HPC	—	10.2	3.8	7/1	7.36 (±0.09)	7.27	−0.09	0.82	0.96
SS	SA	High	8.9	182.7	11/8	7.18 (±0.04)	7.30 (±0.08)	0.12	1.29	1.04
MC	HPC	High	10.9	7.2	12/4	7.21 (±0.02)	7.36 (±0.02)	0.16	1.41	1.06
NF	HPC	Low	4.2	210.6	16/9	7.06 (±0.05)	7.24 (±0.10)	0.18	1.48	1.05
TH	FSA	Low	9.0	536.8	12/4	7.25 (±0.02)	7.43 (±0.04)	0.18	1.48	1.08
JW	FSA	Low	1.5	49.5	12/4	7.13 (±0.04)	7.33 (±0.04)	0.20	1.54	1.07
RC	MM	—	10.0	40.4	4/5	6.83 (±0.02)	7.08 (±0.06)	0.25	1.68	1.05
PR	SA	High	12.8	48	6/2	6.92 (±0.02)	7.19 (±0.03)	0.27	1.77	1.07
TS	HPC	Low	13.6	81.9	5/4	6.95 (±0.06)	7.24 (±0.05)	0.29	1.85	1.08
CT	HPC	Low	9.9	343.6	15/8	6.95 (±0.02)	7.29 (±0.09)	0.34	2.06	1.10
WR	HPC	Low	15.5	88.2	12/2	6.86 (±0.02)	7.22 (±0.12)	0.36	2.13	1.09
LW	FSA	Int./High	5.0	2266.3	12/2	6.97 (±0.08)	7.37 (±0.04)	0.40	2.38	1.13
BH	HPC	Low	11.8	567.4	4/2	7.10 (±0.03)	7.54 (±0.12)	0.44	2.63	1.20
NE	ADC	—	13.0	441.9	12/4	6.97 (±0.04)	7.41 (±0.04)	0.44	2.61	1.15
BV	SA	High	12.0	202.5	12/2	6.68 (±0.06)	7.24 (±0.12)	0.56	3.18	1.12
BT	LPS	Int.	10.0	618	8/2	6.83 (±0.06)	7.49 (±0.11)	0.66	4.15	1.23
GM	NFS	Low	11.2	216.5	8/2	6.92 (±0.01)	7.66 (±0.00)	0.74	5.00	1.34
SBa	FSA	Low/int.	5.0	85.3	12/2	6.48 (±0.01)	7.50 (±0.16)	1.02	8.17	1.28
GS	HPC	—	8.0	125.6	14/1	7.00 (±0.04)	7.37	0.37	2.22	1.12
KW	MYX	Low	10.0	15.7	13/1	7.32 (±0.02)	7.23	−0.09	0.82	0.97
PF	FSA	—	10.0	45.3	11/8	6.85 (±0.01)	7.16 (±0.11)	0.31	1.91	1.07
PC	FSA	—	12.0	28.8	12/2	7.12 (±0.02)	7.50 (±0.05)	0.38	2.30	1.16
RH	FSA	—	5.0	120	13/1	6.89 (±0.03)	7.2	0.31	1.92	1.08
SB	HPC	—	14.0	66.4	8/3	7.23 (±0.05)	7.6	0.37	2.27	1.20
FM	HPC	—	13.0	122.5	15/3	6.93 (±0.03)	7.49 (±0.15)	0.56	3.35	1.21
LR	MYX	—	8.0	84.5	20/8	6.85 (±0.06)	7.06 (±0.08)	0.21	1.54	1.04

[a] ID, identification code; HPC, hemangiopericytoma; MCT, mast cell tumor; FSA, fibrosarcoma; LPS, liposarcoma; SA, undifferentiated sarcoma; NFS, neurofibrosarcoma; ADC, adenocarcinoma; MM, malignant melanoma; Int., intermediate.

[b] —, grade not available.

[c] pH_e/pH_i.

[d] $C_i/C_e = (1 + 10^{pH_i - pK_a})(1 + 10^{pH_e - pK_a})$.

by the Animal Care and Use Committees of both institutions, and owner consent was obtained for all studies on canine patients.

Anesthesia. The dogs were anesthetized with diazepam (0.2 mg/kg, i.v.) and sodium thiopental (12 mg/kg, i.v.). They were intubated, and anesthesia was maintained with inhalation of isoflurane (1.5%) in 100% oxygen. Heart rate, indirect blood pressure, and respiratory rate were monitored every 5 min. Throughout the anesthetic procedure, body temperature was monitored using rectal temperature measurements. To reduce heat loss during the study, animals were kept warm by heating blankets. No significant body temperature loss was observed during the procedure. Lactated Ringer's solution (10 ml/kg/h, i.v.) was given for maintenance fluid administration. Prior to entering the MR suite, the tumor was clipped of any surface hair.

Sequence of pH_i and pH_e Measurements. MRI and MRS studies were performed first, as described below. The MRI scan data were used to assist in placement of pH electrodes in tumor and to direct location of probes away from necrotic areas.

pH_e Measurements. Extracellular pH was determined using combination interstitial needle electrodes (Microelectrode, Inc., Londonderry, NH; Agulian, Hamden, CT), following previously published protocols (20). Prior to each study, the electrodes were calibrated using buffered solutions of pH 6.0, 7.0, 7.4, and 8.0. All tumor measurements were corrected using a calibration curve generated at each study. The calibration was repeated after the study to verify the performance of the pH microelectrode.

The tumor was aseptically prepared. The needle electrode was inserted into the tumor, and pH_e was measured at 0.5–1.0-cm intervals as the needle was advanced. Information from the MR images was used to assist in localization of measurement sites. Several locations were measured in each tumor site and were averaged to obtain mean and SE values for each individual.

pH_i Measurements. In tumor-bearing animals, T_2 MRI and ^{31}P-MRS scans were done to determine tumor location, volume, and pH_i. After obtaining T_1 and T_2 weighted MR imaging studies at 1.5 Tesla (Signa Spectrometer, General Electric Medical Systems, Milwaukee, WI), the tumor region was identified and local magnetic field homogeneity was adjusted using the AUTOSHIM capability of the Signa system using DC offsets applied to the *x*, *y*, and *z* gradients. ^{31}P spectroscopy was

carried out using a 6-cm home-built surface coil of distributed capacitance design. Spatial localization of the spectral information was accomplished with image-correlated chemical shift imaging (21) and with repetition time = 1500 ms and total acquisition time = 13–25 min. The data matrix was 512 complex points in the chemical shift dimension and 8*8*8 in the three spatial dimensions with a FOV of 24 cm, yielding nominal 27-ml volume elements.

The spectroscopic data were transferred to an off-line system (SUN Microsystems, Milpitas, CA) operating the SAGE/IDL software (General Electric Medical Systems, Milwaukee, WI) for reconstruction and extraction of spectroscopic parameters in each volume element (voxel). The data were processed by application of a decaying exponential filter in the chemical shift domain, zero padding to 1024 complex points in the chemical shift domain, and Fourier reconstruction. Frequency independent and linear phase corrections were applied automatically to obtain the real (absorption) component of the spectrum. Baseline correction was accomplished using a sinc deconvolution to account for the time delay for magnetic field gradient encoding. Parameterization was automated, by best fit of lorentzian or lorentzian/gaussian lines (phosphomonoester, inorganic phosphate, phosphodiester, phosphocreatine, γNTP, αNTP, and βNTP) to the extracted real frequency spectrum using a Marquardt algorithm in the SAGE/IDL software. Tissue pH was determined by the frequency difference between the inorganic phosphate and phosphocreatine resonances. pH_i was calculated for each tumor containing voxel and averaged for each dog. The results are reported as averages and SE. These methods have been described previously (19).

pH gradient and Calculation of Drug Concentration Ratios. The pH gradient (intracellular *versus* extracellular) was calculated for each tissue examined using the following equation.

$$pH_{grad} = pH_i - pH_e \quad (A)$$

pH_i and pH_e were the means of all measurements made in each individual.

The effect of pH_{grad} on intracellular/extracellular concentration ratios for drugs with pK_a values of 6.0 and 8.0 were calculated according to the equation developed by Gerweck and Seetharaman (4).

$$C_i/C_e = (1 + 10^{pH_i - pK_a})/(1 + 10^{pH_e - pK_a}) \quad (B)$$

C_i and C_e are equal to the concentrations of drug in the intracellular and extracellular compartments, respectively. The predicted C_i/C_e ratio for the weakly acidic drug chlorambucil was calculated based on pH_e and pH_i measurements from this study and $pK_a = 5.8$ (Fig. 2). Similar predictions were calculated for the topoisomerase I targeting agents CPT and TPT, assuming a pK_a of 6.0. The predicted relative increase in the intracellular levels of CPT and TPT was compared to the relative increase in intracellular levels obtained from previously published *in vitro* studies (Ref. 11; Fig. 3).

RESULTS

Thirty-one tumor-bearing dogs were evaluated in this study. Twenty-eight dogs had soft tissue sarcomas of a variety of histologies (undifferentiated sarcoma, fibrosarcoma, hemangiopericytoma, liposarcoma, neurofibrosarcoma, and myxosarcoma). One dog had an adenocarcinoma, one had a mast cell tumor, and one had a malignant melanoma. The tumor volumes were relatively large, ranging in size from 7 to over 2000 cm^3. Based on the T_1 and T_2 weighted MR images, voxels containing tumor volume, with little or no contaminating normal tissue, were identified and analyzed. The mean number of voxels measured for pH_i per tumor was 3.9 (SD, 2.6). The mean number of sites measured for pH_e per tumor was 10.8 (SD, 3.8).

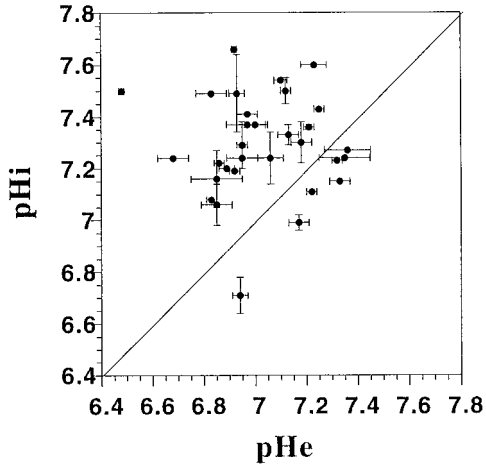

Fig. 1 Relationship of pH_i measured with ^{31}P-MRS, and pH_e measured with interstitial electrodes for tumor tissue in 31 tumor-bearing dogs. All values are means ± SE.

Means of tumor pH_i and pH_e were 7.29 (SD, 0.19) and 7.03 (SD, 0.21), respectively. There was no relationship between either tumor grade or tumor volume and pH_e, pH_i, or pH_{grad} (Table 1). The means of pH_e, pH_i, and pH_{grad} for high/intermediate *versus* low grade tumors were 7.03 (SD, 0.22), 7.27 (0.24), 0.24 (0.36) and 7.01 (0.22), 7.28 (0.14), and 0.27 (0.29), respectively.

There was no relationship between pH_i and pH_e measured in individual tumors (Fig. 1). For the majority of tumors (except two), however, pH_i was maintained at pH 7 or greater, and pH_{grad} was ≥ 0. There were some exceptions, however, in which the gradient was negative. There was considerable variation in the magnitude and direction of pH_{grad} between tumors (Table 1).

Predicted Drug Concentration Ratios. The relative abundance of positive pH_{grad} in this series of tumors leads to the prediction that there will be greater concentrations of drugs with a low pK_a (weak acids) intracellularly than extracellularly, with C_i/C_e ratios as high as 8 (Table 1). On the other hand, the pH gradients do not favor preferential accumulation of drugs with a pK_a that is relatively alkaline.

One way to improve the intracellular concentration of weakly acidic drugs in tumors would be to transiently drop pH_e. The induction of hyperglycemia has been shown to reduce human tumor pH_e by 0.1–0.2 pH units (22). In animal studies using *meta*-iodobenzylguanidine in combination with moderate hyperglycemia tumor pH_e was reduced by 0.7 units (23, 24). If

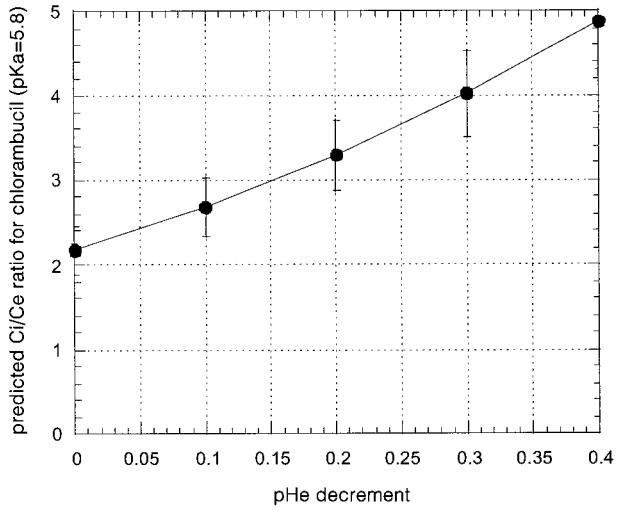

Fig. 2 Mean (SE) calculated tumor intracellular drug concentration ratios for chlorambucil (pK_a is assumed to be 6.0 = 5.8) considering the potential benefits of transient pH_e acidification. The baseline data are indicated by a pH_e decrement of 0. The fraction of cases in each scenario with drug concentration ratios less than 1 is indicated *under* each mean.

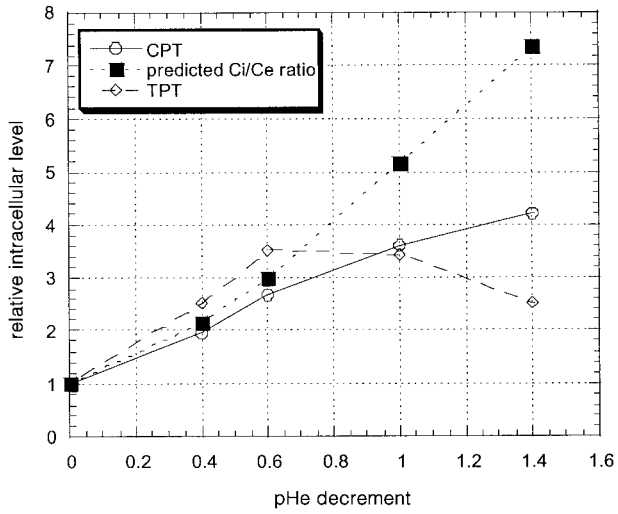

Fig. 3 Predicted relative increase in intracellular uptake of CPT and TPT in comparison to observed *in vitro* increase in intracellular level of the same drugs (data from Ref. 11). CPT and TPT pK_a values are assumed to be 6.0.

one recalculates C_i/C_e ratios for the weakly acidic drug chlorambucil, assuming a preferential drop of 0.1–0.4 pH units in tumor, this increases the predicted mean concentration ratio quite favorably and decreases the fraction of tumors with nonpreferential weakly acidic drug uptake into tumor cells (Fig. 2). At the assumed pH decrement of 0.6 units, predicted CPT and TPT uptake from our model appear to coincide with the observed intracellular drug uptake in the previously published *in vitro* studies (11). However, at a larger pH_e decrement, our model predicts higher drug uptake (Fig. 3).

DISCUSSION

In this paper, we have characterized intracellular to extracellular pH gradients in spontaneous canine tumors. In most cases, the extracellular environment was more acidic (positive gradient). This condition would tend to favor uptake of weakly acidic drugs into tumor cells as predicted by our calculations (Table 1, Fig. 2). This has been demonstrated for chlorambucil, a weak acid with a pK_a value of 5.8 (22, 25). Furthermore, the uptake of 5-fluorouracil is also enhanced at low pH_e conditions (23). However, cytotoxicity of doxorubicin, a weak base, and uptake of mitoxantrone are reportedly reduced at a low pH_e (15, 16, 17).

It has been demonstrated previously that pH gradients exist in rodent tumors, and it has been suggested that this physiological characteristic of tumors could be used to therapeutic advantage (4). Furthermore, the magnitude and direction of pH gradients could represent a source for chemotherapeutic treatment resistance, depending on the pK_a of the drug being used (5). Clinical utilization of this information will depend on how reproducible the pH gradient is in individual tumors.

The majority of these tumors had relatively acidic extracellular pH, but there were exceptions. In addition, the magnitude of the gradient varied widely. These two features of the tumor population led to a wide range of expected drug concentration ratios, from values less than 1 to greater than 8 for a drug with a pK_a of 6.0.

Based on pH measurements in this study, we have predicted an increase in CPT and TPT intracellular uptake in spontaneous canine tumors and compared them to intracellular drug uptake observed in the experiments *in vitro* (11). At smaller pH_e decrements, our predicted drug uptake is not different from the uptake in the *in vitro* studies (Fig. 3). However, at larger pH_e decrements, our model predicts higher drug uptake. We hypothesized that observed differences between *in vitro* and *in vivo* studies are due to differences in the time of acidification. Previously published *in vitro* studies have used acute acidification. In the clinical scenario, however, pH_e is chronically low, and pH_i is maintained nearer a physiological level, as shown in the present study (Table 1). Acute acidification studies could therefore underestimate effect of pH gradient on drug uptake in the clinical setting of a chronically acidic tumor environment.

Some additional advantage in drug uptake could be gained if strategies could be implemented that would acutely and preferentially acidify the extracellular space in tumors. A sample calculation suggested that a 0.2 pH_e unit drop in tumors would create favorable drug concentration ratios in the majority of cases. Acute acidification of human and murine tumors has been accomplished by induction of hyperglycemia alone or in combination with the mitochondrial inhibitor *meta*-iodobenzylguanidine, and the degree of acidification has been near 0.2 pH units (22, 25). In humans, this effect does not occur in normal s.c. tissue (22). However, the effects of hyperglycemia on pH_e of other normal tissues have not been reported. Additional strategies to lower pH_e, such as use of respiratory inhibitors (23) and/or tumor blood flow reduction, could prove useful to further enhance the pH_{grad} in tumors.

The pH data from this paper compare favorably to the

average pH_i for human soft tissue sarcomas reported from our institution (7.24 ± 0.15; Ref. 19), the mean pH_i for human sarcomas reported by Vaupel *et al.* (Ref. 1; pH_i = 7.19; range, 6.9–7.35), the mean pH_e for human soft tissue sarcomas reported by Engin *et al.* (Ref. 26; pH_e = 7.01 ± 0.21), and mean pH_e for human sarcomas reported by *et al.* (Ref. 1; pH_i = 6.69; range, 6.2–6.9). Similarity in pH_i and pH_e between the human and canine tumors suggests that this tumor type has physiological characteristics that are similar to the human counterpart. Further attesting to this conjecture is our prior report demonstrating a relationship between pH_i and treatment outcome in both human and canine soft tissue sarcomas treated with hyperthermia and radiation therapy (19). Thus, one might expect that the range of pH_{grad} described in this paper would be representative of the range seen in human sarcomas.

Most of the dogs in this study are part of thermoradiotherapy trials, in which local control and disease-free survival are the primary end points. In future analyses, we intend to investigate whether there are relationships between pH_e, pH_i, and/or pH_{grad} and treatment outcome.

Cautionary notes arise from this study as well. The lack of direct correlation between pH_e and pH_i suggests that one cannot predict the value of pH_i based on measurement of pH_e alone. Thus, the estimation of drug concentration ratios for any tumor will be dependent on direct measurement of both pH parameters. Caution should also be used in extrapolation of these data to tumors other than sarcomas. For example, Engin *et al.* (26) reported variation in pH_e values between tumors of different histological types. Additional studies are needed to verify the magnitude and direction of pH gradients in other histological types.

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