The Relationship between Intracellular and Extracellular pH in Spontaneous Canine Tumors

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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 (pHi) and extracellular (pHe) 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 pHi and pHe 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, pHi was more acidic than pHe, 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 (pHi - pHe). 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 pHi 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 HCO3~/Cl− exchange mechanism (6, 7). Thus, the extracellular environment tends to be more acidic than the intracellular environment, leading to a pH gradient (pHgrad) across the cell membrane.

The magnitude and the direction of pHgrad across the tumor cell membrane may be important for certain kinds of therapy. For example, it has been speculated that pHgrad may affect intracellular accumulation of weakly acidic or basic drugs, thereby affecting the efficacy of such agents (2, 4, 5). A low pHi 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 pHi 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 is relatively acidic (18), as well as when pH 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 T2 weighted MR3 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.
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<sub>e</sub> and pH<sub>i</sub> 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<sub>e</sub> Measurements. Extracellular pH was determined using combination interstitial needle electrodes (Microelec-

trode, 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<sub>e</sub> 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<sub>i</sub> Measurements.** In tumor-bearing animals, T<sub>2</sub> MRI and 31P-MRS scans were done to determine tumor location, volume, and pH<sub>i</sub>. After obtaining T<sub>1</sub> and T<sub>2</sub> weighted MR imaging studies at 1.5 Tesla (Sigma 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 Sigma system using DC offsets applied to the x, y, and z gradients. 31P 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.

\[
pH_{\text{grad}} = pH_i - pH_e
\]  

(A)  

pH$_i$ and pH$_e$ were the means of all measurements made in each individual.

The effect of pH$_{\text{grad}}$ 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).

\[
\frac{C_i}{C_e} = \frac{1 + 10^{pH_i-pK_a}}{1 + 10^{pH_e-pK_a}}
\]  

(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$_i$ and pH$_e$ 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$_i$ per tumor was 10.8 (SD, 3.8).

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$_i$, pH$_e$, or pH$_{\text{grad}}$ (Table 1). The means of pH$_i$, pH$_e$, and pH$_{\text{grad}}$ 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$_{\text{grad}}$ was $\geq$ 0. There were some exceptions, however, in which the gradient was negative. There was considerable variation in the magnitude and direction of pH$_{\text{grad}}$ between tumors (Table 1).

**Predicted Drug Concentration Ratios.** The relative abundance of positive pH$_{\text{grad}}$ 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$_i$. The induction of hyperglycemia has been shown to reduce human tumor pH$_i$ by 0.1–0.2 pH units (22). In animal studies using meta-iodobenzylguanidine in combination with moderate hyperglycemia tumor pH$_i$ was reduced by 0.7 units (23, 24). If
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 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
The average pH for human soft tissue sarcomas reported from our institution (7.24 ± 0.13; Ref. 19), the mean pH for human sarcomas reported by Vaupel et al. (Ref. 1; pH = 7.19; range, 6.9–7.35), the mean pH for human soft tissue sarcomas reported by Engin et al. (Ref. 26; pH = 7.01 ± 0.21), and the mean pH for human sarcomas reported by et al. (Ref. 1; pH = 6.69; range, 6.2–6.9). Similarity in pH was reported by Engin et al. (Ref. 1; pH = 7.19; range, 6.9–7.35), the mean pH for human sarcomas reported by et al. (Ref. 1; pH = 6.69; range, 6.2–6.9). Similarity in pH and pH gradients 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 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 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, pH gradient, and/or pH gradient and treatment outcome.

Cautionary notes arise from this study as well. The lack of direct correlation between pH 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 described in this paper would be representative of the range seen in human sarcomas.

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