Modelling tumour acidity and invasion

Steven D. Webb*[1], Jonathan A. Sherratt* and Reginald G. Fish†

*Department of Mathematics, Heriot-Watt University, Edinburgh EH14 4AS and †Velindre Hospital, Whitchurch, Cardiff CF7XL, UK

Abstract. The intracellular pH (pH_i) of mammalian cells is tightly regulated by the concerted action of a number of different pumps in the plasma membrane. Despite the acidic extracellular environment (pH_e 6.8–7.0) of some tumours, the pH_i of solid tumours is neutral or slightly alkaline compared to normal tissues (pH_i 7.0–7.4). This gives rise to a reversed pH gradient across the cell membrane between tumours and normal tissue, which has been implicated in many aspects of tumour progression. One such area is tumour invasion: the incubation of tumour cells at low pH has been shown to induce more aggressive invasive behaviour *in vitro*. In this paper we use mathematical models to investigate whether altered proteolytic activity at low pH is responsible for the stimulation of a more metastatic phenotype. We examined the effect of culture pH on the secretion and activity of two different classes of proteinases: the metalloproteinases (MMPs), and the cysteine proteinases (such as cathepsin B). The modelling predicts that, in addition to metalloproteinase activity, the pH-induced peripheral redistribution of cathepsin B could be a major factor involved in the acquisition of a more metastatic phenotype in malignant cells at low pH_e.

2001 The tumour microenvironment: causes and consequences of hypoxia and acidity. Wiley, Chichester (Novartis Foundation Symposium 240) p 169–185

The extracellular pH (pH_e) of tumours is generally more acidic than that of normal tissue, with median pH values of about 7.0 in tumours and 7.5 in normal tissue (Warburg 1930, Tannock & Rotin 1989). This is thought to be a consequence of a compromised tumour vasculature together with an increased use of the glycolytic pathway for energy production, whereby tumour cells preferentially convert glucose and other substrates into lactic acid (Hawkins et al 1992). In contrast, with the advent of ^{31}P magnetic resonance spectroscopy (MRS) imaging for the non-invasive measurements of pH, both human and animal tumour cells have been shown to have a neutral or slightly alkaline intracellular pH (pH_i 7.1–7.2) (Stubbs et al 1994, Vaupel et al 1989). This gives rise to a cellular pH gradient difference between tumours and normal tissue, which provides the basis for pH-dependent selective treatment of cancer (Gerweck & Seetharaman 1996).

[1]Present address: School of Mathematics, University of Minnesota, 127 Vincent Hall, 206 Church St. S.E., Minneapolis, MN 55455, USA

A number of cellular processes are affected by changes in microenvironmental pH, e.g. glycolysis, protein synthesis, DNA synthesis, exocytosis and secretion (Busa 1986). It has been shown that the maintenance of intracellular pH within the range pH 7.0–7.2 appears to be necessary for normal cellular proliferation (Gillies et al 1992). More specifically Martínez-Zaguilán et al (1996) observed that culturing of two human melanoma cell lines at acidic pH (6.8) caused significant increases in both migration and invasion. This suggests that low pH_e may be an important factor involved in the invasive behaviour of tumours.

Tumour invasion and metastasis are the major causes of treatment failure for cancer patients (Liotta & Stetler-Stevenson 1993). These processes can be subdivided into several steps, such as degradation of the basement membrane, intravasation, circulation, extravasation and secondary growth at the target organ (Chambers & Matrisian 1997, Kato et al 1992). It is likely that each of these steps is individually pH sensitive (Martínez-Zaguilán et al 1996). For instance, proteolytic enzymes play an important role in the metastatic process by degrading the extracellular matrix (ECM), thus allowing escape from the primary tumour, and each class of proteinases is pH sensitive (Powell & Matrisian 1996). The ECM-degrading proteinases produced by most tumour cells can be subdivided into three classes: serine, metallo- and cysteine proteinases (Duffy 1992). In general, the enzymes are secreted as latent forms which require extracellular activation, and tumour spread is correlated with increased levels of the activated enzyme (Chambers & Matrisian 1997).

Many studies have shown the importance of matrix metalloproteinases (MMPs) in tumour invasion (Stetler-Stevenson et al 1993, Sato et al 1994), as well as many normal morphogenetic processes such as embryonic development and bone remodelling (Powell & Matrisian 1996). Although MMP activity is optimal in the physiological pH range (Chambers & Matrisian 1997), Martínez-Zaguilán et al (1996) showed that while there was a decrease in the overall amount of MMP activity in cells grown at low pH (6.8), there was a significant increase in the relative amount of active MMP. Rozhin et al (1994) also demonstrated that a reduction of pH_e to 6.5 resulted in a redistribution of cathepsin B, a cysteine proteinase, toward the surface of tumour cells. Furthermore, this pH-induced peripheral redistribution of cathepsin B was accompanied by an enhanced secretion of active cathepsin B. The objective of the present study was to test the significance of these alterations in proteolytic activity at low pH.

In this paper, we present a mathematical model, building on our previous work on pH_i regulation (Webb et al 1999a), for tumour cell–ECM interactions during cancer invasion, focusing on the degradation of ECM components via active proteolytic enzymes. Our work also builds on previous modelling studies by Gatenby (Gatenby & Gawlinski 1996, Gatenby 1996), which predict that altered metabolic properties of tumour cells are a simple but sufficient mechanism of

tumour invasion. We will show that the effects of pH_e on the invasive behaviour of tumour cells are not mediated via changes in MMP activity at low pH. We will also extend our model to reflect the altered distribution and increased secretion of cathepsin B in malignant cells at low pH_e.

Role of pH in ECM proteolysis: metalloproteinases

We propose an ordinary differential equation model, focusing on the role of the matrix MMPs in the process of matrix degradation. We denote the concentrations of the tumour cells, ECM, pro-MMP and active-MMP at time t by $m(t)$, $c(t)$, $p(t)$ and $a(t)$ respectively. To study the effect of pH on the tumour cell dynamics and metastatic potential, we introduce two variables: pH_i and pH_e. For convenience we represent these in the model via the corresponding hydrogen ion concentrations $I(t)$ and $E(t)$ respectively (recall that $pH = -\log[H^+]$).

Steady-state aerobic energy metabolism consumes the same number of H^+ ions as are produced by the hydrolysis of ATP (Busa & Nuccitelli 1984), and as a result has very little effect on pH_i. However, in the absence of oxygen, cells rely on the more inefficient process of anaerobic glycolysis to obtain energy (Vaupel et al 1989, Gatenby 1996). During glycolysis hydrogen ions are formed, which are actively transported outside the cell via membrane-based ion transport mechanisms.

Cells have developed several membrane transport mechanisms for regulating pH (see Roos & Boron 1981). The level of intra- and extracellular pH influences the operation of these exchangers which directly transport either H^+ out of the cell, or HCO_3^- into the cell to neutralize H^+ in the cytosol (Tannock & Rotin 1989). Major transport mechanisms which have been implicated in pH_i regulation include the Na^+/H^+ exchanger, the Na^+ dependent Cl^-/HCO_3^- exchanger, and the cation-independent Cl^-/HCO_3^- exchanger. In our model, we use $u(I, E)$ to denote the combined activity of these exchangers (see Webb et al 1999b, for full details).

Once hydrogen ions are transported outside the cell they are removed from the tissue by the supporting vasculature (Vaupel et al 1989). The vasculature of many tumours is often compromised and unable to supply the nutritional needs of an expanding population of tumour cells, leading to the existence of hypoxic regions within solid tumours (Helmlinger et al 1997). To represent the functional vasculature in our modelling we introduce the parameter V, which represents the extent of vascularity. For simplicity, we assume that the rate at which H^+ ions are removed from the extracellular space is directly proportional to V. We use the term $s_2 = s_2(V)$ to represent this removal (see Fig. 1).

The focus of our modelling is on the increased use of the glycolytic pathway by tumour cells, which occurs even under aerobic conditions (Gatenby 1996). In the absence of quantitative data we describe the rate at which cellular metabolism can cause H^+ ions to accumulate intracellularly as a function $s_1(V)$. We would expect

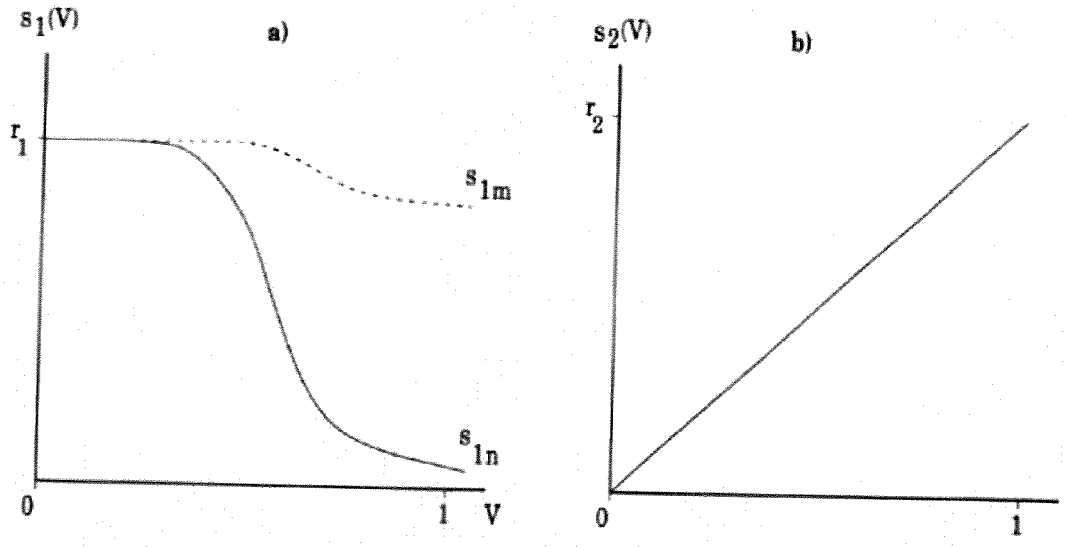

FIG. 1. (a) Schematic representation of the net production of H^+ ions from cellular metabolism of normal cells (s_{1n}), and tumour cells (s_{1m}). We represent the extent of functional vasculature in our modelling by the parameter V. Here, $V=0$ represents a poorly organized functional vasculature, leading to hypoxia. Increasing V corresponds to an increase in oxygen availability. In our model, we take $0<V<1$. We expect $s_1(V)$ to be a monotone decreasing function of V. As V increases $s_{1n}(V)$ falls to very low values, representing that under physiological pH, aerobic metabolism has no net effect on pH_i. In tumour cells, with their high glycolytic rate and consequent net production of H^+ ions, even in the presence of oxygen (i.e. high V), we expect $s_{1m}(V)$ to be significantly greater than zero. (b) The rate of removal of H^+ ions from the interstitial space by convective and/or diffusive transport. We assume that once hydrogen ions are transported outside the cell then the rate at which they are removed from the interstitial space is directly proportional to V. The quantitative values of $s_1(V)$ and $s_2(V)$ are of the order of mM/min.

$s_1(V)$ to be a monotone decreasing function of V: in normal cells $s_1(V)$ falls to very low values at large V, representing that under physiological pH, aerobic metabolism has no net effect on pH_i. As illustrated in Fig. 1, the form of $s_1(V)$ for tumour cells is more gently sloping which represents their high glycolytic activity and consequent production of H^+ ions, even in the presence of oxygen (i.e. high V). In our model, we denote $s_1(V)$ for normal and tumour cells by $s_{1n}(V)$ and $s_{1m}(V)$, respectively. For computational purposes, we have chosen simple specific functional forms possible for $s_1(V)$ and $s_2(V)$, and these are given in Webb et al (1999b).

Several studies have shown that tumour cells are able to survive and even proliferate in low pH_e environments which are ordinarily lethal to corresponding normal cells (Stubbs et al 1994, Gatenby & Gawlinski 1996). Under these conditions the pH_i of normal cells drops to values which are no longer permissive for cellular proliferation (Gillies et al 1992), whereas the pH_i in tumour cells is less sensitive to external pH and is maintained within physiological levels. In general, a slightly alkaline pH_i of about 7.2–7.25 appears to be necessary for normal proliferation (Gillies et al 1992). In our model, we use

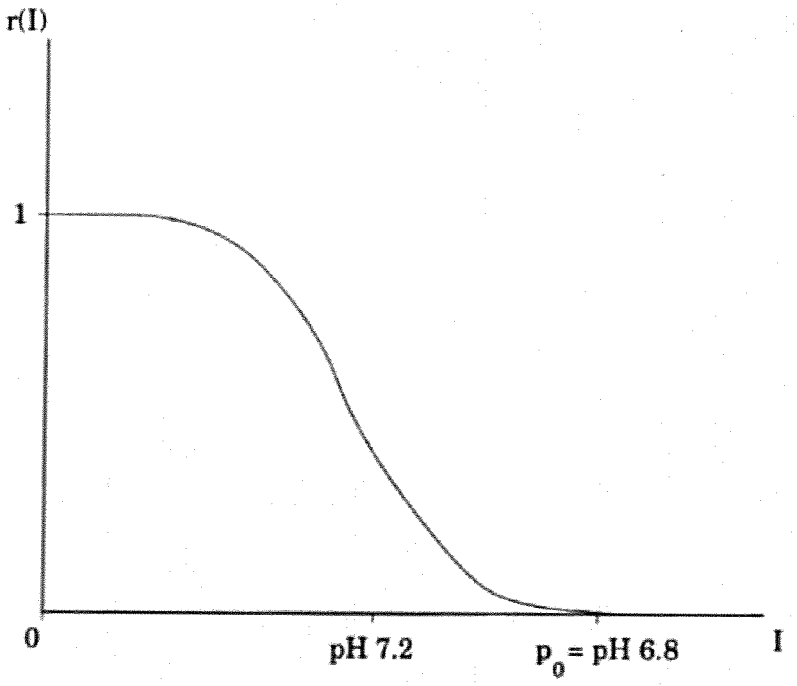

FIG. 2. The qualitative form of the function $r(I)$, which represents the effect of low pH_i on the proliferation rate of the cells. The maintenance of intracellular pH within the physiological pH range (pH 7.2–7.25) is necessary for normal proliferation. If the intracellular pH is lowered below pH 7.2, growth is inhibited. We assume that proliferation can only take place if the pH_i is above a threshold level p_0, which is permissive for normal cellular growth. The value of p_0 varies between cell types but is typically in the pH range 6.6–7.0. We choose $p_0 = pH_i$ 6.8. Note that it is intracellular H^+ ion concentration, rather than pH_i, that is plotted on the horizontal axis; some corresponding pH values are indicated.

the term $r(I)$ to represent the effect of low pH_i on the proliferation rate of the cells. It is assumed that proliferation can only take place if the pH_i is above a threshold level p_0 which is permissive for normal cellular growth (see Fig. 2).

In most cases, metalloproteinases are secreted as latent pro-enzymes that require extracellular activation to exert their proteolytic activity (Powell & Matrisian 1996). The *in vivo* mechanism of normal metalloproteinase activation is unknown, but may involve the action of other MMPs as well as other enzymes, such as plasmin and membrane-type MMP (Vassalli & Pepper 1994). It is likely that the activation of latent metalloproteinases is pH sensitive (Kato et al 1992). For instance, Davis & Martin (1990) observed that 94 kDa progelatinase, a latent pro-MMP, is activated by acid treatment. In our model, we neglect the activations by other enzymes to focus on the effects of pH. We use the term $f_1(E)$ to represent the effect of low pH_e on the autolytic activation of pro-MMP. Once activated, these enzymes can degrade extracellular matrix components. MMP activity is tightly regulated; typically, a metalloproteinase has a bell-shaped pH profile with a maximum activity in the physiological pH range (Kramer et al 1985, Turner 1979), and maintains ~80% of the maximum activity even at pH

6.8. In our model, we use a function $f_2(E)$ to reflect the effect of pH on the proteolysis of ECM.

Microenvironmental pH may also effect MMP secretion (Jang & Hill 1997). For instance, Kato et al (1992) have reported that two human cell lines (A549 and HT1080) secreted a higher level of 90 kDa gelatinase at pH 6.8 compared with pH 7.3. We use the term $f_3(I)$ to represent the effect of pH on the secretion of pro-MMP by the tumour cells.

With these definitions and assumptions, the model system is as follows:

$$\boxed{m = \textit{tumour cells}} \quad \frac{dm}{dt} = \overbrace{k_1 r(I) m\left(1 - \frac{m}{k_2}\right)}^{\text{cell division}} \tag{1a}$$

$$\boxed{c = \textit{ECM density}} \quad \frac{dc}{dt} = \overbrace{-k_3 f_2(E) a c}^{\text{proteolysis}} \tag{1b}$$

$$\boxed{p = \textit{Pro-MMP}} \quad \frac{dp}{dt} = \overbrace{k_4 f3(I) m -}^{\text{MMP secretion}} \underbrace{k_5 p}_{\text{MMP degradation}} \overbrace{-k_6 f_1(E) p}^{\text{MMP activation}} \tag{1c}$$

$$\boxed{a = \textit{active-MMP}} \quad \frac{da}{dt} = \overbrace{k_6 f_1(E) p -}^{\text{MMP activation}} \overbrace{k_7 a}^{\text{MMP degradation}} \tag{1d}$$

$$\boxed{I = \textit{intracellular } \mathrm{H}^+} \quad \frac{dI}{dt} = \underbrace{-u(I, E) +}_{\text{Ion transport}} \overbrace{s_{1m}(V) + \beta E}^{\text{acid loading}} \tag{1e}$$

$$\boxed{E = \textit{extracellular } \mathrm{H}^+} \quad \frac{dE}{dt} = \overbrace{m \cdot [u(I, E)}^{\text{Ion transport}} \underbrace{-s_2(V)E - \beta E].}_{\mathrm{H}^+ \text{ removal by vasculature}} \tag{1f}$$

Here the k_i values are positive constants. The term βE represents the passive movement of H^+ ions into the cell due to the internally negative membrane

potential, we assume β is constant. To describe the evolution of the tumour cell population we use a logistic-type growth, with growth rate k_1 and carrying capacity k_2. For simplicity we represent the activity of MMP inhibitors and active-MMP decay as a single term in the model, $-k_7 a$. The decay of latent pro-MMP is assumed to be linear; with rate constant k_5. Details of functional forms and parameter values are given in Webb et al (1999b).

We solved equation (1) numerically and compared the numerical solutions with data from Martínez-Zaguilán et al (1996), an experimental study which examined the effect of pH on the invasive potential of tumour cells. In this study the culturing of tumour cells at acidic pH (6.8) was found to increase both migratory and invasive behaviour. In their experiments, cells were grown for three weeks at either pH 6.8 or 7.4. Subsequently, cells were transferred to a membrane invasion culture system, and the invasive capabilities of these cells were evaluated in either acidic (pH 6.8) or normal (pH 7.4) conditions. They observed that cells exposed to low pH conditions for three weeks migrate and invade faster, regardless of the pH at which they were tested.

To simulate the incubation of cells in their respective media, we solve equation (1) with the medium fixed at either acidic (6.8) or normal (7.4) pH_e, starting with a small number of tumour cells. The levels of pH_i, tumour cells and proteolytic enzymes rapidly attain their steady state values. Once these values have been obtained, we then use them as initial conditions in a simulation where we test the proteolytic capabilities of these cells. Here, we allow pH_e to vary over time, starting with an initial pH_e of either 6.8 or 7.4 for the culture medium. Our simulations show that during this testing period the ECM is gradually degraded to zero. The numerically calculated ECM profiles in the above cases are illustrated in Fig. 3a.

Our results indicate that culturing cells at low pH (6.8) causes a change of less than 1% in ECM decay rates. This suggests that incubating cells under mildly acidic conditions has very little effect on their invasive behaviour. Further numerical simulations of this model for a wide range of parameter values showed a decrease in the ability of acid cultured cells to degrade ECM, regardless of the pH at which the cells were tested. The reduced ability of cells to degrade ECM in our model solutions is due to a decrease in the overall amount of extracellular MMP activity from cells grown at acidic pH_e (6.8) (relative to pH_e 7.4). Thus, our model predicts that the effects of pH on the invasive potential of tumour cells are not mediated via changes in MMP activity at low pH.

Role of pH in ECM proteolysis: cysteine proteinases

In this section, we consider an alternative model in which we neglect the proteolytic activity of the metalloproteinases and focus on the action of the cysteine proteinases. The differences between the two models is that in the MMP

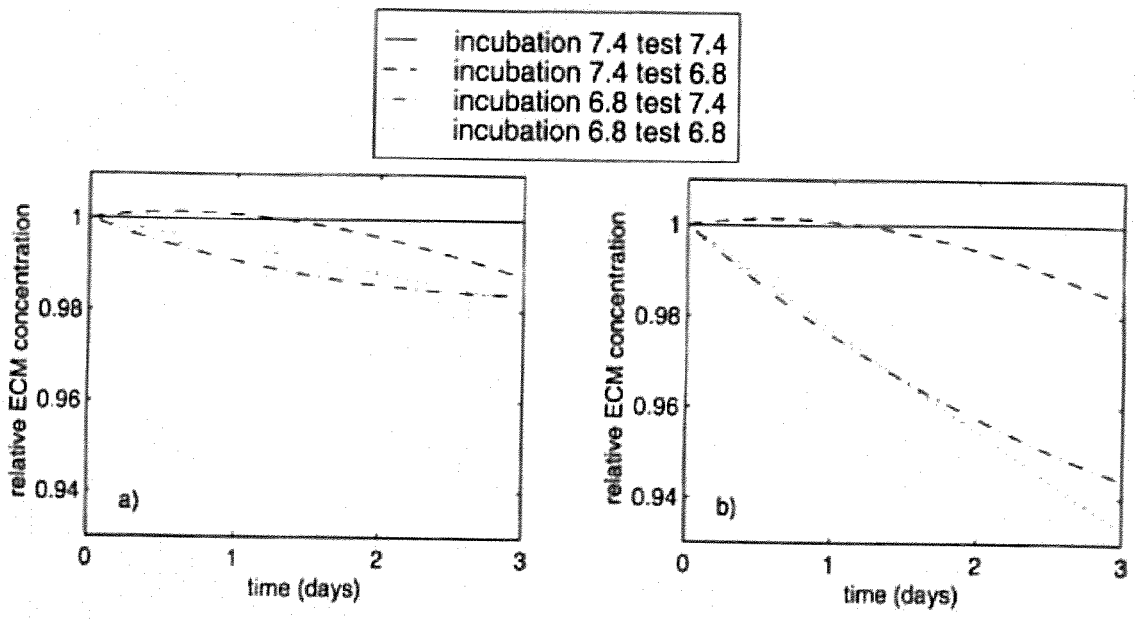

FIG. 3. Numerically calculated decrease in ECM with time from (a) MMP activity and (b) cathepsin activity. We incubate the cells for three weeks with pH_e of the culture medium fixed at either 6.8 or 7.4, with initial conditions corresponding to a small level of tumour cells with an initial pH_i of 7.2. The pH_i, tumour cells, and proteolytic enzymes rapidly attain their steady state values (not shown), which we denote by pH_{i0}, m_0, p_0 and a_0, respectively. Once these values have been obtained we then use them as initial conditions in a simulation where we test the proteolytic capabilities of these cells. Here, we allow the pH_e to vary over time, starting with an initial pH_e of either 6.8 or 7.4. The curves represent the ECM decay profiles for the cells in the four cases; (i) cells grown and tested at normal pH (pH_e 7.4) (—), (ii) cells grown at pH 7.4 and tested with an initial pH_e of 6.8 (- - -), (iii) cells grown at pH_e 6.8 and tested with an initial pH_e of 7.4 (- - -), and (iv) cells grown and tested at acidic pH (6.8) (· · · ·). We plot the decrease in ECM relative to the case where the cells are incubated and tested at normal pH (case (i)). The ECM is gradually degraded to zero. With the metalloproteinases, there is very little quantitative difference between the different cases. In particular, the decrease in ECM when cells are cultured at pH_e 6.8 is very similar to that observed at pH_e 7.4. Thus, the modelling suggests that the effects of pH on the invasive potential of tumour cells are not mediated via changes in MMP activity at low pH. However, our results indicate an increased ability of acid cultured cells to degrade ECM in the cathepsin model. Here, there is a much more rapid decrease in ECM density from acid treated cells compared to when cells are cultured at normal pH (pH_e 7.4). The initial values of the incubation media are $m(0)=0.1$, $a(0)=p(0)=c(0)=0$, $I_m(0)=$pH 7.2. At the end of the incubation time, the invasion capabilities of these cells are tested with the initial conditions $m(0)=m_0$, $a(0)=a_0$, $p(0)=p_0$, $c(0)=1$, $I_m(0)=pH_{i0}$, with $E(0)=pH_e$ 6.8 or 7.4. The parameter values used in this simulation are $k_3k_4k_2/k=15$, $k_5/k_1=1$, $k_6/k_1=3.43$, $k_7/k_1=5\times10^5$ for (a), and $A_3A_4A_2/A_1=15$, and $A_5/A_1=5\times10^4$ for (b), with $r_1=1$, $r_2=14$, $r_3=0.01$, $r_4=107$, $\beta=1$, $V=0.5$ and $a_m=0.4$ (see Webb et al 1999b, for full details).

model there is an overall decrease in the amount of extracellular proteinase activity from cells grown at pH 6.8 (relative to pH 7.4), whereas in the cathepsin model there is a substantial increase (e.g. 10-fold) in the secretion of active proteinase from acid-cultured cells (Rozhin et al 1994). Also, the optimal pH for the proteolytic activity of the cysteine proteinases is very acidic, in the pH range of

2.8–5.0 (Briozzo et al 1988, van der Stappen et al 1996), compared to more physiological pH levels for the maximal MMP activity.

A number of studies have implicated the cysteine proteinases in malignant progression (van der Stappen et al 1996, Chambers et al 1992). For example, substantial increases in the secretion of procathepsin B have been reported in a series of B16 melanoma cell lines (Qian et al 1989). For the secreted procathepsins to participate in tumour cell invasion, they must be activated. As with the metalloproteinases, we expect the extracellular activation of the secretion of procathepsins to be pH sensitive. However, we are not aware of experimental data that would enable these rates to be estimated quantitatively. For this reason, and in order to focus on the pH-induced secretion of active cathepsin-B (based on data in Rozhin et al 1994), we remove the latent form from the model. As with the previous model system, we represent the inhibition of cathepsins and active-cathepsin decay with the single term $-A_5 a$. This model consists of conservation equations for tumour cells, ECM, intracellular and extracellular pH, and active cathepsins. Here, we represent active-cathepsins via the variable $a(t)$. The model system is as follows:

$$\boxed{m = \textit{tumour cells}} \quad \frac{dm}{dt} = -A_1 r(I) m \left(1 - \frac{m}{A_2}\right) \tag{2a}$$

$$\boxed{c = \textit{ECM density}} \quad \frac{dc}{dt} = \overbrace{-A_3 b_1(E) a c}^{\text{proteolysis}} \tag{2b}$$

$$\boxed{a = \textit{active cathepsin}} \quad \frac{da}{dt} = \overbrace{A_4 b_2(I) m}^{\text{cathepsin secretion}} \overbrace{-A_5 a}^{\text{cathepsin decay}} \tag{2c}$$

$$\boxed{I = \textit{intracellular } \mathrm{H}^+} \quad \frac{dI}{dt} = -u(I, E) + S_{1m}(V) + \beta E \tag{2d}$$

$$\boxed{E = \textit{intracellular } \mathrm{H}^+} \quad \frac{dE}{dt} = -m \cdot [u(I, E) - S_2(V) E - \beta E]. \tag{2e}$$

Again, functional forms and parameter values are given in Webb et al (1999b).

We solved this model numerically for a wide range of parameter values. Again we incubated and tested the cells at either acidic (pH_e 6.8) or normal (pH_e 7.4) conditions. As before, the rate of ECM decay was determined by the ability of

cells to degrade ECM material. In Fig. 3b we plot the numerically calculated ECM profiles for the different cases. Our simulations show that culturing cells at pH 6.8 causes an increase of about 5% in ECM decay rates compared to when cells are grown under normal conditions (pH 7.4). In this case, there is a much more rapid decrease in the ECM density.

The important features in the cathepsin model are the more acidic pH value for the optimum proteolytic activity of the cathepsins compared to the MMP case and the increased secretion of active-enzyme from acid cultured cells. These are represented by the functional forms $h_1(E)$ and $h_2(I)$, in the ECM and active cathepsin equations. To investigate the importance of these terms, we varied these functions in turn, and compared the relative change in the model solutions. Our results showed that varying $h_1(E)$ has a negligible effect on the ability of cells to degrade ECM at either acidic or normal pH, indicating that the effect of pH on the proteolytic activity of the cells is not the crucial term in the model. Hence, we can conclude that $h_2(I)$, the function representing the effect of low pH_i on the secretion of active cathepsins, is crucial, and is the key term which accounts for the differences between the two enzyme models.

In our model, we have used a very simple representation of the pH-induced peripheral redistribution of cathepsin B, which is accompanied by the secretion of the active form of this enzyme. With this alternative model, there is good agreement with the experimental data in Martínez-Zaguilán et al (1996), namely that the numerical solutions show a significant increase in the ability of acid cultured cells to degrade ECM, regardless of the pH at which the cells are tested. Our model predicts that the acidic pH-induced redistribution of active cathepsins to the cell surface of malignant cells could be a major factor in the acquisition of a more metastatic phenotype at low pH.

Discussion

Cathepsin B is a lysosomal cysteine proteinase involved in both intracellular and extracellular protein degradation. The amount of cathepsin B secreted varies considerably according to cell type and culture conditions. Rozhin et al (1994) have shown that the reduction of pH_e to 6.5 resulted in a redistribution of cathepsin B-containing vesicles to the surface of tumour cells. Moreover, this resulted in an enhanced secretion of active cathepsin B. Since our modelling suggests that changes in MMP activity at low pH do not have significant effects on invasive behaviour, we considered an alternative system in which we neglected the proteolytic activity of the metalloproteinases and focussed on the acidic pH-induced secretion of active cathepsin B. The main difference between the two models is the effect of pH on the secretion of the two different classes of enzymes; in the MMP model there is an overall decrease in the amount of

extracellular proteinase activity from cells grown at pH 6.8 (relative to pH 7.4), whereas in the cathepsin model there is a substantial increase (e.g. 10-fold) in the secretion of active proteinase from acid cultured cells (Rozhin et al 1994). From numerical solutions of this alternative model we were able to reproduce the quantitative features of the experimental data in Martínez-Zaguilán et al (1996). In particular, we were able to show an increased ability of acid cultured cells to degrade ECM. Furthermore, this result is independent of the pH at which the cells are tested. Our model thus predicts that the pH-induced peripheral redistribution of cathepsin B could be a major factor in the acquisition of a more metastatic phenotype in malignant cells at low pH_e.

Human and animal tumours *in vivo* often contain both hypoxic and well oxygenated areas (Sartorelli 1988) and approximately one unit variations in pH_e between tumour capillaries have been observed in animal model systems (Helmlinger et al 1997). The present theoretical model suggests that, in addition to metalloproteinase activity, the cysteine proteinases play a critical role in tumour progression, a prediction with important implications for the design of anti-invasive therapies.

Acknowledgement

SDW was supported by an earmarked studentship from the Engineering and Physical Sciences Research Council.

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DISCUSSION

Roepe: I am curious about your assumptions regarding the numbers that you used in balancing the function of the lactate proton symporter. Probably because of my own ignorance, I am not aware of a lot of experimental data on that. If the pH gradient is pointing into the cell, then it has to be the lactate gradient that is driving the net outward process: there is no other source of energy as I see it. The lactate concentration inside the cell must be greater than outside. We have some numbers for external lactate. What experimental data exist, if any, that describe the overall thermodynamics of that symport process, and what assumptions are you making when you use these in your modelling?

Webb: There are some good data available for the Na^+:H^+ antiporter and the Na^+-dependent bicarbonate exchanger. The problem is with the lactate anion symporter: the data aren't available. We had to formulate a term for this by looking at the higher amounts of glucose consumed by tumours compared to corresponding normal cells. Also, lactate release is directly related to glucose uptake. If you couple this with the observation that only 40–85% of glucose is taken up and released as lactate, you can translate these data to give you a term representing lactate release in the model.

Roepe: Not precisely. Since it is a logarithmic scale, the pH gradient is important. Your model is making certain predictions about the necessary value of intracellular lactate in order to produce these pH values. If the lactate proton symporter is the driving force, and if we have a number for lactate, your data predict the value of intracellular lactate in the presence of a certain pH gradient for that process to have sufficient capacity to produce these pH values.

Webb: In our model, we did not explicitly consider lactate as a variable. However, we could use our model to predict details of intracellular lactate by comparing the rates of glycolysis with lactic acid release. You are right; in our model there has to be a lactate gradient difference to drive this symporter.

Stubbs: There is a lactate gradient in tumours; this is well known. The intracellular lactate is 2–4 times higher than the extracellular.

Roepe: My point is that this is just about in equilibrium with the proton gradient. This delicate balance may be important.

Webb: Our model is intended to be a simple framework. We also considered looking at physicochemical buffering. There is also a case for short-term homeostasis for the transfer of H^+ into organelles. The reason I showed this model is because despite its simple form it does reproduce the biological observations very well under certain circumstances.

Roepe: How is your model affected if you take into account that CO_2 is an acid? You have rather ignored this. We heard yesterday that this is probably not a good assumption.

Webb: I agree. The reason I didn't take that into account initially was because of the lack of data, which is one of the things that inhibits modelling. There are a lot of available data, but the kind of data we need are limited in supply.

Tannock: I have a comment about the lactate symporter. Certainly there are papers showing the existence of this pathway, but if you acidify cells in tissue culture under conditions that you might think would exist *in vivo*, and you block the two exchangers that you mentioned, then cells correct their acidification extraordinarily slowly. This would suggest that any additional regulatory mechanisms for pH_i are not very active. It doesn't suggest that lactate could have a large role in the control of acidity under the conditions you mimic in tissue culture.

Webb: Doesn't this depend on the experimental set-up and the tumour cell line used?

Roepe: It's important to keep in mind that many of these experiments don't have a lot of glucose.

Gatenby: What is the glucose utilization under those circumstances?

Tannock: The glucose consumption in tissue culture is usually quite substantial. Most cells in tissue culture use glucose and produce lactate. Cells are lazy: if they can get enough energy by using glucose through glycolysis they tend to do this in culture.

Gatenby: This is using acidified medium.

Tannock: It is only acutely acidified.

Rochefort: I am pleased with your general conclusion that cathepsins may be more important than MMPs in cancer! However, I don't totally agree with your model based on cathepsin B secretion.

You use Bonnie Sloane's laboratory data (Rozhin et al 1994) which reported a redistribution of the lysosomes at pH 6.5. These end up closer to the cell membrane due to microtubule-associated translocation as observed by Heuser (1989). We confirmed this translocation, induced by acidic pH, with cathepsin D in MDA-MB231 cells (R. Farnoud & H. Rochefort, unpublished data). However, we have difficulties in showing that an acidic pH increases secretion of an active enzyme. In general, cancer cell lines secrete the proenzyme, and the major problem with cathepsins is their activation, which requires a sufficiently acidic pH.

Webb: The study that I used to obtain the model parameters for the cathepsin proteinases looked at both membrane endosomal fractions and secretion. We have clear data that there was an increased secretion of the activated enzyme (see Rozhin et al 1994).

Rochefort: Perhaps there is a difference between cathepsin B and D. Cathepsin L has also been suggested to play a role.

Gillies: I am surprised that the Na^+/H^+ exchanger cannot be responsible for the differences we see, because its activity is so much higher than any of the other proton transporters.

Webb: Of course, it depends on how we formulate our acid-loading term, and the rate at which H^+ is produced. But for quite a wide range of values, the Na^+/H^+ exchangers couldn't maintain the pH_i of the tumour cells.

Gillies: I have gone through these calculations a number of times. My conclusion is that because of the Nernst equilibrium, there is an inwardly directed hydrogen ion driving force. But the measured permeability for H^+ (about 10^{-4} cm/s) is too small to account for any appreciable acid load. Have you come to the same conclusion?

Webb: The term for passive fluxes was very small. We could have neglected it. Metabolism is the primary acid-loading term.

Ackerman: There has been a lot of work done that defines the point at which the gradient between intracellular and extracellular pH is lost, in a variety of cells. Do you get the right point of collapse in your model if you take *in vitro* cells, start decreasing pH_e and at some point lose the intracellular/extracellular pH gradient?

Webb: It depends on the balance between the acid loading terms and acid extrusion via the different cellular transports in the model.

Ackerman: So if you include cells in your model and start dropping the pH_e, when did the pH_i collapse and equal the pH_e?

Webb: It varies, depending on the primary values. In the specific example I presented, the collapse occurred at pH 7.0–7.1 for normal cells and pH 6.6–6.8 for tumour cells.

Ackerman: I didn't have a good sense of how many adjustable parameters are in the model. What are the fixed parameters?

Webb: There are a number of parameters, and quite a few are variable between cell types. The experimental data were presumably measured under steady-state conditions, and this is how we attained some of our parameter values. We then took data from a cell cultivation study which studied the change in pH_e over time starting from different levels of pH, and then matched our model solutions with the data to get a feel for the order of magnitude estimates of the remaining parameter values in the model.

Tannock: I want to return to a general point that perhaps John Griffiths will be able to answer. I have struggled with this question of the addition of hydrogen ions from the production of carbonic acid. There have been papers that have suggested that this metabolic pathway didn't produce hydrogen ions, and others that have suggested it does. My chemistry has not been good enough to really grapple with this.

Griffiths: The first people who put this theory forward, Hans Krebs, George Alberti and Frank Woods (Krebs et al 1975), suggested that if in the glycolytic

pathway you have glucose going to lactate, the acidifying effect would be nullified by the fact that you are also converting ADP to ATP. The newly formed ATP absorbs hydrogen ions as fast as they are made by the breakdown of glucose. The most detailed exposition of this argument, by Hochachka & Mommsen (1983) has been widely cited. Strictly speaking, what they say is true, but it is irrelevant to the situation that we are all interested in because in the steady state the ADP and ATP are continuously being cycled round and round and their concentrations don't change, whereas the lactate really is increasing in concentration. We don't have a significant net change in the hydrogen ions bound or given up, because there is a fixed pool of ATP and ADP, which is continuously being phosphorylated and dephosphorylated. There are several papers which have refuted this argument (e.g. Wilkie 1979). It only works in the unusual situation involving recovery from an acute period of ischaemia when the concentrations of ADP and ATP actually do change.

Simon: Just to comment on the cathepsin secretion, we have been able to show that if you selectively disrupt acidification through the secretory pathway, with no effects on cytosolic pH or extracellular pH, this substantially increases the secretion of a variety of lysosomal enzymes, including hexosaminidase and cathepsin L. When you disrupt the pH_e, you have to be careful to make sure that the effect you are seeing is directly due to extracellular pH rather than the effect of overall pH regulation within the cell.

Webb: In the study that these data are based on, they asked the question as to whether it was the effect of low pH_e or the effect of low pH_i, which was associated with a decrease in pH_e. To study this they induced acidification of the cell by mechanisms other than low pH_e and found that this was sufficient to induce the movement of lysosomes to the cell surface together with the increased secretion of cathepsin B. Therefore, it was the effect of the low pH_i, rather than pH_e in those particular cells.

Rochefort: In the case of Dr Simon's data, there was also alkalinization of acidic vesicles by very high concentrations of tamoxifen. In this case, tamoxifen might be acting in a similar way to NH_4Cl to facilitate the secretion of lysosomal proenzymes.

Simon: We used a variety of techniques to disrupt the acidification of the secretory pathway, including bafilomycin, concanomycin, monensin and nigericin. I will describe the effects of tamoxifen. Tamoxifen has all the obvious effects of an oestrogen receptor antagonist, but in addition it also disrupts the organellar pH through a number of mechanisms (Altan et al 1999). One of these is by acting through weak base effects, and another is by acting as a Cl^-/H^+ co-transporter (Chen et al 1999). Under conditions where we can show that there is no effect on the cytosolic pH (we measured this by loading 1000 kDa dextrans into the cytosol), we see that with increasing doses of tamoxifen, there is increased hexosaminidase

secreted to the supernatant, and a decreased amount in the cell-associated form. In the case of cathepsin L, we see both the pro form and the mature form being secreted. Tamoxifen treatment has no effect on nuclear pH, and very little on cytosolic pH, but the lysosomes go from pH 5 to pH 7.1, the Golgi and endosomes from pH 6 to pH 6.7, and secretory vesicles from pH 5 all the way up to pH 6.7. In this case the mannose-6-phosphate receptor which normally binds a lot of these things in the Golgi and brings them to the lysosomes, can't dump its cargo. Because of this, it gets saturated and the enzymes are secreted.

Rochefort: This is not acidification of the extracellular environment.

Simon: No, in this case the secretion is the consequence of affecting the pH in the organelles. The extracellular medium may be working to activate the pro form or it may also be having an indirect effect by affecting pH regulation within the cell.

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