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The relationship between extracellular lactate and tumour pH in a murine tumour model of ischaemia–reperfusion

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Summary We have studied the relationship between extracellular lactate ($LACT_e$) and extracellular pH (pH_e) in murine tumours after vascular occlusion (clamping) followed by reperfusion. In tumours occluded at ambient room temperature, $LACT_e$, measured by microdialysis, increased linearly with time and correlated strongly with the acidification of the extracellular compartment (r=0.97, P<0.03, n=4). Significant decrease in $LACT_e$ was evident following removal of occlusion at room temperature and is consistent with vascular reperfusion. Occlusion at 35°C, i.e. to maintain tumour temperature during occlusion, resulted in an initial increase in $LACT_e$, which mirrored a rapid reduction in pH_e. However further reductions in pH_e occurred without increase in $LACT_e$. During vascular occlusion, tumour adenine nucleotide pool decreased and AMP accumulated. AMP subsequently decreased in the 35°C group and this may contribute to the observed differences in accumulation of $LACT_e$, and capacity to recover from vascular occlusion, between the two treatment groups. These data show that extracellular lactate concentration is a good predictor for tumour pH when adequate energy sources are available within the tumour. However, under conditions of more severe stress, resulting in abolition of primary energy stores and cell death, the pH_e continues to decline in the absence of a corresponding accumulation of extracellular lactate. This emphasizes the fact that other processes, apart from lactate production, can contribute to reduction in extracellular pH.

Keywords: tumour pH; lactate; adenine nucleotide; ischaemia; reperfusion

The rapid growth of some tumours results in areas of tumour cells distant from the supplying blood vessel in a microenvironment of reduced oxygenation (hypoxia) and also an accumulation of metabolic products causing extracellular acidosis (Vaupel et al 1989). These characteristics do not normally occur within normal tissues and have been identified as potential targets for tumour therapy, e.g. hypoxia-selective toxins or pH-dependent cytotoxic drugs (Wike-Hooley et al, 1984; Tannock and Rotin, 1989). Recently, novel therapies have been identified that mediate their toxicity by interfering with the tumour vasculature causing vascular stasis, and potentiation of hypoxia or pH-selective chemotherapeutic agents can be achieved directly, if they are administered just before vascular stasis (Brown, 1987; Chaplin and Acker, 1987; Stratford et al, 1987; Parkins et al, 1994*a*).

Since acidic tumour pH is clearly a potential selective therapeutic target for development of novel therapeutic approaches, there is a need to understand the mechanisms that induce the acidic state in tumours. Tumour acidosis has in general been associated with increased glycolysis, which is caused at least in part by the hypoxic environment that exists within tumour tissue. The aim of the present work was to establish whether lactate production is the sole driving force for increased acidosis within tumours under ischaemic conditions.

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We have reported previously that complete vascular occlusion results in tumour cell killing, which is dependent upon both the duration of the occlusion and the temperature of the tumour (Parkins et al, 1994*b*). Blood flow in tumours, unlike that in normal tissues, is depressed for many hours after relatively short periods of vascular occlusion and suggests that tumour vasculature is more susceptible to damage resulting from ischaemia itself or the subsequent reperfusion. It is well known that reduction in cellular energy status during occlusion compromises cellular function and results in intracellular acidification leading ultimately to cell death (McCoy et al, 1995).

It has been known since the early experiments of Warburg (1930) that tumour cells produce lactic acid by aerobic glycolysis. Many tumours use glycolysis to maintain their energy status, and a specific monocarboxylate carrier, through which lactic acid can be effluxed from the cell, has been identified in Ehrlich ascites tumour cells (lactic acid is fully dissociated at physiological pH to lactate ions)(Spencer and Lehninger, 1976). Homeostasis of cellular pH_i is also maintained by efflux of H^+ by the Na^+/H^+ antiport exchanger, which has been shown to be ubiquitous in mammalian cells and is necessary for the growth of some tumours (Grinstein et al, 1989; Rotin et al, 1989). The high rate of lactate production by tumour cells, combined with the poor vascular structure in tumours, results in a significantly acidic extracellular space, which has been investigated using different techniques (Vaupel et al, 1989; Griffiths, 1991; Gillies et al, 1994). In addition, because H^+ and lactate move together on the monocarboxylate carrier, the distribution of H^+ and lactate tend to assume a reciprocal relationship with pH across the plasma membrane (Spencer and Lehninger, 1976; Veech, 1991; Stubbs et al, 1994).

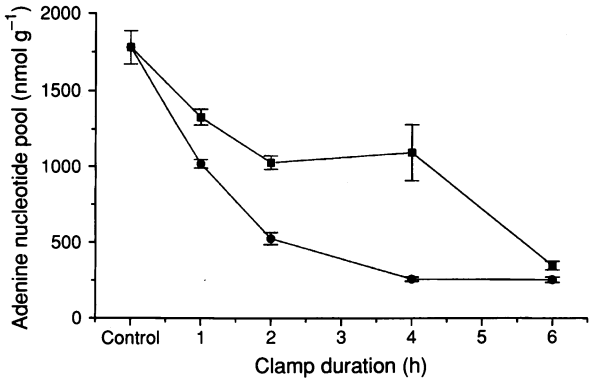

Figure 1 Total adenine nucleotide levels (ATP + ADP + AMP per gram) were measured in freeze-clamped CaNT tumours using HPLC assay. Decreases of total nucleotide pool were greater in those tumours whose temperature was maintained during the period of vascular occlusion up to 6 h ($P < 0.05$ at all time points). Occlusion at room temperature (■), occlusion at 35°C (●) (4–8 mice contribute to each data point; nmol g^{-1} tumour weight ± s.e.m.)

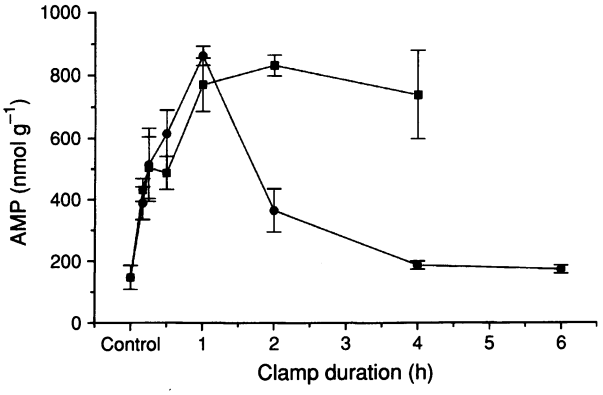

Figure 2 Accumulation of AMP in CaNT tumours during complete vascular occlusion appeared to be independent of tumour temperature during the first hour of vascular occlusion. At longer occlusion times, the breakdown of AMP was temperature dependent, with significant decreases occurring in the 35°C-maintained tumours beyond 1 h clamping ($P < 0.05$). (■), Occlusion at room temperature; occlusion at 35°C (●) (4–8 mice contribute to each data point; nmol g^{-1} tumour weight ± s.e.m)

We have shown previously that extracellular pH (pH_e) is acidic in the CaNT murine tumour (using inserted pH microelectrodes or non-invasive magnetic resonance spectroscopy) with intracellular pH (pH_i) maintained close to neutrality (Stubbs et al, 1992; McCoy et al, 1995). In response to complete vascular occlusion, the pH of both compartments became significantly more acidic and, after many hours of occlusion, both pH_i and pH_e tended to equilibrate to the same value. In the present study, we addressed whether the decreased pH_e observed during ischaemia can be explained entirely by the expected production of lactate. It has previously been assumed that lactate is one of the major determinants of acidity in tumours. In addition, studies in human tumours have indicated that high lactate levels correlate with a high risk of metastasis, underlying their possible role in other biological processes (Schwickert et al, 1995). Thus, if extracellular pH correlates with lactate levels, it might be a useful predictor for such biological effects. However, the simple assumption that lactate is a major determinant of extracellular pH has been challenged by the finding that transfected cells, which cannot produce lactate, still develop an acidic extracellular milieu (Newell et al, 1993).

We have investigated the relationship between extracellular lactate ($LACT_e$) and pH_e in tumours challenged by vascular occlusion and reperfusion at two different ambient temperatures (room temperature and 35°C). Since cellular energy metabolism is required for maintaining ion gradients, we also measured tumour adenine nucleotide levels by high-performance liquid chromatography (HPLC) analysis on freeze-clamped tumours. The recovery of tumour $LACT_e$ levels following removal of the occlusion was also investigated.

MATERIALS AND METHODS

Experimental tumours

The syngeneic murine tumour CaNT, a moderately differentiated breast adenocarcinoma, was used in the mouse strain CBA/Gy f TO aged 12–16 weeks. Cells obtained from a tumour suspension were implanted subcutaneously (s.c.) on the dorsum. Tumours were treated when their geometric mean diameter (g.m.d.) was between 6 and 8 mm (150–300 mg) by application of a metal D-shaped clamp across the skin at the base of each s.c. tumour (Parkins et al, 1994*b*). At least four tumours were used for each data point. In some groups, tumour temperature was maintained by placing the mice in a warm air incubator thermostatically controlled at 35°C (equivalent to the control temperature of superficial subcutaneous tumours).

Tumour $LACT_e$ and adenine nucleotide analysis

The microdialysis and HPLC assays used to measure $LACT_e$ from tumours has been reported previously (Stratford et al, 1995). At various times up to 6 h after vascular occlusion by clamping, animals were killed by cervical dislocation, and the microdialysis probe immediately inserted into the tumour. Briefly, this technique consisted of insertion of the pH microelectrode probe (CMA/12, Biotech Instruments, Herts, UK) through a needle-made hole in the skin into the underlying tumour and dialysing using saline as the dialysate. Separate groups of tumour-bearing mice were used for each of the time points. Microdialysis was also performed up to 3 h after clamp removal. Tumour adenine nucleotide levels were measured by HPLC assay in neutralized extracts of freeze-clamped samples taken after up to 6 h of complete occlusion (see Stratford and Dennis, 1994 for details).

Tumour pH measurement

Tumour extracellular pH (pH_e) was measured as described previously (Parkins et al, 1994*b*), but briefly consisted of insertion of a needle pH microelectrode (MI-402, Microelectrodes Inc., USA) into the tumour at the start of the occlusion period. Animals were anaesthetized (i.p.) with 25 mg kg^{-1} diazepam and 50 mg kg^{-1} ketamine before insertion of the electrode. Continuous readings were recorded for up to 3 h after occlusion had started. No pH data was obtained at later times after occlusion owing to the limited duration of the anaesthetic.

RESULTS

Effect of occlusion on adenine nucleotides

Figure 1 shows the adenine nucleotide levels of CaNT tumours after vascular occlusion by clamping for up to 6 h. The tumours

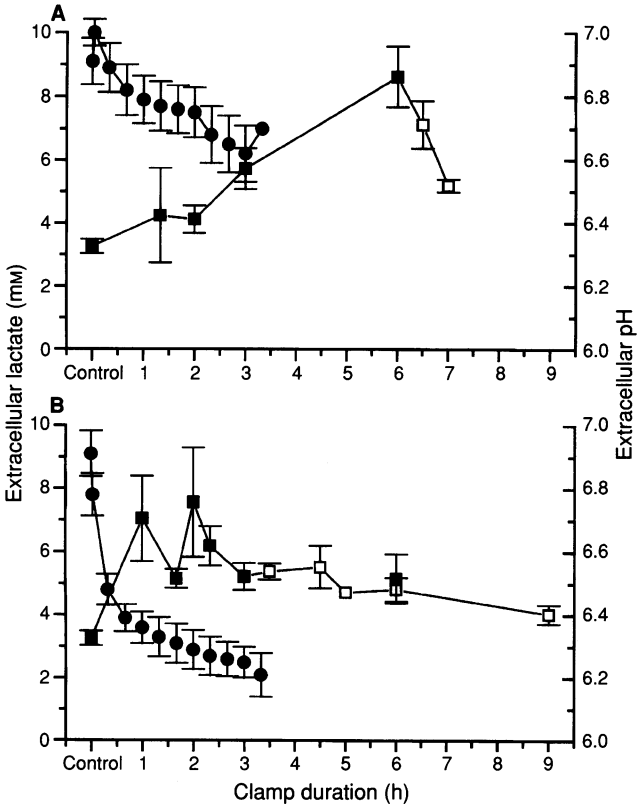

Figure 3 (**A**) After complete vascular occlusion at room temperature, extracellular lactate (■) significantly accumulated in CaNT tumours (P <0.05 at all times exceeding 1.3 h clamp) with a concomitant fall in extracellular pH (●). Tumour $LACT_e$ levels decreased rapidly after the occlusion was removed (□), indicating some recovery of tumour perfusion P=0.069 at 1 h after clamp removal (mean ± s.e.m) (McCoy et al, 1995; Stratford et al, 1995). (**B**) Extracellular pH (●) fell rapidly after complete vascular occlusion in CaNT tumours, with temperature maintenance at 35°C. $LACT_e$ levels (■) were significantly increased at all occlusion times (P < 0.0005), although they were poorly correlated to extracellular pH. This may be indicative of inhibition of metabolic enzymes by low pH in combination with exhaustion of cellular energy levels. No significant reduction of tumour $LACT_e$ levels (□) was observed after removal of the vascular occlusion and this suggests reduced recovery of tumour perfusion (mean ± s.e.m)

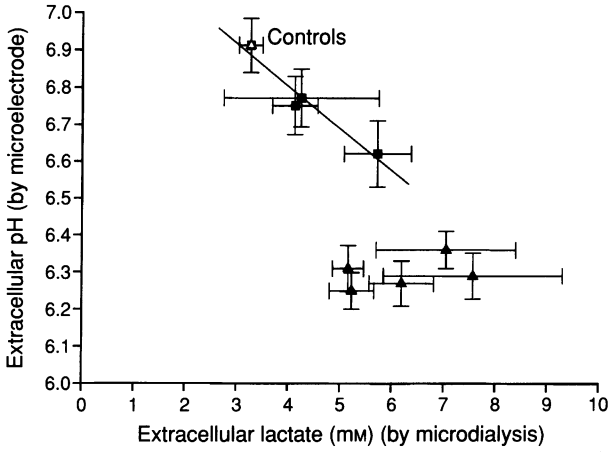

Figure 4 Correlation between extracellular pH (measured by pH microelectrode) and extracellular lactate ($LACT_e$) (measured by microdialysis) in CaNT tumours after vascular occlusion at either room temperature (■) or with temperature maintenance at 35°C (▲). Control samples, open symbols. (Correlation coefficient at room temperature r=0.97, P <0.03; at 35°C r=0.74, P > 0.09)

were either maintained at preocclusive temperature (35°C) or allowed to cool naturally until equilibrated with room temperature. In tumours allowed to cool during occlusion, there was significantly less (P<0.001 at 1, 2 and 4 h) breakdown of total adenine nucleotides, with levels being maintained at more than 50% of the starting value even 4 h after occlusion. These findings were mirrored by tumour AMP (Figure 2), which showed rapid increases during the first hour of occlusion followed by no further increase in the room temperature tumours. However, in the 35°C tumours, the AMP levels, after increasing fourfold at 1 h, decreased again to preocclusion values over the next 3 h, indicating further breakdown of AMP (possibly to inosine and hypoxanthine).

Effect of occlusion on pH_e and $LACT_e$

Tumours allowed to cool to room temperature after occlusion showed a time-dependent decrease in tumour pH_e over a 3-h period (pH_e fell from 6.91 ± 0.07 to 6.62 ± 0.09), during which a corresponding increase in tumour $LACT_e$ was observed (Figure 3A). When the occlusion was removed after 6 h, there was a rapid fall in $LACT_e$ (from 8.62 ± 0.95 to 5.18 ± 0.2), which is consistent with restoration of the tumour blood supply as confirmed independently by radiolabel tracer studies (Parkins et al, 1995; Stratford et al, 1995).

The response to occlusion of the CaNT tumour maintained at 35°C, however, was quite different (Figure 3B). The reduction in tumour pH_e is more rapid in this treatment group, reaching a significantly lower value [pH_e = 6.25 ± 0.09 (P<0.01)] at 3 h after occlusion compared with room temperature (Parkins et al, 1994*b*). During the first hour of occlusion at 35°C, $LACT_e$ increased significantly, although extension of the period of vascular occlusion to 6 h did not result in any further time-dependent increase in $LACT_e$, as was seen in the room temperature-maintained tumours. Removal of the occlusion after 3 h and assay 6 h later, i.e. allowing 6 h for any reperfusion to occur, did not show any significant decrease in tumour $LACT_e$ and may be evidence of a reduced degree of reperfusion in these tumours after this treatment.

At room temperature, there was a significant correlation between the acidification of extracellular pH and extracellular lactate accumulation in the CaNT tumour over the period of study (r=0.97, P<0.03, n=4) (Figure 4). No significant correlation was found between $LACT_e$ and decrease in pH_e in tumours occluded at 35°C (r=0.74, P>0.09, n=6).

DISCUSSION

This study has shown that total vascular occlusion resulted in a time-dependent decrease in tumour cellular adenine nucleotide levels. The decrease is also temperature dependent with significantly lower tumour nucleotide levels achieved by maintenance of the tumour temperature at 35°C, thereby preventing cooling during the clamping. The use of superficial tumours alone in any study would, therefore, underestimate the effect of occlusion and would not reflect the potential anti-tumour effect that would occur if the tumour had been centrally located where temperature would be maintained by surrounding tissue.

In a recent study, we investigated the changes in both intra- and extracellular pH using non-invasive magnetic resonance

spectroscopy (MRS) techniques during complete vascular occlusion. Data from both techniques show that tumours commonly have an acidic extracellular pH compared with the relatively neutral intracellular compartment (McCoy et al, 1995). Intracellular pH is maintained by membrane-based proton transporters, which are indirectly dependent on cellular energy to transport protons into the extracellular space. It is probable, therefore, that the greater breakdown of adenine nucleotides in the 35°C-maintained tumours, in addition to causing loss of ion gradients, would add to the proton load by the release of protons from the breakdown of ATP, causing a lower pH_e under these conditions. In the room temperature-maintained tumours, the loss of adenine nucleotides is much less severe, with relatively higher pH_e for a given period of occlusion, and, more importantly, the levels of AMP are maintained throughout the clamp. When the blood flow is restored AMP can be reconverted to ATP, whereas in the 35°C-maintained tumours not only was the blood flow inadequately restored, but most of the AMP has been irretrievably lost.

The reciprocal relationship between pH_e and $LACT_e$ levels was previously found in tumours occluded at room temperature (Stratford et al, 1995). The correlation is significant for room temperature tumours, but not in this study when the tumour temperature was maintained at 35°C during occlusion. This might be expected on the grounds that the greater breakdown of adenine nucleotides under these conditions causes an increase in H^+ and, thus, a lower pH_e. A key enzyme of glycolysis is phosphofructokinase (PFK), whose action is inhibited when H^+ levels rise (Ui, 1966; Halperin et al, 1969), preventing excessive formation of $LACT_e$ and a precipitous drop in blood pH (acidosis). Such a mechanism could explain the absence of increase in $LACT_e$ seen at 35°C. Our previous studies have shown no loss of clonogenic potential during a 3-h period of occlusion at room temperature (Parkins et al, 1994*b*). However, significant reductions in cell survival are observed after clamp periods of 1 h or more when tumour temperature is maintained. It is of interest to note that the loss of relationship between pH_e and $LACT_e$ is observed in the present study under conditions known to influence cellular integrity and clonogenicity.

The recovery of tumour blood flow following clamp removal has previously been shown to be inversely related to clamp duration; relative tumour perfusion at 1 h following either a 1- or 3-h period of occlusion at room temperature was 70.1% ± 14.6% of control compared with 50.5% ± 6.3% respectively (Parkins et al, 1995). Vascular occlusion at 35°C is significantly more damaging than occlusion at room temperature, so it is likely that recovery of blood flow following vascular occlusion at 35°C will be reduced compared with that at room temperature (Parkins et al, 1994*b*). The observation in this study that tumour $LACT_e$ levels were not reduced in the 35°C tumours following clamp removal may indicate some irreversible vascular damage, thus preventing $LACT_e$ washout. These findings emphasize the important role of glycolysis in determining vascular and tissue function in tumours under conditions of total vascular occlusion. The correlation between $LACT_e$ and pH_e is only evident under ischaemic conditions that elicit no tumour cell death. Clearly under more extreme conditions that elicit marked breakdown of high-energy phosphates and other biochemical changes concomitant with the loss of cellular integrity, this relationship no longer holds. It should be noted that within a particular tumour there may be a correlation between $LACT_e$ and pH_e following an intervention such as ischaemia; however, it is clear that pH_e of different types of tumours may depend on factors other than $LACT_e$. This is emphasized by a recent study using *ras*-transfected fibroblast cells, which are glycolysis deficient (Newell et al, 1993). These variant cells produce approximately 1% of the parental line's production of lactic acid but have similar pH_e ($pH_e = 6.78 \pm 0.04$) compared with the parental line ($pH_e = 6.65 \pm 0.07$) when grown in vivo. These investigations attributed such a finding to the fact that other proton-producing processes contribute to extracellular acidity. The present study shows that accumulation of extracellular lactate is not the only determinant of an acidic environment in solid tumours. Understanding the mechanisms that contribute to tumour acidosis could provide improved understanding of tumour physiology and identify potential targets for therapeutic intervention.

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