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 (LACTe) and extracellular pH (pHe) in murine tumours after vascular occlusion (clamping) followed by reperfusion. In tumours occluded at ambient room temperature, LACTe, 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 LACTe 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 LACTe which mirrored a rapid reduction in pHe. However further reductions in pHe occurred without increase in LACTe. 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 LACTe, 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 pHe 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 (Wiike-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, 1994a).

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.

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, 1994b). 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 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).
We have shown previously that extracellular pH (pHe) is acidic in the CaNT murine tumour (using inserted pH microelectrodes or non-invasive magnetic resonance spectroscopy) with intracellular pH (pHi) maintained close to neutrality (Stubbs et al., 1992; McCoy et al., 1995). In response to complete vascular occlusion, the pHe of both compartments became significantly more acidic and, after many hours of occlusion, both pHi and pHe tended to equilibrate to the same value. In the present study, we addressed whether the decreased pHe 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 (Schwickett 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) and pHe 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 samples. The recovery of tumour LACT 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 T0 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., 1994b). 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 and adenine nucleotide analysis
The microdialysis and HPLC assays used to measure LACT, 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 (pHe) was measured as described previously (Parkins et al., 1994b), 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...
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 preoclusion values over the next 3 h, indicating further breakdown of AMP (possibly to inosine and hypoxanthine).

**Effect of occlusion on pH and LACT.**

Tumours allowed to cool to room temperature after occlusion showed a time-dependent decrease in tumour pH over a 3-h period (pH fell from 6.91±0.07 to 6.62±0.09), during which a corresponding increase in tumour LACT was observed (Figure 3A). When the occlusion was removed after 6 h, there was a rapid fall in LACT (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 is more rapid in this treatment group, reaching a significantly lower value [pH = 6.25±0.09 (P<0.01)] at 3 h after occlusion compared with room temperature (Parkins et al, 1994b). During the first hour of occlusion at 35°C, LACT increased significantly, although extension of the period of vascular occlusion to 6 h did not result in any further time-dependent increase in LACT, 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 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 and decrease in pH 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 intracellular and extracellular pH using non-invasive magnetic resonance imaging.
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 pHr under these conditions. In the room temperature-maintained tumours, the loss of adenine nucleotides is much less severe, with relatively higher pHr 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 converted 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 pHr and LACTr 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 pHr. 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 LACTr and a precipitous drop in blood pH (acidosis). Such a mechanism could explain the absence of increase in LACTr 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., 1994b). 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 pHr and LACTr 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., 1994b). The observation in this study that tumour LACTr levels were not reduced in the 35°C tumours following clamp removal may indicate some irreversible vascular damage, thus preventing LACTr 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 LACTr and pHr is only evident under ischemic 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 LACTr and pHr following an intervention such as ischaemia; however, it is clear that pHr of different types of tumours may depend on factors other than LACTr.

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 pHr (pH = 6.78 ± 0.04) compared with the parental line (pH = 6.65 ± 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 acidity could provide improved understanding of tumour physiology and identify potential targets for therapeutic intervention.

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Extracellular lactate and tumour pH

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