Stable bioenergetic status despite substantial changes in blood flow and tissue oxygenation in a rat tumour

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Summary Experiments on s.c. rat tumours (DS sarcoma) were performed to determine whether chronic or acute changes in tumour perfusion necessarily lead to changes in tissue oxygenation and bioenergetic status since, as a rule, blood flow is thought to be the ultimate determinant of the tumour bioenergetic status. Based on this study, there is clear experimental evidence that growth-related or acute (following i.v. administration of tumour necrosis factor α) decreases in tumour blood flow are accompanied by parallel decreases in tissue oxygenation. In contrast, tumour energy status remains stable as long as flow values do not fall below 0.4–0.5 ml g⁻¹ min⁻¹, and provided that glucose as the main substrate can be recruited from the enlarged interstitial compartment. Perfusion rate seems to play a paramount role in determining energy status only in low-flow tumours or low-flow tissue areas.

Materials and methods

Animals, tumours and surgical procedures

Sprague–Dawley rats (Charles River Wiga, Sulzfeld, Germany; body weight: 310 ± 5 g) were used for experiments. Animals were allowed access to food and water (pH 4.0) ad libitum prior to experiments. Experimental tumours were grown subcutaneously after injection of ascites cells of DS sarcoma into the hindfoot dorsum (Kluge et al., 1992).

Once tumours reached the desired size, animals were anaesthetised with sodium pentobarbital (40 mg kg⁻¹ i.p., Nembutal, Ceva, Paris, France). Catheters were then surgically placed into the thoracic aorta via the left common carotid artery and into the right external jugular vein. During surgical procedures and throughout all experiments, the animals were placed supine on a heated operation pad (rectal temperature 37°C), such that tumour temperature was maintained within the range 34–36°C throughout all experiments. In order to monitor the mean arterial blood pressure (MABP) continuously, the arterial catheter was connected to a Statham pressure transducer (type P 23 ID, Gould, Oxnard, CA, USA). Animals breathed room air spontaneously. Oxygen (P O₂) and carbon dioxide (P CO₂) partial pressures and pH were determined in arterial blood samples (50 μl) at regular time intervals.

Relevant parameters describing tumour perfusion, bioenergetic and oxygenation status were measured either during growth of the s.c. tumours ('chronic' decrease of tumour blood flow) or following acute changes in tissue perfusion. In the latter case, TNF-α was applied in order to induce a significant flow drop within a short period of time.

Measurement of tumour blood flow

Tumour blood flow (TBF) was studied using the ⁸⁵Kr clearance technique. For TBF measurements the indicator (0.1 ml of a solution of ⁸⁵Kr in 0.9% sodium chloride, 37 MBq ml⁻¹, Amersham-Buchler, Braunschweig, Germany) was applied as a bolus injection through the arterial catheter into the thoracic aorta. The registration of the washout process was performed with a Geiger-counting tube con-
connected to a ratemeter (FHT 1100 FAG Kugelfischer, Erlangen, Germany). The method of evaluation of TBF was identical to that described earlier (Kluge et al., 1992). Measurements were performed on tumours of varying sizes at 20 min intervals before application of TNF-\(\alpha\) and at 30 min intervals thereafter over a total time period of 2 h post treatment. In all experiments performed in this study, animals were allowed to stabilise following the surgical procedures. Measurements commenced once constant baseline readings for MABP and flow were obtained for at least 20 min.

**Laser Doppler flowmetry**

A Periflux model PF 3 dual-channel laser Doppler flowmeter was used for this study (2 mW He-Ne laser, wavelength 632.8 nm; Perimed, Stockholm, Sweden). Laser Doppler flow (LDF) signals were continuously recorded from central locations on the tumour surface using a type PF 108 probe. The fibroptic probe was placed above (but not in contact with) the tumour tissue under study. LDF was recorded for 10 min before i.v. administration of TNF-\(\alpha\) or saline (control) and for 90 min thereafter (Kluge et al., 1992).

**Tumour oxygen tension measurements**

Tumour oxygen tension values were determined using polarographic needle electrodes (recessed 12 \(\mu\)m gold in glass cathode; shaft diameter 250 \(\mu\)m) and \(P_O_2\) histography (model KIMOC-6650, Eppendorf, Hamburg, Germany) as described previously (Vaupel et al., 1989a, 1991). Measurements were made either on tumours of varying sizes or before and 120 min after acute flow changes upon TNF-\(\alpha\) application.

**Measurement of global concentrations of adenylate phosphates in perchloric acid extracts**

In order to obtain mean (global) levels of adenylate phosphates, the tumour-bearing hindfoot was rapidly frozen and the tumours (\(n = 12\)) were prepared under liquid nitrogen and stored at \(-80^\circ\)C for further processing. In a first series of experiments, tumours of varying sizes were analysed. In another series, tumours were assayed before or 120 min after administration of TNF-\(\alpha\).

Each deep-frozen tumour was ground to a fine powder and freeze dried. For determination of ATP, ADP and AMP levels, aliquots of freeze-dried tissue were extracted with 0.66 \(M\) perchloric acid, centrifuged and the supernatant neutralised with 2 \(M\) potassium hydroxide. The concentrations of the adenylate phosphates were then determined using reversed-phase high-performance liquid chromatography (HPLC) techniques at 254 \(nm\) (for more details see Kräger et al., 1991; Schaefer et al., 1993). Concentrations were expressed as \(\mu\)mol per g tissue wet weight.

**Determination of microregional ATP distribution**

Before preparing the rapidly frozen tumours for HPLC analysis, approximately 30% of the tumour mass was separated, cut at \(-25^\circ\)C in a cryostat into 5-\(\mu\)m sections and used for ATP bioluminescence measurements to assess the microregional ATP distribution using single-photon imaging and quantitative bioluminescence (for methodological details see Walenta et al., 1992; Schaefer et al., 1993). The spatial resolution gained by this method is about 50 \(\mu\m\), thus revealing information about the intra-tumour variability of the ATP levels in relation to histological details.

**Introduction of acute flow drops through TNF-\(\alpha\)**

Recombinant human TNF-\(\alpha\) (specific activity: 8.2 \(	imes\) 10\(^7\) U per mg of protein; Knoll, Ludwigshafen, Germany) was diluted in isotonic phosphate-buffered saline solution containing 0.5% (w/v) bovine serum albumin (Sigma Chemie, Deisenhofen, Germany). TNF-\(\alpha\) was given into the external jugular vein at a dose level of 1 mg kg\(^{-1}\) over approximately 3 min. The catheter used for the i.v. route was flushed with saline thereafter. Control animals received identical fluid loads (1 ml kg\(^{-1}\) phosphate-buffered saline i.v.). For further details see Kluge et al. (1992).

**Statistical analysis**

Results are expressed as means \(\pm\) s.e. with the numbers of experiments indicated in brackets. Significance was assessed using the paired or unpaired Student’s \(t\)-test, as appropriate. Results were considered as significant if \(P\)-values were less than 5\% (\(P < 0.05\)).

**Results and Discussion**

Like many other experimental tumour systems, tumour blood flow (TBF) and tissue oxygenation significantly decrease in the DS sarcoma with increasing tumour mass (see Figure 1). Starting from a mean TBF value of 0.98 ml g\(^{-1}\) min\(^{-1}\) in the smallest tumours investigated, flow decreased by about 50% in the larger malignancies (\(2P < 0.001\)). This flow drop coincides with a similar decrease in the mean \(P_O_2\) value from 39 to 16 mmHg (\(2P < 0.001\)).

As long as tumour masses do not exceed 1% of the body weight (i.e. biologically relevant tumour sizes), global ATP concentrations and adenylate energy charge remain almost constant. During tumour growth from 0.86 \pm 0.02 to 2.15 \pm 0.04 g, ATP concentrations insignificantly increased from 1.15 \pm 0.10 to 1.37 \pm 0.12 \(\mu\)mol g\(^{-1}\). Similar results were obtained when the microregional ATP distribution was analysed in three tissue sections of three tumours each of three different size groups (mean tumour weights: 0.82 \pm 0.08 g, 1.25 \pm 0.10 g and 2.14 \pm 0.12 g; see Figure 1).

Flow and \(P_O_2\) values in these tumour size ranges are similar to those observed in many normal tissues (Vaupel et al., 1989c) and are seen to be accompanied by a stable energy status. Changes in TBF can influence tissue oxygenation but not ATP concentrations in this tissue. This may be explained by an intensified glycolytic rate as the oxygenation status deteriorates and/or a decreasing number of proliferating cells which compensate for the poorer oxygen supply as the tumours become larger. As long as TBF and/or \(P_O_2\) values do not fall below a certain 'threshold', tumour energy status can be maintained. Under these conditions, glucose has to be considered as the major energy source, which is available in sufficient amounts even under normoglycaemic conditions. Owing to the large interstitial space of those tumours [app-
roximately 50% (v/v); Gullino et al., 1965; Vaupel & Müller-Klieser, 1983; Stubbs et al., 1992), the mean tissue glucose concentration is >1.5 μmol g⁻¹ (‘reservoir function’ of the interstitial space). The missing decrease in high-energy phosphates despite severe restrictions in tumour blood flow during hyperglycaemia as observed in rodent tumours supports this notion (Okunieff et al., 1989b; Krüger et al., 1991; Schaefer et al., 1993). In line with these findings is a recent study of Gerweck et al. (1995) showing that energy status and oxygenation are not closely linked in the presence of glucose.

Similar observations have been described for other experimental tumour systems. In an amelanotic hamster melanoma (A-Mel-3), ATP concentrations remained constant as long as blood flow values were above 0.4 ml g⁻¹ min⁻¹ (Walenta et al., 1992). This finding is based on pixel-to-pixel correlations between microregional ATP concentration and flow data.

In murine FSAII tumours, median P₀₂ values of 10–15 mmHg represent a critical threshold for energy metabolism (Vaupel et al., 1993). At higher median P₀₂ values, ATP levels were relatively constant. On average, median oxygen tensions below 10–15 mmHg coincided with ATP depletion, intracellular acidosis, a drop in the energy charge and rising P₃ (Vaupel, 1992). These conditions were, however, only found when tumour masses were >1.5% of body weight.

Stable bioenergetic status is observed not only during ‘growth-related’ decreases in blood flow or tissue P₀₂ values, but also upon acute falls in TBF following TNF-α administration. This cytokine is known to drastically reduce microcirculatory function (Kluge et al., 1992; Naredi et al., 1993). Starting from TBF values of 98 ± 0.5 ml g⁻¹ min⁻¹ (tumour wet weights: 0.85 ± 0.05 g), TNF-α application resulted in a 50% flow drop within 120 min (Figure 2). Similar changes were observed for the tumour P₀₂ distribution. Despite these substantial changes, ATP levels, phosphocreatine (PCr)/P₃, and β-nucleoside triphosphate (β-NTP)/P₃ ratios remained almost unchanged.

Here again, energy status was stable at mean flow values ≥0.5 ml g⁻¹ min⁻¹, mean oxygen tensions ≥13 mmHg and mean tumour tissue glucose levels ≥1.4 μmol g⁻¹ (Engel & Vaupel, 1993). From the data presented it is concluded that growth-related or acute changes in tumour perfusion are, as a rule, accompanied by parallel alterations of tissue oxygenation.

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**Figure 2** Acute effects of TNF-α (1 mg kg⁻¹ i.v.) on tumour blood flow (TBF, open circles, n = 28), laser Doppler flow (LDF, closed circles, n = 6), mean oxygen partial pressure in tumour tissue (P₀₂, open triangles, n = 12), tumour tissue ATP concentrations (closed triangles, n = 12) and ³¹P-magnetic resonance spectroscopy-derived PCr/P₃ (closed squares), and β-NTP/P₃ ratios (open squares, n = 5). Values are means ± s.e.

In contrast, tumour energy status is stable providing flow values do not fall below a certain threshold (approximately 0.4–0.5 ml g⁻¹ min⁻¹ in the rodent tumour systems investigated). As compensatory mechanisms, an intensified glycolysis due to the recruitment of glucose from the 'interstitial reservoir' and a decrease in the number of proliferating cells, have to be assumed.

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Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; HPLC, high-performation liquid chromatography; LDF, laser Doppler flow; MAPF, mean arterial blood pressure; NTP, β-nucleoside triphosphate; PCr, phosphocreatine, P₃, inorganic phosphate; P₀₂, oxygen partial pressure; TBF, tumour blood flow; TNF-α, tumour necrosis factor α.
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