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Microenvironmental and cellular consequences of altered blood flow in tumours

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Abstract. Tumour angiogenesis is triggered by various signals characteristic of the tumour microenvironment, including low oxygen tension, low extracellular pH and low glucose concentration. Tumour microvasculature is chaotic, producing perfusion heterogeneities which can be visualized by MRI and other modalities. Inefficient perfusion in tumours produces regions of transient and chronic hypoxia. Tumour hypoxia is associated with adverse clinical outcomes and reduced patient survival. Hypoxia may be a factor in activation of extracellular matrix-degrading proteases, and some studies have correlated primary tumour hypoxia with likelihood of tumour cell dissemination. Exposure to hypoxia either induces or selects for cells that are hyperglycolytic, and this in turn produces local acidosis which is also a common feature of solid tumours. Increased glucose uptake in hyperglycolyzing tumour cells is the basis of lesion-visualization in positron emission tomography using ^{18}F-fluorodeoxyglucose. Tumour acidity can reduce the effectiveness of weak-base drugs, but can be exploited to increase the anti-tumour activity of weak-acid chemotherapeutics. Evidence linking tumour acidity with increased activity of several extracellular matrix-degrading enzyme systems is examined. High levels of lactate, another end-product of glycolysis, in primary lesions have been correlated with increased likelihood of metastasis. In the numerous studies correlating hypoxia, acidity and lactate with metastasis, the direction of the causality has not been adequately established. We hypothesize that adoption of a hyperglycolytic phenotype is a necessary feature of carcinogenesis itself, and confers a survival and proliferative advantage to tumour cells over surrounding normal cells. Empirical evidence supporting this ''acid-mediated tumour invasion'' model is discussed.

Tumour microcirculation and perfusion

The vasculature of growing tumours results both from recruitment of pre-existing stromal vessels and from the sprouting and growth of new blood vessels (angiogenesis). Angiogenesis is induced by vascular endothelium-specific growth factors (VEGFs), including at least five members of the VEGF family, four members of the angiopoietin family, and one member of the ephrin family. Many other growth factors that are not vascular endothelium-specific, such as members of the platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) families, and basic fibroblast growth factor (bFGF), are also involved in blood vessel formation. VEGF is the best validated target for anti-angiogenesis, and the most common therapies described so far involve blockade of VEGF, its receptor or downstream intracellular sequelae [1–3]. In particular, the central importance of VEGF-A in tumour vascularization, and its relatively minor role in adult mammals, have made it an attractive target for antiangiogenic therapy of tumours [4].

Angiogenesis is required for tumours to grow beyond 500 μm in diameter [5]. Induction of angiogenesis, *i.e.* the ''angiogenic switch'', is triggered or selected for by various signals, including low pO_2, low pH and low glucose [6]. In normal tissues, long-term hypoxia leads to expression and secretion of VEGF. VEGF expression can be increased in hypoxia by HuR, a protein that binds specifically to a recently-identified binding site on VEGF mRNA, resulting in an increase in the half-life of VEGF mRNA [7]. But the major transcription factor responsible for induction of VEGF production and secretion is hypoxia inducible factor (HIF), a dimer comprised of HIF-1α and HIF-1β. HIF-1β is thought to be constitutively and ubiquitously expressed. HIF-1α is also constitutively expressed, but is continuously degraded in the presence of oxygen, leading to decreased HIF levels in normoxia and high levels in hypoxia. Degradation is mediated by ubiquitin, which is activated by the von Hippel-Lindau ubiquitin ligase [8, 9]. HIF-1 upregulates the transcription of a number of genes connected with glucose uptake and metabolism, iron metabolism, and angiogenesis, including VEGF [10]. Loss of von Hippel-Lindau (VHL) tumour suppressor protein results in full activation of the HIF system, and is the causative event in familial renal cell carcinoma [11]. Elevated HIF-1α expression has been reported in a variety of human tumours and pre-neoplastic lesions [12, 13]. This elevation could simply be an indicator of the existence of hypoxia in most human tumours. Alternatively, it has been theorized that some cancers have constitutively high levels of HIF. In this context it is important to note that HIF-1α accumulation can also be the result of hypoxia-independent mechanisms. Semenza and co-workers have demonstrated that HER2^{neu} [14], insulin-like growth factor 1 [15] and prostaglandin E2 [16] can induce expression of HIF-1α in cancer cells. These effects are on the rate of HIF-1α protein synthesis, in contrast to the effect of hypoxia which acts by inhibition of HIF-1α degradation.

Accumulation of HIF-1 can result in elevated glucose uptake, and this issue will be considered later. Constitutively high levels of HIF may also result in over-production of VEGF, and consequent hypervascularization. Tumour

vessels are structurally and functionally abnormal, with uneven diameter, excessive branching, increased length and tortuosity, multiple arteriovenous shunts and loss of physiological regulation of blood flow, which combine to produce pronounced spatial and temporal heterogeneities in tumour blood flow. Abnormalities in their ultrastructure also make tumour blood vessels leaky [17]. Jain and colleagues have demonstrated that, in some models, up to 15% of tumour blood vessels show presence of cancer cells in the lumen [6]. This has significant implications for tumour metastasis, as increased microvessel density (MVD) is positively correlated with metastasis. It is hypothesized that this is due to increased peritumour lymphatic drain and tumour cell egress from leaky vessels [18, 19], although causality has not been definitely established.

Tumours can be segregated into sub-regions based on perfusion rates: an avascular, necrotic region, an intermediate seminecrotic region, a stabilized microcirculation region, and an advancing front [20]. Poorly developed lymphatics within tumours result in high interstitial fluid pressure (IFP) inside the tumour relative to the tumour periphery and surrounding normal tissue. The average patent vascular surface area per unit tissue weight decreases with tumour growth, and in combination with increased IFP, results in reduced delivery of drug molecules to solid tumours [21, 22]. Pre-radiotherapy IFP has been shown to predict disease-free survival in cervical carcinoma patients independently of tumour oxygenation and other clinical prognostic factors [23], and a high tumour IFP has been associated with increased incidence of metastases in mice [24].

Such heterogeneities in tumour perfusion can be visualized by contrast-enhanced MRI (Figure 1), laser Doppler flowmetry [25] and colour Doppler ultrasound [26], and are common across solid tumours, suggesting that this is a hallmark of tumourigenesis. Heterogeneities in tumour perfusion produce irregular metabolic gradients, particularly gradients in oxygen concentration that can be chronic or intermittent [27]. In a recent comparative study on patients with carcinoma of the cervix, Cooper et al demonstrated that dynamic contrast-enhanced MRI parameters indicative of perfusion efficacy correlated with tumour oxygenation levels measured using the ''gold standard'' Eppendorf polarographic pO_2 histograph system [28].

Almost a century ago, August Krogh modelled capillaries in muscle as capable of servicing annular cylinders of surrounding tissue, and calculated that oxygen could be supplied out to a distance of 200 μm from a vessel [29]. Assuming a uniform flow of oxygenated blood in homogeneously spaced vessels, he predicted that muscle would be adequately oxygenated even at 20 times the basal metabolic demand. Secomb et al have modelled the relatively chaotic microvasculature in a mammary adenocarcinoma xenograft grown in a rat dorsal skin flap chamber, and concluded that the intermittent and heterogeneous nature of tumour microcirculation makes the occurrence of chronically and acutely hypoxic microregions in tumours more likely than what is predicted by the Krogh model [30]. Helmlinger et al [31] have employed optical imaging techniques to simultaneously visualize pH and pO_2 gradients in tumour xenografts grown in dorsal skin flap chambers in mice. In support of the early observations of Thomlinson and Gray on human lung carcinoma [32] and the calculations of Secomb et al [30], they identified hypoxic regions that were within 80 μm of the nearest vessel wall. Intervessel pH and pO_2 gradients exhibited considerable heterogeneity, and while pH profiles were stable, pO_2 profiles were time-variant. Correlation between pH and pO_2 was generally poor, and hypoxic regions were detected in regions with normal pH ($\geqslant$7.2). However, the majority of pO_2 measurements in regions with pH $<$7.0 indicated hypoxia or anoxia [31]. In agreement with these observations, Harris and co-workers found that expression of VEGF and CA9, two hypoxia-inducible genes, was negligible within 80 μm of microvessels in bladder cancer specimens [33].

Exposure to hypoxia either induces or selects for cells that are hyperglycolytic, and this can result in significant local acidosis which is also a common feature of solid tumours [34]. We now examine some of the consequences of this adverse microenvironment on disease progression and treatment response.

Consequences of tumour hypoxia

Structurally and functionally compromised vasculature and disrupted diffusion conditions result in heterogeneous perfusion to tumours, with inadequate supply of oxygen (hypoxia) to regions within most tumours [17, 35]. As discussed by Hockel and Vaupel in a recent review, a single oxygen tension cannot be used to identify hypoxia, and functional definitions of the hypoxic threshold range from 45 to 50 mm Hg O_2 at the venous end of capillaries,

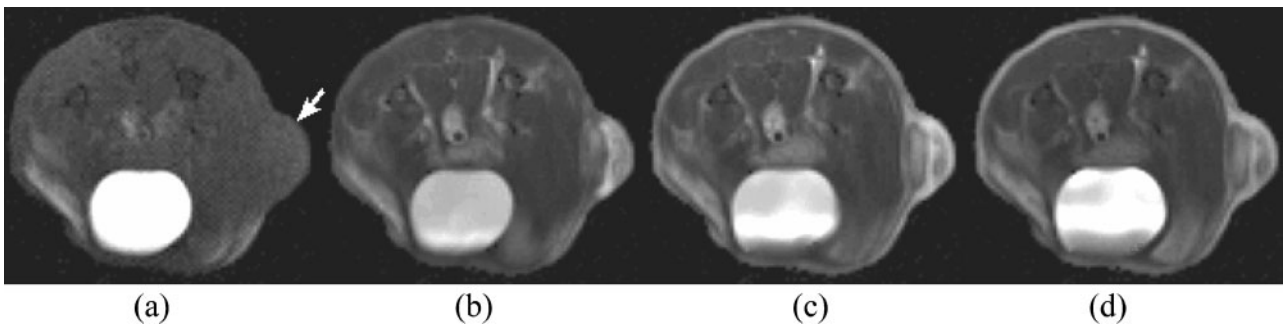

Figure 1. Axial T_1 weighted MRI images of a SCID mouse bearing an MCF-7 human breast cancer xenograft in the flank (arrow): (a) pre-Magnevist®, (b) 10 min post-contrast, (c) 20 min post-contrast and (d) 30 min post-contrast. The heterogeneity of enhancement is indicative of non-uniform wash-in and wash-out kinetics of the contrast agent. Such heterogeneity is routinely observed in both animal and human tumours, and illustrates tumour perfusion heterogeneity.

to 0.5 mm Hg O_2 in mitochondria, to 0.02 mm Hg O_2 at cytochromes. *In vitro*, hypoxia-induced gene expression occurs at pO_2 below 15 mm Hg [36].

Hypoxia increases cell death via apoptosis in a *p53*-dependent manner, but this does not seem to require *de novo* expression of death-promoting genes. Giaccia and co-workers [37] propose that the transrepressor activity, but not the transactivation activity, of *p53* comes into play in hypoxia and other stresses, resulting in diminished expression of certain target genes and consequent signalling for apoptotic cell death. They have proposed that hypoxia creates an evolutionary pressure to select for cells with diminished apoptotic potential [38]. The manifold effects of *p53* also include suppression of tumour angiogenesis by various mechanisms, including upregulation of thrombospondin-1 [39], degradation of HIF-1α [40], transcriptional suppression of VEGF expression [41], and down-regulation of bFGF-binding protein expression [42]. Loss of *p53* function will promote angiogenesis by removal of these inhibitory signals. This provides pressure for selection of *p53*-defective cells under hypoxia.

HIF-1α itself may be a general mediator of hypoxia- and hypoglycaemia-driven apoptosis. Carmeliet et al have found that hypoxia and hypoglycaemia reduce proliferation and increase apoptosis in wild-type (HIF-1$\alpha^{+/+}$) embryonic stem (ES) cells, but not in ES cells with inactivated HIF-1α genes (HIF-1$\alpha^{-/-}$) [43]. Loss of HIF-1α was found to reduce hypoxia-induced VEGF expression, impair vascular function, and lower perfusion, resulting in hypoxic microenvironments within ES cell-derived tumours. Interestingly, tumour growth was not retarded by loss of HIF-1α, presumably owing to reduced hypoxia-induced apoptosis and increased stress-induced proliferation [43].

In general, tumour hypoxia is associated with adverse clinical outcomes, likelihood of tumour recurrence and metastases, and shorter patient survival. Hypoxia has been implicated as a factor in drug resistance and metastasis (reviewed by Rofstad [44]). An association between hypoxia in the primary tumour and likelihood of secondary dissemination has been noted in uterine cervical cancer [45] and soft tissue sarcomas [46]. Hill and co-workers have found that exposure of tumour-bearing rodent hosts to cyclical, but not chronic, hypoxia produces a significant increase in metastasis to the lungs [47]. This is significant in light of the intermittent disruptions in perfusion that are observed in tumours [27].

The effect of hypoxia on tumour cell metastasis may be via activation of extracellular proteases such as the urokinase-type plasminogen activator (uPA) system. This system consists of the serine proteinase uPA, the urokinase receptor (uPAR), the serpin inhibitors α_2-anti-plasmin, PAI-1 and PAI-2, and plasminogen. Plasminogen is the inactive form of the serine proteinase plasmin, and its activation is catalyzed by uPA. Plasmin has a wide substrate specificity, and can degrade a wide variety of extracellular matrix (ECM) proteins, including fibronectin, vitronectin and fibrin. Plasmin can also catalyze the activation of several matrix metalloproteinases (MMPs), which in turn break down the collagen components of the ECM. Overexpression of the uPA-uPAR system would therefore allow tumour cells to deploy the matrix-degrading activities of plasmin and MMPs in their immediate neighbourhood [48]. *In vitro* studies have demonstrated upregulation of uPAR and increased activity in *in vitro* invasion assays in MDA-mb-231 breast cancer cells following exposure to hypoxia [49]. uPAR expression in microvascular endothelial cells is also reported to be increased by hypoxia, and has been linked to their ability to form tube-like structures *in vitro* [50]. *In vivo* studies have demonstrated a correlation between immunohistochemical staining for uPAR and pimonidazole staining for hypoxia, and a correlation of the two with incidence of lymph node metastases in a mouse model of human melanoma has been observed [51]. In addition, Semenza and co-workers have recently demonstrated that HIF-1 regulates the expression of proteins potentially involved in basement membrane invasion, including matrix metalloproteinase 2 (MMP2), cathepsin D and uPAR, and cells transfected with HIF-1α exhibited increased invasiveness in an *in vitro* Matrigel® invasion assay [52].

Another molecule of interest is interleukin-8 (IL-8), a cytokine with angiogenic activity which is also an autocrine growth factor for many tumour cells. Shi et al [53] used variants of COLO 357 human pancreatic cancer cells that express differing levels of IL-8 to demonstrate that it plays a significant role in both local tumour growth and metastasis in this tumour model. Exposure of the cells to hypoxia and acidosis *in vitro* resulted in increased levels of IL-8 expression. *In vivo*, elevated IL-8 levels were found in tumour cells surrounding necrotic areas, a microenvironment which might be expected to be hypoxic and acidic. In human melanoma xenografts in mice, Rofstad and Halsor also found IL-8 staining in cells surrounding necrotic regions, and this staining was co-localized with pimonidazole, a hypoxia marker. IL-8 positive foci were also found in non-necrotic regions, and these were also co-localized with pimonidazole-stained foci. The incidence of pulmonary metastases was correlated to the extent of hypoxia in the primary tumour in this model, suggesting a cause-effect relationship between hypoxia (or its physiologic or metabolic sequelae) and metastasis. Anti-VEGF or anti-IL-8 treatment reduced the incidence of metastases without altering the occurrence of hypoxic foci in the primary tumour [54].

An additional mechanism linking hypoxia and increased metastasis is evident in a recent study by Niizeki et al, who found that expression of autocrine motility factor (AMF) and random *in vitro* motility of pancreatic ductal adenocarcinoma cells are increased by hypoxia in a HIF-dependent manner [55]. AMF is a molecule that stimulates directional mobility (chemotaxis) and random motility (chemokinesis). Interestingly, AMF is identical to the glycolytic enzyme phosphohexose isomerase [56]. We will address the possible association between glycolysis and metastasis in a subsequent section.

The role of hypoxia in radioresistance of cancer cells has been known for almost a century, and was confirmed in patients in 1988 by Gatenby et al [57] who measured significant differences in pO_2 between tumours of responding and non-responding patients. Efforts to improve the radiosensitivity of hypoxic tumours by increasing tumour oxygenation have been reported. Secomb et al [58] have modelled oxygen transport in tumours and calculated that improvement of tumour oxygenation cannot be reliably achieved by increasing systemic pO_2 and tumour blood flow. However, their simulations indicate that tumour hypoxia can be largely eliminated by decreasing demand

for oxygen by a relatively modest degree. In a follow-up to that prediction, Dewhirst and colleagues demonstrated in animal models that combined hyperglycaemia and hyperoxia achieve greater improvements in tumour oxygenation than hyperoxia alone, with the hyperglycaemia presumably serving to decrease oxygen demand by enabling increased glycolytic output (the "Crabtree Effect") [59]. Another avenue to overcome hypoxic radioresistance is the development of agents that sensitize hypoxic tissue to radiation. An example is Tirapazamine, which induces single- and double-strand breaks in DNA when reduced to its active free radical form in hypoxic regions [60]. Tirapazamine has been shown to be effective at sensitizing mouse tumours to fractionated radiation [61], and phase III clinical trials are ongoing. The MRI-visible gadolinium texaphyrin has been demonstrated to increase the lifetime of hydroxyl radicals created after exposure to ionizing radiation, in an oxygen-independent manner [62], and is currently in phase III clinical trials. Yet another strategy to exploit tumour hypoxia is by means of bioreductive prodrugs that are activated by enzymes such as DT-diaphorase in reducing environments, in order to provide selectivity towards hypoxic cells [63].

Consequences of tumour acidity

The microenvironment of most solid tumours is acidic [34]. The origins of this extracellular acidity are thought to lie in the chaotic nature of tumour vasculature, increased glycolytic flux in tumour cells, increased export of protons from tumour cells exhibiting aggressive intracellular pH control [64], diminished buffering capacity of tumour interstitial fluid [65], and diffusion-limited rates of transport of lactic acid [66] and protons [67] from the interstitium into the vasculature. VEGF expression in cancer cells is known to be upregulated by acidosis [68] and it has been shown in human glioma xenografts in mice that this effect of low pH is independent of hypoxia, although the effects were not additive [69]. Analogous to hypoxia [38], an acidic extracellular pH (pH_e) provides a selective pressure towards outgrowth of apoptosis-impaired cells [70].

Also analogous to hypoxia, an acidic pH_e has been implicated in tumour metastasis. One mechanism by which an acidic tumour pH may increase tumour invasion and metastasis is by increasing the expression and/or activity of extracellular matrix-degrading proteases such as collagenases [71, 72]. There have also been many reports showing that a number of transformed cell lines secrete lysosomal proteases with acidic pH optima, such as the cathepsins B, D and L [73–76]. Sloane and colleagues [74] have demonstrated that exposure to a mildly acidic pH_e of 6.5 results in the redistribution of lysosomes to the plasma membrane, followed by increased secretion and extracellular activity of the cysteine proteases cathepsins B, D, and L. It has been suggested that an acidic pH_e may enhance redistribution of active cathepsin B to the surface of malignant cells followed by secretion, thereby facilitating invasion [77, 78]. High levels of uPA and uPAR in tumour tissue are correlated with poor prognosis in many different cancers [79]. uPA is initially released from tumour cells in the inactive form pro-uPA. Activation of uPAR-bound-pro-uPA is by cathepsin L, which has an acidic pH optimum [80]. Thus, an acidic tumour microenvironment may lead to increased uPA activity in tumours. In a vicious cycle, the increased activities of cathepsin D and L in the acidic tumour microenvironment may exacerbate the inadequate perfusion in tumours, by generation of angiogenesis inhibitors angiostatin [81] and endostatin [82] from proteolysis of plasminogen and collagen in the ECM.

Long-term culture of melanoma cell lines at pH 6.8 has been shown to result in enhanced cell migration and invasion as measured by the membrane invasion culture system, and an increased production of the active forms of the MMPs gelatinases A and B [83]. Kato and colleagues found that the highly metastatic B16 mouse melanoma cells do not secrete significant amounts of gelatinase/type IV collagenase in neutral culture medium (pH 7.1–7.3), but secrete high levels of a 103 kD gelatinase when cultured at a pH range of 5.4–6.1 [84]. These results indicate that acidic conditions can potentiate the ability of tumour cells to invade through type IV collagen, a major constituent of basement membrane.

Another important component of basement membrane is heparan sulphate proteoglycan, which contains heparan sulphate oligosaccharides [85]. Degradation of these heparan sulphate chains is believed to be important for penetration of the basement membrane, and a correlation between heparanase activity and lung colonization potential has been demonstrated in murine melanoma cells by Nakajima et al [86]. Gilat et al have reported that at physiological pH mammalian heparanases function as adhesion molecules, while at acidic pH they function as enzymes, degrading heparan sulphate proteoglycans [87]. Toyoshima and Nakajima purified and characterized a human heparanase [88]. They report that this heparanase has an optimal pH of 4.2 and negligible activity above pH 7.0. Their data also indicate that significant heparanase activity persists at pH 6.0–6.5, suggesting that the acidic microenvironment of tumours may activate the basement membrane degrading properties of tumour heparanases. All in all, a substantial and growing body of evidence links acid pH with increased activity and/or secretion of several ECM-degrading enzymes.

Tumour acidity also impinges adversely on therapy. Since the early work of Trowell [89], many have looked at extracellular pH as a modulator of tumour response to radiation therapy. These studies have shown that low pH_e reduces cellular sensitivity to radiation therapy [89–91]. It was initially hypothesized that this protective effect was due to a decrease in the fraction of proliferating tumour cells at an acidic pH_e. However, Holahan et al [92] demonstrated that the radioprotective effects imparted to tumour cell by an acidic pH_e were not solely mediated by a reduction in the number of cycling cells at the time of treatment. Cells placed in an acidic environment following radiation therapy experienced a radioprotective effect similar to that originally observed by Trowell. It was demonstrated that this radioprotective effect was due to reduced fixation of radiation-induced DNA damage [93].

Tumour pH also affects chemotherapy. The anti-tumour activities of several weakly-ionizing chemotherapeutic drugs are known to be affected by tumour pH. This is typically ascribed to the phenomenon of "ion trapping", but can also be due to the influence of the plasmalemmal pH gradient on facilitated transport of drug into the cell. The extracellular acidity of tumours imparts a physiological

drug resistance to tumours against weak-base drugs. In our lab, we have employed chronic and acute sodium bicarbonate-induced alkalosis to circumvent this drug resistance and enhance the anti-tumour activities of the weak-base drugs doxorubicin [94] and mitoxantrone [95]. *In vitro* studies using radiolabelled drugs indicate that mitoxantrone undergoes classic and substantial ion-trapping, while results with doxorubicin were equivocal [96]. *In vivo*, $NaHCO_3$-induced alkalinization produced a significant redistribution of mitoxantrone to tumours, but did not have a significant effect on the tumour uptake of doxorubicin. This difference in the behaviour of these two drugs could be explained on the basis of differences in the octanol-water partition coefficients of their charged forms [97]. Hence, the increase in anti-tumour activity of doxorubicin produced by sodium bicarbonate co-treatment that we have observed [94] must be due to a mechanism other than increased drug retention in the tumour. Perhaps, as observed *in vitro*, the subcellular distribution of doxorubicin or the activity of topoisomerase are affected by pH_e alterations [98]. Our results indicate that induction of metabolic alkalosis using sodium bicarbonate co-treatment can produce a net gain in the therapeutic index of mitoxantrone, and to a lesser extent, doxorubicin.

The acidity of the tumour microenvironment can be exploited to increase the activities of weak-acid drugs, and strategies have been developed to enhance tumour acidity for this purpose. For example, the anti-tumour activity of 5-fluorouracil was enhanced by respiratory acidosis induced by carbogen breathing [99]. Enhancement of extracellular acidity in tumours by hyperglycaemia potentiated the action of chlorambucil, another weak-acid drug, in human tumour xenografts [100]. The *in vitro* cytotoxicity of melphalan has been shown to be enhanced by acidosis and hypoxia [101]. Acute acidosis by itself has recently been shown to cause tumour shrinkage in an isolated limb perfusion model of melanoma in rats, in a manner that requires the activity of nitric oxide synthase. This was additive with the anti-tumour activity of melphalan [102]. Thus, the acidic microenvironment of tumours presents both a challenge and an opportunity for cancer treatment by chemotherapy. A more complete discussion of the ramifications of tumour acidity on chemotherapy and drug resistance is presented elsewhere [103].

A recent study on cervical cancer patients found a significant correlation between degree of overexpression of the glucose transporter Glut-1 and frequency of occurrence of tumour pO_2 values below 2.5 mm Hg [104]. In the same study, absence of staining for Glut-1 in the biopsy samples predicted metastasis-free survival. While a growing body of such evidence indicates that hypoxia and acidosis are associated with increased incidence of metastases, interpretation of these observations is confounded by the fact that hypoxic regions are also likely to be acidic and contain high levels of lactate due to elevated glycolysis. High lactate levels in primary lesions of head and neck tumours and cervical cancer correlate with increased likelihood of development of nodal and distant metastases, and lowered patient survival [105, 106]. In cervical cancer patients, no correlation was found between clinical staging or pathohistological grading of the tumours, and ATP, glucose or lactate levels. However, the disease-free survival of patients with low tumour lactate values was significantly higher than for patients with high tumour lactate values [106]. Rofstad and coworkers have investigated the possibility that the higher likelihood of metastases is due to enhanced neovascularization at such lactate ''hot spots''. However, they did not find a correlation between lactate levels and vascularity and incidence of metastases. Alternatively, it is possible that high lactate is simply a marker of hypoxia and as already discussed, tumour hypoxia is a predictor of metastatic potential. However, a poor correlation is reported between lactate concentration and oxygen tension in advanced cervix carcinoma [107]. In the only such study to date, a magnetic resonance spectroscopy (MRS) study of a rodent model of glioma found no correlation between the spatial distributions of pH and lactate [108]. A possible explanation is that most of the MR-visible lactate in gliomas is intracellular [109].

In all of these studies correlating hypoxia, acidity and/or lactate with metastasis, the direction of the causality has not been adequately established. We now investigate the possibility that a glycolytic phenotype is a precursor to an aggressive phenotype.

Hyperglycolysis in tumours

Cellular metabolism of glucose may occur aerobically or anaerobically. In aerobic metabolism, glucose is converted to CO_2 and H_2O via the tricarboxylic acid (TCA) cycle with the generation of about 36 moles of ATP per mole of glucose consumed. In anaerobic glycolysis, glucose is metabolized to lactic acid, producing 2 moles each of ATP and H^+ ions per mole of glucose [110]. For fundamental thermodynamic reasons [111], the efficiency of aerobic metabolism is achieved at the cost of decreased maximum rate, and ATP production by the respiratory pathway rapidly saturates at high levels of glucose or limited oxygen supply. In the lower-yield anaerobic pathway, more of the energy from glucose degradation is used to drive the reaction, allowing a greater maximum rate of metabolism. Thus, in the presence of adequate glucose, the net ATP production rate of the anaerobic pathway can be similar to that of the aerobic route despite the relative inefficiency. Normal mammalian cells under physiological conditions utilize high-yield aerobic glucose metabolism, but can adapt to periods of hypoxia by elevating the anaerobic pathway, provided the transition to hypoxia is gradual and allows for induction of response mechanisms such as HIF. The energy cost of this transition is substantial, as the output of ATP per mole of glucose is reduced by over 90%. To compensate for this decreased efficiency, glycolytic flux must increase several-fold.

The pioneering work of Warburg in the 1920s [112] first demonstrated consistent alteration in tumour glucose metabolism. Transformed cells *in vivo* and *in vitro* typically rely on anaerobic pathways to generate ATP from glucose even in the presence of abundant oxygen, and a rough correlation between degree of malignancy and glycolytic rate has long been noted [113]. The decreased efficiency of anaerobic metabolism is compensated by increased glucose flux, maintaining energy production sufficiently in excess of basal metabolic demands to allow for cellular proliferation. The necessary increase in glucose flux can be accomplished through an increase in the number and distribution of glucose transporters on the cell membrane,

and this has been observed in many cancers [114–116]. A correlation between overexpression of the GLUT-1 glucose transporter, and tumour aggressiveness, patient prognosis and survival has been noted in a variety of cancers [117–124]. In a recent retrospective study of biopsy specimens from cervical cancer patients, GLUT-1 expression intensity was found to correlate to tumour grade and also to distance from stromal blood supply. Comparison of normal, pre-neoplastic and tumour sections indicated that GLUT-1 expression is a late phenomenon in neoplastic transformation in this cancer type [125]. A progressive increase in GLUT-1 staining intensity between benign, borderline and invasive ovarian epithelia has also been reported [126]. There is evidence that the transition to malignancy in colonic epithelia is accompanied by a switch from use of short-chain fatty acids to glucose as primary energy source [127].

Expression of GLUT-1 is regulated by HIF-1, and it has been immunohistochemically shown to co-localize with hypoxia in cervix carcinoma [104]. HIF has also been shown to regulate expression of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2-6-bisphosphatase-3, which controls the synthesis and degradation of fructose-2,6-bisphosphate, a major regulator of glycolytic flux [128]. Expression of other glycolytic enzymes is also regulated by HIF [129]. Overexpression of the GLUT-3 isotype, which has a low K_m for glucose that permits substrate uptake in a low glucose environment, has also been reported to correlate with reduced patient survival in laryngeal carcinoma [130]. An increase in the number of low K_m membrane transporters would increase the maximum rate of glucose transport into the cell.

This metabolic transition and the associated increase in glucose transport may form the biological basis for positron emission tomography (PET) of tumours with ^{18}F-fluoro-deoxy-glucose (^{18}F-FDG), which has confirmed substantially increased glucose uptake in the vast majority of primary and metastatic tumours when compared with normal tissue [131–135]. ^{18}F-FDG uptake measured by PET has been shown to correlate with the extent of GLUT-1 and hexokinase I expression, but not hexokinase II or III expression, in breast cancer [136, 137]. ^{18}F-FDG PET imaging has also demonstrated a direct correlation between aggressiveness (and prognosis) and the rate of tumour glucose consumption [131, 132]. Furthermore, alterations in glucose uptake appear to parallel response to tumour therapies. For example, reduction in glucose uptake is observed within 24 h following administration of hormone therapy in prostate and breast cancer [138, 139], a response that precedes by several days any measurable change in tumour volume. An important caveat is that not all the increase in uptake of ^{18}F-FDG in tumours is attributable to tumour cells. Recently, by comparing results from immunocompetent and athymic tumour-bearing mice, before and after methylprednisolone pretreatment, Kobayashi and colleagues [140] concluded that a significant portion of the enhanced ^{18}F-FDG uptake observed in tumours may be ascribed to activated lymphocytes infiltrating the tumour stroma.

One consequence of the high glucose consuming "glycolytic phenotype" is increased tumour acid production. Initially it was hypothesized that this would result in substantially lower tumour intracellular pH (pH_i) [141]. However, it is now clear from MRS and other measurements that pH_i of tumour cells is typically identical to or even higher than that of non-transformed or normal cells [65, 66, 94, 95, 141, 142]. This is due to increased H^+ export from tumour cells resulting from upregulation of the Na^+/H^+ antiport, Na^+-dependent anion exchange, vacuolar-type H^+-ATPases and other membrane transporters [143–145]. This is likely a significant factor resulting in the extracellular acidosis typically found in tumours, as already discussed. Upregulation of pH_i regulatory mechanisms has been identified as an early, necessary, and sometimes sufficient, step in the malignant transformation of mammalian cells [64, 143, 146].

In colon and oesophageal cancer [147, 148], constitutive alterations in cellular metabolism are relatively late events in the multistep somatic evolution of the malignant phenotype. For example, in colorectal carcinogenesis, glucose uptake as measured by ^{18}F-FDG PET remains normal in small polyps and most large polyps, but is uniformly increased in invasive cancers, suggesting that development of the glycolytic phenotype is coincident with the transition from non-invasive to invasive tumour growth dynamics [127, 149]. The preponderance of data indicate that virtually all metastatic cancers take up ^{18}F-FDG avidly, suggesting that adoption of a glycolytic phenotype is a common (necessary) feature of carcinogenesis itself.

The basis for this metabolic transformation remains unclear. Warburg hypothesized that it was the result of an acquired error in respiration—an error that ultimately caused acquisition of the malignant phenotype [150]. However, this "Warburg hypothesis" has been clearly shown to be untrue [151]. Most likely, the glycolytic phenotype arises in tumour cells as a transient adaptation to the cyclical periods of hypoxia caused by heterogeneous perfusion, as outlined in previous sections. However, the near-universal observation of increased glucose utilization in human cancers suggests that ultimately these transient, adaptive, changes in glucose metabolism result in a constitutive upregulation of the glycolytic phenotype due to environmental factors other than hypoxia alone. We hypothesize that adoption of the glycolytic phenotype is the result of underlying Darwinian selection dynamics in which cellular phenotypes with metabolic properties best suited to the extant microenvironment have a selective proliferative advantage resulting in clonal expansion.

Connections and models

The altered metabolism and microenvironment of invasive cancers results from the complex interactive dynamics of neoplastic populations with each other, and with adjacent normal tissue [152]. Carcinogenesis is often described as somatic evolution, as transformed cells are subject to random and accumulating genetic mutations, producing new phenotypes that interact with environmental selection parameters. Those cellular alterations conferring a proliferative advantage are rewarded with clonal expansion [153–155]. The resulting Darwinian competition among the mutant populations produces a steady phenotypic drift from the cells of origin. Mathematical models of carcinogenesis [156, 157] demonstrate that favourable cellular traits confer selective growth advantage by releasing the cell from extant proliferative constraints. This includes degradation of normal

inhibitory signalling pathways and upregulation of growth promoters. In addition, mutations that affect the microenvironment may be favoured if they induced death or growth inhibition of adjacent normal cells, degradation of the extracellular matrix to facilitate invasion, or recruitment of normal mesenchymal support.

The near-universal evolution of increased glycolysis by invasive cancers would initially appear surprising in terms of this Darwinian "survival of the fittest", because it produces two unfavourable results: (1) inefficient energy production with greater demand for glucose acquisition and; (2) increased acid by-products from glycolysis that must be excreted into the environment. These changes in cell metabolism both increase basal cellular energy requirements and create a local extracellular microenvironment that is both acidic and hypoglycaemic.

Mathematical analysis provides an answer to this conundrum by demonstrating that environmental perturbations resulting from glycolysis, in fact, confer subtle proliferative advantages on the tumour cells by providing a mechanism for invasion of normal adjacent tissue. The "acid-mediated" tumour invasion model [158–160] posits the following sequence: Transformed tumour cells with increased glycolysis and acid excretion alter the microenvironment by substantially reducing intratumoural pH_e. The H^+ ions in the tumour extracellular space diffuse along concentration gradients into adjacent normal tissues. Acidification of the extracellular environment leads to destruction of the normal tissue. This includes killing of normal cells due to caspase-mediated activation of *p53*-dependent apoptosis pathways [70, 161], promotion of angiogenesis through acid-induced expression of VEGF and IL-8 [53, 68], extracellular matrix degradation by proteolytic enzymes such as cathepsin B [74, 77], and inhibition of immune function [162]. Upregulation of H^+ export mechanisms [143–145] confers a survival advantage to tumour cells over normal cells in an acidic microenvironment. Furthermore, mutations in *p53* or downstream effectors allow tumour cells to remain actively mitotic in relatively acidic pH_e [70]. Thus, the tumour edge can be envisioned as a travelling wave extending into normal tissue following a parallel travelling wave of increased microenvironmental acidity [158].

Detailed simulations of the tumour-host interface have been computed using mathematical models based on acid-mediated tumour invasion. A full presentation of these results can be found elsewhere [158–160]. Briefly, this analysis reproduces many experimental and clinical observations including a transition from benign to malignant tumour growth coincident with acquisition of the angiogenic phenotype. A hypocellular gap at the tumour-host interface is predicted to occur in some tumours, and has been demonstrated in 70% of pathological samples from human squamous cell carcinoma of the head and neck [158] (Figures 2 and 3). Finally, the models predict that perturbations of the tumour microenvironment through systemic acidosis may slow, or even reverse, tumour growth. An interesting supporting observation that has recently emerged from a retrospective study of renal cell carcinoma patients is that patient survival following removal of the primary lesion by unilateral nephrectomy was strongly correlated to the degree of loss of renal function and resulting metabolic acidosis [163]. Hence, alteration of systemic pH may have direct anti-tumour

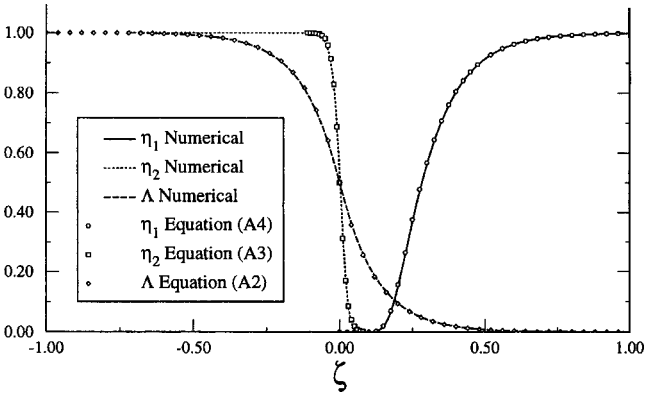

Figure 2. Wave-front profiles of the tumour-host interface generated from computer simulations and analytic solutions of coupled partial differential equations used to model the acid-mediated tumour invasion hypothesis. Details of this analysis are available in reference [158]. The wave-fronts are propagating left to right with a speed of about 0.03 mm day^{-1}. Normal tissue is identified as η1 with the boundary of normal cells receding before the advancing wave-fronts of propagating tumour, identified as η2, and the accompanying acid gradient, identified as Λ. Note the predicted acellular gap between the edges of the tumour and normal tissue. The mathematical models predict this gap will occur under some conditions as a result of rapid death of normal cells in the region of most severe extracellular acidosis.

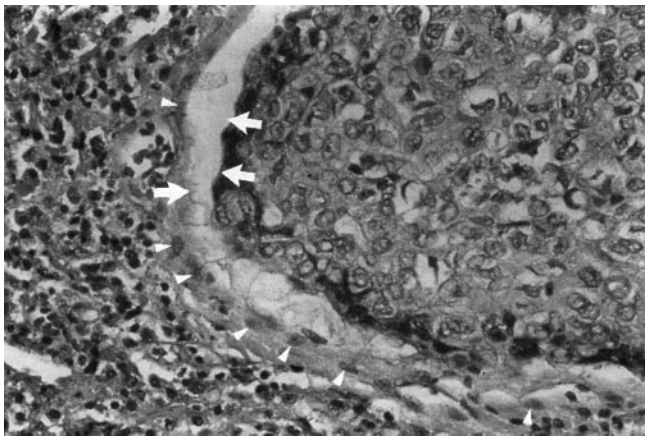

Figure 3. Heamotoxylin and eosin stained micrographs of the tumour-host interface of a formalin-fixed specimen from human squamous cell carcinoma of the head and neck. An acellular gap between the tumour and normal tissue edges is identified (arrows), consistent with the predictions of the mathematical model (*cf.* Figure 2). Note the dying normal cells just beyond this acellular gap (arrowheads) presumably due to acid-induced apoptosis.

consequences by altering the tumour microenvironment. In this context it is worth reiterating the observations of Kelley et al [102], who found that acidosis induced by isolated limb perfusion produced a significant decrease in tumour burden in a melanoma xenograft model.

Conclusions

Cancer imaging has provided important clues about its nature: heterogeneous perfusion, increased glucose uptake, hypoxia and acidity. These environmental parameters are

highly correlated with tumour aggressiveness, suggesting that these are fundamental aspects of tumours. The influence of these microenvironmental parameters on the natural progression of cancers can be modelled and be shown to strongly influence tumour progression via natural selection/somatic evolution. These mathematical models are also useful in that they predict the effects of altering the tumour microenvironment, agreeing with empirical observations in the case of chemotherapy with weakly-ionizing drugs, and also in at least one retrospective study of long-term outcome in metastatic renal cell carcinoma following cytoreductive nephrectomy.

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