The role of ascorbate in the regulation of HIF-1 in cancer cells

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Abstract

Cancer cells within a solid tumour are often under considerable metabolic stress, which would normally signal cell death. However, cancer cells are able to adapt and survive their inhospitable microenvironment by activating the transcription factor hypoxia-inducible factor (HIF)-1. HIF-1 drives the transcription of hundreds of genes involved in various processes known to drive cell survival and tumour progression, as well as metastasis and chemo- and radio-resistance, and is an established independent prognostic indicator in many cancer types.

HIF-1 is constantly synthesised in all cells, but its activity is kept controlled by post-translational hydroxylation, causing it to ‘switch off’ when it is not needed. The hydroxylation reactions are performed by a group of enzymes known as the HIF-hydroxylases that specifically require ascorbate (vitamin C) as a co-factor for activity. This suggests that ascorbate-deficiency may result in over-activation of the HIF-1 response and thus, tumour progression. Despite this, ascorbate has received relatively little attention as a possible inhibitor of the HIF-1 response.

It was therefore the aim of this thesis to investigate whether intracellular ascorbate levels can significantly influence HIF-1 activity in cancer cells, and whether this mechanism has any clinical relevance. These questions were addressed using multiple approaches. In vitro studies were performed to monitor the optimal intracellular ascorbate concentrations required to inhibit HIF-1 induction by various means. In addition, clinical samples from two tumour types (endometrial and colorectal) were analysed for markers of HIF-1 activation, and related to the tissue ascorbate content and clinico-pathological data. Furthermore, pharmacokinetic data was obtained describing what plasma ascorbate concentrations are needed for its optimal distribution in avascular tumour tissue.

The results presented in this thesis have shown a clear and consistent relationship between low intracellular ascorbate levels and high HIF-1 activation in cancer cells. The in vitro
studies showed that ascorbate was able to dramatically inhibit HIF-1, induced by various means, and that intracellular concentrations greater than 1 mM are optimal for this effect.

That these results could have clinical relevance was shown by the analysis of human tumour tissues, where low levels of tissue ascorbate were associated with higher HIF-1 activation, high tumour grade, tumour necrosis and larger tumour size. These results were markedly similar between endometrial and colorectal tumours. Furthermore, poor disease-free survival in colorectal cancer patients was associated with low tumour ascorbate content. Further to this, pharmacokinetic data indicated that a constant, saturated plasma ascorbate concentration is necessary to avoid ascorbate-deficiency in avascular regions of a tumour.

There is considerable scepticism surrounding the use of ascorbate to treat cancer, with little mechanistic or clinical evidence to inform this practice. However, the results presented in this thesis support a role for ascorbate in regulating HIF-1 and tumour progression, and this mechanism is worthy of further clinical investigation.
Acknowledgements

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### Abbreviations

<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>2-OG</td>
<td>2-oxoglutarate</td>
</tr>
<tr>
<td>ACSRC</td>
<td>Auckland Cancer Society Research Centre</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Angiopoietin-like protein 4</td>
</tr>
<tr>
<td>ARD</td>
<td>Ankyrin repeat domain</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived cell</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Bcl2/adenovirus E1B 19 kDa interacting protein 3</td>
</tr>
<tr>
<td>CAD</td>
<td>C-terminal activation domain</td>
</tr>
<tr>
<td>CODDD</td>
<td>C-terminal oxygen-dependent degradation domain</td>
</tr>
<tr>
<td>C-P4H</td>
<td>Collagen prolyl-4-hydroxylase</td>
</tr>
<tr>
<td>CSTBC</td>
<td>Cancer Society Tissue Bank, Christchurch</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DFO</td>
<td>Desferrioxamine</td>
</tr>
<tr>
<td>DHA</td>
<td>Dehydroascorbate</td>
</tr>
<tr>
<td>DMOG</td>
<td>Dimethyloxalylglycine</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylene triamine pentaacetic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>FIH</td>
<td>Factor inhibiting HIF</td>
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<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>HEPES</td>
<td>Hydroxyethyl piperazine ethane sulfonic acid</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HPLC-ECD</td>
<td>High performance liquid chromatography – electro-chemical detection</td>
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<td>Hypoxia response element</td>
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<td>HRP</td>
<td>Horse-radish peroxidase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>L1CAM</td>
<td>L1 cell adhesion molecule</td>
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<td>Lysyl oxidase</td>
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<td>MCL</td>
<td>Multicellular layer</td>
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<td>NAD</td>
<td>N-terminal activation domain</td>
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<td>N-terminal oxygen-dependent degradation domain</td>
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</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
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<td>SVCT</td>
<td>Sodium-dependent vitamin C transporter</td>
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<td>TBST</td>
<td>Tris-buffered saline with Tween®-20</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial cell growth factor</td>
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Chapter 1: Introduction

As cancer cells proliferate, their rapid growth results in tissue hypoxia and metabolic stress. As a result, there is a need for an increased blood supply to support the expanding cell mass with nutrients and oxygen. In an attempt to drive new blood vessel formation (angiogenesis), and restore metabolic homeostasis, the cancer cells release soluble factors, such as vascular endothelial growth factor (VEGF), and they also alter their metabolic pathways to maximise energy production and cell survival under stress. Many of these processes are under the control of the transcription factor hypoxia-inducible factor (HIF)-1, which controls the expression of VEGF and numerous proteins involved in cell metabolism and survival. Activation of HIF-1 enables cancer cells to thrive and supports the growing tumour mass, forming a heterogeneous and complex organ. HIF-1 is therefore central to tumour development and its regulation is vital to controlling tumour progression. The subject of this thesis is the mechanism of HIF-1 regulation and the role played by ascorbate in this process. Therefore, the following introduction will explore the relevance of HIF-1 to tumour biology, how it is regulated and the potential for ascorbate to influence this.

1.1 Tumour hypoxia and the microenvironment

The tumour microenvironment is now recognised as a significant enabling factor in tumour growth and spread (1). One of the most common and well characterised features of the tumour microenvironment is hypoxia. Tumour tissue oxygenation has been determined using polarographic needle electrodes, endogenous protein markers and exogenous probes. These have associated tumour hypoxia with worse outcome and it is now an established independent prognostic indicator in multiple cancer types (2, 3). Even surgically removed hypoxic tumours are associated with poorer patient outcomes, indicating that hypoxic tumours represent an inherently more aggressive phenotype and may be associated with an increased risk of metastasis (4).
The molecular basis behind the biological consequences of tumour hypoxia is now becoming better understood. Perhaps the most far-reaching mediator in the hypoxic response, or rather the microenvironmental response, is the transcription factor HIF-1. HIF-1 is activated under conditions of cell stress, including hypoxia, and drives the transcription of a diverse and numerous set of genes involved in several cell survival pathways (Figure 1.1), the ultimate aim being to restore oxygen and energy homeostasis. In normal tissues under hypoxic stress this is a valuable protective mechanism. However, it provides an advantage for cancer cells, which is clearly undesirable for the host.

In a growing tumour, hypoxia initially occurs due to rapidly proliferating cancer cells outgrowing their local blood supply. This triggers the so-called ‘angiogenic switch’ where tumour cells release pro-angiogenic factors to restore adequate blood supply (5). However, in a tumour the result is often a highly disorganised and abnormal microvasculature, comprising dilated and tortuous vessels with disturbed arterio-venous flow dynamics (6). As the tumour grows, so does the extent of aberrant blood flow and metabolism, leading to an acidic and inflammatory extracellular milieu, and further regions of hypoxia (5). The hypoxic tumour microenvironment is highly conducive for Darwinian selection pressures to play out, leading to clonally selected populations of ‘survivor’ cancer cells (7).

Normal tissue has a pO$_2$ of around 38 mmHg and hypoxia occurs at pO$_2$ <10 mmHg (8). Spatial, or chronic, hypoxia is a common feature of solid tumours (6, 9) with the calculated diffusion distance of O$_2$ in tumour tissue being reported to range from 41 to 183 µm, and the median inter-vessel distance reported at ~257 µm (based on dorsal skin flap window experiments in rat tumours) (9). In addition to this, significant temporal fluctuations in pO$_2$, primarily caused by changes in erythrocyte flux (10), can affect up to 50% of the tumour microvasculature over periods of tens of minutes (11, 12). Therefore, hypoxia occurs heterogeneously throughout the tumour with both chronic and acute hypoxia affecting tumour cells in different ways (8).
1.1.1 Responses to tumour hypoxia

In normal, untransformed cells, hypoxia is a significant stress and can signal apoptotic death through up-regulation of p53 (13). However, if cells have acquired a p53 mutation, which occurs in up to 50% of malignancies, they become apoptosis-resistant under hypoxia (14). In solid tumours, these survivor cells can remain subjected to continuous hypoxic insult, and this has been shown to substantially increase genomic instability and mutagenicity (15). This has been demonstrated in experimental mouse models in which hypoxia dramatically increased spontaneous metastases due to hyper-mutagenicity and DNA repair breakdown (8, 16). These results correspond with the clinical observations that hypoxic tumours have a worse prognosis, and more recently, many of the processes behind this increased tumour aggressiveness have been attributed to the activation of HIF-1.
Figure 1.1: Protein products of HIF-1 target genes. Activation of the transcription factor HIF-1 results in up-regulation of numerous genes involved in pathways of cell metabolism, angiogenesis and cell survival. To date, hundreds of genes for which expression is controlled by HIF-1 have been identified. Prominent members of key pathways are shown. From Schofield & Ratcliffe, 2004 (17).

1.2 Hypoxia-inducible factor

HIF-1 was identified following a search for the regulator of the gene encoding human erythropoietin (EPO), which stimulates erythrocyte production in response to systemic hypoxia, such as at high altitude. The EPO gene was found to have a specific enhancer region
that HIF-1 bound to in a hypoxia-inducible manner (18). This led to the further characterisation of the structure and function of HIF-1 as a transcription factor involved in the wider response to tissue hypoxia (19-22). HIF-1 is beneficial in normal tissues, where it induces an adaptive response to changes in oxygen supply and cellular metabolism that enables cell survival. However, when activated in cancer cells, these same adaptive responses are unfavourable as they promote tumour progression. Hence, the activity and regulation of HIF-1 are now being closely examined in the hopes of curbing its pro-survival effects in tumours.

1.2.1 Structure and function

HIF-1 is a ubiquitously expressed, heterodimeric transcription factor comprising a labile, regulatory α-subunit and a stable β-subunit (also known as aryl hydrocarbon receptor nuclear translocator). They are both basic helix-loop-helix (bHLH; DNA binding domain) proteins and belong to the Per-Arnt-Sim (PAS; heterodimerisation domain) family (19). HIF-1α contains four regulatory domains: the N- and C-terminal oxygen-dependent degradation domains (NODDD and CODDD) which regulate protein stability, and the N- and C-terminal activation domains (NAD and CAD) which regulate co-activation (Figure 1.2). HIF-1 binds to a specific DNA sequence known as the hypoxia response element (HRE), which is an asymmetric E-box motif consisting of the consensus core sequence 5’-RCGTG-3’ (23). The HIF-1α CAD is essential for recruiting transcriptional co-activators p300 and CREB binding protein at the HRE.
Figure 1.2: Domain structure of HIF-1α and HIF-1β. The functional regions are shown: bHLH (basic helix-loop-helix) and PAS (Per-Arnt-Sim, containing repeating A and B sequences) domains control DNA binding and heterodimerisation, respectively. NODDD (N-terminal oxygen-dependent degradation domain), CODDD (C-terminal oxygen-dependent degradation domain), NAD (N-terminal activation domain) and CAD (C-terminal activation domain) regulate protein stability and co-activation. Proline and asparagine hydroxylation at these domains inhibits HIF-1α (see section 1.2.2). From Schofield & Ratcliffe, 2004 (17).

There are two other known HIFα isoforms (HIF-2α and HIF-3α) that are also hypoxia-inducible and that dimerise with HIFβ, but which have distinct roles in the hypoxic response. Whereas HIF-1α is typically involved in the acute hypoxic response, primarily up-regulating metabolic genes (24), HIF-2α is involved in the chronic hypoxic response and controls genes relating to differentiation and stem cell control (24). There is comparatively little information on the involvement of HIF-3α in the hypoxic response but, depending on its splice variant, it can negatively regulate HIF-1α by heterodimerisation (17, 25). Both HIF-1α and HIF-2α were found to bind significantly and with similar affinity to HRE sites throughout the genome, but have distinct target gene expression profiles (23, 26). This indicates a high degree of regulation of HIF transcriptional activity involving post-translational modifications, epigenetic restrictions and differential interactions with co-activators. HIF-1α is the most studied isoform and is typically associated with tumour biology. It is currently a primary target for the development of small molecule inhibitors to be used in cancer therapy and is therefore the isoform that will be the focus of this thesis.
1.2.2 HIF-1 regulation by hydroxylation

The activation state of HIF-1 is controlled by the stability and activity of the α subunit. HIF-1α is under dual control, with both HIF-1α protein stability and transcriptional activity being regulated by hydroxylation reactions (Figure 1.3). HIF-1α protein is constantly synthesised in most cells in the body, and under normal, physiological conditions is rapidly degraded (27). This occurs following hydroxylation of proline residues 402 and 564 on the NODDD and CODDD, respectively (28, 29) (Figure 1.2). This modification initiates binding of the tumour-suppressor von Hippel-Lindau protein (pVHL), which recruits an E3 ubiquitin ligase complex (30, 31). HIF-1α is subsequently ubiquitinated, which targets it to the 26S proteasome for degradation.

A further hydroxylation event on asparagine 803 on the CAD of HIF-1α (Figure 1.2) prevents its co-activation with p300, thus preventing transcriptional activation (32). These events combine to enable a rapid response to cell stressors, where a decrease in hydroxylation activity immediately halts HIF-1α degradation, both allowing the protein to accumulate and also to activate a transcriptional response (33). The activation of HIF-1 is, therefore, dependent upon the activity of the hydroxylases responsible for modification of the HIF-1α subunit.

The proline and asparagine hydroxylase enzymes that catalyse these reactions are collectively known as the HIF-hydroxylases. They are dependent on oxygen for activity, and therefore link a lack of oxygen in the cell to an active HIF-1 response (Figure 1.3) (29, 32). They are also dependent on Fe^{2+}, 2-oxoglutarate [2-OG; a tri-carboxylic acid (TCA) cycle intermediate] and ascorbate (17). Thus, other cell stressors which also occur as a result of poor vascularity, such as metabolic disturbance and oxidative stress, are able to induce HIF-1. Therefore, the HIF-hydroxylases are the metabolic sensors that control the hypoxic response. Their activity and substrate requirements, particularly their need for ascorbate as a co-factor, are a central theme of this thesis, and will be discussed in detail in sections 1.4 and 1.5.
As well as regulation by hydroxylation, other modifications that affect HIF-1α activity have been reported and these may also affect tumour biology. HIF-1α is known to be acetylated by sirtuin 1, which may contribute to its protein stabilisation (34). Likewise, nitrosylation of HIF-1α by nitric oxide may stabilise the protein (34). SUMO (small ubiquitin-like modifier) isoforms 1-3 bind to the HIF-1α N/CODDD and this has been shown to either increase HIF-1α protein stability (35, 36), have no effect (37), or cause pVHL binding and subsequent degradation independent of hydroxylation (38). SUMOylation has also been reported to either increase (35, 36) or decrease (37) HIF-1 transcriptional activity. Hence, it remains unclear as to whether SUMOylation results in increased or decreased HIF-1 activity.
HIF-1α protein stability can also be regulated by modification of pVHL. E2-endemic pemphigus foliaceus ubiquitin carrier protein was found to ubiquitinate pVHL, causing its degradation and thus, indirectly, affecting the normoxic stabilisation of HIF-1α (39). This was associated with increased tumour cell proliferation, invasion and metastasis (39). Furthermore, pVHL can be reversibly sequestered in the nucleolus during metabolic acidosis (a common feature of solid tumours), causing stabilisation of HIF-1α in the presence of oxygen (40). Hypoxic acidosis has been shown to result in sequestered pVHL that was not reversed by reoxygenation, enabling a prolonged HIF-1 response (40).

Several oxygen- and pVHL-independent degradation pathways have been identified that also control HIF-1α. Receptor of activated protein kinase C (RACK1) acts similarly to pVHL by binding to HIF-1α and recruiting the E3 ubiquitin ligase complex leading to HIF-1α degradation (41). It competes with heat-shock protein 90 (a molecular chaperon necessary for correct folding of the PAS domain) for binding at the PAS domain of HIF-1α (41). Homodimerisation of RACK1 is essential for binding and this is inhibited by dephosphorylation by calcineurin – a Ca²⁺-activated phosphatase (42). Thus, high [Ca²⁺]i can lead to HIF-1α stabilisation. More recently, a gene product of SHARP1, associated with reduced risk of triple-negative breast cancer metastasis, was shown to directly present HIF-1α to the 26S proteasome for degradation and inhibit production of HIF-1 target genes (43).

1.2.4 **HIF-1α protein synthesis**

In addition to post-translational regulation of HIF-1α, its synthesis is primarily regulated at the translational level, downstream of growth factor signalling (44). Activation of the PI3K-Akt and MAPK pathways initiate phosphorylation events leading to the increased ribosomal affinity for HIF-1α mRNA essential for translation initiation (45). Oncogene activation or tumour-suppressor loss of function often activate these pathways and can thus increase HIF-1α protein synthesis, counteracting its degradation (44). Hypoxia rapidly shuts down cellular protein translation machinery, but HIF-1α protein continues to be synthesised, although the mechanisms behind this are yet to be clarified (45).
The studies described above highlight that there are many levels of regulation controlling HIF-1 activity. Some of these may be relevant to the tumour microenvironmental stress response. However, whereas the significance of the various means of HIF-1α regulation are yet to be elucidated, it is the hydroxylation-mediated degradation and inactivation that is most widely acknowledged as the primary restraint of the HIF-1 response, with alterations in HIF-1 hydroxylation likely to result in major changes in the activity of this important transcription factor.

1.3 HIF-1 and tumour biology

So far, several hundred HIF-1 target genes have been identified (23), involving numerous cell survival and metabolic pathways, some of which are represented in Figure 1.1. When activated in a growing tumour, the subsequent effects of the wide-ranging HIF-1 response provide a selective survival advantage, facilitating tumour progression. However, our understanding of the overall biological consequences of HIF-1 activity in the complex setting of a tumour is still evolving.

1.3.1 Clinical evidence for HIF-1 activation

Numerous studies have examined human tumour tissue for HIF-1α protein using immunohistochemistry and have correlated the level of protein expression with clinical outcomes (Table 1.1). Similar to what has been observed with tumour hypoxia, it appears that HIF-1α is an independent prognostic indicator for worse outcomes in many cancers. In addition, HIF-1 has been implicated in the progression of carcinogenesis, with pre-neoplastic hepatocytes over-expressing HIF-1α as an early change in mouse hepatocarcinogenesis (46). Furthermore, HIF-1α and specific target gene expression correlated with increased malignancy in human endometrial tissue, from normal to premalignant to adenocarcinoma (47) and increased with each clinical stage of endometrial adenocarcinoma (48). Similarly, HIF-1α protein was detected in human gastric tissue, with levels increasing from gastritis to metaplasia to dysplasia to adenocarcinoma (49).
Table 1.1: Human tumour types in which increased HIF-1α protein levels have been demonstrated, and associated clinical prognosis.

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Prognosis</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Breast</td>
<td>Poor</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>(51)</td>
</tr>
<tr>
<td></td>
<td>Poor (lymph node negative tumours)</td>
<td>(52)</td>
</tr>
<tr>
<td></td>
<td>Poor (lymph node negative tumours)</td>
<td>(53)</td>
</tr>
<tr>
<td>Endometrial, stage I</td>
<td>Poor</td>
<td>(54)</td>
</tr>
<tr>
<td>Endometrial, type I</td>
<td>No prognostic association; increased stage,</td>
<td>(55)</td>
</tr>
<tr>
<td></td>
<td>grade, invasion and metastasis</td>
<td></td>
</tr>
<tr>
<td>Endometrial, type II</td>
<td>No association</td>
<td>(55)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>Poor</td>
<td>(56)</td>
</tr>
<tr>
<td></td>
<td>Poor (only with mutated p53)</td>
<td>(57)</td>
</tr>
<tr>
<td></td>
<td>Poor (serous tumours)</td>
<td>(58)</td>
</tr>
<tr>
<td>Cervical</td>
<td>Poor</td>
<td>(59)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>(60)</td>
</tr>
<tr>
<td>Bladder</td>
<td>Poor</td>
<td>(61)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>Poor</td>
<td>(62)</td>
</tr>
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<td>(63)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>(64)</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>Poor</td>
<td>(65)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>(66)</td>
</tr>
<tr>
<td>Gastric adenocarcinoma</td>
<td>No association</td>
<td>(67)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>(49)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>(68)</td>
</tr>
<tr>
<td>GIST</td>
<td>Poor</td>
<td>(69)</td>
</tr>
<tr>
<td>Oropharyngeal SCC</td>
<td>Poor</td>
<td>(70)</td>
</tr>
<tr>
<td>Head and neck SCC</td>
<td>Favourable</td>
<td>(71)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>(72)</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>(73)</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Favourable</td>
<td>(74)</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>Poor</td>
<td>(75)</td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>Poor</td>
<td>(76)</td>
</tr>
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GISt, gastrointestinal stromal tumour; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer.
The implication from these studies is that increased HIF-1$\alpha$ protein will generally reflect increased gene expression and, as HIF-1 is known to induce many processes involved in tumour progression, the increased gene expression could explain its association with worse patient outcomes (Table 1.1). The following sections detail the information to date regarding the role of HIF-1 in driving the processes associated with tumour progression, highlighting the extent of its involvement in tumour biology, and why its regulation is of such importance.

### 1.3.2 Effects of HIF-1 on apoptosis and autophagy

One of the key traits of malignant cells is the ability to overcome cell death pathways and become apoptosis-resistant. HIF-1 is known to be involved in this process, but can be both pro- and anti-apoptotic, depending on the genetic and signalling background of the cell. HIF-1$\alpha$ has been shown to stabilise wild-type p53 leading to induction of apoptosis (78). In addition, the HIF-1 target genes Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) and NOXA are BH3-only proteins that can be involved in pro-apoptotic signalling (79). On the other hand, HIF-1 induction was found to protect HepG2 cells against apoptosis by serum deprivation and oxidative stress (80). Furthermore, several proteins involved in apoptosis signalling, including NF-$\kappa$B, survivin, Bak, Bax, Bcl-xL and Bcl-2, have been found to be regulated by HIF-1 to result in inhibition of apoptosis or promotion of cell survival (81). Despite the varied responses, it appears that in the majority of cancer cells HIF-1 prevents apoptosis and promotes the ‘survivor’ phenotype, particularly when coincident with mutated p53 (81).

Another mechanism used by tumour cells to overcome cell death in an inhospitable microenvironment is autophagy, or ‘self-digestion’. This process involves catabolism of intracellular components for recycling in an attempt to stave off cell death under stress. The highly specific HIF-1 target gene, BNIP3, is thought to activate autophagy, and also mitophagy – the specific digestion of mitochondria (79). Originally identified as a pro-apoptotic, mitochondrial BH3-only protein (82), BNIP3 is known to induce necrosis-like cell death by opening the mitochondrial permeability transition pore, a function independent of its atypical BH3 domain (83). However, more recent studies have shown BNIP3 to be essential
for autophagy and mitophagy, promoting adaptive cell survival (84-86). Although BNIP3 was found to be epigenetically silenced in highly aggressive pancreatic, colorectal and gastric tumours (79), its expression has been associated with poor prognosis in NSCLC (87), breast cancer (88) and endometrial cancer (89). Furthermore, BNIP3 can be sequestered in the nucleus (87, 88) preventing its mitochondrial insertion and cell death signal, and nuclear BNIP3 has been shown to transcriptionally repress apoptosis-inducing factor to overcome cell death (90). It is likely that HIF-1-dependent BNIP3 drives the selection of death-resistant cells, and also promotes adaptive survival through autophagy and mitophagy, while other tumourigenic factors dominate in the case of BNIP3 silencing.

1.3.3 HIF-1 and chemo- and radio-resistance

A major challenge in cancer therapy is the ability of the cells to develop resistance to current treatments. Cancer cells often overcome the cytotoxic effects of chemo- and radio-therapy, where HIF-1 is known to play a substantial role in this (81). In vitro studies have determined that HIF-1-dependent BNIP3 up-regulation is responsible for hypoxia-induced resistance to etoposide treatment, and this is due to mitochondrial enlargement conferring protection against apoptosis (91-93). However, there are many other known mechanisms by which HIF-1 mediates hypoxia-induced chemo-resistance, including inducing drug efflux pumps (94), inhibiting cell senescence (95), inhibiting DNA damage (96) and decreasing mitochondrial activity and ROS production (97). On the other hand, HIF-1 is known to inhibit the oncogene MYC, resulting in cell cycle arrest and inhibition of DNA repair genes, both of which may enhance some chemotherapeutics (34). Nevertheless, HIF-1 remains generally associated with chemo-resistance in cancer cells and this is yet another reason for its association with poor clinical outcomes.

Tumour hypoxia is classically associated with resistance to radiotherapy (98). This is hypothesised to be due to decreased production of oxygen-derived free radicals by ionising radiation, thereby preventing DNA strand breaks (8, 98). However, lack of oxygen may not be the sole cause of poor responses to radiotherapy, as HIF-1 has been associated with radio-resistance independent of tumour oxygenation in xenografts (99). In addition, in vitro studies
have shown that siRNA inhibition of HIF-1α significantly enhanced radio-sensitisation in PC3 cells (100), while HIF-1 induction conferred radio-resistance to HeLa cells (101). In contrast, the degree of basal levels of HIF-1 in several cancer cell lines did not correlate to radio-responsiveness (102), however the response with induced HIF-1 was not measured. Overall, it appears that HIF-1 is associated with radio-resistance, but the mechanisms involved remain to be clarified.

1.3.4 HIF-1 and angiogenesis

A major mechanism by which malignant tumours are able to thrive is through the formation of new blood vessels, or angiogenesis (1). This is activated very early in the tumourigenic process and is also a common feature in established tumours in order to maintain nutrient supply and waste disposal (103). Increased microvessel density is associated with HIF-1α protein expression and poor prognosis in many human cancers, and this is due to several pro-angiogenic target genes that are up-regulated by HIF-1 (Figure 1.1), the most notable being VEGF (104, 105). VEGF is commonly overproduced in tumours, resulting in rapid and aberrant proliferation of the microvasculature (106). Vascular endothelial cells rely on VEGF for survival and outgrowth, and several currently used anti-VEGF therapies exploit this to arrest tumour vascularisation (106). More recently, the efficacy of this approach is being questioned, as inhibiting tumour vascularity can result in increased hypoxia and this may encourage further hypoxia/HIF-driven clonal selection (107). Furthermore, studies have shown that improving the tumour vasculature can restore tumour oxygenation and drug delivery and can reduce metastatic potential (107, 108). These developments demonstrate that although VEGF and angiogenesis are clearly critical factors in tumour biology, how they can be optimally exploited remains to be realised (109).
1.3.5 HIF-1 and metastasis

HIF-1 has recently been implicated as a driver of metastasis, and may contribute to the clinical associations between tumour hypoxia, increased metastasis and poor survival (2, 4). In support of this, HIF-1α knockdown was found to result in significantly reduced extravasation of cancer cells, decreased metastatic colony formation (Figure 1.4) and reduced secondary metastatic spread and cell scattering \textit{in vivo} (110). In this study, the gelatinase matrix metalloproteinase 9, which degrades the basement membrane and is crucial for metastasis, was identified as a HIF-1 target gene (110). Importantly, these pro-metastatic effects of HIF-1 are independent of its pro-survival effects as HIF-1 did not confer a survival advantage to this cell line under hypoxia (110).

![Figure 1.4: Knockdown of HIF-1α in L-CI.5s cells inhibits liver metastases in mice.](image)

The image shows that stained metastatic foci in mouse liver lobes are dramatically reduced in HIF-1α-silenced L-CI.5s cells compared to control L-CI.5s cells seven days after intravenous inoculation of these cancer cells. shNT is non-targeted short hairpin RNA and shHif-1α is short hairpin RNA against HIF-1α. Bar, 1cm. From Schelter, et al., 2010 (110).
These results emphasise the potential for HIF-1 to promote metastasis by activating the expression of target genes crucial for cell motility and invasion. Another specific HIF-1 target gene that has been identified as essential for breast cancer metastasis is lysyl oxidase (LOX) (111, 112), a protein secreted from breast cancer cells that is involved in the formation of a mature extracellular matrix (ECM) by increasing collagen cross-linking. It is also involved in cell adhesion and motility and LOX knockdown completely prevented cell movement \textit{in vitro} (111) and was required for hypoxia-dependent cellular migration and invasion and spontaneous metastases in mice (111). Localisation of LOX protein in the lung tissue was necessary for bone marrow-derived cell (BMDC) recruitment to form a pre-metastatic niche, allowing cancer cell focal adhesion and growth in mice (112) and the LOX protein was found to be elevated in human breast cancer tissue compared to surrounding normal tissue (112). Its presence was associated with poor progression-free and overall survival, particularly in oestrogen receptor-negative tumours (111). Furthermore, LOX was found to increase HIF-1\(\alpha\) protein synthesis via the Akt pathway, thus providing a positive feedback loop promoting metastasis (113).

Additional HIF-1 target genes, angiopoietin-like 4 (ANGPTL4) and L1 cell adhesion molecule (L1CAM), have also been implicated in breast cancer metastasis (114). ANGPTL4 promoted the hypoxic invasion of cancer cells through endothelial cells (EC) by inhibiting EC-EC interaction, and L1CAM enhanced cancer cell-EC interaction, resulting in increased lung metastases in mice in a HIF-1-dependent manner (114).

Together, these studies provide compelling evidence that suggests HIF-1 is a driving force of metastasis, not only by preparing the pre-metastatic niche at distant tissues, but also by promoting cancer cell migration to, and colonisation at, these sites. As metastasis is responsible for the majority of cancer deaths, the HIF-1-dependent processes behind it are highly attractive targets for therapies at both early and late stages of disease.
1.3.6  **HIF-1 and tumour metabolism**

Under normal biological conditions, ATP is efficiently produced aerobically via the TCA cycle and oxidative phosphorylation, where oxygen is used as the terminal electron acceptor, producing 36 moles of ATP per mole of glucose. In the case of hypoxic cancer cells, ATP production is maintained despite a lack of oxygen by utilising the glycolytic pathway, producing just 2 moles of ATP per mole of glucose. Therefore, in order to maintain adequate ATP production, tumour glucose consumption is dramatically increased (5). Although this a primary response to hypoxia, it can occur despite adequate oxygen availability, which is known as the ‘Warburg effect’, or aerobic glycolysis, a typical feature of aggressive cancers (115).

Not surprisingly, HIF-1 is at the centre of cancer metabolism, where it promotes a highly glycolytic phenotype (116). The entire glycolytic pathway is up-regulated by HIF-1, with glucose transporters (e.g. GLUT-1) and the glycolytic enzymes being target genes of HIF-1 (115, 117) (Figure 1.5). Another HIF-1 target gene is pyruvate dehydrogenase kinase-1, which prevents the conversion of pyruvate to acetyl-CoA for entry into the TCA cycle (Figure 1.5) and this has been shown to diminish mitochondrial oxygen consumption and increase hypoxic cell survival *in vitro* (118). HIF-1 can decrease mitochondrial biogenesis by counteracting MYC (119) and mitochondrial mass can be reduced by more than 50% via BNIP3-mediated mitophagy (86). Hence, when HIF-1 is active, many pathways can combine to shut down oxidative phosphorylation and activate glycolysis (Figure 1.5).

As pyruvate cannot enter the TCA cycle, it is converted to lactate by lactate dehydrogenase A and exported out of the cell by monocarboxylate transporter 4, both processes that are also up-regulated by HIF-1 (Figure 1.5). Other cancer cells are able to take up this lactate for use as an oxidative energy source preferentially over glucose, resulting in increased glucose availability for the glycolytic cells (120). The HIF-1 target gene carbonic anhydrase 9 converts CO$_2$ to extracellular carbonic acid and this, together with increased lactate efflux, results in significant acidification of the extracellular environment (121). An acidic tumour microenvironment is thought to be another selection pressure for cancer cells and can promote
invasion and metastasis by increasing the activity and/or secretion of ECM-degrading enzymes (5).

Figure 1.5: Glycolytic and oxidative metabolism in cancer cells. Cancer cells activate the glycolytic pathway and increase glucose consumption and lactate production and efflux, via HIF-1 target genes (yellow ovals). Green ovals represent tumour suppressors within the tri-carboxylic acid cycle, whose loss of function is associated with increased HIF-1 activation (see section 1.4.2). GLUT1, glucose transporter-1; Gly Enz, glycolytic enzymes; LDHA, lactate dehydrogenase A; MCT4, monocarboxylate transporter 4; PDK1, pyruvate dehydrogenase kinase 1; PDH, pyruvate dehydrogenase; CS, citrate synthase; ACON, aconitase; IDH, isocitrate dehydrogenase; αKGDH, α-ketoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; FH, fumarate dehydrogenase; MDH, malate dehydrogenase. From Semenza, 2010 (116).
In summary, HIF-1 is undoubtedly a key mediator of many survival processes in tumour biology, driving tumour progression and resulting in poor clinical outcomes. This central role of HIF-1 to tumour biology makes it a highly attractive target for the treatment of many malignancies. Consequently, understanding the mechanisms that control HIF-1 activation is essential, and can provide opportunities for targeted intervention. Hydroxylation of HIF-1α is the primary mechanism effectively ‘switching off’ HIF-1 activation, and the enzymes responsible for these reactions are therefore highly likely to be important targets for cancer treatment. The role of ascorbate in controlling these hydroxylation reactions, and its effect on the HIF-1 response will be examined in this thesis.

1.4 The HIF-hydroxylases

As described earlier (Figure 1.3), regulation of the HIF-1 transcriptional response is mediated by hydroxylation of HIF-1α, catalysed by the HIF-hydroxylases. This alters key protein-protein interactions, and inactivates HIF-1. Proline hydroxylation increases pVHL binding affinity by over 1000-fold and thereby triggers HIF-1α protein destruction (122, 123). In addition, asparagine hydroxylation prevents the otherwise tight binding of HIF-1α to its co-activator p300 to thwart HIF-1 transcriptional activity (124). There are three known prolyl-hydroxylases – prolyl hydroxylase domain containing (PHD) 1-3 (28, 29) and one known asparaginyl-hydroxylase – factor inhibiting HIF (FIH) (32), that control HIF-1.

The three PHD isoforms have different activities in relation to HIF-1α hydroxylation, which is thought to be due to differences in areas of the enzyme involved in substrate binding (125). PHD2 is generally acknowledged as the primary mediator of HIF-1α protein degradation, with PHD1 and 3 more likely to be involved in fine tuning of the HIF-1-response (27). The difference in substrate affinity may also be reflected in the subcellular localisation of each isoform, with PHD1 detected exclusively in the nucleus, PHD2 (and FIH) in the cytoplasm and PHD3 in both compartments (126). This allows proximate hydroxylation and degradation of HIF-1α synthesised in the cytoplasm via PHD2 and to a lesser extent, PHD3 (126). Furthermore, PHD2 and 3 are significantly up-regulated in response to hypoxia and
this serves to rapidly suppress the HIF-1 transcriptional response as soon as oxygen becomes available (127-129).

The HIF-hydroxylases belong to a growing family of enzymes known as the 2-oxoglutarate (2-OG)-dependent dioxygenases, which includes the collagen prolyl 4-hydroxylase (C-P4H), the JmjC subfamily of histone demethylases, the DNA demethylase AlkB and the ten-eleven translocation proteins which hydroxylate 5-methyl cytosine (130). The discovery of these enzymes has highlighted the growing importance of post-translational hydroxylation reactions in cell signalling, and suggests that the 2-OG-dependent dioxygenases can drive changes in protein structure and function and gene expression in response to cellular environmental cues.

1.4.1 Structure and reaction cycle

The HIF hydroxylases contain a core structural motif consisting of eight β-strands arranged in a ‘jelly-roll’ or double-stranded β-helix, surrounded by α-helices, which characterises the 2-OG-dependent dioxygenase family (33) (Figure 1.6A). The PHDs are predominantly monomeric (125), whereas FIH is a homodimer (131) (Figure 1.6A). The highly conserved active site is positioned within the double-stranded β-helix and contains a non-haem iron that is coordinated by a ‘facial triad’ of two histidines and one aspartate with the remaining three coordination sites occupied by labile water molecules (33) (Figure 1.6B). This is thought to give the catalytic iron a relatively exposed and flexible arrangement compared to haem oxygenases, allowing for a wide range of catalytic oxidations. However, it may also make the active site more prone to auto-oxidation (132). The co-substrate, 2-OG, binds to the iron in a bidentate manner as depicted in Figure 1.6B, where FIH is shown with 2-OG, and PHD2 with a competitive inhibitor (the interaction is predicted to be analogous) (33, 125).
Figure 1.6: Protein and active site structures of FIH and PHD2. Protein structures of FIH and PHD2 are shown (A), with FIH as a homodimer and PHD2 as a monomer. The double-stranded β-helix motifs are shown in red. FIH’s dimerisation domain is in yellow and α-helices are light/dark blue. The active sites (B) are shown with Fe$^{2+}$ as a black sphere and 2-oxoglutarate (with FIH) or a competitive inhibitor (with PHD2) shown in grey. From Ozer & Bruick, 2007 (33).

The catalytic cycle of the HIF-hydroxylases is thought to be conserved throughout the 2-OG-dependent dioxygenase family (133), and is outlined in Figure 1.7, using FIH as an example. 2-OG binding occurs first, allowing entry of the prime substrate, HIF-1α, which displaces a water molecule from Fe$^{2+}$. This allows binding of molecular O$_2$ to Fe$^{2+}$ which catalyses the oxidative decarboxylation of 2-OG to succinate and generates a highly reactive ferryl-oxo
species (133). This oxidative intermediate causes hydroxylation of HIF-1$\alpha$, resulting in formation of Fe$^{3+}$, which is reverted back to Fe$^{2+}$ followed by release of the substrates and CO$_2$, and the cycle can repeat. One atom of the O$_2$ is incorporated into hydroxy-HIF-1$\alpha$ and the other into succinate. The CO$_2$ is derived from the oxidative decarboxylation of 2-OG to succinate (33, 130). Ascorbate is a necessary co-factor for this reaction, with one thought being that it is required to maintain Fe$^{2+}$ in its reduced state. The HIF-hydroxylase K$_m$ values for ascorbate are relatively high (140–260 µM (134, 135); Table 1.2), indicating high intracellular requirements and sensitivity to ascorbate loss. However, the exact role of ascorbate in the HIF-hydroxylase reaction is unclear and will be discussed in detail in section 1.5.

Figure 1.7: Proposed HIF-hydroxylase reaction cycle using FIH as an example. The co-substrate, 2-oxoglutarate, binds to the active site Fe$^{2+}$ followed by the primary substrate, HIF-1$\alpha$. Molecular O$_2$ binds to Fe$^{2+}$ to generate an oxidising intermediate which hydroxylates HIF-1$\alpha$. Blue, FIH active site amino acid side chains (His-Asp-His); dark green, 2-oxoglutarate; black, HIF-1$\alpha$ (asparagine); light green, succinate and CO$_2$ – reaction products. From Ozer & Bruick, 2007 (33).
1.4.2 Regulatory mechanisms

Inhibition of the HIF-hydroxylases results in activation of HIF-1. Therefore, the molecules necessary for the HIF-hydroxylase catalytic cycle (O$_2$, Fe$^{2+}$, 2-oxoglutarate, ascorbate) provide clues as to the kind of stimuli that can activate the HIF-1 response. Decreased oxygen availability (hypoxia) is typically associated with HIF-1 activation, and this is reflected in the HIF-hydroxylase’s requirements for molecular O$_2$ with reported K$_{m}$ values for the PHDs of 230-250 µM; (Table 1.2) slightly above dissolved O$_2$ concentrations in air (200 µM) (134). On the other hand, FIH has a much lower reported K$_{m}$ for O$_2$ (90 µM; Table 1.2) (135) indicating that it is likely to require more severe hypoxia to be inactivated. The HIF-hydroxylases are considered to function as direct oxygen sensors, linking cellular hypoxia to activation of the HIF-1 transcriptional response in order to maintain homeostasis.

Iron is also a necessary co-factor for the HIF-hydroxylases and its substitution/depletion can disrupt HIF-hydroxylase activity and activate the HIF-1 response. The addition of Co$^{2+}$ and Ni$^{2+}$ ions and iron chelators such as desferrioxamine (DFO) robustly induce HIF-1, and this is thought to be through poisoning of the hydroxylases via removal of enzyme-bound iron (17, 29, 136). The apparent K$_{m}$ values for Fe$^{2+}$ are low, with 0.03 µM for PHD1 and 2, 0.1 µM for PHD3 (137) and 0.5 µM for FIH (Table 1.2) (135). This suggests tight binding of Fe$^{2+}$ to the HIF-hydroxylase active site, despite the coordination chemistry predicting a labile arrangement (132). However, whether Fe$^{3+}$ produced during enzymatic cycling has the same tight binding as Fe$^{2+}$ is unknown.

2-OG (also known as α-ketoglutarate) is an intermediary metabolite of the TCA cycle (Figure 1.5) and is a co-substrate in the HIF-hydroxylase reaction. Other 2-oxoacids from the TCA cycle, such as succinate and fumarate, can compete with 2-OG to inhibit HIF-hydroxylase activity and induce HIF-1 in vitro (138). Pyruvate, oxaloacetate and malate have also been shown to have similar HIF-1-inducing effects, and may cause significant basal HIF-1 activation under normoxic conditions (139, 140). HIF-1 activity can be increased by mutations in the TCA cycle enzymes succinate dehydrogenase and fumarate dehydrogenase, which cause a build-up of their normal substrates succinate and fumarate, respectively (141) (Figure 1.5). Furthermore, mutations in isocitrate dehydrogenase-1 can inhibit 2-OG
formation (Figure 1.5) to also inhibit the HIF-hydroxylases and activate HIF-1 (142). These mutations then have the potential to drive non-hypoxic HIF-1 activity and tumourigenesis (116), and have been clinically associated with the susceptibility to renal cancer and paragangliomas (142). This may confer not only an oxygen-sensing role to the HIF-hydroxylases, but also a metabolic-sensing role, where increases in the levels of glucose metabolites result in increased expression of HIF-1-regulated glycolytic genes (Figure 1.5). However, exactly which 2-oxoacids are relevant, and to what degree, is unknown under physiological conditions.

Table 1.2: Reported Km values for the HIF-hydroxylases and C-P4H. Values were determined using human recombinant enzymes in crude cell extract or using purified enzyme. Adapted from Ozer & Bruick, 2007 (33).

<table>
<thead>
<tr>
<th></th>
<th>PHD1</th>
<th>PHD2</th>
<th>PHD3</th>
<th>FIH</th>
<th>C-P4H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>230a</td>
<td>250a</td>
<td>230a</td>
<td>90c</td>
<td>40d</td>
</tr>
<tr>
<td>Fe2+</td>
<td>0.1b</td>
<td>0.03b</td>
<td>0.03b</td>
<td>0.5c</td>
<td>2d</td>
</tr>
<tr>
<td>2-oxoglutarate</td>
<td>55a</td>
<td>60a</td>
<td>60a</td>
<td>25c</td>
<td>20d</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>140a</td>
<td>180a</td>
<td>170a</td>
<td>260c</td>
<td>300d</td>
</tr>
</tbody>
</table>


1.4.3 Other mechanisms of HIF-hydroxylase inactivation

It has been argued that the HIF-hydroxylases can be inactivated by production of reactive oxygen species produced by mitochondria in response to hypoxia (144). There is evidence that HIF-hydroxylase activity can be inhibited by H₂O₂, inducing HIF-1 in a process requiring functional mitochondria, although the precise mechanisms behind this are unclear (139, 145-147). Conversely, mitochondrial H₂O₂ production has been shown to be decreased in response to hypoxia (148) and another study found no association between levels of reactive oxygen species (DCF fluorescence) and HIF-1 target gene expression (149). There may be a distinct role for H₂O₂ in regulating HIF-hydroxylase activity that remains to be further clarified.
1.4.4 PHDs vs. FIH

Despite being grouped together as the HIF-hydroxylases, the PHDs and FIH have slightly different catalytic properties (Table 1.2), allowing differential regulation of HIF-1α protein stabilisation and transcriptional activity, respectively. Structural analyses have revealed a conserved iron-binding motif between the PHDs and FIH. However, the opening to the active site of PHD2 is narrower than that of FIH (125), and this may explain the unusual tight binding of Fe^{2+} and 2-OG to PHD2 (150). In addition, PHD2 and FIH utilise two distinct residues to bind 2-OG, which suggests evolutionary divergence between the two enzymes (125). Indeed, FIH was found to have significant homology with the JmjC family of enzymes (151). The more open conformation of FIH’s active site is proposed to make it more vulnerable to oxidation, while also allowing a wider range of substrates, as opposed to PHD2 which is highly selective for HIFα (152). FIH also hydroxylates ankyrin repeat domain containing (ARD) proteins with similar affinity to HIF-1α, and these include IκBα and the intracellular domain of Notch-1 (153). Aside from the potential regulatory mechanisms on these proteins themselves, it is thought that the ARD pool may compete with HIF-1α for hydroxylation by FIH, thus fine tuning the hypoxic response (154).

1.5 Ascorbate and hydroxylase/HIF-1 regulation

The HIF-hydroxylases act as sensors of cellular metabolism, with decreased availability of fundamental metabolites such as oxygen, iron and 2-OG resulting in activation of the HIF-1 transcriptional response. HIF-1 target gene products, such as EPO, VEGF, transferrin and the glycolytic enzymes, are up-regulated to restore supply of these metabolites and therefore cellular homeostasis. Because ascorbate is also a required co-factor, its availability may also affect this, however it has received comparatively little attention as a regulator of the HIF-hydroxylases and of HIF-1 activation.
1.5.1 Ascorbate and 2-OG-dependent dioxygenase activity

The majority of data on ascorbate and hydroxylase activity has come from a series of studies in the 1970-80s on purified C-P4H in the context of collagen proline-hydroxylation. The dependence of C-P4H on ascorbate for activity provided an explanation for some of the symptoms of scurvy, with a severe deficiency of ascorbate (vitamin C) resulting in a reduction of the rate of peptidyl-proline hydroxylation performed by C-P4H, inhibiting formation of the tertiary structure of mature collagen and initiating subsequent deterioration of connective tissues (155-157). These early studies on C-P4H may be relevant to the HIF-hydroxylases, as the active site and the catalytic cycle are thought to be highly conserved among 2-OG-dependent dioxygenases (133, 158).

Ascorbate was found to be important, if not essential, for C-P4H activity, with an optimal concentration of 1-2 mM (143, 155, 159-162). In the absence of ascorbate, C-P4H could catalyse only ~15-30 reaction cycles over 8 seconds before the reaction ceased, and addition of 1 mM ascorbate to the reaction mixture rescued 100% of enzyme activity (157). Another study found C-P4H had a 54% lower initial reaction rate in the absence of ascorbate, which decreased to ~8% of total activity within 30 seconds with subsequent ascorbate addition able to rescue partial activity (155). On the other hand, others have found no C-P4H activity without ascorbate, demonstrating an absolute requirement for this co-factor (161, 162). Furthermore, ascorbate was needed for activity prior to 2-OG addition, suggesting that under constant turnover conditions, a permanent ascorbate presence is required (155, 161).

Although these results may be influenced by variation in co-substrate concentrations or order of addition, it is clear that ascorbate has a significant effect on hydroxylase activity, with recombinant PHD2 activity having also been shown to increase dose-dependently with ascorbate (163).

The mechanism by which ascorbate enhances hydroxylase activity has been thought to be due to the specific reduction of enzyme-bound Fe\(^{3+}\) to Fe\(^{2+}\). Uncoupled reaction cycles may be the primary cause of iron oxidation and although these represented only 0.7% or 1.25% of total C-P4H activity, this was found to be sufficient to oxidise enzyme-bound iron (143, 162). This oxidation could be reversed by ascorbate (155, 160, 162). Ascorbate is not
stoichiometrically consumed during hydroxylase activity (161, 162), but one study found that it was stoichiometrically consumed specifically during uncoupled reaction cycles, supporting its involvement in protecting against this mode of enzyme inactivation (160). However, in another study, using recombinant PHD2, there was a significant increase in ascorbate-reversible Fe$^{3+}$ only when the prime substrate was added, suggesting coupled turnover may also oxidise the iron (150).

Despite ambiguity regarding the mechanism of ascorbate in the hydroxylase reaction cycle, it has been shown to be highly specific for optimising hydroxylase activity. Other reducing agents including dithiothreitol (DTT), L-cysteine and tetrahydrofolic acid, were unable to replace ascorbate for C-P4H activity (157, 161, 162). In addition, a recent study has examined HIF-hydroxylase (purified recombinant PHD2 and FIH) activity in relation to ascorbate and other reductants and, similar to C-P4H, ascorbate was necessary for full substrate hydroxylation, substantially increasing both the initial rate and extent of HIF-1α domain hydroxylation (164). Glutathione and DTT were not able to substitute for ascorbate, although DTT was able to partially enhance PHD2 hydroxylase activity (164). This suggests that the role of ascorbate is unlikely to be that of a general antioxidant to maintain the free iron pool in a reduced state and, in support of this, addition of catalase did not affect HIF-hydroxylase activity either (164).

Ascorbate may be structurally specific to the hydroxylase active site, although an exact binding position is unknown. There is some support for its direct binding to the active site iron in C-P4H, where it may act as an inner-sphere reductant for the iron (143, 159). However, a study using a plant member of the 2-OG-dependent dioxygenases family, anthocyanidin synthase, reported a plausible ascorbate binding site within the enzyme-substrate complex (165). This provides an alternative possible mechanism for ascorbate, where it may stabilise the enzyme-substrate interaction to facilitate hydroxylase activity. The effect of ascorbate structural analogues on PHD2 and FIH activity revealed that the ene-diol reducing moiety of ascorbate is essential for hydroxylase activity, but the mechanism of action in the hydroxylase reaction cycle is still unclear (164).
1.5.2 Ascorbate and HIF-1 regulation

There is a paucity of data on the direct role of ascorbate on HIF-hydroxylase activity, particularly using native enzyme and substrate in live cells. Similarly, there are few reports on the effect of ascorbate on HIF-1 itself. Separate *in vitro* studies have shown that ascorbate (25, 100 or 500 µM in culture medium) was able to suppress HIF-1α protein stabilisation and transcriptional activity due to pyruvate and oxaloacetate (140), CoCl\(_2\) (166, 167), DFO (167) and insulin-like growth factor or insulin (167). The response to hypoxia is less clear, with some of these studies reporting that ascorbate could inhibit HIF-1 at 1-3% O\(_2\) (166-168), while other results showed no effect of ascorbate at ≤1% O\(_2\) (140, 167). Ascorbate was also able to completely block basal HIF-1α present in oncogenically activated cells that had mutant p53 and PTEN, indicating that it is able to enhance HIF-hydroxylase catalytic capacity in order to cope with the increase in HIF-1α synthesis (167). Analysis of the effect of ascorbate on both HIF-1α protein levels and transcriptional activity in relation to various hydroxylase inhibitors/HIF-1-inducers is lacking.

One *in vivo* study has measured xenograft growth in mice of P493 cells that constitutively expressed stabilised HIF-1α (CA5 mutants; partial deletion of oxygen-dependent degradation domain) (169). Ascorbate supplementation of the animals resulted in significant inhibition of wild-type tumour growth, with no effect on CA5 tumours (Figure 1.8) (169). Interestingly, the mice in this study were able to synthesise their own ascorbate, and the control group also had reduced tumour growth compared to the CA5 group (Figure 1.8) (169). This indicates that ascorbate can inhibit HIF-1-mediated tumour growth in mice, and that higher concentrations have a greater effect. However what plasma and tissue concentrations were achieved was not reported. To my knowledge, there is currently no information relating ascorbate levels, HIF-1 activity and tumour pathology from human tissues, other than what is presented in this thesis.
While it is evident that ascorbate has potential as a regulator of HIF-1, a clearer understanding of its role in HIF-1 activation in human cancer cells is required. Generally, HIF-1 activation is monitored using HIF-1α protein levels only, but its transcriptional activity is also regulated by hydroxylation and the effect of ascorbate on this mechanism needs investigation. Further to this, analysis of human tumour tissue for markers of HIF-1 activity in relation to ascorbate levels will determine whether this mechanism is indeed relevant in human cancers and worthy of further investigation. These issues will be addressed in this thesis.

### 1.6 Ascorbate in human biology

As the HIF-hydroxylases have relatively high requirements for ascorbate (Table 1.2), high intracellular ascorbate concentrations must be maintained to sustain hydroxylase activity and thereby prevent over-activation of HIF-1. In most organisms, ascorbate is synthesised from glucose. Animals produce ascorbate in the liver or kidneys, from which it is transported to
the plasma for distribution to the rest of the body (170). However, primates, including humans, as well as guinea pigs and some species of bat are unable to synthesise it due to evolutionary loss of the terminal synthetic enzyme, gulonolactone oxidase (170). Therefore ascorbate, or vitamin C, is an essential nutrient we must obtain from our diet, with the development of scurvy if intake is inadequate.

In 1747, James Lind, a surgeon with the British Royal Navy, performed what is considered the first human clinical trial in an attempt to treat scurvy. While at sea, he used a group of 12 scurvy-stricken sailors and administered various potential remedies, including cider, vinegar or sea water. However, the only patients to recover were those who received lemons and oranges. The curative anti-scorbutic compound that the citrus fruits contained was identified almost 200 years later as hexuronic acid, which was then named ascorbic acid (ascorbate) for its anti-scurvy properties (171). In the 1970’s ascorbate was shown to be an essential co-factor in collagen hydroxylation, which provides some explanation of the many symptoms of scurvy including gum disease, bleeding and poor wound healing (156, 157).

1.6.1 Biochemistry of ascorbate

Ascorbate has diverse roles in human biology, and many of its known functions are attributable to its action as an electron donor. Ascorbate has two ionisable hydroxyl groups with pKₐ values of 4.2 and 11.6, meaning at physiological pH, it is present as the ascorbate monoanion (Figure 1.9) (172). It readily undergoes two consecutive, reversible, one-electron oxidations, resulting in the ascorbate radical and dehydroascorbate (DHA; Figure 1.9) (172). The ascorbate radical is relatively unreactive, but two molecules will dismutate at a high rate, generating one ascorbate and one DHA (173, 174). Ascorbate radical and DHA are easily reduced back to ascorbate enzymatically by glutathione- or NADPH-dependent reductases (173, 174), but if not reduced, DHA will undergo spontaneous and permanent decay (175). Ascorbate is an excellent antioxidant, both thermodynamically and kinetically, and is able to neutralise many highly reactive oxidising species as well as recycling other biologically important reductants, such as tocopherol and glutathione (reaction 1) (176). Ascorbate is therefore known as a terminal small molecule antioxidant (176).
At physiological pH, ascorbate exists as the ascorbate monoanion and can undergo two consecutive, reversible, one-electron oxidations to produce the ascorbate radical and dehydroascorbate, respectively. Adapted from Kall, 2003 (172).

\[
\text{AscH}^- + X^* \rightarrow \text{Asc}^+ + \text{XH}
\]  

(1)

Ascorbate also interacts with iron, and has long been known to increase its intestinal absorption by reducing insoluble ferric iron complexes to stable, soluble, ferrous chelates that are readily absorbed (177, 178). It has also been shown to modestly chelate iron intracellularly (179), which supports its specific role in stabilising and maintaining enzyme-bound Fe\(^{2+}\) for various hydroxylase reactions. Paradoxically, the combination of ascorbate and free iron (or other transition metals) can initiate chain reactions generating highly reactive oxidants such as the hydroxyl radical (176). However, as most transition metal ions are sequestered by carrier proteins, this effect is not considered likely to be biologically significant (173, 180).

In addition to its general antioxidant role, ascorbate has many other functions in the body. Similar to C-P4H and the HIF-hydroxylases, several other iron- or copper-containing enzymes also require ascorbate as a co-factor and it may function as an electron donor for the active site metals (181). These enzymes are involved in carnitine synthesis, conversion of dopamine to noradrenaline and \(\alpha\)-amidation of pro-hormones (173, 181). Ascorbate may also affect the functioning of the immune system, where it is known to decrease histamine levels, increase complement protein C1q formation, enhance immune cell function and regulate prostaglandins (173). These activities cover a range of important biological functions. The
addition of the HIF-hydroxylases to the list of enzymes for which ascorbate is a co-factor significantly increases the scope of its biological activities and suggests that it is highly desirable to maintain adequate tissue levels for optimal health.

1.6.2 Cellular ascorbate uptake

Tissue ascorbate levels vary significantly and this is generally thought to reflect a functional requirement. The highest levels are found in the adrenal medulla where noradrenaline is synthesised and in the pituitary gland where many hormones are produced (182). The brain also has high levels and is the last organ to be depleted during deficiency (183), and many other tissues, including white blood cells, contain high concentrations. Tissue ascorbate levels are maintained by specific active transporters identified as sodium-dependent vitamin C transporters (SVCTs), with two known isoforms – SVCT1 and SVCT2 (184). SVCT1 is thought to be specifically involved in gastrointestinal absorption and renal reabsorption and SVCT2, being expressed in most tissues, is thought to be responsible for whole body cellular uptake (185).

Plasma ascorbate saturation from gastrointestinal absorption is ~100µM and the SVCTs transport it against a concentration gradient resulting in intracellular concentrations up to 100-fold higher than circulating plasma levels (186, 187). This results in the accumulation of millimolar concentrations into cells, provided there are adequate circulating levels (188).

DHA can also be taken up into cells by facilitative transport using glucose transporters (GLUTs), particularly GLUT-1 and GLUT-3 (189). Once inside the cell, DHA is rapidly reduced back to ascorbate (185). However, this mode of ascorbate accumulation is unlikely to contribute significantly to intracellular ascorbate levels under physiological conditions as the amount of DHA accounts for less than ~5% of total ascorbate in biological fluids (190). In addition, transport via the GLUTs is in competition with glucose, which is generally present in higher concentrations (190). An exception to this may be in highly oxidising microenvironments, such as the inflammatory milieu of neutrophils and monocytes, which
express high levels of GLUTs and preferentially take up DHA (191). This process could also occur within the tumour microenvironment (192) where GLUT levels may be elevated. However, the issue of ascorbate accumulation into tumour tissue has never been explored. This will be an aim of this thesis.

1.6.3 Pharmacokinetics of ascorbate

Intracellular ascorbate concentrations depend on the circulating plasma concentration available to the cells for uptake. The apparent K_m values for the SVCTs have been determined in various cultured human cell lines, and range between 65-237 µM for SVCT1 and 8-62 µM for SVCT2, the primary mediator of intracellular uptake (193). An optimal plasma level to achieve tissue saturation is ~70-80 µM, which corresponds to a dietary intake of ~200 mg per day. If plasma levels fall below ~20 µM tissue accumulation becomes significantly impaired (194). This suggests that access to concentrations in the range of those found in plasma is required to sustain intracellular levels. Whether cells in a growing tumour, with poor access to the vasculature, are able to accumulate sufficient intracellular ascorbate is unknown. In addition, several studies have shown that cancer patients have significantly lower plasma ascorbate levels compared to healthy controls (195-200) which may limit tumour cell uptake. Therefore, there is a high probability that tumour tissue may have low ascorbate levels which in turn can promote HIF-1 activation. Investigation of this hypothesis is a focus of this thesis.

Given the difficulty of accessing the plasma supply, it is possible that better delivery of ascorbate to tumour cells may require supra-physiological plasma levels. However, plasma levels are tightly controlled and do not normally exceed ~100µM with dietary intake (194). The SVCTs tightly regulate the plasma concentration, as they have saturable transport kinetics at both the intestine and kidney to limit absorption and reabsorption (193), therefore excess oral ascorbate is eliminated via the gastrointestinal tract. Intravenous administration of ascorbate bypasses this tight control and can yield plasma levels up to 100-fold higher with maximum levels of up to 15 mM (Figure 1.10) (201). Whether these high concentrations
would significantly increase delivery to tumour cells or cellular uptake is unknown, but investigation of this possibility may be of particular relevance to cancer.

Figure 1.10: Predicted plasma ascorbate concentrations following oral or intravenous administration. Pharmacokinetic modelling from healthy subjects predicts a maximum plasma ascorbate concentration of ~200 µM following high-dose oral supplementation (A) whereas intravenous administration can achieve up to 15 mM ascorbate (B). From Padayatty, et al., 2004 (201).
1.7 Ascorbate and cancer

Ascorbate has a controversial history in relation to cancer treatment. In the 1970s, Ewan Cameron and Linus Pauling used both intravenous and oral ascorbate to treat 100 advanced cancer patients and found significant improvements in survival time and quality of life (202, 203). Subsequently, the Mayo Clinic sought to further investigate their findings by performing randomised, double-blind, placebo-controlled studies, using only oral ascorbate, initially with 60 advanced cancer patients who had received prior chemotherapy (204), or later in 100 colorectal cancer patients who had not received prior treatment (205). These clinical trials found no difference between ascorbate and the placebo, and ascorbate as a cancer therapy was effectively dismissed. Recent pharmacokinetic data showing the much higher plasma concentrations achievable by intravenous administration, as used by Cameron and Pauling’s studies, but not the Mayo Clinic (Figure 1.10), has reignited debate surrounding ascorbate’s potential in cancer treatment. Today, its high-dose administration to cancer patients is widespread (206), and there is an urgent need for mechanistic and clinical evidence to inform this practice.

The renewed interest for a role for ascorbate in cancer has generated a growing body of in vitro and animal studies to determine its effectiveness against cancer cell survival and tumour growth, particularly using high millimolar doses (186). One hypothesis to support the use of high-dose ascorbate is the finding that at these pharmacological concentrations, achievable with intravenous administration, it may act as a prodrug to deliver extracellular \( \text{H}_2\text{O}_2 \) that is selectively toxic to cancer cells (207). At millimolar levels, ascorbate can interact with protein-bound transition metals leading to ascorbate radical accumulation that has been proposed to interact with \( \text{O}_2 \) to produce superoxide which then dismutates to \( \text{H}_2\text{O}_2 \) (208). These reactions can occur only in the interstitial fluid, but not in plasma, and are independent of intracellular concentrations (207, 208). However, exactly which extracellular proteins are involved has not yet been shown. Furthermore, the ascorbate radical chemistry may be different in hypoxic tumour tissue where \( \text{H}_2\text{O}_2 \) production may be ineffectual in regions of low oxygen. Further to this, cancer cells may have sufficient antioxidant defences similar to, if not greater than, normal cells (209, 210). Nevertheless, these studies on the pharmacological, extracellular, pro-oxidant role of ascorbate against tumour cells are an
important insight into a potential mechanism that has been lacking since the early clinical trials.

Given its ability to regulate the HIF hydroxylases, it would seem reasonable to hypothesise that an additional mechanism of ascorbate against cancer cells is through its inhibition of HIF-1. It is likely that the optimal functioning of the HIF-hydroxylases requires physiological intracellular ascorbate concentrations, and these have been shown to decrease tumour growth in mice (211, 212). These studies used *Gulo-/-* mice which lack the ability to synthesise ascorbate, thus allowing for comparison between ascorbate-deficient and supplemented mice. Although these studies did not measure HIF-1, they support the hypothesis that optimal intracellular ascorbate levels can inhibit tumour growth, where ascorbate’s effect on HIF-1 is likely to contribute to this (169).

These studies strongly suggest that ascorbate may have anti-cancer activity. However, in the context of regulating HIF-1 activation, these effects have only been modestly studied. In addition, whether ascorbate levels in human tumours correlate with HIF-1 activation and whether this could affect tumour growth conditions has not been investigated. In my thesis I aim to address these issues.

### 1.8 Thesis aims

HIF-1 is ‘switched off’ by two distinct hydroxylation reactions that require ascorbate as a cofactor. Therefore, maintenance of a sufficient concentration of intracellular ascorbate in a growing tumour may suppress the HIF-1 response. This has led to the hypothesis that an inadequate vascular network could deprive tumour cells of a sufficient ascorbate supply, resulting in exacerbated HIF-1 activation.

The aims of this thesis are to address this question using a number of approaches. Specifically:
1. The effect of intracellular ascorbate concentrations on the induction of HIF-1 can be investigated using cultured cells. An initial aim was, therefore, to measure the precise intracellular ascorbate concentrations accumulated by a number of different cancer cells lines (Chapter 3).

2. Whether intracellular ascorbate concentrations have an effect on the HIF-1 activation profile in response to a variety of inducing conditions was of interest. The aim was to determine the effect of ascorbate on HIF-hydroxylase activity/HIF-1 induction in cultured cancer cells. Both HIF-1α protein stabilisation (PHD activity) and transcriptional activity (FIH activity) were monitored (Chapter 4).

3. To extend the hypothesis that there is a relationship between low cellular ascorbate levels and HIF-1 activation in human cancer, the analysis of human tumour tissues was required. The aim was to quantify ascorbate content and markers of HIF-1 activity in tissue samples from endometrial (Chapter 5) and colorectal (Chapter 6) cancer.

4. To investigate whether avascular regions of a tumour are indeed likely to be deprived of ascorbate, data on the extravascular transport kinetics of ascorbate through tumour tissue was acquired using an in vitro pharmacokinetic model (Chapter 7).
Chapter 2: Materials and Methods

Materials and equipment used are listed in Table 2.1. The H₂O used in all experiments, and for making buffers and reagents, was ultrapure, sourced from a Heal Force Ultrapure water system (Acorn Scientific Ltd., Auckland, NZ).

Table 2.1: Materials and specialist equipment used including suppliers and sources.

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## Chapter 2: Materials and Methods

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**Specialist equipment**

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2.1  Cell culture

Three immortal cancer cell lines were obtained from the ATCC (Ishikawa, WiDr, Jurkat). Ishikawa cells are endometrioid adenocarcinoma, WiDr are colon adenocarcinoma and Jurkat cells are T cell leukaemia. Human umbilical vein endothelial cells (HUVECs) are primary cells and were used within 4-5 passages. HUVECs were extracted by collagenase from human umbilical veins from normal donors after informed consent and ethical approval from the Upper South B Regional Ethics Committee, New Zealand (166).

Sterile technique was used throughout all cell handling procedures. All cell lines used were cultured in their appropriate medium containing 10% FBS, penicillin (1,000 U/ml) and streptomycin (1,000 µg/ml) and incubated at 37°C in a humidified atmosphere containing 5% CO$_2$. Ishikawa cells were grown in MEM-alpha medium (no nucleosides), WiDr cells in DMEM high glucose medium and Jurkat cells in RPMI 1640 medium. HUVECs were grown in Medium 199 supplemented with endothelial cell growth factor and heparin on gelatin-coated plasticware (166). Adherent cells (Ishikawa, WiDr, HUVEC) were routinely passaged when confluent by dissociation from their flasks using TrypLE® solution, diluted 1:5 with fresh medium and re-seeded at ~0.2 x 10$^6$ cells/mL. Jurkat cells were maintained in suspension at a density of ~0.5 – 0.8 x 10$^6$ cells/mL and diluted into fresh medium as required.

2.2  Cell volume

Intracellular water volume of each cell line was measured using the method of Kletzien et al. (213) This method utilises a radiolabeled, metabolically inert glucose compound (3-O-methyl[$^3$H]-D-glucose) and assumes the intracellular glucose concentration equilibrates equivalently between intracellular and extracellular compartments, thus allowing calculation of the intracellular water volume. Ishikawa, WiDr and HUVEC cells were seeded onto 12-well plates and grown to ~90% confluency. The culture medium was aspirated and cells were washed with 10 mM phosphate buffered saline (pH 7.6; PBS), and 2 mL warmed, 3-O-methyl[$^3$H]-D-glucose solution (250 µM in Hanks buffer without glucose) was added for 20
minutes at 37°C. For each cell type, quadruplicate wells were tested and from each well, 100 µL of the solution was sampled in duplicate and added to 2 mL of scintillation fluid (Ultima Gold XR) for determination of the extracellular concentration. The stock solution used in each experiment was also sampled.

The remaining solution was aspirated and the cells were washed twice in 2 mL PBS containing 0.1 mM phloretin, which prevents efflux of the radiolabeled glucose from the cells while also removing any extracellular radioactivity. 100 µL of the last wash was sampled in duplicate to ensure no extracellular radioactivity contributed to the intracellular counts. The remaining PBS/phloretin was aspirated and cells were lysed in the wells by adding 100 µL 0.2 M NaOH. The whole cell lysate was added to 2 mL of scintillation fluid. Four additional wells were treated with Hanks buffer (without glucose) only and were used for cell counting after trypsinisation. Samples were analysed on a Packard Tri-Carb® liquid scintillation counter.

As Jurkat cells grow in suspension, the same experiment was performed in 1.7 mL Eppendorf tubes in a volume of 1 mL. Cells were pelleted, washed in PBS/phloretin twice before the cells were counted and the pellet was lysed in NaOH, as above.

The 3-O-methyl[^3]H]-D-glucose concentration used was based on optimal counts measured: 100 µL solution contained 60,000 – 70,000 cpm under the conditions used for detection. Cell counts were in the general range of 0.3 – 0.7 x 10^6 per well, which would give ~600 – 700 cpm per cellular sample assuming a minimum of 1 µL intracellular volume.

The intracellular volume was determined from these measurements as follows: the original stock concentration (250 µM) was first re-calculated based on a half-life of 4537 days for[^3]H (e.g. after 220 days, stock = 242 µM). The cpm of the stock was then used with this concentration to give cpm/nmol of 3-O-methyl[^3]H]-D-glucose. This was used to determine the intracellular and extracellular concentrations and thus the intracellular water volume per
$10^6$ cells (using 100 µL sample in 2 mL scintillation fluid volumes throughout). The calculations used are demonstrated below.

- $\text{cpm} / \times \text{nmol} = \text{cpm/nmol in stock solution}$.
- Extracellular conc. (nmol/µL) = (cpm in medium/cpm stock solution) / $x$ µmol stock.
- Intracellular conc. (nmol/$10^6$ cells) = [cellular cpm/(cpm/nmol)] / cell count.
- µL/$10^6$ cells = intracellular conc./extracellular conc.

### 2.3 Intracellular ascorbate uptake

Adherent cells were seeded on 24-well plates and grown to ~90% confluence. Various concentrations of sodium-$L$-ascorbate were added to the culture medium overnight (or a series of time points). Fresh, 100 mM stock sodium-$L$-ascorbate solution was prepared in PBS, filter-sterilised and a 10 mM dilution was prepared using sterile PBS. This allowed volumes of 5 – 25 µL per mL of medium to be added to achieve extracellular concentrations of 50 – 1000 µM.

After incubation with ascorbate, the cells were washed twice in PBS to remove any extracellular ascorbate and the cells were detached, collected, counted, then pelleted. The supernatant was aspirated, 50 µL 0.54 M perchloric acid (containing 50 mM DTPA) added, to precipitate protein while liberating and stabilising intracellular ascorbate, and 50 µL H$_2$O added as a diluent. Samples were vortexed for ~10 seconds before immediate freezing and storage at -80°C. Before analysis, samples were thawed and the protein pelletted. The supernatant was then analysed by high performance liquid chromatography (HPLC) as described below. The amount of ascorbate per $10^6$ cells was related to the available intracellular water volume to give an estimate of intracellular concentration.
2.4 Ascorbate HPLC

All ascorbate measurements were performed using HPLC with electro-chemical detection (HPLC-ECD) (214). The mobile phase was 80 mM sodium acetate, pH 4.8 (using glacial acetic acid) containing 0.54 mM DTPA and 0.017% (v/v) n-octylamine and run isocratically at 1.2 mL/min. A Waters 600E multi-solvent delivery system was used with an Aqua 5µm, 150 x 4.6 mm, C18 column and an ESA Coulochem® II detector (guard cell, 300 mV; electrode 1, 200 mV; sensitivity, 20 µA). A loop size of 20 µL was used and samples ran for 7 minutes each, either injected manually or from an autosampler (maintained at 4°C; acquired in 2011). Ascorbate eluted at ~3 minutes with no other interfering peaks (Figure 2.1). Chromeleon 6 software (Dionex/Thermo Fisher Scientific, Sunnyvale, CA, USA) was used for data acquisition, integrating the ascorbate peaks and calculating the peak area.
Figure 2.1: Representative ascorbate peaks obtained from HPLC-ECD analysis. A, representative chromatogram from fresh sodium-L-ascorbate standard (40 μM or 0.8 nmol). B, representative chromatogram obtained from a tissue sample (note different scales).
Ascorbate standard curves (Figure 2.2) were prepared fresh for each run using sodium-L-ascorbate in 77 mM perchloric acid containing 25 mM DTPA. The standard ascorbate concentration was verified by spectrophotometry, where a 50 µM solution should have an absorbance of 0.493 at 245 nm. Standards were serially diluted 1:1 from 40 to 1.25 µM.

![Ascorbate Standard Curve](image)

**Figure 2.2:** **Representative ascorbate standard curve.** Sodium-L-ascorbate standards were diluted from a fresh 50 µM solution verified by spectrophotometry and analysed by HPLC-ECD.

2.5 **HIF-1-induction**

HIF-1 was inducible in all cell lines (~90% confluence) by exposing the cells to different inhibitors of the HIF-hydroxylases. Under normal culture conditions the cells were ascorbate-deficient, and when required, were pre-loaded with ascorbate overnight as described in
section 2.3. CoCl$_2$, NiCl$_2$, DMOG and DFO stock solutions (100 mM) were dissolved in H$_2$O and added separately to the culture medium. Incubation with the cells was for four or eight hours, then the medium was removed, the cells washed with PBS and lysed in the wells using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (60 mM Tris pH 4.8, 2% SDS, 20% glycerol, 0.1 M DTT, complete mini protease inhibitor cocktail and bromophenol blue) at a volume of 100 µL per ~1 x 10$^6$ cells. Cell lysates were scraped from the well with a rubber scraper, collected into Eppendorf tubes and frozen immediately at -20°C. Exposure of cells to decreasing oxygen tensions was achieved by incubation in a hypoxic chamber at 37°C, humidified and gassed with N$_2$ and 5% CO$_2$, and maintained using a calibrated ProOx C21 gas controller fitted to the chamber. Upon removal, the cells were lysed as described above within 30 seconds and extracts frozen at -20°C.

2.6 Clinical samples

All human tissue samples were donations to the Cancer Society Tissue Bank, Christchurch (CSTBC), and were approved for use by the CSTBC board. All tissue donors gave informed, written consent allowing research on their samples and access to medical records. Ethical approval was granted by the Upper South B Regional Ethics Committee, New Zealand.

2.6.1 Endometrial tumour and normal samples

Endometrial tumour tissue samples were obtained from women undergoing hysterectomy for the removal of endometrial tumours. As part of surgical resection, normal endometrial tissue was also obtained from each patient, providing paired normal and tumour tissue for each case. Surgical samples, excess to that required for diagnosis, were flash-frozen and stored at -80°C within an hour of being resected. All associated clinical and pathology data was stored in a secure relational database by the CSTBC curator. A total of 51 normal and tumour sample pairs were analysed (102 total tissue samples).
2.6.2 Colorectal tumour and normal samples

Colorectal tumour tissues were obtained following surgical removal of tumours, with normal tissue samples coming from adjacent tissue removed with the tumour as part of resection. A total of 50 normal and tumour tissue pairs were analysed (100 total tissue samples). As with the endometrial samples, banked tissues were dissected and frozen at -80°C within an hour of collection.

2.6.3 Tissue sample preparation

Frozen tissue samples were ground to a fine powder with liquid nitrogen in a mortar and pestle sitting on dry ice. The ground tissue was briefly thawed to measure the wet weight, then 200 µL cold 10 mM phosphate buffer (pH 7.4) was added to make a homogenous tissue suspension. Figure 2.3 describes how the tissue homogenates were processed. For each sample, a 40 µL aliquot was removed for DNA analysis and frozen at -20°C. A further 40 µL aliquot was removed for ascorbate analysis and 40 µL 0.54 M perchloric acid containing 50 mM DTPA was immediately added to precipitate the protein and was immediately frozen at -80°C. The remaining 120 µL was frozen at -20°C for Western blotting and the appropriate volume of sample buffer was added so that they were balanced for DNA content (12 µg DNA per 100 µL sample for endometrial tissues or 15 µg DNA per 100 µL for colorectal tissues).
Figure 2.3: **Schematic of tumour and normal tissue sample processing.** The tissue homogenate was divided into aliquots for different analyses.

### 2.7 DNA assay

Endometrial and colorectal tissue samples were analysed for DNA content, as an estimate of the cellularity of the tissue, by monitoring binding to propidium iodide. A 40 µL aliquot of the homogenate obtained after tissue grinding was diluted to 1 mL with 10 mM phosphate buffer (pH 7.4). In order to free the DNA with as little chemical interference as possible, the solution was rapidly freeze-thawed three times using ethanol/dry ice and a 37°C water bath then sonicated for ~20 seconds. This procedure was previously shown to yield the greatest amount of DNA (IGM Molenaar and MCM Vissers, unpublished results).

In order to achieve readings from each sample within a linear standard range, the samples were serially diluted 1:1 twice. A standard curve of DNA was prepared using purified calf thymus DNA (Figure 2.4), the concentration of which was verified by monitoring the
absorbance at 280 nm using a Nanodrop analyser. Standards were serially diluted 1:1 from 40 to 1.25 µg DNA per mL in 10 mM phosphate buffer (pH 7.4). 100 µL of each sample and standard was added per well in triplicate to a black 96-well microplate. Propidium iodide was added (1 mg/mL) for 10 mins, RT in the dark and subsequent fluorescence was measured on a POLARstar fluorescent plate reader at 544-590 nm.

![Figure 2.4: Representative DNA standard curve. Purified calf thymus DNA was used as a standard, measured by propidium iodide fluorescence emission at 590 nm.](image)

**Figure 2.4:** Representative DNA standard curve. Purified calf thymus DNA was used as a standard, measured by propidium iodide fluorescence emission at 590 nm.
2.8 Western blotting

Protein levels of HIF-1α, BNIP3, GLUT-1 and β-actin were monitored in both clinical and in vitro samples using Western blotting.

2.8.1 SDS-PAGE

All samples were lysed in SDS-PAGE sample buffer (60 mM Tris pH 4.8, 2% SDS, 20% glycerol, 0.1 M DTT, complete mini protease inhibitor cocktail and bromophenol blue), heated in a boiling water bath for five minutes and subjected to SDS-PAGE, based on the Laemmli method (215), using a Bio-Rad Mini-PROTEAN system. A 3% acrylamide stacking gel was used together with the appropriate percent acrylamide resolving gel as per Table 2.4. Gels were run in Tris/glycine buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, with 0.1% (w/v) SDS at 200 V until the dye front was near the bottom (usually ~40-45 minutes).

2.8.2 Immunodetection

Resolved proteins were electrophoretically transferred from acrylamide gels to PVDF membranes using Mini-Trans-Blot® Cells in cold Western blotting buffer (25 mM Tris, 192 mM glycine, pH 8.3, with 10% methanol) for 50-70 minutes. Non-specific binding sites were then blocked in 5% (w/v) non-fat milk powder or 1% (w/v) BSA in Tris buffered saline with 0.05% (v/v) Tween®-20 (TBST), as per Table 2.4. The membrane was then incubated in primary antibody (10 mL) overnight at 4°C then washed in several changes of TBST for at least 20 minutes before adding horseradish peroxidase-conjugated (HRP) secondary antibody (10 mL) for 1 h, RT and washed again. β-actin was used as a loading control by re-probing each membrane with anti-β-actin primary antibody after detection of the protein of interest and two washes. All antibodies were diluted in TBST and the concentrations used for Western blotting are shown in Table 2.4.
2.8.3 Quantification of protein expression by Western blot in clinical samples

Chemiluminescent detection was performed using ECL™ Plus detection reagent and the signal was captured using a Bio-Rad ChemiDoc XRS gel documentation system and quantified by densitometry (Quantity One® 4.6.1 software; Bio-Rad Laboratories, Hercules, CA, USA). This involved normalisation of each sample to the same positive control, which was included on each gel, to account for inter-blot variation. In addition, β-actin was used as a loading control to account for intra-blot variation.

Table 2.4 Conditions and antibodies used for Western blotting.

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<td>7</td>
<td>5% milk, 1 h</td>
<td>Goat polyclonal anti-HIF-1α 1:2,500 Mouse monoclonal anti-HIF-1α 1:1,000</td>
<td>Rabbit anti-goat-HRP 1:20,000 Goat anti-mouse-HRP 1:2,500</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>10</td>
<td>5% milk, 1 h 1% BSA, 1 h</td>
<td>Rabbit polyclonal anti-GLUT-1 1:3,000</td>
<td>Goat anti-rabbit-HRP 1:20,000</td>
</tr>
<tr>
<td>BNIP3</td>
<td>12</td>
<td>5% milk, 1 h</td>
<td>Goat polyclonal anti-BNIP3 1:1,000</td>
<td>Rabbit anti-goat-HRP 1:20,000</td>
</tr>
<tr>
<td>β-actin</td>
<td>N/A</td>
<td>N/A</td>
<td>Mouse monoclonal anti-β-actin 1:10,000</td>
<td>Goat anti-mouse-HRP 1:2,500</td>
</tr>
</tbody>
</table>

2.9 VEGF ELISA

VEGF protein levels were measured in cell culture medium or tissue homogenate using a commercial ELISA kit which supplied all antibodies and human VEGF standard (216). Capture antibody (mouse anti-human VEGF; 100 µL of 1 µg/mL) was added to 96-well high binding enzyme immunoassay plates (Costar EIA) overnight, RT. The wells were washed out
with wash buffer [PBS with 0.05% (v/v) Tween®-20; PBST] three times, then 300 µL of blocking buffer [1% (w/v) BSA in PBS, 0.2 µm filtered] was added for 1-2 h, RT and the wash procedure repeated. 100 µL of samples and standards (diluted in blocking buffer if necessary) were added in duplicate to each well and incubated for 2 h, RT. The wash step was repeated and 100 µL biotinylated detection antibody (goat anti-human VEGF; 0.1 µg/mL) was added for 2 h, RT and washed again. 100 µL of streptavidin-HRP (diluted in blocking buffer depending on kit instructions) was added for 20 min, RT, in the dark, then washed again. Chromogenic peroxidase substrate solution (TMB One-Step substrate) was added (100 µL) for 20 min, RT, in the dark before adding 50 µL of 1 M H₂SO₄ to stop the reaction. Absorbance was read immediately at 450 nm with background correction at 540 nm on a SoftMax Pro spectrophotometric plate reader. VEGF protein concentration in the samples was calculated from the standard range using a 4-parameter logistic (4-PL) standard curve.

2.10 HIF-1α DNA-binding

In order to examine the level of transcriptionally active HIF-1 in vitro, ascorbate-deficient or pre-loaded Ishikawa cells were treated with 100 µM CoCl₂ for eight hours, and the nuclear extract analysed for DNA-bound HIF-1α using a commercial ELISA. This assay captures HIF-1α protein that is bound to a specific, biotinylated double-stranded oligonucleotide containing a consensus HIF-1α binding site, which is then detected using streptavidin-HRP.

2.10.1 Nuclear extract preparation

After treatment, cells were detached with TrypLE®, pelleted, washed in PBS, counted, pelleted and all PBS removed. The cell pellet was then solubilised in ice cold lysis buffer A [10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1% (v/v) NP40, PhosSTOP phosphatase inhibitor cocktail and Complete Mini protease inhibitor cocktail] at 1 mL per 2.5 x 10⁷ cells and gently pipetted up and down to mix before centrifugation at 16,000 x g for 5 minutes at 4°C. The cytosolic supernatant was discarded and ice cold lysis buffer B [20 mM
HEPES, 1.5 mM MgCl$_2$, 420 mM NaCl, 0.5 mM DTT, 25% (v/v) glycerol, PhosSTOP phosphatase inhibitor cocktail and Complete Mini protease inhibitor cocktail] was added at 1 mL per 1 x 10$^8$ cells, vortexed and incubated on ice for 20 minutes before centrifugation at 16,000 x g for 5 minutes at 4°C. The nuclear supernatant was recovered and used for protein determination and the HIF-1α DNA-binding ELISA.

2.10.2 Protein assay

Sample protein concentration was determined using a Bio-Rad DC protein assay against a standard curve of BSA. In duplicate, 5 µL of samples and standards were added to each well of a 96-well microplate. 25 µL of reagent A was added followed by 200 µL of reagent B and incubated for 15 minutes, RT. Absorbance was read at 750 nm on a SoftMax Pro spectrophotometric plate reader.

2.10.3 DNA-binding ELISA

Costar EIA 96-well plates were coated with 100 µL of capture antibody (4 µg/mL) per well and incubated overnight, RT. Plates were washed out three times with PBST and 300 µL of blocking buffer [5% (w/v) BSA in PBST] was added for 1-2 h, RT and washed again. The ELISA kit provided a solution of biotinylated double-stranded oligonucleotide (ds-oligo’) containing a consensus HIF-1α binding site, and this (3 µL) was incubated with 50 µg of each nuclear extract sample, with the volume made up to 30 µL with lysis buffer B, for 30 minutes, RT. Selected samples had 3 µL of an unlabeled ds-oligo’ added to determine specificity by competition. 200 µL of blocking buffer was added to each sample, and 100 µL was added in duplicate to the plate and incubated for 2 h, RT. The plates were washed again and 100 µL streptavidin-HRP was added for 20 minutes, RT, in the dark then washed five times. TMB One-Step substrate solution was added (100 µL, 20 minutes, RT, in the dark) and the reaction stopped by adding 50 µL of 1 M H$_2$SO$_4$. Absorbance was measured immediately at 450 nm with background correction at 540 nm on a SoftMax Pro spectrophotometric plate reader.
2.11 HIF-1α immunohistochemistry

A formalin fixed, paraffin embedded tissue microarray of 62 endometrial tumour 1 mm cores (33 from the same tumours additionally analysed in this thesis) was obtained from the CSTBC and immunostained for HIF-1α according to the protocol developed by Mackenzie et al. (217). Sections were cut on a microtome to 3 µm thickness onto charged glass slides. Slides were baked for ~1-2 h at 60°C to maximise tissue adhesion. While still warm, slides were deparaffinised and rehydrated by placing in xylene for 5 minutes twice (consecutive baths) then 95% ethanol for 5 minutes twice then in H₂O for one minute twice and set aside in PBS. Slides were incubated in antigen retrieval buffer [10 mM Tris, 1 mM EDTA and 0.05% (v/v) Tween®-20, pH 9.0] for 40 minutes in a water bath at 90°C. When cooled, slides were rinsed in PBST twice.

The staining procedure was performed using a commercial kit (HRP-DAB tissue staining kit – mouse) which supplied all reagents except the primary antibody. All incubations were carried out at RT in a humidified chamber and excess reagent was carefully drawn off using the corner of a tissue after each step. Using a Dako Pen, a water-repellent ring was drawn around each section and peroxidase blocking reagent was added for 5 minutes then gently rinsed off with PBS. Serum, avidin and biotin blocking reagents were added consecutively for 15 minutes each and were rinsed with PBS after the avidin and biotin blocking only.

Primary antibody (monoclonal mouse anti-HIF-1α; 1:25 in Dako antibody diluent) was added overnight at 4°C. Negative controls included omitting the primary antibody or adding non-immune mouse IgGκ (1 mg/mL) in Dako antibody diluent as an isotype control. Primary antibody was rinsed off with PBS and slides were washed by soaking in PBS twice followed by PBST for 5 minutes each. Biotinylated secondary antibody was added for 60 minutes, slides washed, then streptavidin-HRP added for 30-60 minutes and slides washed. DAB chromogenic substrate solution was added and rinsed off with H₂O after ~20 minutes or when staining was visualised microscopically. Counterstaining with haematoxylin and eosin and coverslipping was performed using a Leica autostainer.
Scoring of HIF-1α immunostaining was carried out by three individuals, where a relative score of 0-6 was given for both intensity and coverage of staining then multiplied together allowing a maximum score of 36. The average of the three independent scores was used.

2.12 Ascorbate extravascular transport

Studies on the diffusion of ascorbate through multicellular layers were performed at the Auckland Cancer Society Research Centre (ACSRC) in collaboration with Dr. Kevin Hicks using a model system of extravascular drug transport in tumour tissue (218, 219). The multicellular layer (MCL) and diffusion chamber apparatus is depicted in Figure 2.5.
Cross-section of one pair of chambers showing the donor (D) and receiver (R) compartments (6.6 mL final volume in each) separated by a multicellular layer (MCL), which generally grows up to 150 µm in thickness. The Teflon support membrane is shown adjacent to the MCL with a 30 µm thickness. The D and R compartments are magnetically stirred and gassed. The whole apparatus contains 3 pairs of chambers in a row. Ascorbate was added to the donor compartment and 100 µL samples taken using a syringe from both donor and receiver compartments, via the sampling ports, over 5 h. Image courtesy K.O. Hicks (220).

2.12.1 Multicellular layers

HT29 cells were maintained in monolayer in MEM-alpha medium (no nucleosides) with 5% FBS, with weekly passaging. Multicellular layers were grown on sterile collagen-coated Teflon support membranes in Millicell-CM inserts with a sterile polyethylene ring attached around the insert for flotation. HT29 cells were seeded at 1 x 10⁶ cells per insert in 0.5 mL volume and floated in a large reservoir of medium (MEM-alpha medium with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin) for ~6 h at 37°C and 5% CO₂ to allow cell
adhesion. The inserts were then submerged in the medium using a stainless steel wide-mesh grid and incubated at 37°C for 3 days in sealed, magnetically stirred jars.

2.12.2 Ascorbate flux

The MCLs were inspected microscopically to verify uniform growth across the support. They were then inserted into the diffusion apparatus between two compartments (donor and receiver) of culture medium (6.6 mL; Figure 2.5), containing 10 µM EDTA to prevent ascorbate oxidation. Both compartments were magnetically stirred in a 37°C water bath and gassed with 95% N₂ and 5% CO₂ or 95% O₂ and 5% CO₂. The flux of ¹⁴C-urea was used to determine the thickness of each MCL and as a marker of transcellular diffusion and ³H-mannitol flux was measured as marker of paracellular diffusion (221). Fresh sodium-L-ascorbate was added to the donor compartment at concentrations of 0.1, 0.5, 1 and 10 mM, and 100 µL samples of both the donor and receiver compartments were taken using a syringe over five hours to monitor the flux through either the bare Teflon support (i.e. in the absence of cells) or through the MCL. Solutions of both of the radiolabeled internal standards (¹⁴C-urea and ³H-mannitol) were added together with the ascorbate to the donor compartment. Samples were added to 100 µL 0.54 M perchloric acid (containing 50 mM DTPA). Of this, 50 µL was taken for scintillation counting in 3 mL scintillation fluid (Emulsifier Safe) by a Packard Tri-Carb® liquid scintillation counter. The remaining 150 µL was immediately frozen at -80°C for ascorbate measurement by HPLC-ECD as described in section 2.4.

2.12.3 Modelling of flux data

Concentration-time data of ¹⁴C-urea, ³H-mannitol and ascorbate flux through MCLs were fitted to a diffusion model to obtain the MCL thickness (using the known diffusion coefficient of urea) or the diffusion coefficient through tissue (for mannitol and ascorbate), as previously described (218, 219), using the Ficks 2nd law equation for reaction diffusion in a plane slab with ascorbate assumed to diffuse via the paracellular route (equations 1 and 2). The flux experiments were performed on MCLs grown in ascorbate-containing medium (MEM-alpha
is formulated with 50 µM ascorbate) and the cells had high intracellular ascorbate concentrations. Therefore, the ascorbate data was fitted as simple diffusion by nonlinear regression using a custom written FORTRAN program as previously described (218, 219). All MCL diffusion data were fitted with the compound’s diffusion coefficient in the support membrane \( D_{sup} \) fixed at the average value measured in separate stability experiments, where flux was measured through the bare support membrane only.

\[
\frac{\partial C_e}{\partial t} = D_{MCL} \frac{\partial^2 C_e}{\partial x^2} - \frac{\varphi_i}{\varphi_e} \left( \frac{V_{max1} C_e}{K_{m1} + C_e} + \frac{V_{max2} C_e}{K_{m2} + C_e} - k_r C_i \right) \\
\frac{\partial C_i}{\partial t} = \frac{V_{max1} C_e}{K_{m1} + C_e} + \frac{V_{max2} C_e}{K_{m2} + C_e} - k_r C_i - k_i C_i
\]

where \( D_{MCL} \) is the diffusion coefficient in the MCL and \( \varphi_i, \varphi_e \) are the intracellular and extracellular volume fractions in the MCL.

Cell uptake data, obtained from HT29 monolayers, was modelled by Dr. Kevin Hicks, assuming low and high affinity transport into the cell (Michaelis-Menten parameters), including rate constants for passive diffusion out and intracellular turnover (equation 3), while loss from the medium is described by equation 4. The model was fitted to the cell uptake data using Phoenix™ WinNonlin® v6.2 (Pharsight Corp.). Simulations of tumour tissue penetration and cellular uptake were performed using Mathematica V9 (Wolfram Research Inc.) with the same reaction-diffusion equations, assuming cells were ascorbate-deficient, in the same planar geometry as the MCL.
\[
\frac{\partial C_i}{\partial t} = \frac{V_{\text{max}1} C_e}{K_{m1} + C_e} + \frac{V_{\text{max}2} C_e}{K_{m2} + C_e} - k_r C_i - k_i C_i
\]

(3)

\[
\frac{\partial C_e}{\partial t} = -\frac{\phi_i}{\phi_e} \left( \frac{V_{\text{max}1} C_e}{K_{m1} + C_e} + \frac{V_{\text{max}2} C_e}{K_{m2} + C_e} - k_r C_i \right)
\]

(4)

where \( C_i \) and \( C_e \) are the intracellular and extracellular concentrations, \( V_{\text{max}1}, V_{\text{max}2}, K_{m1}, K_{m2} \) are the Michaelis-Menten parameters for high and low affinity uptake respectively, \( k_r \) is the rate constant for passive diffusion out of the cell, \( k_i \) is a rate constant for intracellular turnover, and \( \phi_i, \phi_e = 1 - \phi_i \) are the intracellular and extracellular volume fractions for the experiment.

2.13 Data analysis

Figures were generated using Microsoft Excel or SPSS 19-x for Windows (SPSS Inc., Chicago, IL, USA). Data were assessed as either parametric or non-parametric according to the Kolmogorov-Smirnov and Shapiro-Wilk tests for normality. The appropriate tests for significance were performed (\( \alpha \) level set to 0.05) using SPSS. For non-parametric data, the Wilcoxon signed-rank test (paired data), Mann-Whitney test (unpaired data) and/or Spearman’s correlation coefficient (all two-tailed) were used. For parametric data, Student’s \( t \)-test (two-tailed; paired or unpaired) was used. The Jonckheere-Terpstra test for ordered data (one-tailed) (222) and linear regression analysis were also utilised.
Chapter 3: Intracellular ascorbate concentrations in cancer cells

*in vitro*

The results presented in this chapter have been included in:

**Kuiper C, Dachs GU, Currie MJ and Vissers MCM.** Intracellular ascorbate can prevent induction of the HIF-1 transcriptional response in cancer cells. Manuscript in preparation.

3.1 Introduction

Physiological intracellular ascorbate concentrations in humans are achieved from an optimal plasma concentration of ~70-80 µM, with ascorbate primarily existing in its reduced form (194). Provided cells are continually exposed to these circulating levels, they will accumulate ascorbate up to millimolar levels against a concentration gradient using the vitamin C transporter, SVCT2, which is highly specific for L-ascorbate (184). Most tissues will concentrate ascorbate within a range of approximately 1 to 10 mM (182, 223, 224). Therefore, under optimal, healthy conditions, most cells in the body will have a constant supply of reduced ascorbate to draw on to maintain a high intracellular concentration.

Even in animals that generate their own vitamin C, synthesis only occurs in liver cells (and sometimes kidney cells). Therefore, most mammalian cells in culture, aside from animal liver cells, cannot generate ascorbate, and as cell culture media do not generally contain an ascorbate supplement, cell culture conditions generally reflect a deficiency state (166). By addition of ascorbate to the medium, it can be determined whether cells are able to accumulate ascorbate *in vitro* to reach an intracellular plateau at a biologically relevant level (i.e. millimolar concentrations).
Typically, cellular ascorbate content is measured relative to cell number or total protein, and does not reflect the intracellular concentration that exists in the available water space within the cells. As it is the intracellular concentration that is biologically relevant, particularly in relation to HIF-hydroxylase activity, calculation of the intracellular ascorbate concentration is required. Therefore, accurate measurement of the intracellular water volume, together with the ascorbate content is needed.

3.1.1 Chapter aims

The aim of this chapter was to determine whether cancer cells in culture are able to reach physiologically relevant intracellular ascorbate concentrations and to establish the conditions under which this occurs. Specifically, this chapter will examine:

1. The intracellular water volumes of four cell lines to allow calculation of intracellular ascorbate concentrations.

2. The optimal time and extracellular concentration required to achieve maximal and durable intracellular ascorbate loading.

3.1.2 Experimental approach

Intracellular ascorbate uptake was monitored in three cancer cell lines: Ishikawa (endometrial adenocarcinoma), WiDr (colorectal adenocarcinoma) and Jurkat (T cell lymphoma), and also in normal, primary endothelial cells (HUVEC). The cells were supplied with fresh sodium-L-ascorbate added to the media at various concentrations for specific times under normal culture conditions (see section 2.3). The cultures were generally ~90% confluent. Upon harvesting, the cells were counted, pelleted and the ascorbate extracted into perchloric acid for measurement by HPLC-ECD as described in section 2.4.
For each cell line, the intracellular water volume was determined by uptake of a radiolabeled, metabolically inactive, glucose compound (3-O-methyl[3H]-D-glucose; see section 2.2). This compound equilibrates evenly between all compartments in the culture well and the proportion inside the cell is used to estimate the available water space. This measurement was used, together with the measured cellular ascorbate content, to calculate the intracellular ascorbate concentration as described in section 2.2.

3.2 Results

3.2.1 Intracellular water volume

The volume of the intracellular water space was determined for Ishikawa, WiDr, Jurkat and HUVEC cells and expressed as µL/10⁶ cells (Table 3.1). The assay gave consistent results and provides a reliable estimate of the intracellular water volume in each cell line (213). There were considerable differences between the cell lines, with Ishikawa and WiDr cells having the largest water volumes, and HUVECs having approximately half the volume of these cells. The intracellular volume of Jurkat cells was much less by comparison.

Table 3.1: Intracellular water volume calculated for four cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Intracellular water volume (µL/10⁶ cells) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ishikawa</td>
<td>2.46 ± 0.18 (n=4)</td>
</tr>
<tr>
<td>WiDr</td>
<td>2.81 ± 0.16 (n=3)</td>
</tr>
<tr>
<td>Jurkat</td>
<td>0.34 ± 0.08 (n=3)</td>
</tr>
<tr>
<td>HUVEC</td>
<td>1.29 ± 0.36 (n=2)</td>
</tr>
</tbody>
</table>

n, number of independent experiments.
3.2.2 *Time course and stability of cellular ascorbate uptake in vitro*

To establish an optimal incubation time with ascorbate, and ensure it was stable intracellularly, the cellular ascorbate content was monitored in WiDr cells over 30 hours, following addition of 500 µM ascorbate to the culture medium. Figure 3.1 shows that ascorbate added to the medium was accumulated by the cells within the first 8 hours of incubation. There was no further cellular uptake after this point, and the amount taken up remained in the cells for the remainder of the 30 hour incubation period. As the cells doubled during the incubation period, the intracellular ascorbate content decreased reciprocally (Figure 3.1). Therefore, as the decline in ascorbate content reflects its further cellular distribution, there was no overall loss of ascorbate observed. Based on these data, it appears that incubation with ascorbate overnight (~16 hours) results in intracellular levels that were stable for at least a further 24 hours.

![Figure 3.1: Ascorbate uptake from culture medium and its intracellular stability over time.](image)

*Figure 3.1: Ascorbate uptake from culture medium and its intracellular stability over time.* Fresh sodium-L-ascorbate (500 µM) was added to the culture medium of WiDr cells then incubated for up to 30 hours. The solid line shows the intracellular ascorbate uptake (left-hand y-axis; data are mean ± SD of 3 replicates). The dashed line shows the cell count for each time point (right-hand y-axis) with a reciprocal increase in relation to the ascorbate content of the cells near 16 hours. The accumulated ascorbate remained stable intracellularly for the remainder of the 30 hour time period.
3.2.3 Intracellular ascorbate concentrations achievable under culture conditions

Incubation of Ishikawa, WiDr, Jurkat and HUVEC cells with increasing doses of fresh, sodium-L-ascorbate in the culture medium overnight (~16 hours), resulted in a dose-dependent increase in intracellular uptake (Figure 3.2). The maximum absolute ascorbate content of the different cell lines varied, and ranged from 2.0 to 8.6 nmol/10^6 cells between Ishikawa cells and HUVEC, respectively.

The absolute ascorbate content of the cells (nmol/10^6 cells) was related to the intracellular water volume (µL/10^6 cells; Table 3.1) to give the intracellular ascorbate concentration for each cell line (mM; Figure 3.2). The cancer cell lines accumulated varying intracellular ascorbate concentrations, with a maximum of 0.8, 1.1 and 12 mM for Ishikawa, WiDr and Jurkat cells, respectively. Normal, primary HUVEC reached a maximum concentration of 2.2 mM.

For Ishikawa cells, the intracellular concentration after 16 hours was the same as the extracellular concentration at every dose, indicating that these cells did not concentrate ascorbate. In contrast, WiDr, Jurkat and HUVEC concentrations exceeded the extracellular levels, indicating these cell lines are able to concentrate ascorbate intracellularly (Figure 3.2).

Intracellular levels reached a plateau in HUVEC, and were approaching steady-state saturation in WiDr and Jurkat cells. From these data, the maximum intracellular concentration that is closest to saturation, and achievable under culture conditions, required addition of 1 mM ascorbate to the medium for WiDr cells and 0.5 mM ascorbate for Jurkat cells and HUVEC. Addition of 1 mM to Ishikawa cells was used. Some cell death was observed in Jurkat cells at the 1 mM dose, and this is reflected in the relative variability of the data at this point. For this reason, and because these cells reached high intracellular levels, 0.5 mM was thereafter used as the optimal loading dose.
Figure 3.2: Intracellular ascorbate concentrations of three cancer cell lines (Ishikawa, WiDr and Jurkat) and one primary cell type (HUVEC). Increasing concentrations of fresh sodium-L-ascorbate were added to the culture media overnight (~16 hours) and the intracellular content measured using HPLC-ECD. The left-hand y-axis shows the absolute cellular ascorbate uptake and from this, the intracellular ascorbate concentrations (right-hand y-axis) were calculated from the intracellular water volume. Data are means ± SEM of 3-5 independent experiments.
3.3 Discussion

These results have demonstrated that adding ascorbate to the culture medium is sufficient for stable intracellular uptake to within a physiological range. With the exception of Ishikawa cells, the cell lines reached a maximal uptake that was dose-independent. Ishikawa cells may be capable of much higher intracellular levels, however at extracellular doses greater than 1 mM, toxicity in the medium can occur via the generation of $\text{H}_2\text{O}_2$ (207). Therefore, these cells may have been at sub-optimal levels. The results obtained here have established a protocol for the incubation time and optimal extracellular concentration of ascorbate required to achieve maximal intracellular levels for each cell line. As such, for subsequent experiments, cells were incubated overnight (~16 hours) with addition of 1 mM ascorbate to the medium for Ishikawa and WiDr cells and 0.5 mM for HUVEC and Jurkat cells. All cell lines examined achieved concentrations that are within a physiological range that can satisfy the HIF-hydroxylase $K_m$ requirements (up to 260 µM). Therefore, the protocol is appropriate for subsequent examination of the HIF-1 response in ascorbate-deficient compared to ascorbate-loaded cells.

The intracellular water volume measured for each cell line is in the range of previous approximations of cell volume (166, 225). Ishikawa and WiDr cells are both adenocarcinoma cells of epithelial origin, and had similar water volumes (2.46 and 2.81 µL/10$^6$ cells, respectively). These values are in close agreement with the reported cell volume for CaCo-2 cells (colorectal adenocarcinoma) of 2.61 µL/10$^6$ cells (225), confirming the reliability of this method. HUVEC had less volume at 1.29 µL/10$^6$ cells, which may reflect the flat endothelial morphology of these cells. Jurkat cells had a relatively small water volume (0.34 µL/10$^6$ cells) which is comparable to a previous estimate of 0.20 µL/10$^6$ cells (226), and is in agreement with the known morphology of these small cells which contain very small cytoplasmic compartments (227).

That the intracellular ascorbate concentrations achieved here are physiologically relevant is suggested by what is known of in vivo levels. Accurate measures of ascorbate concentrations in healthy human organs are difficult to obtain, hence exact intracellular concentrations in vivo are largely unknown. However, there are estimates from autopsy tissues showing low
millimolar levels (~0.5-3 mM) in kidney, liver, spleen, pancreas, adrenal glands and pituitary gland (182). These are likely to be underestimates as the concentration is calculated from the tissue weight, not water volume, and the method of quantitation is out-dated. More recent intracellular ascorbate concentrations from healthy blood cells show levels of ~1-4 mM in immune cells and platelets (223), and up to 10 mM in neurons (224). Therefore, from the available information, it would appear that the maximal intracellular ascorbate concentrations achieved here can be considered physiological.

Tissue ascorbate accumulation can become severely impaired if plasma levels fall below ~20 µM (194), and this is also seen in Figure 3.2, where lower extracellular concentrations resulted in poorer intracellular uptake. However, it would appear that the conditions required to achieve physiological intracellular levels in vitro are different to those found in vivo. The results obtained here show that supra-physiological levels in the medium (up to 1 mM) are necessary for adequate accumulation, for when 50 µM (reflective of plasma levels) was added, cellular uptake was well below saturation. This may be due to oxidation of ascorbate, as it was added as a bolus dose to the culture medium and is likely to oxidise over time, unlike in plasma, where it is constantly maintained in its reduced state. However, there may be other unknown differences between in vivo and in vitro conditions that further affect relative uptake. Therefore, for the same intracellular levels to be achieved in vitro as found in vivo, cultured cells will have a shorter exposure time to reduced ascorbate, while also requiring a higher concentration.

There was significant variation in maximal intracellular ascorbate concentrations between cell lines, ranging from 0.8 mM in Ishikawa cells to 12 mM in Jurkat cells. This may be a result of changes in ascorbate transporter activity across different cell lines. In addition to reduced ascorbate uptake via SVCT2, the oxidised form, DHA, can also be taken up via GLUTs, and this may occur in vitro. Normal lymphocytes have been reported to saturate at ~4 mM (223), but Jurkat cells may be more dependent on DHA for uptake, allowing them to accumulate higher levels of ascorbate than their normal counterparts in vivo (226). In addition, as cultured cells are grown in ascorbate-free medium, the expression of SVCT2 may be up-regulated, increasing the ascorbate uptake capacity upon addition to the medium. However, it is unknown what levels of SVCT2 are expressed in the cells examined here, although it has
been shown to vary widely among breast cancer cell lines (228). This is likely to contribute to variation in ascorbate uptake capacity and may contribute to the relatively low uptake observed in Ishikawa cells.

Cells within a solid tumour may not have sufficient ascorbate supply, which may be due to lack of a functional vasculature or low circulating levels, both of which can occur in patients with solid tumours (194-197, 199, 200). In addition to potential differences in ascorbate transport capacity, this may result in sub-optimal intracellular ascorbate levels in tumour cells \textit{in vivo}. These low intracellular ascorbate levels in cancer cells may be sufficient to suppress HIF-hydroxylase activity and over-activate HIF-1, and this hypothesis will be explored in the following Chapters 4, 5 and 6.

\section*{3.4 Chapter summary}

This chapter has demonstrated that ascorbate is available to cells for uptake under standard culture conditions, and remains stable intracellularly. Physiological intracellular ascorbate concentrations were achieved, appropriate for further experiments monitoring HIF-1 activation (Chapter 4).
Chapter 4: The effect of intracellular ascorbate on HIF-1 induction in cultured cancer cells

The results presented in this chapter have been included in:


### 4.1 Introduction

The HIF-hydroxylases are responsible for regulating HIF-1 activity by two distinct pathways. Proline hydroxylation by the PHDs targets HIF-1α for destruction (30) and asparagine hydroxylation by FIH prevents HIF-1 co-activation (32). Hence, both HIF-1α protein stabilisation and the transcriptional activity of HIF-1 can be independently regulated. The HIF-hydroxylases require intracellular ascorbate for optimal catalytic activity (164), and this suggests that ascorbate deficiency may result in over-activation of HIF-1.

In addition to ascorbate, the HIF-hydroxylases require iron, molecular oxygen and 2-oxoglutarate (2-OG) for activity (17). Therefore, the HIF-1-response can be activated by depriving the hydroxylases of any one of these substrates, which can occur under conditions of hypoxia, metabolic disturbance or iron deprivation. A small number of studies have shown that ascorbate is able to counteract HIF-1 induction *in vitro* (140, 166, 167, 229) and this has been demonstrated using various HIF-hydroxylase inhibitors (Co²⁺, Ni²⁺, 2-OG analogues, hypoxia or growth factors). However, these studies have generally been carried out with a single set of conditions and the intracellular ascorbate concentrations have generally not been determined.
To date, there has not been a comprehensive examination on the effects of ascorbate on HIF-1 activation that includes a comparison of different conditions of activation. Therefore, a cohesive investigation of the HIF-1-response in multiple cell lines containing known, physiological ascorbate concentrations is warranted. This will determine whether intracellular ascorbate can maintain HIF-hydroxylase activity (both the PHDs and FIH) during deprivation of different co-substrates, and thereby restrain the HIF-1 transcriptional response. Furthermore, such an investigation could shed light on the potential mechanistic role of ascorbate in maintaining HIF-hydroxylase activity.

4.1.1 Chapter aims

The aim of the experiments described in this chapter was to investigate whether physiological, intracellular ascorbate concentrations can influence various modes of HIF-1 induction across multiple cell lines. This includes the effect on both HIF-1α protein stabilisation and HIF-1 transcriptional activity. Specifically, this chapter will examine:

1. The effect of intracellular ascorbate on HIF-1α protein stabilisation induced by various means of HIF-hydroxylase co-substrate deprivation, reflective of PHD activity.

2. The effect of ascorbate on HIF-1 transcriptional activity in response to various means of HIF-hydroxylase co-substrate deprivation, reflective of FIH activity.

3. The potential mechanistic role of ascorbate in maintaining HIF-hydroxylase activity by comparing its effect on different inhibitors in the same cell lines.

4.1.2 Experimental approach

Three cancer cell lines (Ishikawa, WiDr and Jurkat) and one primary cell line (HUVEC) were used for these experiments (see section 2.1). All were pre-loaded overnight (~16 h) with ascorbate to achieve optimal intracellular concentrations as described in Chapter 3, and the
HIF-1 response of ascorbate-deficient or pre-loaded cells was measured following addition of HIF-hydroxylase inhibitors.

Four different methods of HIF-hydroxylase inhibition/HIF-1 induction were utilised to interfere with each enzyme co-substrate. The active site iron was compromised with the divalent metal ions Co$^{2+}$ and Ni$^{2+}$ and the iron chelator desferrioxamine (DFO) (229). Metabolic disturbance was mimicked using the cell-permeable, competitive 2-oxoglutarate analogue, dimethyloxalylglycine (DMOG) (140). The oxygen tension was manipulated by incubation in a controlled atmosphere hypoxic chamber. Cells were incubated separately with these HIF-1-inducers for either 4 hours to monitor HIF-1α protein stabilisation, or 8 hours to monitor HIF-1 target gene production.

Whole cell lysate was analysed for HIF-1α protein by Western blot and for production of HIF-1-specific target genes: BNIP3 protein (by Western blot) and VEGF protein (secreted and measured by ELISA). HIF-1α DNA-binding activity was also measured using an ELISA method that detects HRE-bound HIF-1α.

### 4.2 Results

#### 4.2.1 Dose-response of ascorbate on HIF-1α protein stabilisation

Initially using WiDr cells, HIF-1α protein levels were monitored in response to each HIF-1-inducer following pre-loading with increasing doses of ascorbate. HIF-1α was consistently detected as a 120 kDa band in accordance with its published molecular weight (22). Figure 4.1 shows that ascorbate is able to inhibit HIF-1α protein stabilisation by each inducer, and that there is a dose response, with the highest concentration (1000 µM) resulting in the greatest inhibitory effect. This concentration reflects a healthy physiological level of ascorbate, as shown in Chapter 3, being equivalent to ~1.1 mM intracellularly, whereas 250 µM and 50 µM result in ~0.7 mM and ~0.2 mM respectively (see Figure 3.2). Basal levels of HIF-1α in control cells as well as inducible protein were inhibited (Figure 4.1).
Figure 4.1: Dose-dependent inhibition of HIF-1α protein by ascorbate in WiDr cells. Representative Western blots are shown (from 2 independent experiments) of HIF-1α and β-actin as a loading control from WiDr cells pre-loaded with increasing ascorbate concentrations showing a decline in HIF-1α protein levels, in untreated control cells and in response to different means of HIF-hydroxylase inhibition/HIF-1 induction.
4.2.2 The effect of ascorbate on HIF-1α protein induced by iron competition

Under normal culture conditions, without supplemental ascorbate, HIF-1α protein levels increased in response to the iron competitors Co^{2+}, Ni^{2+} and DFO. Figure 4.2 shows representative Western blots of the effect of ascorbate on levels of induction in Ishikawa and Jurkat cells. There was a clear dose-dependency of each agent on HIF-1α stabilisation (Figure 4.2) and the other cell lines tested showed similar results (not shown). As shown for WiDr cells (Figure 4.1), ascorbate pre-loading prevented the small amount of basal HIF-1α protein accumulation in Ishikawa cells and almost completely prevented HIF-1α protein stabilisation in response to even the highest doses of the iron competitors (Figure 4.2A-C). Ascorbate-loaded Jurkat cells and HUVEC had completely undetectable levels of HIF-1α protein in the presence of iron competitors (Figure 4.2D-F and Figure 4.3), while ascorbate-loaded Ishikawa and WiDr cells had detectable, but significantly reduced HIF-1α protein levels compared to ascorbate-deficient cells (Figure 4.3; p<0.05).
Figure 4.2:  **HIF-1α protein levels in ascorbate-deficient compared to ascorbate-loaded cells following induction by iron competitors.** Representative Western blots (from 2-3 independent experiments) of HIF-1α and β-actin as a loading control from Ishikawa (A-C) and Jurkat (D-F) cells. HIF-1α protein accumulates dose-dependently with each iron competitor in the absence of ascorbate. After ascorbate-loading, Ishikawa cells contained minimal amounts of stabilised HIF-1α at all doses of iron-competitors used and Jurkat cells generally had undetectable HIF-1α protein. Ascorbate pre-loading was achieved with 1 mM fresh sodium-L-ascorbate in the medium for Ishikawa cells and 0.5 mM for Jurkat cells.
Figure 4.3: Quantification of HIF-1α protein induction by iron competitors in ascorbate-deficient compared to ascorbate-loaded cells for all cell lines tested. Densitometry of HIF-1α from 3-4 independent experiments in different cell lines shows ascorbate-loaded cells (black bars) have significantly reduced or undetectable, HIF-1α protein levels compared to ascorbate-deficient cells (grey bars) in response to iron competition. Cells were incubated for 4 hours with 100 µM DFO (A), 100 µM CoCl₂ (B) or 1 mM NiCl₂ (C). Ascorbate pre-loading was achieved by adding 1 mM fresh sodium-L-ascorbate to the medium for Ishikawa and WiDr cells and 0.5 mM for Jurkat cells and HUVEC. Data are means ± SEM. *p<0.05 by paired Student’s t-test (2-tailed).
4.2.3 The effect of ascorbate on HIF-1α protein induced by metabolic disturbance

Addition of DMOG to the cultured cells resulted in a dose-dependent increase in HIF-1α protein levels. Figure 4.4 shows representative Western blots of the induction in Ishikawa and Jurkat cells, with similar levels observed for WiDr cells and HUVEC (not shown). Ascorbate was able to diminish HIF-1α induction by DMOG in the cancer cell lines (Figure 4.4), although this reduction was not as dramatic as that seen with iron competition (Figure 4.3). A significant reduction in HIF-1α protein induced by DMOG treatment was seen in ascorbate-loaded WiDr and Jurkat cells compared to ascorbate-deficient cells (Figure 4.5; p<0.05). The response in Ishikawa cells was similar but slightly more variable (p=0.15) and, in contrast to the effects seen in the cancer cell lines, there was no effect of ascorbate in DMOG-treated HUVEC (p=0.30; Figure 4.5).
Figure 4.4: HIF-1α protein levels in ascorbate-deficient compared to ascorbate-loaded cells following induction by DMOG. Representative Western blot (from 2-3 independent experiments) of HIF-1α from Ishikawa cells (A) and Jurkat cells (B) are shown. Ascorbate-loaded cells have reduced HIF-1α at all doses of DMOG used. Ascorbate pre-loading was achieved by adding 1 mM fresh sodium-L-ascorbate to the medium for Ishikawa cells and 0.5 mM for Jurkat cells.
Chapter 4: The effect of intracellular ascorbate on HIF-1 induction in cultured cancer cells

4.2.4 The effect of ascorbate on HIF-1α protein induced by hypoxia

In response to lowered oxygen tension, Ishikawa cells were very sensitive to even a small reduction in oxygen, and at relatively high levels of O₂ (10%), HIF-1α protein levels were increased (Figure 4.6A). On the other hand, HUVEC had a more ‘normal’ response, with induction of HIF-1α requiring more physiological levels of hypoxia at O₂ levels of 2.5% (Figure 4.6C). In Ishikawa cells, ascorbate loading was able to reduce HIF-1α protein stabilisation at O₂ levels ≥ 10% (Figure 4.6A). Ascorbate-loaded Jurkat and WiDr cells accumulated less HIF-1α protein at physiological O₂ levels, and showed a significant decrease in HIF-1α protein levels at oxygen tensions of 1% or 5%, respectively (Figure 4.7). In HUVEC, there was no effect of ascorbate under hypoxic conditions (Figure 4.7).
Figure 4.6: **HIF-1α protein levels in ascorbate-deficient compared to ascorbate-loaded cells following induction by hypoxia.** Representative Western blots of HIF-1α (from 2-3 independent experiments) from Ishikawa cells (A), Jurkat cells (B), and HUVECs (C) under conditions of increasing hypoxia. Ishikawa cells showed greater sensitivity to reduced oxygen tension compared to Jurkat cells and normal HUVEC, with HIF-1α induction apparent even above 10% oxygen. Ascorbate decreased HIF-1α induction in Ishikawa cells at oxygen tensions ≥ 10% and in Jurkat cells at 2.5% and 1% oxygen. There was no effect of ascorbate loading in HUVEC. Ascorbate pre-loading was achieved by adding 1 mM fresh sodium-L-ascorbate to the medium for Ishikawa cells and 0.5 mM for Jurkat cells and HUVEC.
Figure 4.7: Quantification of HIF-1α protein induction by hypoxia in ascorbate-deficient compared to ascorbate-loaded cells for all cell lines tested. Densitometry of HIF-1α from 3-4 independent experiments in each cell line shows Jurkat and WiDr ascorbate-loaded cells (black bars) have lower HIF-1α protein levels compared to ascorbate-deficient cells (grey bars) at 1% (A) or 5% (B) oxygen, respectively. Ascorbate pre-loading was achieved by adding 1 mM fresh sodium-L-ascorbate to the medium for Ishikawa and WiDr cells and 0.5 mM for Jurkat cells and HUVEC. Data are means ± SEM. *p<0.05 by paired Student’s t-test (2-tailed).
4.2.5 *The effect of ascorbate on DNA-binding activity of HIF-1*

The HIF-1 transcriptional response reflects both PHD and FIH activity, with FIH preventing HIF-1 co-activation and binding to the promoter region. Using a DNA-binding ELISA, Ishikawa cells showed increased HIF-1 DNA-binding activity in response to induction by CoCl$_2$, and this was prevented by pre-loading cells with intracellular ascorbate (Figure 4.8).

![Bar graph showing HIF-1 DNA-binding activity](image)

**Figure 4.8:** HIF-1 DNA-binding activity in ascorbate-deficient compared to ascorbate-loaded Ishikawa cells treated with CoCl$_2$. Cells were treated with 100 µM CoCl$_2$ for 4 hours, or left untreated (control). Nuclear protein extracts were isolated and incubated with a biotin-labelled oligonucleotide corresponding to the HIF-1 consensus sequence and subsequent binding detected by ELISA. The CoCl$_2$-treated extracts were also incubated with an unlabelled oligonucleotide as a competitive control (Comp). Ascorbate pre-loading (black bars) prevented induction of HIF-1 DNA-binding compared to ascorbate-deficient cells (grey bars). Ascorbate pre-loading was achieved by adding 1 mM fresh sodium-L-ascorbate to the medium. Data are means ± SD from 2 independent experiments. *p<0.05 by paired Student’s t-test (2-tailed).
4.2.6 The effect of ascorbate on production of the HIF-1 target gene, BNIP3

Figure 4.9 shows representative Western blots of BNIP3 in Ishikawa cells, where it was detected as a doublet band, with the lower band migrating at ~30 kDa as predicted for BNIP3 (82). In Ishikawa, Jurkat cells and HUVEC, BNIP3 protein levels increased in response to all HIF-1 inducers used (Figure 4.10). In Ishikawa cells, ascorbate pre-loading decreased BNIP3 protein production and it remained at control levels under all conditions of HIF-1-induction (Figure 4.10; p<0.05). A similar effect was seen in ascorbate-loaded Jurkat cells and HUVEC, with significantly reduced BNIP3 protein levels in response to all HIF-1-inducers used (Figure 4.10; p<0.05). The one exception to this was HUVEC at 1% O₂ where ascorbate did not affect BNIP3 protein expression.

Figure 4.9 also shows corresponding HIF-1α protein levels in Ishikawa cells after 8 hours of induction, showing a greater inhibitory effect of ascorbate on production of HIF-1-dependent BNIP3 than on HIF-1α protein levels. In this experiment, HIF-1α protein was measured after 8 hours of incubation with the different inducers as compared with 4 hours for previous experiments, with a reduction in DMOG-induced HIF-1α seen here at 8 hours. Notably, at these concentrations of DMOG and oxygen, ascorbate had a lesser, and sometimes minimal, effect on HIF-1α protein levels (Figure 4.10 and also see Figures 4.4 and 4.6). Therefore, despite there being residual HIF-1α protein in the cells, intracellular ascorbate can inhibit HIF-1 transcriptional activity.
Figure 4.9: BNIP3 compared to HIF-1α protein levels in ascorbate-deficient compared to ascorbate-loaded Ishikawa cells following exposure to HIF-1 inducers. Representative Western blots (from 3-4 independent experiments) of BNIP3, HIF-1α, and β-actin as a loading control, from Ishikawa cells show prevention of BNIP3 induction in ascorbate-loaded cells in response to all HIF-1-inducers used. This occurs despite HIF-1α protein being present in the cell. Cells were incubated for 8 hours with 100 µM of CoCl₂, NiCl₂, DFO or DMOG, or 1% O₂. Ascorbate pre-loading was achieved by adding 1 mM fresh sodium-L-ascorbate to the medium.
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Figure 4.10: Quantification of BNIP3 protein induction in ascorbate-deficient compared to ascorbate-loaded cells following exposure to HIF-1 inducers. Densitometry of BNIP3 from 3-4 independent experiments in 3 different cell lines shows prevention of BNIP3 induction in ascorbate-loaded cells in response to all HIF-1-inducers used (except in HUVEC at 1% O\textsubscript{2}). Cells were incubated for 8 hours with 100 µM of CoCl\textsubscript{2}, NiCl\textsubscript{2}, DFO or DMOG, or 1% O\textsubscript{2}. Ascorbate pre-loading was achieved by adding 1 mM fresh sodium-L-ascorbate to the medium for Ishikawa cells and 0.5 mM for Jurkat cells and HUVEC. Data are means ± SEM. *p<0.05 by paired Student’s t-test (2-tailed).
4.2.7 The effect of ascorbate on HIF-1-mediated VEGF production

A similar trend as seen with BNIP3 protein was observed when secreted VEGF protein levels were measured in Ishikawa cells. VEGF protein secretion was increased in response to all HIF-1-inducers, but this was suppressed by ascorbate with a significant reduction seen in CoCl$_2$- and DMOG-treated cells (Figure 4.11; $p<0.05$). In WiDr cells, VEGF levels were not increased by HIF-1-induction using iron competition, and only minimally increased with DMOG and hypoxia (Figure 4.11), suggesting it is unlikely there is any HIF-1 dependent expression of VEGF in these cells. Notably, there was no effect of ascorbate on VEGF expression in WiDr cells, which supports the specificity of ascorbate for affecting HIF-1-dependent processes in Ishikawa cells.
Figure 4.11: VEGF protein expression in ascorbate-deficient compared to ascorbate-loaded cells following incubation with HIF-1 inducers. Ishikawa cells increased VEGF production in response to all HIF-1-inducers and this was diminished in ascorbate-loaded cells (black bars) compared to ascorbate-deficient cells (grey bars). WiDr cells did not increase production of VEGF in response to HIF-1-induction by iron competition, only minimally with DMOG and hypoxia, and ascorbate did not affect this. Cells were incubated for 8 hours with 100 µM of CoCl$_2$, NiCl$_2$, DFO or DMOG, or 1% O$_2$, and secreted VEGF measured in the medium by ELISA. Ascorbate pre-loading was achieved by adding 1 mM fresh sodium-L-ascorbate to the medium. Data are means ± SEM of 4-6 independent experiments. *p<0.05 by paired Student’s t-test (2-tailed).
4.3 Discussion

These results have shown that physiological levels of ascorbate in cancer cells can prevent or reduce HIF-1α protein accumulation, DNA-binding and transcriptional activity induced under a variety of conditions. Ascorbate affected HIF-1α protein stabilisation to some extent under almost all conditions of induction, although the degree of inhibition varied with the cell line and HIF-1-inducer used. The greatest effect was in counteracting the iron competitors with almost complete inhibition across a range of concentrations. In contrast to the effect on HIF-1α protein stabilisation, HIF-1-dependent gene expression was consistently inhibited by ascorbate across all cell lines and inducers tested.

4.3.1 HIF-1α protein induced by iron competition and possible mechanisms for ascorbate in the HIF-hydroxylase reaction cycle

Intracellular ascorbate had a profound inhibitory action against HIF-1α protein accumulation in all cell lines in the presence of the iron competitors Co^{2+}, Ni^{2+} and DFO. This suggests that ascorbate may be supporting HIF-hydroxylase activity by somehow maintaining the active site iron. This may be a structural/chelating effect, an antioxidant effect to keep the iron reduced, or both. Indications are offered from the results presented here for the mechanism of ascorbate in promoting HIF-hydroxylase activity and are explored below.

It is still unclear how transition metal ions such as Co^{2+}, known as a ‘hypoxia mimetic’, interfere with hydroxylase activity. Inhibition is considered to be a result of poisoning of the enzyme by substitution of the iron at the active site with other metals that are unable to participate in the reaction cycle. However, although the coordination chemistry of the hydroxylase active site suggests the iron is loosely bound (17), there is also evidence to the contrary. Notably, addition of excess iron could not recover enzyme activity (151) and this, together with low reported K_m values for Fe^{2+} [0.03 and 0.5 µM for PHD2 (137) and FIH (135), respectively], suggests tight binding. It has also been suggested that substitution of Fe^{2+} by Ni^{2+} is unlikely to occur in cells as the modelled activation energy required was too high (230). There may be an alternative metal binding site that inactivates the enzymes, with two atoms of Zn^{2+}, Cd^{2+} and Cu^{2+} being able to bind to PHD2 (231). However, the primary
metal activators of HIF-1, Co\(^{2+}\) and Ni\(^{2+}\), were found to only bind once, as did Fe\(^{2+}\) (231). Furthermore, in a related enzyme, histone demethylase JMJD1, Ni\(^{2+}\) was found to substitute the active site iron (232), and as the active site is highly conserved among these enzymes (158), this may also occur in the HIF-hydroxylases. Although it remains to be resolved exactly how different transition metal ions are able to induce the HIF-1-response, it appears probable that Co\(^{2+}\) and Ni\(^{2+}\) ions could replace the active site iron.

It was of interest that ascorbate affected HIF-1-induction by DFO in an almost identical manner to CoCl\(_2\) and NiCl\(_2\). DFO specifically chelates Fe\(^{3+}\) (233), which is formed during the hydroxylase reaction cycle, likely removing it from the enzyme and causing its inhibition. Ascorbate may be bound to Fe\(^{3+}\) during the enzymatic cycle in a structurally specific manner (230) and may act to stabilise Fe\(^{3+}\) in the active site while also rapidly reducing it back to Fe\(^{2+}\) for continuous enzyme activity. This effect may also be relevant to transition metals which may replace lost Fe\(^{3+}\) in the absence of ascorbate. As the low K\(_m\) values for iron refer to Fe\(^{2+}\), not Fe\(^{3+}\), it is plausible that ascorbate deficiency permits the free loss of bound Fe\(^{3+}\) from the enzyme rather than its being retained and recycled back to Fe\(^{2+}\). This mechanism may also utilise the ability of ascorbate to chelate intracellular iron (179), and it may act to structurally stabilise the iron in the active site, preventing its free loss, as well as keeping it reduced.

There is evidence from early studies of pure C-P4H that ascorbate acts to sustain the active site iron (as opposed to free iron) in the reduced state in order to maintain enzyme cycling, and this is a role highly specific to ascorbate (155, 157). A more recent study using human recombinant PHD2 and FIH have also demonstrated a specific requirement for ascorbate with the activity of FIH being more dependent (164). Other potent antioxidants such as glutathione and DTT were not able to substitute for ascorbate in the reaction, although DTT was able to partially enhance PHD2 activity (164). In a mouse model, it was found that ascorbate could suppress HIF-1-mediated tumourigenesis, however this effect was mimicked by the antioxidant N-acetylcysteine [NAC; (169)]. To my knowledge, there is no data on the effect of NAC on HIF-hydroxylase activity. Other antioxidants may be able to partially support HIF-hydroxylase activity, but ascorbate is clearly most effective, and this supports its likely role in chelating and reducing enzyme-bound iron.
4.3.2 The role of ascorbate in preventing HIF-1α induction by DMOG and hypoxia

A lesser, but significant, effect of ascorbate on HIF-1α was seen with DMOG and lowered O$_2$ availability. This likely reflects its ability to optimise PHD activity. This is also reflected in the decreased basal HIF-1 activation in ascorbate-loaded cancer cells, which is thought to be caused by increased glycolytic intermediates interfering with 2-OG binding (140). Unlike the iron competitors, there may not be a direct effect of ascorbate in counteracting these inhibitors and, therefore, the level of HIF-1 inhibition was less marked. Ascorbate is unlikely to have a major impact under conditions of stringent hypoxia, as the PHDs have an absolute requirement for oxygen (33). However, at slightly higher, more physiological tissue oxygen levels (1-5%, as seen here), ascorbate was able to suppress the HIF-1 response (166, 167), and this may have important implications in preventing the proliferation of HIF-driven cancer cells at early stages of tumourigenesis.

Ascorbate-loaded Ishikawa cells had a variable response to DMOG, and no response to oxygen tensions below 10%. This was a lesser effect than was seen with the other cancer cell lines tested and may reflect differences in the intracellular ascorbate levels. As noted in Chapter 3, the ascorbate levels in Ishikawa cells may have been sub-optimal, and it is possible that HIF-1α may have been further inhibited under these conditions of metabolic stress had they been able to accumulate ascorbate to a higher concentration.

4.3.3 The effect of ascorbate on the PHDs vs. FIH

BNIP3 was measured as a specific marker of HIF-1 transcriptional activity, as it is up-regulated by HIF-1 with high fidelity (79) and has been closely linked with the hypoxic response and poor prognosis in clinical samples (87, 89). That ascorbate was able to prevent BNIP3 production in response to all the HIF-1-inducers used, even under conditions where HIF-1α protein was present, suggests that the PHDs and FIH have differential dependencies on oxygen and ascorbate for activity. FIH appears to be more similar to C-P4H than the PHDs in terms of co-factor requirements (135). The K$_m$ of FIH for ascorbate is relatively high (~260µM; C-P4H ~300µM) compared to the PHDs (140-180µM), making FIH more sensitive to ascorbate loss (see Table 1.2). The results from this chapter support these data,
and suggest an important role for ascorbate in maintaining FIH activity to dampen HIF-1 transcripational activity.

The $K_m$ of the PHDs for oxygen is relatively high (230-250µM) compared to FIH (~90µM; C-P4H ~40µM), meaning the PHDs may be more sensitive to fluctuations in $pO_2$ (see Table 1.2). Indeed, enzymatic activity of FIH was maintained at oxygen levels that would inactivate the PHDs (234). Together with the differential requirements of the PHDs and FIH for ascorbate, this may explain its increased efficacy in preventing HIF-1 transcriptional activity at 1% $O_2$ despite the increased stabilisation of HIF-1α protein in Ishikawa cells.

Secreted VEGF protein levels were also measured in response to HIF-induction and ascorbate, and there was a similar response as with BNIP3, with the HIF-1-dependent increase in VEGF production being inhibited by ascorbate. However, VEGF is less HIF-1-specific than is BNIP3, having multiple other regulatory mechanisms (106), and in WiDr cells, which did not increase VEGF protein secretion in response to the HIF-1-inducers, ascorbate had no effect. This observation supports the specificity of the ascorbate-mediated inhibition of HIF-1-dependent gene transcription in other cell lines.

#### 4.3.4 Clinical relevance

Adequate intracellular ascorbate may have significant implications for the prevention of HIF-1-dependent tumour growth. This could be particularly relevant to nickel-induced carcinogenesis, which may occur through occupational and environmental exposure, and is thought to be driven by activation of HIF-1 (136, 235). In a recent study, ascorbate-deficient mice were 40% more susceptible to nickel-induced carcinogenesis than wild-type mice and ascorbate supplementation significantly prolonged the tumour latency period (212). Therefore, maintaining adequate ascorbate levels may be sufficient to prevent or slow down this mechanism of carcinogenesis. In addition to these studies, I have shown that ascorbate was able to curb HIF-1 transcriptional activity under conditions of hypoxia and metabolic disturbance, and may therefore be able to disrupt the HIF-1-mediated survival mechanisms of...
cells in established tumours. Such a shift away from the ‘survivor’ phenotype may be sufficient to help overcome chemo-resistance (81) as well as preventing further tumour spread.

Ascorbate is clearly an effective regulator of HIF-1α protein and transcriptional activity. However, measurement of its direct effect on HIF-hydroxylase activity in live cells is needed to verify this. In addition, whether this effect of ascorbate on inhibiting HIF-1-dependent processes is sufficient to impact on cancer cell survivability requires investigation. Furthermore, the analysis of human cancer tissues for a relationship between ascorbate and HIF-1 activation is necessary, and will determine if there is indeed any real clinical relevance to this mechanism. This will be examined in the following two chapters.

4.4 Chapter summary

The data presented in this chapter have demonstrated that physiological, intracellular ascorbate concentrations can significantly reduce, if not prevent, induction of HIF-1α protein by iron competition and, to a lesser extent, metabolic disturbance and hypoxia in cancer cells. In addition, intracellular ascorbate can prevent induction of HIF-1 transcriptional activity under all conditions of HIF-1-induction, which suggests that FIH is more dependent on ascorbate for activity than are the PHDs. This also highlights the importance of monitoring HIF-1 transcriptional activity and not measuring HIF-1α protein only, as this may be inactive.

The marked and similar effect of ascorbate on Co^{2+}, Ni^{2+} and DFO-induced HIF-1 suggests that its role is to protect the active site iron of the HIF-hydroxylases. This could be by preventing the free loss (and substitution) of Fe^{3+}, while also reducing it back to Fe^{2+} for continued enzyme cycling.
Chapter 5: Measurement of ascorbate levels and HIF-1 activation in human endometrial tumours

The results presented in this chapter have been included in:


5.1 Introduction

The use of ascorbate to treat cancer is widespread (206), despite a lack of mechanistic evidence to support this practice. Animal studies have indicated that increasing the supply of ascorbate can inhibit tumour growth (169, 211, 212), but only one of these studies has linked this effect to inhibition of HIF-1 (169). Intracellular ascorbate has been shown to modulate the HIF-1 response in cancer cells, as described in my previous studies in vitro (Chapter 4), and also reported by others (140, 166, 167). To my knowledge, no studies have examined the link between ascorbate and HIF-1 in clinical samples.

Here in Christchurch, we have access to a large cancer tissue bank (Cancer Society Tissue Bank, Christchurch; CSTBC) containing high quality, meticulously stored tissue samples from cancer patients collected since 1998 (236). This valuable resource offered the opportunity to examine a variety of human tumour tissue. Each tumour is banked together with a sample from adjacent normal tissue from the same patient. Therefore, each tumour sample has its own internal control, allowing comparisons to be made between individuals. In addition, clinical and pathological information for each case is stored in a secure relational
database, allowing experimental data to be analysed together with tumour pathology and patient clinical data.

Samples from the CSTBC were used to investigate whether there is a relationship between tumour ascorbate content and activation of HIF-1 in human cancer. This investigation was based on the hypothesis that solid tumours may have compromised delivery of ascorbate to some regions, causing a deficiency state that could exacerbate HIF-1 activation, thereby enhancing tumour growth and spread. Therefore, it was determined whether the ascorbate content of human tumour tissue was related to HIF-1 activity and/or tumour pathology.

Analyses in this chapter were carried out using human endometrial tumour tissue samples. Endometrial cancer is generally categorised into two types: type I tumours are of endometrioid morphology and are often low grade, whereas type II tumours predominantly have serous or clear cell morphology and are generally high grade (237). It is a relatively common cancer in post-menopausal women, being the fourth most common malignancy in women in the developed world (237). In addition, endometrial cancer is known to be driven by an active hypoxic response (54), with disease progression and poor prognosis being linked to HIF-1α and VEGF levels (54, 89, 238). Therefore, this cancer was chosen as a suitable initial model for the investigation of ascorbate and the HIF-1 response.

5.1.1 Chapter aims

The aim of this chapter was to examine human endometrial tumour and adjacent normal tissue samples obtained from the CSTBC for markers of HIF-1 activation and ascorbate content, and to relate these measures to clinico-pathological data. Specifically, this involved:

1. Measurement of protein levels of both HIF-1α and specific, pro-survival downstream gene products to represent the level of activation of the HIF-1 response in tumour and normal tissue samples.
2. Quantification of cellular ascorbate levels in tumour compared to normal tissue from the same patient and relating this to the degree of HIF-1 activation in the tumour.

3. Comparison of the expression of HIF-1 activity markers and ascorbate content with clinico-pathological features of the tumours.

5.1.2 Experimental approach

Frozen tissue samples were ground to a fine powder in liquid nitrogen, providing tissue wet weight ~20-100 mg, and then 10 mM phosphate buffer (pH 7.4) was added to make a homogeneous tissue suspension (see section 2.6.3). This homogenate was used for all analyses.

In order to obtain quantifiable data that is representative of the entire tumour sample, Western blotting or ELISA of the tissue homogenate was used for protein measurements. In addition, a formalin-fixed, paraffin-embedded tissue microarray (prepared by the CSTBC) containing endometrial tumour (n=96) and some normal (n=10) tissue cores was used to verify HIF-1α protein expression and localisation. Thirty three tumour samples on the microarray were common to the samples studied by western blotting.

Protein levels of HIF-1α and three pro-survival downstream gene products – BNIP3, GLUT-1 and VEGF were quantified, and used together as a marker of HIF-1 activity. The ascorbate content of the tissue was measured using HPLC-ECD, a highly sensitive assay, providing accurate and reproducible measurements down to approximately 500 nM (214). This was related to the cellularity of each sample as measured by the DNA content (propidium iodide fluorescence) and the calculated ascorbate levels were related to the extent of HIF-1 activation in each sample.
5.2 Results

5.2.1 Tumour samples

Tumour and normal tissue samples were from women undergoing hysterectomy for removal of endometrial tumours (51 cases). The sample set contained approximately equal numbers of FIGO grades 1, 2 and 3 tumours, representing increasing solid growth and lack of differentiation (239), with the grade 3 group including three cases of carcinosarcoma and 10 cases of serous and/or clear cell adenocarcinoma. Table 5.1 describes the clinico-pathological features of the sample set.
Table 5.1  Clinico-pathological features of endometrial tumour sample set.

<table>
<thead>
<tr>
<th>Pathological parameter</th>
<th>n (%)</th>
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<tbody>
<tr>
<td><strong>FIGO grade (solid growth pattern)</strong></td>
<td></td>
</tr>
<tr>
<td>1 (&lt;5%)</td>
<td>18 (35)</td>
</tr>
<tr>
<td>2 (6-50%)</td>
<td>15 (30)</td>
</tr>
<tr>
<td>3 (&gt;50%)</td>
<td>18 (35)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>1A – confined to uterus, &lt;50% myometrial invasion</td>
<td>20 (39)</td>
</tr>
<tr>
<td>1B – confined to uterus, &gt;50% myometrial invasion</td>
<td>10 (19)</td>
</tr>
<tr>
<td>2 – invasion of cervical stroma, contained within uterus</td>
<td>8 (16 )</td>
</tr>
<tr>
<td>3A – serosal and/or adnexal invasion</td>
<td>2 (4)</td>
</tr>
<tr>
<td>3B – vaginal and/or parametrial invasion</td>
<td>0 (0)</td>
</tr>
<tr>
<td>3C – lymph node metastasis</td>
<td>4 (8)</td>
</tr>
<tr>
<td>4 – bowel and/or bladder invasion, and/or distant metastasis</td>
<td>7 (14)</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
</tr>
<tr>
<td>Endometrioid adenocarcinoma</td>
<td>38 (74)</td>
</tr>
<tr>
<td>Serous or clear cell component</td>
<td>10 (20)</td>
</tr>
<tr>
<td>Carcinosarcoma</td>
<td>3 (6)</td>
</tr>
<tr>
<td><strong>Tumour size</strong></td>
<td></td>
</tr>
<tr>
<td>≤ 50 mm</td>
<td>32 (63)</td>
</tr>
<tr>
<td>&gt; 50 mm</td>
<td>17 (33)</td>
</tr>
<tr>
<td>ND</td>
<td>2 (4)</td>
</tr>
<tr>
<td><strong>Myometrial invasion</strong></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Inner half</td>
<td>26 (51)</td>
</tr>
<tr>
<td>Outer half</td>
<td>22 (43)</td>
</tr>
<tr>
<td><strong>Necrosis</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>9 (18)</td>
</tr>
<tr>
<td>Yes</td>
<td>18 (35)</td>
</tr>
<tr>
<td>ND</td>
<td>24 (47)</td>
</tr>
<tr>
<td><strong>Lymph/vascular invasion</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>33 (65)</td>
</tr>
<tr>
<td>Yes</td>
<td>18 (35)</td>
</tr>
</tbody>
</table>

FIGO, International Federation of Gynecology and Obstetrics. ND, no data.
5.2.2 Stability of ascorbate in dissected and stored tissue

To determine whether the protocol for tissue banking resulted in a loss or variation in ascorbate content, tissue from a healthy mouse liver (post-mortem collection) was used to simulate the procedure used by the tissue bank. The liver was dissected into pieces (~20 mg) that were left at room temperature for 0-7 hours. At each time point, the tissue pieces were either analysed immediately or frozen at -80°C for 21 weeks for comparison.

Figure 5.1 shows that long term freezer storage of the tissue had no effect on the tissue ascorbate content, with fresh and stored samples having the same amount. The amount of time to process samples was also monitored and there was no loss of ascorbate in samples kept at room temperature for up to 5 hours (Figure 5.1). Also, there was no apparent loss of ascorbate after long term storage of these samples. This suggests that the tissue banking procedure, which is completed within approximately one hour of tumour resection, does not significantly affect ascorbate levels in the tissue samples.

The stability of ascorbate in the homogenised endometrial tumour tissue extracts was also measured and there was no difference between a newly extracted sample (5.75 ± 0.15 mg/100g) and the same extract after four days storage at -80°C (5.85 ± 0.01 mg/100g). Identical levels of ascorbate were also found when repeat measurements were made on two samples after a further seven months of freezer storage (11.22 vs. 11.19 mg/100g; 7.22 vs. 6.68 mg/100g).
Figure 5.1: Stability of ascorbate in tissue left at room temperature and stored at -80°C. A healthy mouse liver was dissected into pieces and left at room temperature for up to 7 hours. At each time point, tissue pieces were either analysed immediately (■) or frozen at -80°C for 21 weeks and then analysed (♦). This simulates the tissue banking procedure protocol (tumours were dissected and stored within an hour by the CSTBC). There was no loss of ascorbate after long term storage, and up to three hours spent at RT did not affect ascorbate levels. Data are means ± SD of triplicate samples. RT, room temperature.

5.2.3 Determination of cell density in tumour tissue and normal tissue

As normal and tumour endometrial tissues can vary considerably in their composition, the samples were standardised for cell content. Tumour tissue contained significantly less collagen than normal tissue (tumour: normal tissue hydroxyproline ratio was 0.20 ± 0.16, n=20, p=0.037; data courtesy MCM Vissers), indicating a lower cell content in normal tissue than in tumour samples per unit weight. This was confirmed by measuring the DNA content of the tissue homogenates, with tumour tissue containing significantly more DNA than normal tissue (p=0.005; Figure 5.2). There was no difference between tumour grades.
The DNA content was used to standardise the normal and tumour tissue specimens for cell content, as the intracellular levels of ascorbate are relevant to HIF-1 activity (described in Chapter 3). This was necessary for the accurate comparison of subsequent ascorbate measurements, Western blot analyses and VEGF assays, as all samples were balanced for cell content (DNA). The reliability of this method was confirmed by even loading of the housekeeping protein β-actin (as shown in Figures 5.5, 5.6 and 5.7), indicating that equal cellular protein content was achieved.

Figure 5.2: DNA content of endometrial tumour and normal tissue samples according to tumour grade. Tumour tissue had significantly higher DNA content than normal tissue (all grades combined; **p=0.005 by Wilcoxon signed-rank test). There was no difference in DNA content between tumour grades. n=51 cases of normal and tumour (18 are grade 1, 15 are grade 2 and 18 are grade 3). Data are means ± SEM.
5.2.4 Localisation of HIF-1α protein in endometrial tumour sections

As previous studies have exclusively used immunohistochemistry (IHC) to analyse HIF-1 activation (47, 54, 89, 240), the presence and localisation of HIF-1α was confirmed on a tissue microarray containing endometrial tumour cores and some normal tissue cores. To initially validate the IHC protocol, renal cell carcinoma sections were used as a positive control. Figure 5.3 shows strong nuclear immunodetection of HIF-1α with the primary antibody and no staining when the primary antibody was replaced with non-immune mouse IgGκ.

Figure 5.3: Renal cell carcinoma sections used as a positive control for HIF-1α immunohistochemistry. There is no HIF-1α immunostaining when the primary antibody is substituted with non-immune mouse IgGκ (A). Upon inclusion of the primary antibody, nuclear HIF-1α protein is detected as seen by brown staining (B). 200X magnification.
When detected in the endometrial tumour cores, HIF-1α was seen either throughout the entire section or in a small number of cells in the nuclei of glandular tissue (Figure 5.4A,B). HIF-1α was also seen surrounding areas of necrosis, with both cytoplasmic and nuclear staining (Figure 5.4C). Normal tissue sections were negative (Figure 5.4D) and had clearly different morphology with very little glandular tissue. They also often contained some myometrium, whereas tumour sections were primarily composed of glandular tissue and areas of necrosis.
Figure 5.4: HIF-1α protein levels in endometrial tumour and normal tissue core sections. Immunohistochemical localisation of HIF-1α from representative tumour sections showing positive staining in glandular tumour tissue (A, B) mainly in nuclei (inset; arrows). Perinecrotic HIF-1α staining was also seen (C; arrows). Normal endometrial sections were negative and contained very little glandular tissue (D). Necr, necrosis. Bar, 200 μm.
5.2.5 HIF-1α protein levels in endometrial normal and tumour tissue

Analysis of normal and tumour tissue homogenates by Western blotting showed that HIF-1α was readily detected in 37/48 tumours (77%), while weak expression was seen in 13/48 normal tissues (27%; Figure 5.5). Densitometry of the bands showed significantly higher HIF-1α protein in tumour compared to normal tissue (p<0.001; Figure 5.5), with no difference seen between tumour grades.
Figure 5.5: HIF-1α protein levels in endometrial normal and tumour samples. A, representative Western blot of HIF-1α protein from three cases of paired normal (N) and tumour (T) tissues (the numbers represent different samples, paired as N and T from the same patient), with β-actin as a loading control. The first lane contains the positive control (CoCl₂-treated A431 cell lysate; Ctrl) used for quantification and standardisation of each blot. There was little or no detection of HIF-1α in normal tissues and strong or weak expression in tumours. B, boxplot of HIF-1α densitometry in grade 1, 2 and 3 normal and tumour tissues, showing increased levels in tumour compared to normal tissue (**p<0.01, by Wilcoxon signed-rank test). HIF-1α bands were normalised against the positive control as a reference point for between-blot analysis and with β-actin used as a loading control for between-sample analysis. n=48 cases of normal and tumour samples (18 are grade 1, 14 are grade 2 and 16 are grade 3).
5.2.6 Quantification of HIF-1 target gene products in endometrial normal and tumour tissue

To determine the transcriptional activity of HIF-1 in the tumours, protein levels of three specific, pro-survival HIF-1 target gene products (GLUT-1, BNIP3 and VEGF) were measured. GLUT-1, measured by Western blotting, was detected in 46/48 tumours (96%) and 43/48 normal tissues (90%), with significantly higher expression in tumour compared to normal tissue (p<0.001; Figure 5.6) and increasing expression with tumour grade (p=0.029). BNIP3 protein was monitored in Western blots as a 30 kDa band (82) and this revealed expression in 44/46 tumours (96%) and 41/46 normal tissues (89%). BNIP3 was significantly higher in tumour compared to normal tissue (p<0.001; Figure 5.7), but did not change with grade. Tissue VEGF protein, measured by ELISA, was detectable in 44/44 tumours (100%) and 37/44 normal tissues (84%). Levels were significantly elevated in tumour tissue (p<0.001; Figure 5.8) and tended to increase with tumour grade (p=0.225).
**Figure 5.6:** GLUT-1 protein levels in endometrial normal and tumour samples. **A**, representative Western blot of GLUT-1 protein from three cases of paired normal (N) and tumour (T) tissues (the numbers represent different samples, paired as N and T from the same patient), with β-actin as a loading control. The first lane contains the positive control (CoCl$_2$-treated A431 cell lysate; Ctrl) used for quantification of each blot. There is minimal detection in normal tissues and strong or weak expression in tumours. **B**, boxplot of GLUT-1 densitometry in grade 1, 2 and 3 normal and tumour tissues, showing increased levels in tumour compared to normal tissue (*p<0.05, by Wilcoxon signed-rank test), and increasing expression with grade (p=0.029). GLUT-1 bands were normalised against the positive control and β-actin for each blot. n=49 cases of normal and tumour samples (18 are grade 1, 15 are grade 2 and 16 are grade 3).
Figure 5.7: BNIP3 protein levels in endometrial normal and tumour samples. A, representative Western blot of BNIP3 protein from three cases of paired normal (N) and tumour (T) tissues (the numbers represent different samples, paired as N and T from the same patient), with β-actin as a loading control. BNIP3 was detected as a doublet band, with the lower band (~30 kDa) used for densitometry. The first lane contains the positive control (CoCl₂-treated A431 cell lysate; Ctrl) used for quantification of each blot. There is minimal detection in normal tissues and variable expression in tumours. B, boxplot of BNIP3 densitometry in grade 1, 2 and 3 normal and tumour tissues, showing increased levels in tumour compared to normal tissue (**p<0.01, by Wilcoxon signed-rank test). BNIP3 bands were normalised against the positive control and β-actin for each blot. n=47 cases of normal and tumour samples (17 are grade 1, 14 are grade 2 and 16 are grade 3).
5.2.7 Ascorbate accumulation in tumour compared to surrounding normal tissue

The ascorbate content of normal and tumour tissue homogenates was measured and normalised against the DNA content of each sample, which reflects the cellular ascorbate content of normal compared to tumour tissues. Figure 5.9 shows the distribution of ascorbate in normal and tumour tissue with no overall difference seen (p=0.682).
In order to examine the relative ability of tumour tissue to accumulate ascorbate, tumour levels were standardised against the surrounding normal tissue from the same patient and expressed as a ratio (tumour: normal) for each case. There was a significant difference in the tumour: normal ascorbate ratio between tumour grades, with the ascorbate ratio decreasing with increasing tumour grade ($p=0.010$; Figure 5.10). In Grade 1 tumours, the ascorbate levels compared to the paired normal tissue were highly variable and not significantly different. Grade 2 tumours and matched normal tissue had approximately equal amounts, whereas grade 3 tumours had significantly less ascorbate than paired normal tissue ($p=0.028$). The difference between grades was not due to variation in DNA content, as this did not differ between grades (Figure 5.2). Grade 3 tumours had a significantly lower ratio compared to grade 1 tumours ($p=0.006$; Figure 5.10). These results indicate that high grade tumour tissue has reduced capacity to accumulate ascorbate relative to surrounding normal tissue.
Chapter 5: Measurement of ascorbate levels and HIF-1 activation in human endometrial tumours

5.2.8 Relationship between ascorbate, HIF-1 activity and tumour pathology

To determine whether ascorbate levels in the tumour tissue related to the activation of the HIF-1 pathway, the absolute ascorbate content in the tumour samples was compared to levels of HIF-1α and its target proteins (Figure 5.11). There was considerable variation in the tumour tissue ascorbate levels: the tumours ranged from 1.02-32.76 mg/100g with a median of 6.68 mg/100g and the normal tissue from 0.04-23.13 mg/100g with a median of 4.95 mg/100g. These levels are similar to uterine ascorbate levels in healthy women, reported to
be 10-20 mg/100g tissue (241). The lower limit of the samples suggests that many were relatively ascorbate-deficient. Therefore, an ascorbate level either greater or lower than 6.7 mg/100g (median level in tumours) was used to classify the samples as either ascorbate-deficient or ascorbate-replete. Although high grade tumours had low tumour: normal ascorbate ratios (Figure 5.10), each grade contained samples with both high and low tumour ascorbate content (Figure 5.11).

VEGF protein levels were significantly higher in the low ascorbate tumours (p=0.001; Figure 5.11D). A similar trend was seen with HIF-1α, GLUT-1 and BNIP3 proteins, with the highest levels seen in the low ascorbate tumours, whereas the high ascorbate tumours had low levels of these proteins (Figure 5.11). There were very few, if any, samples that had both high ascorbate content and a high level of any of these HIF-1-related proteins, suggesting there may be a relationship between ascorbate and activation of the HIF-1 response.
Figure 5.11: Tumour tissue ascorbate content in relation to protein levels of HIF-1 activation markers. Scatterplots show ascorbate content of individual tumour samples in relation to levels of four markers of HIF-1 activation: HIF-1α (A), GLUT-1 (B), BNIP3 (C) and VEGF (D). The low ascorbate tumours have the highest expression of the protein markers, with very few, if any, having both high ascorbate and high protein marker expression. n= 48 for HIF-1α, n=49 for GLUT-1, n=47 for BNIP3, n=44 for VEGF.
To assess whether low tumour ascorbate content is associated with activation of the HIF-1 pathway, the combined results for each tumour sample were analysed: the expression levels of HIF-1α, BNIP3, GLUT-1 and VEGF were normalised (as percent maximum expression) then added together to give each tumour a “HIF-1 pathway score”. This was verified by correlation with the individual protein levels (Table 5.2). When the HIF-1 pathway score was related to the tumour ascorbate status, it was found that ascorbate-deficient tumours had significantly higher HIF-1 pathway scores than ascorbate-replete tumours (p=0.007; Figure 5.12). Tumour VEGF protein levels were also associated with a low tumour: normal ascorbate ratio (p=0.022; Table 5.2), as well the overall tumour ascorbate content as seen earlier in Figure 5.11D. Together, this suggests that low tissue ascorbate levels are associated with significant up-regulation of the HIF-1 pathway.
Table 5.2: Spearman’s correlations between tumour HIF-1 activation markers, tumour size and ascorbate content in endometrial tumours.

<table>
<thead>
<tr>
<th></th>
<th>Ascorbate (µg/100mg tissue)</th>
<th>Tumor: normal ascorbate ratio</th>
<th>HIF-1α band density</th>
<th>GLUT-1 band density</th>
<th>BNIP3 band density</th>
<th>VEGF protein (pg/µg DNA)</th>
<th>HIF-1 pathway score</th>
<th>Tumour size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate (µg/100mg tissue)</td>
<td>r</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>p</td>
<td>n</td>
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</tr>
<tr>
<td>Tumor: normal ascorbate ratio</td>
<td>r</td>
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<tr>
<td>HIF-1α band density</td>
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<td></td>
<td></td>
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<td>p</td>
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<tr>
<td>GLUT-1 band density</td>
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<td>p</td>
<td>n</td>
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<tr>
<td>BNIP3 band density</td>
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<td></td>
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<td>p</td>
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<tr>
<td>VEGF protein (pg/µg DNA)</td>
<td>r</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>HIF-1 pathway score</td>
<td>r</td>
<td></td>
<td></td>
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<tr>
<td>Tumour size (mm)</td>
<td>r</td>
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</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). r, Spearman’s rho; p, significance; n, number of samples.
Further examination of the data revealed that, in tumour tissue, HIF-1α correlated with GLUT-1 (p=0.037) and VEGF (p=0.031) and tended to correlate with BNIP3 (p=0.090; Table 5.2). GLUT-1 and BNIP3 were strongly associated with each other (p=0.001; Table 5.2). Both HIF-1α and BNIP3 protein levels were associated with larger tumour size (p=0.012, 0.037, respectively; Table 5.2). In addition, tumours positive for lymph/vascular invasion tended to have higher levels of both HIF-1α (p=0.088) and GLUT-1 (p=0.068) than tumours without lymph/vascular invasion. Taken together, these results provide evidence for the activation of HIF-1 and downstream gene expression in endometrial tumours, and indicate that the HIF-1 pathway is activated in higher grade, invasive and larger tumours.

In relation to tumour ascorbate content and tumour pathology, there was no association between ascorbate content and clinical stage. However, there was a tendency for ascorbate-deficient tumours to be larger in size (Figure 5.13A), similar to the relationship with HIF-1 activation (Figure 5.12). In addition, tumours positive for necrosis (10/18 of these were grade 3) had significantly lower ascorbate levels than tumours without necrosis (Figure 5.13B).
Figure 5.12: Tumour ascorbate content in relation to HIF-1 pathway activation in endometrial tumours. The level of HIF-1α, GLUT-1, BNIP3 and VEGF proteins (markers of HIF-1 activity) were each normalised (as percent maximum expression) and each tumour sample was given a score equal to the combined expression of all four proteins (HIF-1 pathway score) which reflects HIF-1 pathway activation. **A**, scatterplot showing individual tumour samples by grade. The low-ascorbate tumours have the highest HIF-1 pathway activation. The dashed reference line is the median ascorbate content (6.7 mg/100g tissue) used to classify tumours as ascorbate-deficient or ascorbate-replete. **B**, ascorbate-replete tumours (n=22) have significantly lower HIF-1 pathway scores than ascorbate-deficient tumours (n=22; p=0.007 by Mann-Whitney test).
Figure 5.13: Relationship between tumour ascorbate content and tumour size and necrosis in endometrial samples. 

A, scatterplot of tumour size according to ascorbate content showing that larger tumours tend to have low ascorbate (p=0.284). 

B, boxplot showing tissue ascorbate levels are significantly lower in tumours positive for necrosis (n=18) compared to tumours without necrosis (n=9), by Mann-Whitney test (p=0.031).
5.3 Discussion

The results of this chapter provide the first direct evidence for a relationship between ascorbate content and an activated HIF-1 pathway in human tumours. Early claims that ascorbate could retard tumour progression (202, 203) were met with scepticism in the absence of a plausible mechanism of action, but the discovery that HIF-1 is controlled by ascorbate-dependent hydroxylation has provided a plausible mechanistic link between tissue ascorbate content and tumour metabolism. However, until now there has been no evidence to support a relationship between HIF-1, ascorbate and tumour progression in humans.

The methods used in this study have allowed for quantification of intracellular ascorbate and HIF-1 activity and for direct comparison with normal tissue from the same patient. To account for differences in tissue composition, DNA content and β-actin were used to reflect tissue cell content (242). The use of Western blot or ELISA to measure HIF-1α and its target gene proteins, respectively, also allowed for a less subjective quantification than is achievable with the more commonly used IHC. However, Western blotting is considered semi-quantitative, and the signal can come from multiple cell types. Nevertheless, Western blotting is representative of the whole tumour sample and the presence and nuclear localisation of HIF-1α was confirmed with IHC, providing further evidence for its transcriptional activity in tumour tissue.

Using human endometrial tumour tissue, it was found that ascorbate accumulation decreased with increasing tumour grade and that high grade tumours had up to 40% less ascorbate than matched adjacent normal tissue (tumour: normal ascorbate ratio). This ratio was also associated with increased VEGF protein levels, as was the overall tumour ascorbate content. Increased levels of other markers of HIF-1 activation (HIF-1α protein, GLUT-1 and BNIP3) all showed an inverse relationship with ascorbate, and when these markers were combined as the HIF-1 pathway score, ascorbate-deficient tumours showed significantly higher HIF-1 scores.
There was strong peri-necrotic HIF-1α staining present in tumour tissue, and interestingly, tumours positive for necrosis also had significantly lower ascorbate than necrosis-negative tumours. As HIF-1 was shown to be active in these regions, this provides another link between low ascorbate content and HIF-1 activation in endometrial tumours. There was also a tendency for low ascorbate tumours to be bigger in size. When taken together, these associations provide evidence to suggest that having inadequate tissue ascorbate levels may promote tumour progression as a result of poor control of HIF-1.

Endometrial tumours are highly angiogenic and the expression of VEGF has been associated with poor outcome (238). That VEGF was elevated in tumour tissue, and that it tended to increase with tumour grade, supports these observations. Furthermore, the correlation between VEGF and HIF-1α protein levels suggests that this increase in VEGF is HIF-1-dependent. GLUT-1 levels also correlated with HIF-1α and BNIP3 proteins, and increased with tumour grade. This increase in glucose transport is likely to be a major contributor to the excessive glycolysis and lactic acid production known to predominate in aggressive solid tumours (243).

The tendency for HIF-1-dependent gene expression to increase with tumour grade was apparent despite there being no variation in HIF-1α protein levels. This suggests that HIF-1α protein levels alone are not representative of its activity and may reflect the dual control of HIF-1, with proline hydroxylation controlling HIF-1α protein levels and asparagine hydroxylation by FIH independently controlling transcriptional activity (32, 33). As seen in Chapter 4, FIH may be more dependent on ascorbate for activity and it is therefore likely that HIF-1-mediated gene expression would be more responsive to low levels of ascorbate than HIF-1α protein stabilisation. This is reflected in the HIF-1 pathway score being higher in low ascorbate tumours and may also explain the significant inverse correlation between VEGF protein expression and ascorbate levels. This implies that low levels of cellular ascorbate may exacerbate the production of VEGF and promote tumour angiogenesis and growth.
The decrease in relative ascorbate accumulation with increasing tumour grade may reflect poor accessibility of the tumour cells to the plasma supply. Ascorbate is delivered via the circulation and uptake is dependent upon plasma levels, accessibility and activity of the transporter, SVCT (194). Hence, in a tumour with a poorly functioning vasculature, ascorbate uptake could be compromised. In addition, variation in SVCT expression or activity could also influence ascorbate uptake. The activity of the SVCTs is reduced by as much as 70% when the extracellular pH falls to 6.5 (190) and this could have a significant impact in the more acidic environment of the tumour (244). Increased levels of the glucose transporters is unlikely to influence ascorbate accumulation as these transport dehydroascorbate which is present in only minimal quantities in biological tissue (190). In addition, as glucose concentrations are far higher than ascorbate/DHA concentrations, competition for GLUT-1 is more likely to favour glucose transport (190).

Some grade 1 tumours contained much higher levels of ascorbate than the adjacent normal tissue from the same patient. Grade 1 endometrioid adenocarcinoma tissue is mainly composed of glandular tissue, as they are relatively well differentiated tumours (239). Because tissues vary widely in their ability to accumulate ascorbate (182), it is possible that ascorbate is distributed differently between myometrium, stroma and glandular tissue. Therefore, this result may reflect the different tissue types, as normal, post-menopausal uterine endometrial tissue biopsies can contain myometrium (smooth muscle) and are predominantly endometrial stromal tissue, with very few glands.

These results indicate that low cellular ascorbate accumulation could result in increased HIF-1 activity in human tumours. This was predicted from the results shown in Chapter 4, where optimal intracellular levels of ascorbate can substantially curb HIF-1 activation and subsequent gene product expression. In addition, these results support the concept that restoring tumour tissue ascorbate levels could limit the expression of pro-survival and angiogenic factors that promote tumour growth and poor outcome. These are the first data linking ascorbate and HIF-1 in human cancer and additional investigations in other tumour types is necessary to determine whether this could be a wider phenomenon.
5.4 Chapter summary

The data in this chapter have shown that HIF-1α protein and three of its pro-survival targets (GLUT-1, BNIP3 and VEGF) were all up-regulated in endometrial tumour tissue, and tended to increase with tumour grade, size and invasiveness. It was found that the ability of high grade endometrial tumour tissue to accumulate ascorbate relative to adjacent normal tissue was significantly impaired. In addition, the overall ascorbate content of endometrial tumours was inversely associated with HIF-1 pathway activation providing evidence that ascorbate may influence HIF-1 activation in human cancers. In addition, the ascorbate content and markers of HIF-1 activity can be reliably and quantitatively measured in stored tissue bank tumour and normal samples.
Chapter 6: Measurement of ascorbate levels and HIF-1 activation in human colorectal tumours

The results presented in this chapter have been included in:

Kuiper C, Dachs GU, Munn D, Currie MJ and Vissers MCM. Decreased ascorbate levels in human colorectal tumors are associated with increased hypoxia-inducible factor-1 activity and poorer disease-free survival. Manuscript submitted.

6.1 Introduction

I have shown that intracellular ascorbate levels are closely linked to the HIF-1 transcriptional response in vitro (Chapters 3 and 4), and results from Chapter 5 have shown a significant association between low ascorbate levels and high HIF-1 activation in human endometrial tumour samples. In addition, high grade tumours had reduced capacity to accumulate ascorbate compared to adjacent normal tissue, and low ascorbate levels were also associated with necrotic tumours and larger tumour size. These results support the hypothesis that inadequate ascorbate uptake by tumour cells may have an impact on tumour development.

The results obtained from the analysis of endometrial tumours in Chapter 5 were, to my knowledge, the first to examine the relationship between ascorbate and HIF-1 activation in human tissue. To determine whether the association found in endometrial cancer also applies in other human tumours, I have undertaken investigation of an additional tumour type. Colorectal cancer is second only to breast cancer for worldwide prevalence, and has an approximate mortality rate of 50% (245), making this a highly pertinent type of cancer to study. This is particularly relevant in New Zealand, where incidence rates of colorectal cancer are among the highest in the world (245).
Like endometrial cancer, colorectal cancer is known to have increased HIF-1α and VEGF expression that is associated with poor patient outcomes (62-64). This makes the HIF-1 pathway particularly relevant to colorectal cancer and it was therefore chosen as a second model to extend the analysis from endometrial cancer. As with the endometrial cancer study, the CSTBC was utilised, and 50 samples of colorectal cancer tissue, along with adjacent normal tissue from each patient, were obtained for study. In addition, survival data was obtained for the patients in this sample set as part of a summer studentship (by Delwyn Munn, 2012/2013), which was examined in relation to the tissue ascorbate content and level of HIF-1 activation.

6.1.1 Chapter aims

The aim of this chapter was to determine whether the relationship found in endometrial cancer also holds true for other tumour types. Hence, similar analyses were performed for HIF-1 activation and ascorbate content as were previously performed on endometrial tumour tissue samples, this time using colorectal cancer with paired normal tissues. These results were then to be compared with the results obtained from endometrial tumour samples reported in Chapter 5. Specifically, this chapter involved:

1. Measuring the degree of HIF-1 activation in human colorectal tumour samples, and paired normal tissue, and relating this to the cellular ascorbate content.

2. Assessing the expression of HIF-1 activity markers and ascorbate content in relation to clinico-pathological features of the tumours.

3. Analysis of patient survival data in relation to their tumour ascorbate content and degree of HIF-1 activation.
6.1.2 Experimental approach

Human colorectal cancer and adjacent normal tissue samples were obtained from the CSTBC (n=50 cases). The same approach as in Chapter 5 was used to process and analyse the tissue samples, which involved grinding the frozen tissue under liquid nitrogen and creating a homogeneous suspension (see section 2.6.3). The stability of ascorbate and detection of proteins in the samples using this protocol has been verified (Chapter 5). Protein levels of HIF-1α, GLUT-1 and BNIP3 were monitored by Western blotting, and VEGF by ELISA. These proteins were quantified and related to the ascorbate content (measured by HPLC-ECD), using DNA as a marker of cellularity.

The collation of patient records and survival data was part of a summer studentship, performed by Delwyn Munn (medical student, University of Otago) with permission from the human ethics committee as described in section 2.6. Statistical analyses of patient survival data against biochemical parameters, and generation of Kaplan-Meier curves, was performed by Dr. John Pearson (biostatistician, University of Otago).

6.2 Results

6.2.1 Tumour samples

All tissue was obtained as part of surgical resection of colorectal tumours with both tumour and adjacent normal tissue samples taken from each patient. Following surgical excision, the tumour was dissected and the samples frozen by the CSTBC within approximately one hour. Table 6.1 describes the clinico-pathological features of the sample set. Most samples were from the colon, with only one rectal tumour sample. The samples were from both male and female patients (42% and 58%, respectively), with a variety of tumour grades, stages and sizes.
Table 6.1 Clinico-pathological features of colorectal tumour sample set.

<table>
<thead>
<tr>
<th>Clinico-pathological parameter</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grade</strong></td>
<td></td>
</tr>
<tr>
<td>1 (well differentiated)</td>
<td>9 (18)</td>
</tr>
<tr>
<td>2 (moderately differentiated)</td>
<td>20 (40)</td>
</tr>
<tr>
<td>3 (poorly differentiated)</td>
<td>21 (42)</td>
</tr>
<tr>
<td><strong>AJCC stage</strong></td>
<td></td>
</tr>
<tr>
<td>1 – submucosal (T1) or muscularis (T2) involvement</td>
<td>7 (14)</td>
</tr>
<tr>
<td>2A – invasion of subserosa or beyond (T3)</td>
<td>12 (24)</td>
</tr>
<tr>
<td>2B – invasion of adjacent organs or perforation of visceral peritoneum (T4)</td>
<td>6 (12)</td>
</tr>
<tr>
<td>3A – T1 or T2 and metastasis to 1-3 regional lymph nodes (N1)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>3B – T3 or T4 and N1</td>
<td>11 (22)</td>
</tr>
<tr>
<td>3C – any T and metastasis to 4 or more regional lymph nodes (N2)</td>
<td>10 (20)</td>
</tr>
<tr>
<td>4 – any T, any N, with distant metastasis (M1)</td>
<td>3 (6)</td>
</tr>
<tr>
<td><strong>Tumour position</strong></td>
<td></td>
</tr>
<tr>
<td>Right ascending colon</td>
<td>17 (34)</td>
</tr>
<tr>
<td>Left descending colon</td>
<td>5 (10)</td>
</tr>
<tr>
<td>Transverse colon</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Caecum</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Sigmoid colon</td>
<td>9 (18)</td>
</tr>
<tr>
<td>Rectum</td>
<td>1 (2)</td>
</tr>
<tr>
<td>ND</td>
<td>14 (28)</td>
</tr>
<tr>
<td><strong>Tumour size</strong></td>
<td></td>
</tr>
<tr>
<td>≤ 50 mm</td>
<td>32 (64)</td>
</tr>
<tr>
<td>&gt; 50 mm</td>
<td>17 (34)</td>
</tr>
<tr>
<td>ND</td>
<td>1 (2)</td>
</tr>
<tr>
<td><strong>Necrosis</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>23 (46)</td>
</tr>
<tr>
<td>Yes</td>
<td>7 (14)</td>
</tr>
<tr>
<td>ND</td>
<td>20 (40)</td>
</tr>
<tr>
<td><strong>Lymph/vascular invasion</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>32 (64)</td>
</tr>
<tr>
<td>Yes</td>
<td>17 (34)</td>
</tr>
<tr>
<td>ND</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

AJCC, American Joint Committee on Cancer; ND, no data.
6.2.2 Cell density of normal and tumour tissue samples

The DNA content of the colorectal normal and tumour samples was measured as a marker of the cell content. This also allowed the samples to be standardised for cell content for subsequent Western blot and ascorbate analyses. Figure 6.1 shows the DNA content of the samples, with tumour tissue having significantly higher DNA content than normal tissue, with no difference seen between tumour grades.

![DNA content of colorectal tumour and normal tissue samples](image)

**Figure 6.1:** DNA content of colorectal tumour and normal tissue samples. Tumour tissue has significantly higher DNA content than normal tissue (all grades combined; **p<0.001, by paired Student’s t-test). There was no difference in DNA content between tumour grades. Data are means ± SEM of 50 normal and tumour tissues, where 9 are grade 1, 20 are grade 2 and 21 are grade 3.
6.2.3 HIF-1α and target gene protein levels

HIF-1α protein was detected by Western blotting as a 120 kDa band in 29 of 50 tumours (58%) and in 4 of 50 normal tissues (8%). Densitometry of the bands showed significantly higher levels of HIF-1α protein in tumour compared to normal tissue (p<0.001; Figure 6.2). GLUT-1 was detected in 36 of 50 tumours (72%) and 34 of 50 normal tissues (68%) with no difference in levels found between normal and tumour samples (Figure 6.3). BNIP3 was detected as a 30 kDa doublet band in 38 of 50 tumours (76%) and 21 of 50 normal tissues (42%) and was significantly higher in tumour compared to normal tissue (p=0.004; Figure 6.4). VEGF protein was detectable in 50 of 50 tumours (100%) and 49 of 50 normal tissues (98%) with significantly higher levels in tumour than normal tissue (p<0.001; Figure 6.5).
Figure 6.2: HIF-1α protein expression in normal and tumour colorectal tissue samples. Representative Western blots of HIF-1α from normal and tumour tissue samples are shown, including a positive control (CoCl₂-treated Jurkat whole cell lysate; Ctrl), followed by a boxplot of densitometry results from all samples (n=50) normalised to the same positive control for each protein and to β-actin loading control. Tumour tissue had significantly higher HIF-1α protein than normal tissue (p<0.001, by Wilcoxon signed-rank test).
Figure 6.3: GLUT-1 protein expression in normal and tumour colorectal tissue samples. Representative Western blot of GLUT-1 from normal and tumour tissue samples are shown, including a positive control (endometrial tumour sample #92; Ctrl), followed by a boxplot of densitometry results from all samples (n=50) normalised to the same positive control for each protein and to β-actin loading control. There was no difference in GLUT-1 levels between normal and tumour samples (p=0.310, by Wilcoxon signed rank test).
Figure 6.4: BNIP3 protein expression in normal and tumour colorectal tissue samples. Representative Western blot of BNIP3 from normal and tumour tissue samples are shown, including a positive control (endometrial tumour sample #80; Ctrl), followed by a boxplot of densitometry results from all samples (n=50) normalised to the same positive control for each protein and to β-actin loading control. BNIP3 protein was significantly higher in tumour tissue compared to normal tissue samples (p=0.004, by Wilcoxon signed-rank test).
**Figure 6.5:** VEGF protein levels in normal and tumour colorectal tissue samples. VEGF protein, as measured by ELISA, shows significantly higher levels in tumour compared to normal tissues (n=50 sample pairs; p<0.001, by Wilcoxon signed-rank test).

As established in previous chapters, ascorbate can affect both HIF-1α protein levels and transcriptional activity via the PHDs and FIH, respectively. Therefore both parameters were combined to assess the activation of the HIF-1 pathway in relation to ascorbate content. As with the endometrial tumours, the level of each protein (HIF-1α, GLUT-1, BNIP3 and VEGF) was normalised (to percentage of maximum expression) and the mean of all four values for each sample was taken to give a HIF-1 pathway score. The HIF-1 pathway score was significantly higher in tumour compared to normal tissue (Figure 6.6; p<0.001).
Figure 6.6: **HIF-1 pathway activation in normal and tumour colorectal tissue samples.**
The HIF-1 pathway score was determined for each sample from the combined analysis of HIF-1α, GLUT-1, BNIP3 and VEGF levels, where the individual scores were normalised as percentage of maximum expression and the mean taken (n=50 cases). This measure was significantly higher in tumour compared to normal tissue (p<0.001, by Wilcoxon signed-rank test).

6.2.4 **Ascorbate content in normal and tumour tissue samples**

As it is the intracellular level of ascorbate that is relevant to the control of HIF-1, an estimate of the cellularity of the tissue is necessary. Therefore, the ascorbate measurements were standardised against the DNA content of the samples and expressed as nmol/µg DNA. There was a strong correlation between DNA content and absolute ascorbate levels (mg/100g tissue) in both normal and tumour samples (Spearman’s rho = 0.547 and 0.482, respectively; p<0.001), suggesting that most of the ascorbate in the samples was, in fact, intracellular. The results demonstrate that ascorbate levels (nmol/µg DNA) in the tumour tissue were significantly lower than in normal tissue (p<0.001; Figure 6.7).
Figure 6.7: Ascorbate content of normal and tumour colorectal tissue samples. Tissue ascorbate levels are significantly lower in tumour compared to normal tissue samples (p<0.001 by paired Student’s t-test, 2-tailed). Data are means ± SEM of 50 normal and tumour cases.

Ascorbate was also expressed as the ratio of tumour: normal tissue levels (nmol/µg DNA) to reflect the ability of the tumour to accumulate ascorbate relative to adjacent normal tissue in each individual. The median tumour: normal ascorbate ratio was 0.70, which was significantly different from normal tissue (p=0.001). This difference was attributable to the high grade tumours, with the median tumour: normal ascorbate ratios being 0.910, 0.695 and 0.610 for grade 1, 2 and 3 tumours, respectively (Figure 6.8). This did not reflect a difference in the cellularity of the three tumour grades, as each had the same DNA content (Figure 6.1). These results indicate that high grade colorectal tumour tissue has a reduced capacity to accumulate ascorbate compared to surrounding normal tissue.
Figure 6.8: Tumour tissue ascorbate content relative to surrounding normal tissue of colorectal samples. Boxplot of the ratio of ascorbate in tumour tissue compared to the adjacent normal tissue from the same patient (ascorbate ratio), by grade, showing high grade tumours have significantly less ascorbate than surrounding normal tissue (n=50). †, p < 0.01, normal vs. tumour, by Wilcoxon signed-rank test.
6.2.5  Relationship between tumour ascorbate content and HIF-1 activation markers

In order to determine whether tumour ascorbate levels relate to the degree of HIF-1 activation, the measured parameters were compared (Figure 6.9). An inverse trend was seen between the tumour ascorbate content and the expression of each protein, and this was significant for VEGF (Figure 6.9D, \( p=0.012 \); linear regression). A similar relationship was observed using Spearman’s correlations (Table 6.2). This analysis revealed a strong inverse relationship between the HIF-1 pathway score and ascorbate levels in tumour tissue, which was relevant to both the overall tumour ascorbate content (\( p=0.001 \)), and the tumour: normal ascorbate ratio (\( p=0.009 \); Table 6.2). In addition, the tumour ascorbate content was inversely correlated to VEGF (\( p=0.023 \)) and BNIP3 (\( p=0.033 \)) protein levels (Table 6.2). The tumour: normal ascorbate ratio was also inversely correlated to VEGF (\( p=0.031 \)) and GLUT-1 (\( p=0.011 \)) protein levels (Table 6.2).
Figure 6.9: Scatterplots comparing tumour tissue ascorbate content and markers of HIF-1 activation in colorectal samples. Protein levels of HIF-1α (A), GLUT-1 (B), BNIP3 (C) and VEGF (D) tended to inversely relate to the tumour ascorbate content (regression lines shown). There was a significant relationship between lower tumour ascorbate and higher VEGF levels.
Table 6.2: Spearman’s correlations between colorectal tumour HIF-1 activation markers, tumour size and ascorbate.

<table>
<thead>
<tr>
<th></th>
<th>Ascorbate content (nmol/µg DNA)</th>
<th>Tumor: normal ascorbate ratio</th>
<th>HIF-1α band density</th>
<th>GLUT-1 band density</th>
<th>BNIP3 band density</th>
<th>VEGF (pg/mg tissue)</th>
<th>HIF-1 pathway score</th>
<th>Tumor size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate content</td>
<td>r</td>
<td>.651**</td>
<td>.006</td>
<td>-.223</td>
<td>-.302*</td>
<td>-.321*</td>
<td>-.445**</td>
<td>-.256</td>
</tr>
<tr>
<td>r (nmol/µg DNA)</td>
<td>p</td>
<td>.000</td>
<td>.970</td>
<td>.120</td>
<td>.033</td>
<td>.023</td>
<td>.001</td>
<td>.076</td>
</tr>
<tr>
<td>Tumor: normal ascorbate ratio</td>
<td>r</td>
<td>.651**</td>
<td>.112</td>
<td>-.357*</td>
<td>-.134</td>
<td>-.305*</td>
<td>-.364**</td>
<td>-.112</td>
</tr>
<tr>
<td>p</td>
<td>.000</td>
<td></td>
<td>.437</td>
<td>.011</td>
<td>.354</td>
<td>.031</td>
<td>.009</td>
<td>.445</td>
</tr>
<tr>
<td>HIF-1α band density</td>
<td>r</td>
<td>-.223</td>
<td>.112</td>
<td>.026</td>
<td>.135</td>
<td>.153</td>
<td>.284**</td>
<td>-.069</td>
</tr>
<tr>
<td>p</td>
<td>-.120</td>
<td></td>
<td>.437</td>
<td>.011</td>
<td>.351</td>
<td>.288</td>
<td>.046</td>
<td>.385</td>
</tr>
<tr>
<td>GLUT-1 band density</td>
<td>r</td>
<td>-.357*</td>
<td>.058</td>
<td>.012</td>
<td>.124</td>
<td>.781**</td>
<td>.083</td>
<td>-.069</td>
</tr>
<tr>
<td>p</td>
<td>.033</td>
<td></td>
<td>.970</td>
<td>.691</td>
<td>.932</td>
<td>.933</td>
<td>.045</td>
<td>.637</td>
</tr>
<tr>
<td>BNIP3 band density</td>
<td>r</td>
<td>-.302*</td>
<td>-.134</td>
<td>.135</td>
<td>.058</td>
<td>.124</td>
<td>.781**</td>
<td>.572</td>
</tr>
<tr>
<td>p</td>
<td>-.305*</td>
<td></td>
<td>.354</td>
<td>.351</td>
<td>.691</td>
<td>.393</td>
<td>.393</td>
<td>.572</td>
</tr>
<tr>
<td>VEGF (pg/mg tissue)</td>
<td>r</td>
<td>-.321*</td>
<td>-.305*</td>
<td>.153</td>
<td>.012</td>
<td>.124</td>
<td>.493**</td>
<td>.172</td>
</tr>
<tr>
<td>p</td>
<td>-.023</td>
<td></td>
<td>.031</td>
<td>.288</td>
<td>.932</td>
<td>.393</td>
<td>.000</td>
<td>.236</td>
</tr>
<tr>
<td>HIF-1 pathway score</td>
<td>r</td>
<td>-.445*</td>
<td>-.364*</td>
<td>.284*</td>
<td>.284*</td>
<td>.781**</td>
<td>.493**</td>
<td>.057</td>
</tr>
<tr>
<td>p</td>
<td>-.001</td>
<td></td>
<td>.046</td>
<td>.045</td>
<td>.000</td>
<td>.000</td>
<td>.000</td>
<td>.695</td>
</tr>
<tr>
<td>Tumor size (mm)</td>
<td>r</td>
<td>-.256</td>
<td>-.112</td>
<td>.127</td>
<td>-.069</td>
<td>.083</td>
<td>.172</td>
<td>.057</td>
</tr>
<tr>
<td>p</td>
<td>.076</td>
<td></td>
<td>.445</td>
<td>.385</td>
<td>.637</td>
<td>.572</td>
<td>.236</td>
<td>.695</td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed).  * Correlation is significant at the 0.05 level (2-tailed).  r, Spearman’s rho; p, significance; n=50 for all calculations, except tumour size (n=49).

The relationship between tumour ascorbate content and the HIF-1 pathway score according to tumour grade for each sample is shown in Figure 6.10. This shows that, despite high grade tumours having lower ascorbate compared to surrounding normal tissue (Figure 6.8), there were samples with high and low overall levels in each grade, and this was related to the HIF-1 pathway score (Figure 6.10A). When samples were separated according to the median ascorbate content (0.13 nmol/µg DNA), the ascorbate-replete tumours had significantly lower HIF-1 pathway scores (Figure 6.10B).
Figure 6.10: Low tumour ascorbate content and high HIF-1 pathway activation in colorectal tumour samples. A, scatterplot showing tumour tissue ascorbate levels are inversely related to the HIF-1 pathway score (solid regression line). Dashed reference line shows the median tumour ascorbate level (0.13 nmol/µg DNA) used to classify tumours as ascorbate-deficient or ascorbate-replete. B, boxplot showing ascorbate-deficient tumours (n=27) have significantly higher HIF-1 pathway scores than ascorbate-replete tumours (n=23); p=0.005, by Mann-Whitney test.
6.2.6 Ascorbate content and tumour pathology

There was an association between tumour size and ascorbate content, with larger tumours having less ascorbate (Figure 6.11A). There was no association between the ascorbate content or HIF-1 markers and AJCC stage or lymph/vascular invasion status of the tumours. There was a tendency for tumours positive for necrosis to have less ascorbate (6/7 were grade 3), with data for only 30 out of 50 tumours (Figure 6.11B; p=0.067).
Figure 6.11: Tumour ascorbate levels in relation to tumour size and necrosis in colorectal samples. A, scatterplot showing an inverse relationship between tumour ascorbate levels and tumour size. B, tumours positive for necrosis (n=7) tended to have lower ascorbate levels than tumours with no detectable necrosis (n=23); p=0.067, by unpaired Student’s t-test, 2-tailed. Data are means ± SEM.
6.2.7 Patient survival data in relation to tissue ascorbate content and HIF-1 pathway score

Of the 50 tumour samples analysed, the outcome of all the patients was determined as both overall survival and time to progression (disease-free survival; starting after tumour resection surgery). Stage 4 patients were excluded (n=3), as they had metastasis on presentation and their disease was considered as already progressed, and one patient was excluded as metastasis was discovered during surgery. In addition, patients who did not recover from surgery (i.e. died within 10 days of surgery) were excluded (n=2), leaving a total of 44 patients that were included. Figure 6.12 shows Kaplan-Meier plots of disease-free survival according to tumour and normal tissue overall ascorbate content, the tumour: normal ascorbate ratio for each patient and the HIF-1 pathway score, where each parameter was divided into ‘high’ (n=22) and ‘low’ (n=22) categories based on the median value. Table 6.3 summarises the statistical observations obtained from Cox proportional hazard modelling for disease-free survival, showing a significant risk of low tumour tissue ascorbate.

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Hazard ratio</th>
<th>CIL</th>
<th>CIU</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour tissue ascorbate</td>
<td>0.135</td>
<td>0.44</td>
<td>0.19</td>
<td>0.98</td>
<td>0.04</td>
</tr>
<tr>
<td>(nmol/ug DNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal tissue ascorbate</td>
<td>0.208</td>
<td>1.92</td>
<td>0.88</td>
<td>4.15</td>
<td>0.10</td>
</tr>
<tr>
<td>(nmol/ug DNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour: normal ascorbate</td>
<td>0.709</td>
<td>0.50</td>
<td>0.23</td>
<td>1.09</td>
<td>0.08</td>
</tr>
<tr>
<td>ratio (nmol/ug DNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1 pathway score</td>
<td>11</td>
<td>1.88</td>
<td>0.86</td>
<td>4.13</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Estimates from Cox proportional hazard models (dichotomous at the median level). CIL and CIU, lower and upper 95% confidence intervals, respectively. P, significance.

Log rank tests showed a significant association between low tumour tissue ascorbate content and shorter time to disease progression (p=0.006), as shown in the Kaplan-Meier plot in Figure 6.12A. In addition, there was a similar trend with the tumour: normal ascorbate ratio
(Figure 6.12C; p=0.064). A high HIF-1 pathway score tended to associate with shorter time to progression, although this was not significant (Figure 6.12D; p=0.234). Of interest was the trend for shorter disease-free progression time to be associated with a higher normal tissue ascorbate level, although this was not significant (Figure 6.12B; p=0.614).

**Figure 6.12:** Disease-free survival of colorectal cancer patients according to ascorbate and HIF-1 measurements. Each parameter was divided into ‘high’ (n=22) and ‘low’ (n=22) categories based on the median value. The proportion of disease-free patients was plotted over time since their tumour resection surgery. Each step down on the figures represents a patient whose cancer has progressed, and each tick mark represents cessation of follow-up of a patient. **A,** low tumour tissue ascorbate content is significantly associated with shorter disease-free time (p=0.006; median=0.135 nmol/µg DNA). **B,** normal tissue ascorbate content shows a trend for patients with low levels to have longer progression-free time, although this is not significant (p=0.614; median=0.208 nmol/µg DNA). **C,** the tumour: normal ascorbate ratio also suggests that low relative tumour ascorbate content is associated with shorter disease-free time (p=0.064; median=0.709). **D,** a high HIF-1 pathway score tended to be associated with shorter disease-free time, although not significantly (p=0.234; median=11).
Log rank tests on the overall survival data did not show any associations or trends (Figure 6.13) for either the tumour tissue ascorbate content ($p=0.581$), normal tissue ascorbate content ($p=0.738$), tumour: normal ascorbate ratio ($p=0.682$) or the HIF-1 pathway score ($p=0.686$). A larger sample size is needed to fully evaluate overall survival.

**Figure 6.13: Overall survival of colorectal cancer patients according to ascorbate and HIF-1 measurements.** Each parameter was divided into ‘high’ (n=22) and ‘low’ (n=22) categories based on the median value. The proportion of surviving patients was plotted over time since their tumour resection surgery. Each step down on the figures represents a patient who died, and each tick mark represents cessation of follow-up of a patient. **A,** tumour tissue ascorbate content. **B,** normal tissue ascorbate content. **C,** the tumour: normal ascorbate ratio. **D,** HIF-1 pathway score.
6.3 Discussion

These results confirm my previous findings in endometrial cancer samples, linking decreased ascorbate availability to high HIF-1 activation (Chapter 5). In addition, there was a significant association between low tumour tissue ascorbate content and shorter disease-free progression time, which is a strong indication that it may play a role in tumour pathology. The similarity between the results obtained from endometrial and colorectal tumours is striking, particularly considering a relatively modest sample size of 50 cases. Both tumour types displayed up-regulation of the HIF-1 pathway that was almost identically higher in the low-ascorbate tumours. Low ascorbate levels were similarly observed to occur in larger-size and necrotic tumours. HIF-1 is known to be active in perinecrotic regions (as seen in Chapter 5), where there is a great deal of selection pressure on cells to survive in this inhospitable microenvironment (7), therefore increasing the ascorbate supply to these regions may help overcome the survival response.

The data presented in this chapter provide additional direct evidence for the potential importance of intracellular ascorbate in the development and progression of human tumours. This represents an important first step in addressing the question of whether sub-optimal intracellular ascorbate concentrations leads to over-activation of the HIF-1 transcriptional response in tumour cells in vivo. If this is the case, then it may be reasonable to determine whether increasing ascorbate availability to cancer cells can in fact decrease the HIF-1 response sufficiently to either inhibit tumour formation and growth, or overcome HIF-1-mediated survival processes, such as chemo-resistance, in existing tumours.

The recent acquisition of patient survival data revealed patterns consistent with the hypothesis that low tumour ascorbate content may lead to tumour progression in association with an over-activate HIF-1 pathway. This was seen in the disease-free survival curves, where a shorter time to disease progression tended to associate with a high HIF-1 pathway score and a low tumour: normal ascorbate ratio, and was significantly associated with having low tumour ascorbate content. To my knowledge, this is the first data to assess patient outcome in relation to tissue ascorbate and is a strong indicator of the potential impact ascorbate may have on HIF-1-dependent tumour progression. However, there was an inconsistent trend with
high normal tissue ascorbate content being associated with shorter time to disease progression, although this was a relatively small effect that was non-significant. Further investigation in a larger population will determine if this is a real phenomenon.

HIF-1 is particularly relevant to colorectal cancer, as HIF-1α levels have been identified as an independent indicator of poor prognosis in a study of 731 prospective patient samples (62). In addition, whereas HIF-1α alone was not significant, in combination with two of its target genes (CXCR4 and VEGF) HIF-1α was associated with distant metastasis and poor disease-free survival (246). In another study, high VEGF expression was also found to be associated with poor survival in colorectal cancer, whereas HIF-1α was borderline significant (247). These studies highlight the dual control of the HIF-1 transcriptional response by the PHDs and FIH, and the importance of measuring HIF-1α protein levels and its downstream gene products in combination, which has been done in this thesis. That both VEGF and BNIP3, but not HIF-1α, protein levels were inversely correlated to the tumour ascorbate content agrees with the observation that FIH may be more dependent on ascorbate for activity that the PHDs (33). Thus, as seen in Chapter 4, and suggested here and in Chapter 5, ascorbate may have a greater influence on curbing HIF-1 transcriptional activity than it does on protein stabilisation of HIF-1α.

Sufficient accessibility of ascorbate to tumour tissue may be a limiting factor for its therapeutic value. This is demonstrated by the result that in both endometrial and colorectal tumours, high grade tumours had up to ~40% less ascorbate than adjacent normal tissue from the same patient, indicating a diminished ability to accumulate ascorbate. The reasons for this are unclear, but one possibility may be that the cells in high grade tumours may have decreased access to a functional blood supply. Solid tumours typically have a highly disorganised and abnormal microvasculature (6) and this may be more common in poorly differentiated tumours (i.e. high grade). Although high grade tumours often have increased microvessel density (248), the vessels may be not be adequately functional, as arterio-venous flow dynamics are often disturbed in these tumours (10, 12). This concept is supported by the observation that the majority of the tumours positive for necrosis (indicative of poor blood supply and tumour hypoxia) were grade 3. Furthermore, the tumour: normal ascorbate ratio
was inversely correlated with GLUT-1 and VEGF protein levels, possibly indicating a response to a poor vascular supply by increasing VEGF and GLUT-1 to restore nutrient access.

In addition to the issue of accessibility of ascorbate to tumour cells, the decreased levels found in some tumours may reflect circulating concentrations that are inadequate for sufficient tissue penetration. In humans, it is unknown how plasma concentrations can influence diffusion into tissues and whether sufficient concentrations can be achieved through dietary intake or whether intravenous administration may be necessary. Maximum plasma ascorbate levels reach only ~100 µM through gastrointestinal absorption, whereas intravenous administration can result in plasma concentrations above 10 mM (201), and this may be necessary for sufficient extravascular diffusion. Pharmacokinetic data on ascorbate in tumour tissues at various plasma concentrations would be particularly valuable.

Several studies have investigated tumour tissue ascorbate levels compared to normal tissue, with variable results. Brain tumours appear to contain significantly less ascorbate than normal brain tissue (210, 249) whereas breast (250), oral (198), skin (251) and lung (195) cancers had up to 2-3 times more ascorbate than corresponding normal tissues. However, it is important to note that as ascorbate is primarily intracellular, and as tumour tissue is more cell dense than normal tissue (242, 250), these increases in ascorbate may not be reflective of the amount per cell. The results in this chapter confirm this, as when the tissues were normalised for DNA content, there was significantly less cellular ascorbate in tumour tissue. In addition, heterogeneity of the tumour tissue may belie any overall differences, and cells within some regions of the tumour may be ascorbate-deficient. If these regions exist because of their distance from functioning vasculature, then ascorbate deficiency may coincide with tumour hypoxia to further amplify HIF-1 transcriptional activity.

If ascorbate is to be considered as a potential cancer therapy, there needs to be a clear understanding of its pharmacokinetics and mechanism of action in tumour tissue before appropriately designed clinical trials begin. Moreover, the dosing regimen would need to be
clarified in order to achieve effective tumour tissue ascorbate levels. As there is considerable scepticism regarding the usefulness of ascorbate in treating cancer, addressing these important mechanistic issues will improve the interpretation of previous clinical research and direct future human studies.

6.4 Chapter summary

The data presented in this chapter are consistent with the hypothesis that tumours which are unable to accumulate sufficient ascorbate have an overactive HIF-1-response and a worse patient outcome. Colorectal tumours had an up-regulated HIF-1 pathway that was associated with low tumour ascorbate levels and high grade colorectal tumours had a significantly impaired ability to accumulate ascorbate compared to adjacent normal tissue. These results are strikingly similar to those from endometrial tumours and indicate that low ascorbate levels are related to an active HIF-1 response in human cancers. Moreover, ascorbate availability to tumour cells may be limited, particularly in HIF-1 active regions (e.g. perinecrotic) in high grade tumours, and this may further enhance HIF-1-mediated cell survival. Furthermore, that low tumour tissue ascorbate was associated with shorter time to disease progression indicates that increasing tumour tissue ascorbate levels may be beneficial.
Chapter 7: Ascorbate pharmacokinetics in avascular tumour tissue

The results presented in this chapter were obtained in collaboration with Dr. Kevin O. Hicks at the Auckland Cancer Society Research Centre.

7.1 Introduction

Solid tumours are known to contain regions of poor perfusion and hypoxia (2). This can be caused by high inter-vessel distances that can average around 250 µm, whereas the calculated diffusion distance of oxygen through tumour tissue can range from only 41-183 µm (9). In addition to a lack of oxygen, delivery of nutrients, such as ascorbate, is also likely to be impaired in these regions. Furthermore, it is common for cancer patients to have low plasma concentrations of ascorbate (195-200). These factors may result in some regions of the tumour becoming ascorbate-deficient, thereby depriving the HIF-hydroxylases of sufficient ascorbate for optimal activity, serving to exacerbate the HIF-1 transcriptional response.

That this occurs has been suggested in the previous two chapters, with many clinical tumour tissue samples containing low levels of ascorbate, and this was associated with increased activation of the HIF-1 pathway. Of particular interest was the observation that both endometrial and colorectal tumours of a high grade had a reduced capacity to accumulate ascorbate compared to surrounding normal tissue. This result indicated that despite the same available plasma concentrations, some tumours cannot acquire the same cellular levels.

This led to the hypothesis that due to the likely nature of these tissues being dense with a poor vascular supply, some areas of the tumour may have limited access to ascorbate for sufficient
cellular uptake. However, to my knowledge, there is currently no data describing the extravascular diffusion of ascorbate through tissue and, therefore, whether a lack of blood supply is indeed likely to be a limiting factor for cellular uptake. It was therefore an aim of this chapter to obtain pharmacokinetic data on the diffusion of ascorbate through tumour tissue. This could provide information regarding what plasma concentrations are needed for maximum tissue penetration and cellular uptake, and inform as to whether physiological plasma levels from dietary intake is sufficient for this to occur, or whether intravenous administration of pharmacological concentrations is required.

An *in vitro* pharmacokinetic model was used that employs a linear three-dimensional cell culture technique known as multicellular layers (MCLs). The MCLs are a model for the extravascular compartment, where they reflect tumour-like tissue. They are made by growing cells on a porous Teflon support membrane (252) to form diffusion limited structures up to several hundred µm in thickness, with many features in common with spheroids (253), including central hypoxia and necrosis (254). However, MCLs have a planar structure, making them particularly amenable to drug diffusion studies (255), where they are used within a specialised diffusion chamber (see section 2.12 and Figure 2.5). This *in vitro* model was developed by Hicks et al., at the ACSRC, where it is used extensively to test the pharmacokinetics of DNA- and hypoxia-targeted anti-cancer compounds, some of which are currently undergoing clinical testing (3, 218, 220).

### 7.1.1 Chapter aims

The aim of this chapter was to utilise the MCL diffusion model to determine what plasma ascorbate concentrations are optimal for adequate tumour tissue penetration and cellular uptake to support HIF-hydroxylase activity. Specifically, this involved:

1. Monitoring the flux of ascorbate through MCLs and calculation of its diffusion coefficient through tissue. The flux of two radiolabeled internal standards, $^{14}\text{C-urea}$
and \(^3\)H-mannitol, were also monitored to calculate the MCL thickness and ensure its integrity for each experiment.

2. Modelling of the data to simulate ascorbate penetration and cellular uptake in tumour tissue, which will indicate optimal plasma concentrations required for penetration and uptake at different tissue depths.

7.1.2 Experimental approach

MCLs were grown using the colorectal cancer cell line HT29, from which WiDr cells are derived (256). The MCLs were inserted into the diffusion chamber apparatus, separating the stirred, gassed (5% CO\(_2\) and 95% N\(_2\)) donor and receiver compartments (see Figure 2.5). Ascorbate, and \(^{14}\)C-urea and \(^3\)H-mannitol, were added to the donor compartment and measured in the receiver compartment over 5 hours. Intracellular ascorbate uptake data was obtained from HT29 cells in monolayer over 5 hours at 0.1% O\(_2\) following the same protocol as used in Chapter 3 (described in section 2.3). The diffusion data and cellular uptake kinetics were incorporated into the diffusion model (described in detail in section 2.12.3).

Simulations of ascorbate tissue penetration and cellular uptake were performed assuming a distance of 100 µm or 200 µm from the capillary. This models a situation intermediate between radial diffusion out of a central blood vessel (Krogh cylinder) and diffusion into a tumour cord from surrounding blood vessels. Two scenarios were simulated. One was of continuous ascorbate infusion for 24 hours, which models a constant intake, either at physiological plasma concentrations (10-100 µM) or pharmacological concentrations (1 mM or 10 mM). The second scenario was an intravenous bolus dose of pharmacological concentrations, followed by plasma concentration decay, with a half-life of 30 minutes (201). Single cell data modelling and simulations were kindly performed by Dr. Kevin Hicks at the ACSRC.
7.2 Results

7.2.1 The flux of urea as a marker of diffusion and MCL thickness

The flux of $^{14}$C-urea was used to calculate the thickness of each MCL, as well as being a marker of transport through the MCL, and an internal standard for subsequent experiments measuring stability and bare support flux of ascorbate without MCLs present. Figure 7.1 shows representative flux curves of urea through the bare support, and through the MCL. The measured diffusion coefficient of urea through the bare support ($D_{\text{sup}}$; $1.77 \times 10^{-6} \pm 0.04 \text{ cm}^2 \text{ s}^{-1}$; n=10) was identical to the historical average ($1.77 \times 10^{-6} \pm 0.03 \text{ cm}^2 \text{ s}^{-1}$) (K.O. Hicks, personal communication). The measured $^{14}$C-urea diffusion coefficient through MCLs ($D_{MCL}$; $4.5 \times 10^{-7} \pm 0.23 \text{ cm}^2 \text{ s}^{-1}$; historical data) (257) was used to calculate the MCL thickness for each experiment, which ranged from 70.9 µm to 132.5 µm (n=19), with $D_{\text{sup}}$ fixed at $1.77 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. 
Figure 7.1:  Flux of $^{14}$C-urea as a marker of transcellular diffusion. Representative flux curves of urea through the bare Teflon support membrane (A), or through a multicellular layer (B), showing overlay of the raw data (symbols) and the fitted curves generated by the model (lines). $^{14}$C-urea was added to the donor compartment and the concentration was measured in both the donor and receiver compartment and represented here relative to the initial concentration in the donor compartment (fraction diffused). The constant mass balance shows no loss of the compound as it diffuses over the 5 hour time period. Diffusion is relatively rapid through the bare support, and is slowed by the presence of the multicellular layer.
7.2.2  The flux of mannitol as a marker of paracellular diffusion

The diffusion of $^{3}$H-mannitol through each MCL was monitored as a marker of paracellular diffusion and MCL integrity. Figure 7.2 shows representative flux curves for $^{3}$H-mannitol through the bare Teflon support and through a MCL. The measured $D_{\text{sup}}$ was $1.14 \pm 0.04 \times 10^{-6}$ cm$^{2}$ s$^{-1}$ (n=10), which was used to fit the $D_{\text{MCL}}$, calculated as $2.56 \pm 0.06 \times 10^{-7}$ cm$^{2}$ s$^{-1}$ (n=19), which was in line with the historical average ($2.1 \pm 0.1 \times 10^{-7}$ cm$^{2}$ s$^{-1}$) (257).

Diffusion of mannitol through the bare supports was slightly slower than urea due to its higher molecular weight (182 g mol$^{-1}$ and 80 g mol$^{-1}$, respectively). In addition, as mannitol can only diffuse paracellularly, its diffusion through the MCLs is also slower than urea, which is assumed to pass through cell layers to a greater extent.
Figure 7.2: Flux of $^3$H-mannitol as a marker of paracellular diffusion. Representative flux curves of mannitol through the bare Teflon support membrane (A), or through a multicellular layer (B), showing overlay of the raw data (symbols) and the fitted curves generated by the model (lines). $^3$H-mannitol was added to the donor compartment and the concentration was measured in the donor and receiver compartment. The constant mass balance shows no loss of the compound as it diffuses over the 5 hour time period. Diffusion is relatively fast through the bare support, and is markedly slowed by the presence of the multicellular layer.
7.2.3 The flux of ascorbate and determination of its diffusion coefficient

Figure 7.3 shows representative flux curves of ascorbate through the bare support or through the MCL. Four ascorbate concentrations were used: 0.1 mM (n=2), 0.5 mM (n=10), 1 mM (n=5) and 10 mM (n=2). These concentrations represent the maximum plasma level achievable by dietary intake (0.1 mM), or bolus intravenous administration of ~1 g (0.5 mM), ~3 g (1 mM) or ~50 g (10 mM) doses (201). Ascorbate was found to be unstable under highly oxic conditions (95% O₂), with a half-life of ~2 h in the medium (Figure 7.3B). Therefore, all experiments were carried out under anoxia which prevented ascorbate oxidation in the medium as seen by the constant mass balance in Figure 7.3A,C.

The \( D_{\text{sup}} \) for ascorbate was calculated as \( 0.81 \pm 0.08 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \) (n=7), and \( D_{\text{MCL}} \) as \( 2.13 \pm 0.11 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1} \) (n=19). Ascorbate appeared to diffuse as a non-reactive compound (under anoxia) via the paracellular route, slightly slower than mannitol, which has a very similar molecular weight (176 g mol⁻¹ and 182 g mol⁻¹, respectively).
Figure 7.3:  Flux of ascorbate through a bare support or multicellular layer.  
Representative flux curves of ascorbate through the bare Teflon support membrane under anoxia (A), or with 95% O$_2$ (B), and through a multicellular layer (C) under anoxia.  Raw data (symbols) and the fitted curves generated by the model (lines) are shown.  The 95% O$_2$ data was not amenable to modelling, therefore the raw data alone is shown, and indicates ascorbate is unstable in oxygen.  Fresh sodium-L-ascorbate (0.5 mM) was added to the donor compartment and the concentration was measured in the donor and receiver compartment.  The constant mass balance shows no major loss of ascorbate as it diffuses over the 5 hour time period under anoxia.  Similar to mannitol, diffusion is markedly slowed by the presence of the multicellular layer.
The lower $D_{MCL}$ of ascorbate compared to mannitol suggests some cellular uptake or metabolism may be occurring. The average, approximate intracellular ascorbate concentrations for the whole MCL were calculated as 0.15, 0.31 and 0.56 mM for donor concentrations of 0.1 mM, 1 mM and 10 mM, respectively (calculated assuming the same cell volume as WiDr cells of 2.81 $\mu$L/10$^6$ cells) after 5 hours, indicating increased cellular uptake at higher concentrations. Figure 7.4A shows a decrease in ascorbate flux at the 10 mM dose, which may be accounted for by cellular uptake.

To further quantify this observed reduction in diffusion while accounting for differences in MCL thickness, the fitted $D_{MCL}$ was plotted as a function of the initial ascorbate concentration measured in the donor compartment (Figure 7.4B). This shows a slight reduction in the ascorbate $D_{MCL}$ with increasing concentrations. However, this is not significant ($p=0.180$; Figure 7.4), indicating that all concentrations of ascorbate have similar diffusion coefficients through tissue, although more data is needed to confirm this. The data indicates that the slightly lower $D_{MCL}$ at the 10 mM dose is due to a thicker MCL, and supports the model’s assumption of simple diffusion when fitting $D$. 
Figure 7.4: Flux of increasing concentrations of ascorbate through MCLs. A, the diffused fraction of the donor concentration as measured in the receiver compartment is shown, where the highest donor concentration appears to diffuse more slowly. The average is shown for each concentration with the measured data (symbols) and fitted curves (lines). B, the diffusion coefficient of each experiment is plotted against the measured initial donor concentration and shows that the 10 mM dose appears to have a lower diffusion coefficient through tissue, however this is not significant (p=0.180). n=2 for 0.1 mM, n=8 for 0.5 mM, n=5 for 1 mM and n=2 for 10 mM.
7.2.4 Constraining the model: obtaining parameters to describe cellular ascorbate uptake and efflux in monolayers

In order to model intracellular ascorbate concentrations along with its diffusion in tumour tissue, cellular uptake kinetics were obtained in single cells, as has previously been done for drug uptake using this model (218). Figure 7.5 shows intracellular ascorbate uptake in HT29 cells in monolayer culture over 5 hours at 0.1% O\textsubscript{2} with various measured, initial extracellular concentrations. The rate of intracellular uptake increased with the available extracellular concentration, but was clearly capacity-limited as the uptake ratios fell markedly with concentration ($C_i/C_e$ of 8.1, 1.4 and 0.3 for measured $C_e$ of 0.1 mM, 0.8 mM and 20 mM respectively). This was not due to depletion in the medium as ascorbate was stable under these conditions and total cellular uptake at 5 hours only accounted for 2.3%, 0.5% and 0.1% of the initial extracellular ascorbate at 0.1 mM, 0.8 mM and 20 mM, respectively. Consequently this cell uptake data was modelled as capacity limited transport (with Michaelis-Menten transport parameters). These data also support the increased rate of cellular uptake at the 10 mM dose.
Figure 7.5: Ascorbate uptake rate of HT29 cells in monolayer with increasing extracellular concentrations. HT29 cells were equilibrated at 0.1% O\textsubscript{2} and incubated for 5 hours with increasing concentrations of fresh sodium-L-ascorbate in the medium (n=3 replicates at each concentration). The intracellular ascorbate concentration was calculated using the water volume of WiDr cells (2.81 µL/10\textsuperscript{6} cells; a derivative of HT29). The rate of intracellular ascorbate uptake increases with the available extracellular concentration but decreases with time despite essentially constant extracellular concentrations. Symbols show the ascorbate measurements and the lines show the fitted cell uptake model. From this, Michaelis-Menten transport parameters were fitted and added into the diffusion model for simulating transport in tumours.

In addition to cellular transport kinetics, parameters describing ascorbate efflux and intracellular stability were included into the model, based on data previously obtained from WiDr cells in monolayer. The small intracellular instability term was necessary to account for the lack of equilibration between C\textsubscript{i} and C\textsubscript{e} at late times in the 20 mM experiments. Figure 7.6 shows intracellular ascorbate loss over time following removal of extracellular ascorbate. In the absence of extracellular ascorbate in the medium, intracellular ascorbate has a half-life of approximately 4 hours. The model predicted a slightly shorter half-life in the absence of extracellular ascorbate of 3.3 hours, but gave the best fit to all the data (Figures 7.5 and 7.6).
simultaneously. In the presence of extracellular ascorbate, intracellular ascorbate remains stable for at least 30 hours, as shown in Chapter 3 (Figure 3.1).

Figure 7.6: Ascorbate efflux from WiDr cells in ascorbate-free medium. WiDr cells in monolayer were grown with 1 mM ascorbate in the medium for 48 hours under standard culture conditions, followed by replacement with ascorbate-free medium. The intracellular ascorbate loss was then monitored over 18 hours. Intracellular ascorbate measurements are shown (○; means ± SD, n=3 replicates) with the fitted model data (line).

7.2.5 Modelling of ascorbate diffusion through tumour tissue and intracellular levels

The penetration and cellular uptake of ascorbate in tumour tissue was modelled as a function of time using the tissue cell volume fraction of 0.517 for HT29 MCLs (258) and assuming that cells were initially ascorbate-deficient. Table 7.1 summarises the various parameters included in the model.
Table 7.1: Parameters included in the model of ascorbate diffusion and cellular uptake in tumour tissue.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
<th>SE</th>
<th>SE%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{MCL}$</td>
<td>$2.13 \times 10^{-7}$</td>
<td>cm$^2$ s$^{-1}$</td>
<td>0.1058</td>
<td>5</td>
</tr>
<tr>
<td>$V_{max1}$</td>
<td>0.0055</td>
<td>mM min$^{-1}$</td>
<td>0.0016</td>
<td>28</td>
</tr>
<tr>
<td>$K_{m1}$</td>
<td>0.012</td>
<td>mM</td>
<td>0.0101</td>
<td>87</td>
</tr>
<tr>
<td>$V_{max2}$</td>
<td>0.052</td>
<td>mM min$^{-1}$</td>
<td>0.0054</td>
<td>10</td>
</tr>
<tr>
<td>$K_{m2}$</td>
<td>5.15</td>
<td>mM</td>
<td>2.0556</td>
<td>39</td>
</tr>
<tr>
<td>$k_i$</td>
<td>0.0042</td>
<td>min$^{-1}$</td>
<td>0.0030</td>
<td>71</td>
</tr>
<tr>
<td>$k_r$</td>
<td>0.0032</td>
<td>min$^{-1}$</td>
<td>0.0032</td>
<td>98</td>
</tr>
</tbody>
</table>

$V_{max1}$ and $K_{m1}$ are for high affinity transport, $V_{max2}$ and $K_{m2}$ are for low affinity transport. $k_i$ is the rate constant for intracellular turnover, and $k_r$ is the rate constant for passive efflux. For equations see section 2.12.3.

The first scenario simulated was of a constant infusion of both physiological and pharmacological plasma concentrations. Figure 7.7A summarises the expected extracellular and intracellular ascorbate levels with a range of physiological plasma concentrations at tissue depths of both 100 µm and 200 µm. It appears that cellular uptake reaches a plateau after ~8 hours of constant exposure. At low plasma concentrations (10 µM), ascorbate penetration is minimal, and is completely unavailable for cellular uptake at 200 µm tissue depth (Figure 7.7A). A similar result is seen for plasma levels of 40 µM, with both tissue penetration and cellular uptake impaired at 100 µm and 200 µm. At 100 µM in the plasma (saturable level with oral ingestion), ascorbate concentrations can reach between 45 µM and 60 µM at 100 µm or 200 µm, respectively, with cellular uptake being only slightly lower than that of cells adjacent to the capillary (Figure 7.7B).
Figure 7.7: Simulations of ascorbate tissue penetration and cellular uptake over time following a constant infusion at physiological levels. The ascorbate diffusion model was simulated using three physiological plasma ascorbate concentrations to predict the extracellular tissue levels (EC) and intracellular concentrations (IC) achievable with each dose at either 100 µm or 200 µm tissue depths, or in cells adjacent to the capillary (IC 0 µm). The three concentrations represent either deficient (A), average (B) or saturated (C) plasma levels.
Pharmacological infusion of 1 mM ascorbate increases both penetration and cellular uptake, with little difference between the 100 µm and 200 µm tissue depths and intracellular concentrations now over 1 mM (Figure 7.8A). At 10 mM plasma levels, tissue levels also reach pharmacological concentrations and intracellular ascorbate levels are almost 10-fold higher compared to 100 µM plasma levels, reaching ~5 mM at both tissue depths (Figure 7.8B).

Figure 7.8: Simulations of ascorbate tissue penetration and cellular uptake over time following a constant infusion at pharmacological levels. The ascorbate diffusion model was simulated using two pharmacological plasma ascorbate concentrations, 1 mM (A) and 10 mM (B), achievable with intravenous administration, to predict the extracellular tissue levels (EC) and intracellular concentrations (IC) at either 100 µm or 200 µm tissue depths, or in cells adjacent to the capillary (IC 0 µm).
A common scenario is that of an intravenous bolus dose, as opposed to constant infusion, to achieve pharmacological plasma concentrations between 1 mM and 10 mM, with a half-life of 30 minutes (201), and these simulations are shown in Figure 7.9. As with the constant infusion (Figure 7.8), tissue penetration at these concentrations is considerable, with ascorbate able to diffuse to reach ~50% of the plasma level at 200 µm. However, these peak plasma and tissue levels rapidly decline over ~2 hours. As cellular uptake reaches a plateau after ~8 hours, intracellular levels are much lower, and only reach millimolar levels at the 10 mM plasma bolus dose.
Figure 7.9: Simulations of ascorbate tissue penetration and cellular uptake over time following bolus intravenous administration. The ascorbate diffusion model was simulated using two initial plasma ascorbate concentrations (1 mM and 10 mM) to predict the extracellular tissue levels (EC) and intracellular concentrations (IC) achievable with each dose at either 100 µm or 200 µm tissue depths, or in cells adjacent to the capillary (IC 0 µm). A, 1 mM plasma ascorbate achievable with ~1 g intravenous dose. B, 10 mM plasma ascorbate achievable with ~50 g intravenous dose.

Figure 7.10 shows the simulated ascorbate concentrations according to distance from the capillary at steady-state following 24 hours of constant infusion of five concentrations. These data show that ascorbate is likely to diffuse non-linearly through tissue. At low plasma ascorbate concentrations (10 µM), there is a gradient through tissue, and relatively proximal cells may become deficient. At average plasma levels of 40 µM, there is still a considerable intracellular concentration gradient. At saturable physiological plasma levels (100 µM),
despite there being a substantial tissue concentration gradient, under steady-state conditions, intracellular levels are similar throughout the tissue plane. These simulations support the hypothesis that at physiological plasma levels, ascorbate penetration is likely to be impaired, with intracellular levels in avascular tissue only reaching the physiological, millimolar range at constant plasma levels near 100 µM.
Figure 7.10: Simulated concentration gradient of ascorbate in tumour tissue at steady-state following 24 hours constant infusion. Modelled extracellular and intracellular ascorbate concentrations are shown according to tissue depth/distance from capillary. Physiological plasma ascorbate levels (below 100 µM) may result in impaired tissue penetration and low intracellular concentrations. This is markedly improved at concentrations ≥ 100 µM.
7.3 Discussion

The results presented in this chapter are, to my knowledge, the first data to describe ascorbate diffusion through tumour tissue. Utilising a highly relevant \textit{in vitro} pharmacokinetic model employing MCLs has enabled the simulation of ascorbate penetration and cellular uptake in avascular, hypoxic tumour tissue. The data obtained suggest that at physiological plasma levels (~40 µM), cellular uptake is likely to be impaired due to insufficient penetration, and that constant, saturated plasma levels (~100 µM) can overcome this. The common scenario of bolus intravenous administration can increase plasma levels to between 1 mM and 10 mM (201), maximising tissue penetration. However, the combination of a short plasma half-life as a result of renal clearance (259), together with slow cellular uptake, means intracellular levels may remain relatively low under these conditions. Therefore, as ascorbate is highly stable in plasma (260), a longer infusion time of ~4 hours may enable sufficient time for the slow cell uptake to occur while maximising exposure to distant cells.

The data modelling performed here is limited by the strength of the parameters incorporated into the model. Rate constants for intracellular uptake and stability were based on available data from \textit{in vitro} experiments (Figures 7.5 and 7.6). However, further investigation of the extracellular and intracellular metabolism of ascorbate is needed to obtain a more robust measure of $k_i$, as this parameter can substantially alter the output by the model. In addition, ascorbate may be prone to oxidation in tumour tissue, and plasma levels may rise and fall as it is absorbed/excreted. Therefore, useful future parameters may describe ascorbate/DHA ratios, and the effect of oxygen tension on this. The lack of concentration dependence of the ascorbate $D_{\text{MCL}}$, together with slow cellular uptake, supports the assumption that uptake and cell turnover has little effect on tissue diffusion when cells already contain high ascorbate levels, and this could be further tested by performing flux experiments in ascorbate-deficient cells. The $K_m$ for ascorbate uptake modelled here (11 µM) is in the lower range of previous calculations for SVCT2 of 8-62 µM in various other cell lines (193), and as such, estimates of intracellular uptake represent conservative examples. For these reasons, the simulations presented here provide very preliminary data on ascorbate diffusion through tumour tissue, which can be strengthened particularly by further defining intracellular turnover ($k_i$).
These results are highly relevant to cancer patients, as their plasma ascorbate levels are known to be lower than healthy controls. Studies have shown mean plasma levels of 15 µM in lung cancer patients compared to 47 µM in healthy controls (196), and another study found similar results with a mean plasma concentration of 18 µM in patients with oral squamous cell carcinoma compared to 57 µM in healthy controls (198). In addition, a study of 50 cancer patients found 30% had levels below 11 µM (200). It has been shown here that plasma levels of 10 µM, which may be quite common, results in drastically impaired tumour tissue penetration, therefore intracellular levels are almost certain to be at deficiency levels at both 100 µm and 200 µm distances. This agrees with what was seen in clinical samples of endometrial and colorectal tumours, where tumour tissue of a high grade contained significantly less ascorbate than the surrounding normal tissue (Chapters 5 and 6).

If optimal intracellular ascorbate concentrations are to be achieved in all cells within a tumour, multi-gram intravenous infusion may increase distant tissue levels enough to adequately increase cellular uptake, particularly as the data indicate that the cell uptake rate increases at high concentrations. Bolus intravenous administration resulting in plasma levels between 1 mM and 10 mM overcomes the tissue penetration issue, however the short half-life of ascorbate at these levels means that, particularly at around the 1 mM concentration, there may not be enough time for those distant cells to accumulate millimolar levels. Therefore, if a bolus dose is to be used, 10-50 g may be the most effective dose, providing peak plasma levels of ~6-13 mM for 2-4 hours (201), which is sufficient for millimolar cell uptake. However, if a longer infusion were given of ~4 hours, much lower doses could be used of only 1-3 g to give plasma concentrations of 1-2 mM (201), which is sufficient for tissue penetration and cellular uptake.

Interestingly, a constant, high physiological ascorbate concentration of 100 µM (saturable plasma level from gastrointestinal absorption) showed that although there is still an extracellular tissue concentration gradient, intracellular levels were similar throughout the tissue plane, being close to the millimolar range. This plasma level can be achieved through dietary means alone, without the need for intravenous administration. However, intravenous dosing may be useful to bolster intracellular levels above 1 mM, which may further dampen
an active HIF-1 response. It has been shown here that intravenous ascorbate administration can produce pharmacological levels of ascorbate in tumour tissue (~5 mM after a bolus dose), and this is thought to have a selective, pro-oxidant effect on tumour cells (261), although this mechanism remains to be clinically tested.

Ascorbate appeared to diffuse non-linearly through tissue, and may follow a similar pattern to that of oxygen (262). Therefore, it is possible that the association between low ascorbate and high HIF-1 activation means that ascorbate is a marker of tissue oxygenation status, rather than affecting HIF-1 activity. However, this is unlikely as HIF-1 is known to be activated under a variety of non-hypoxic conditions, such as metabolic disturbance (116). Nevertheless, further studies are required to clarify whether ascorbate plays a direct role in regulating the HIF-hydroxylases, and thus HIF-1 activity, and to determine whether its effect is powerful enough to yield significant clinical results in HIF-1-dependent tumours.

7.4 Chapter summary

The results from this chapter provide evidence that ascorbate diffuses slowly through tumour tissue. Intravenous administration of ascorbate in pharmacological doses can overcome poor tissue penetration, however an infusion over ~4 hours, rather than a bolus dose, is needed for sufficient intracellular uptake to occur. On the other hand, dietary intake alone may be sufficient, as continuous maintenance of a saturated physiological plasma level near 100 µM is likely to result in intracellular ascorbate concentrations that are close to the millimolar range required to fully support HIF-hydroxylase activity.
Chapter 8: Discussion and Conclusions

The activation of HIF-1 has numerous wide-ranging effects in cancer cells to support their survival in an inhospitable tumour microenvironment. There is currently a focus on developing targeted therapies to inhibit the HIF-1 transcriptional response, thereby rendering cancer cells susceptible to death, particularly during chemo- and radio-therapy (263). The HIF-hydroxylases that ‘switch off’ HIF-1 are a prime target for this, whereby enhancing their activity can suppress the HIF-1 response. Ascorbate is specifically required for optimal activity (164), and as a non-toxic, widely available molecule, it presents a highly attractive opportunity to optimise HIF-hydroxylase, and minimise HIF-1, activity in cancer cells. It was the aim of this thesis to investigate whether intracellular ascorbate can significantly affect the HIF-1 response in cancer cells, and whether this mechanism has clinical relevance.

8.1 Summary of findings

The results presented in this thesis have shown a clear and consistent relationship between low intracellular ascorbate levels and high HIF-1 activation in cancer cells. My in vitro studies showed that intracellular ascorbate levels in the millimolar range have the greatest inhibitory effect on both HIF-1α protein levels and HIF-1 transcriptional activity. It was also apparent that ascorbate may have a greater effect on inhibiting HIF-1 target gene production than HIF-1α protein. That these results could have clinical relevance was suggested by the analysis of clinical samples from both endometrial and colorectal tumours, where low levels of tissue ascorbate were associated with a higher HIF-1 activation score, high tumour grade, tumour necrosis and larger tumour size. Furthermore, poor disease-free survival in colorectal cancer patients was linked to low tumour ascorbate content and a tendency for a high HIF-1 activation score. I have also presented novel, preliminary pharmacokinetic data on ascorbate diffusion and uptake in avascular tumour tissue, which will assist in determining whether
insufficient ascorbate delivery under physiological conditions could contribute to tumour cells becoming ascorbate-deficient.

8.1.1 The intracellular ascorbate requirements of cancer cells for HIF-1 inhibition

I found that cancer cells in vitro can accumulate physiological intracellular ascorbate concentrations to millimolar levels. However, there was a wide variety among the cell lines tested, with intracellular ascorbate concentrations ranging from 0.8 mM to 12 mM, after incubation with 1 mM in the culture medium. This indicates that the uptake capacity of some cancer cells may be either enhanced or impaired, and may be related to the degree of ascorbate transporter (SVCT2) activity, although this was not measured. Interestingly, the effect of ascorbate on inhibiting HIF-1α was more marked in cells that contained very high intracellular concentrations (Jurkat cells; 12 mM intracellular ascorbate) than in cells with relatively low uptake (Ishikawa cells; 0.8 mM intracellular ascorbate). In addition, there was a dose-response of ascorbate on HIF-1α inhibition in WiDr cells, with the maximum intracellular level of 1.1 mM having the greatest effect. This suggests that having intracellular ascorbate concentrations greater than 1 mM is optimal for suppressing HIF-1.

These observations are in line with the ascorbate $K_m$ requirements for the HIF-hydroxylases of 140-260 µM (see Table 1.2), where an optimal concentration for continuous activity is often ten-fold higher than the $K_m$, in this case ~1-3 mM. Similarly, early studies using purified C-P4H (also a 2-OG-dependent dioxygenase enzyme) found that an optimal ascorbate concentration of 1-2 mM was needed for full enzyme activity (143, 155, 159-162). Therefore, a low ascorbate uptake capacity in some cancer cells may limit their intracellular levels to less than 1 mM, and this may be related to variable expression or activity of SVCT2 (228). Sub-optimal intracellular ascorbate levels may then prevent optimal HIF-hydroxylase activity and result in over-activation of HIF-1.
8.1.2 Ascorbate availability to tumour tissue and clinical sample analyses

Low intracellular ascorbate levels may not only result from a low uptake capacity, but also from low circulating levels available for uptake. Pharmacokinetic modelling suggests that at physiological plasma concentrations (<100 µM), tumour tissue at a distance ≤200 µm from a capillary could well be ascorbate deficient. This effect would be especially pronounced when plasma levels are deficient (~10 µM), a state which is common in cancer patients (196, 198, 200). Therefore, in patients with solid tumours, ascorbate deficiency in plasma and in the tissue may result in exacerbated HIF-1 activity and promote tumour progression, particularly in those with HIF-1-dependent tumours.

That this may indeed occur clinically was indicated by the finding that both endometrial and colorectal tumour tissue samples had increased HIF-1 activation scores when low in ascorbate. In addition, high grade tumour tissue (most de-differentiated; both endometrial and colorectal) had a significantly reduced capacity to accumulate ascorbate compared to surrounding normal tissue from the same patient. This data, to my knowledge, is the first to show relative tumour ascorbate content and markers of HIF-1 in human tumours. These results were markedly similar between the two tumour types, and were readily apparent even with a relatively small sample size of 51 and 50 cases for endometrial and colorectal tumours, respectively.

Analysis of survival data in the colorectal sample cohort revealed a significant association between poor disease-free survival and low tumour tissue ascorbate, and tended to be associated with a high HIF-1 score. This is a strong indicator that low tumour ascorbate levels may be associated with HIF-1-dependent tumour progression. Together, these analyses provide a strong foundation for further clinical studies into this mechanism.
8.1.3 The effect of ascorbate on HIF-1-dependent pro-survival processes

A consistent relationship was observed between low intracellular ascorbate and high levels of the HIF-1 target genes BNIP3 and VEGF, as seen both in in vitro studies and from clinical sample analyses. This was observed despite a more modest relationship with HIF-1α protein, and is in line with the high dependence of FIH for ascorbate, which controls HIF-1 transcriptional activity (135). Furthermore, HIF-1α protein is known be regulated by other mechanisms than proline hydroxylation by the PHDs (see section 1.2.3), which could somewhat offset the effect of ascorbate on PHD-mediated degradation of HIF-1α.

BNIP3 is one of the most highly specific HIF-1 target genes, and is an accurate marker of HIF-1 transcriptional activity (79). As well as VEGF, BNIP3 showed one of the strongest relationships with ascorbate, with its expression being prevented under all conditions of HIF-1-induction in vitro, and closely linked to tumour tissue ascorbate levels. It has been shown that BNIP3 could prevent apoptosis in glioma cells by transcriptionally repressing apoptosis-inducing factor (90). Interestingly, another study has shown that ascorbate pre-loading (at physiological levels) was, conversely, able to activate apoptosis-inducing factor-mediated cell death in breast cancer cells (264). This effect was blocked by MG132 (264), a proteasome inhibitor that is known to stabilise HIF-1α. Therefore, a mechanism by which ascorbate may act to inhibit tumour cell survival could be via inhibition of HIF-1-induced BNIP3, and this would be of interest to investigate further.

As with BNIP3, a consistent and strong relationship was found between VEGF protein levels and cellular ascorbate, particularly in the clinical samples. High VEGF levels in both endometrial and colorectal tumour samples were strongly related to low tumour ascorbate levels, as well as a low tumour: normal ascorbate ratio. This may simply reflect an association, where hypoxic regions with poor vascularity could have both reduced access to ascorbate, while also up-regulating VEGF to compensate. However, it may also signify poor control of HIF-1 (activated by hypoxic and/or non-hypoxic mechanisms), whereby low ascorbate levels may be promoting HIF-1-dependent VEGF expression. This was shown to occur in vitro, where ascorbate was able to specifically inhibit HIF-1-dependent VEGF production.
The ability of ascorbate to inhibit VEGF could potentially influence tumour vascularisation. Angiogenesis is known to occur very early in the tumourigenic process, and is often an aberrant process yielding dysfunctional vessels (103). It has been suggested that ‘normalisation’ of the tumour vasculature, rather than total inhibition, may be more effective in treating tumours, as it discourages hypoxia-mediated clonal selection (109). This ‘normalisation’ would involve a more modest inhibition of angiogenic signals (109). Ascorbate could potentially contribute to this effect by inhibiting HIF-1-specific production of VEGF, and possibly other pro-angiogenic HIF-1 target genes. Therefore, high ascorbate levels may inhibit the formation of a dysfunctional tumour vasculature early in tumour development and may also contribute to vascular ‘normalisation’ in developed tumours.

8.2 Clinical implications and further studies

8.2.1 Previous clinical studies and mouse models using ascorbate to treat cancer

There have been some clinical studies using multi-gram, intravenous, pharmacological ascorbate administration in advanced cancer patients (203, 265, 266). The early studies performed by Cameron and Pauling, with 100 patients compared to 1000 controls, showed a beneficial response in terms of both survival time and quality of life (203 112). However these studies were criticised for having a poorly matched control group, and the subsequent Mayo Clinic trials (double-blinded, randomised and controlled but using only oral ascorbate) returned negative results (204), effectively dismissing ascorbate as a potential treatment.

The recent discovery that intravenous ascorbate administration can provide 100-fold higher plasma levels compared to oral intake has renewed interest in its potential in treating cancer (201). Four credible case studies (including prostate cancer, renal cell carcinoma, bladder cancer and B-cell lymphoma) have been reported showing intravenous ascorbate had substantial anti-cancer activity in these advanced cancer patients (267, 268), three of which were evaluated in accordance with the National Cancer Institute Best Case Series guidelines (268). However two recent studies, both with 24 patients and no control population, have
shown no objective response, with the exception of three patients who had stable disease (265, 266). None of these studies have monitored tissue ascorbate levels or any biological markers other than toxicity parameters, and have been performed in an unselected patient population. Nevertheless, studies have found that high-dose ascorbate is remarkably non-toxic and well-tolerated in patients with normal renal function (206, 265, 266), a rare and valuable trait in a potential cancer therapy. This, together with evidence that some cancer patients may benefit from ascorbate treatment, should be grounds for further rigorous clinical investigations, based on sound hypotheses.

Recent animal studies have investigated the effect of ascorbate and tumour growth, all using different dosing regimens and tumour models (211, 212, 269-271). However, they have consistently shown an anti-tumour effect of ascorbate supplementation in mice. Mouse studies that used pharmacological ascorbate dosing (intravenous or intraperitoneal) showed a reduction in tumour growth rate and volume (261, 269-271). Other studies have used the Gulo-/ mouse model, in which the animals cannot synthesise ascorbate, to investigate the effect of physiological ascorbate levels (211, 212), and have also shown inhibition of tumour growth in orally ascorbate-supplemented mice. A very recent Gulo-/ study showed that physiological ascorbate supplementation dramatically reduced tumour metastases and necrosis, features that were associated with MMP-9 (a HIF-1 target gene) expression and tumour invasiveness (272). Serum VEGF levels were also markedly reduced (272). These mouse studies support a role for ascorbate in inhibiting tumour progression, and although the mechanisms behind some of these effects may need further clarification, the inhibition of HIF-1 is likely to contribute (169).

8.2.2 Mechanisms behind the use of ascorbate in cancer treatment

One emerging mechanistic rationale behind the anti-tumour effects of ascorbate at pharmacological levels is that it exerts a pro-oxidant effect in the extracellular fluid which is selectively toxic to cancer cells, and that this requires up to 5 mM ascorbate in the tumour tissue (261). The pharmacokinetic data presented here, shows that these levels are achievable.
in avascular tumour tissue with multi-gram intravenous administration. However, these extracellular levels also considerably increased the intracellular ascorbate concentrations. Therefore, the resultant increase in intracellular levels may serve to diminish HIF-1 activity and inhibit tumour progression, and is an additional mechanism to the extracellular pro-oxidant hypothesis. Future clinical trials should monitor both tumour tissue ascorbate levels and markers of HIF-1 activity in a selected patient population with HIF-1-dependent tumours (for example, endometrial, colorectal and gastric cancers; see Table 1.1) to determine if this mechanism is exploitable for these patients.

The promise of ascorbate in treating cancer may lie in its combined use with other chemotherapeutics. HIF-1 is known to be a driver of both chemo- and radio-resistance (81). Therefore, boosting intracellular ascorbate levels in the tumour may inhibit this effect and enhance the effectiveness of current treatments. There has been some concern that ascorbate, which is a versatile antioxidant, may in fact counteract the oxidative damage against cancer cells caused by some current cancer therapies, thereby limiting their effectiveness (273). However, this hypothesis is based on an effect that can be demonstrated in vitro, but is largely untested in vivo. Notably, recent in vitro and in vivo studies have shown that ascorbate can, in fact, enhance the effectiveness of chemotherapy (271, 274, 275). Pre-treatment of prostate cancer cells with physiological ascorbate concentrations significantly reduced the IC_{50} values of docetaxel and 5-fluorouracil (275) and pharmacological dosing of ascorbate in mice synergised with gemcitabine resulting in significantly inhibited tumour growth (274). In addition, a recent phase I clinical trial of high-dose intravenous ascorbate with gemcitabine and erlotinib in advanced pancreatic cancer has shown no adverse effects of including ascorbate (276). To my knowledge, no studies have examined the effect of intracellular ascorbate on HIF-1-induced treatment resistance. It would be of interest to study the response of tumour cells with an active HIF-1 response to a range of chemotherapeutics, and monitor drug effectiveness in ascorbate-deficient or pre-loaded cells.
8.2.3 Ascorbate as a chemo-preventive

Ascorbate may play a role in inhibiting HIF-1-dependent tumour progression not only in established tumours, but also early in the process of tumour formation. An expanding cancer cell mass can rapidly spread beyond the diffusion distance of oxygen (~80 µm) (9). This initial hypoxic stress can activate HIF-1, allowing these cells to successfully adapt, where without this survival response a cell mass is unlikely to grow beyond ~1-2 mm in diameter (277). Therefore, maintaining high physiological ascorbate levels may sufficiently inhibit the HIF-1 response to shift the balance of signals towards cell death, and may prevent a growing mass becoming established.

Moreover, ascorbate may influence the activity of other 2-OG-dependent dioxygenases which have roles in epigenetic control and DNA repair (130), and this may be one of the links between the large amount of epidemiological evidence for ascorbate in cancer prevention (278). The ascorbate requirement of not only the HIF-hydroxylases, but the entire 2-OG-dependent dioxygenase family of enzymes, and the impact on cancer, is a field of research waiting to be explored.

8.3 Conclusion

Ascorbate may have multiple benefits in limiting the cancer burden, where its inhibitory effect on HIF-1 could be of value at all stages of tumour progression. Having a high tissue ascorbate level may help to prevent formation of solid tumours, slow tumour growth rates, inhibit aggressive tumour behaviour and even aid in the treatment of established cancers. Despite growing interest in ascorbate as a cancer treatment, there remains a great deal of controversy over its clinical use. The mainstream standpoint has lingered from the negative clinical studies from the 1970s, while many alternative practitioners widely administer ascorbate without sufficient clinical evidence to inform this practice. However, this controversy is unnecessary and should be replaced with the objective evaluation of data obtained from systematic inquiry, with an understanding of the underlying mechanisms. The studies in this thesis represent such an inquiry, and strongly encourage further investigation of ascorbate in inhibiting HIF-1-dependent tumour progression.
Chapter 9: References


