Low Ascorbate Levels Are Associated with Increased Hypoxia-Inducible Factor-1 Activity and an Aggressive Tumor Phenotype in Endometrial Cancer

Caroline Kuiper[1], Ilona G.M. Molenaar[4], Gabi U. Dachs[2], Margaret J. Currie[2], Peter H. Sykes[3], and Margreet C.M. Vissers[1]

Abstract

Activation of the transcription factor hypoxia-inducible factor (HIF)-1 allows solid tumors to thrive under conditions of metabolic stress. Because HIF-1 is switched off by hydroxylation reactions that require ascorbate, inadequate intracellular ascorbate levels could contribute to HIF-1 overactivation. In this study, we investigated whether the ascorbate content of human endometrial tumors [known to be driven by HIF-1 and vascular endothelial growth factor (VEGF)] influenced HIF-1 activity and tumor pathology. We measured protein levels of HIF-1α and three downstream gene products [glucose transporter 1 (GLUT-1), Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), and VEGF], as well as the ascorbate content of tumor and patient-matched normal endometrial tissue samples. HIF-1α and its downstream gene products were upregulated in tumor tissue, with the highest levels being present in high-grade tumors. High-grade tumors also had reduced capacity to accumulate ascorbate compared with normal tissue; however, all grades contained tumors with low ascorbate content. Tumors with the highest HIF-1α protein content were ascorbate deficient. Low ascorbate levels were also associated with elevated VEGF, GLUT-1, and BNIP3 protein levels and with increased tumor size, and there was a significant association between low tissue ascorbate levels and increased activation of the HIF-1 pathway ($P = 0.007$). In contrast, tumors with high ascorbate levels had lesser levels of HIF-1 activation. This study shows for the first time a likely *in vivo* relationship between ascorbate and HIF-1, with low tumor tissue ascorbate levels being associated with high HIF-1 activation and tumor growth. *Cancer Res; 70(14); 5749–58. ©2010 AACR.*

Introduction

The role of vitamin C (ascorbate) in cancer has been a subject of controversy for decades. The early work of Cameron and Pauling in the 1970s, in which high doses of i.v. and oral ascorbate were given to patients with advanced cancer, resulted in claims that ascorbate could improve outcome (1, 2). However, a subsequent randomized and placebo-controlled study from the Mayo Clinic, using only oral ascorbate, found no significant benefit of ascorbate and apparently refuted Cameron and Pauling's work (3). More recently, interest in vitamin C has been renewed following the demonstration that millimolar concentrations can be selectively toxic to

cancer cells (4–6) and that these concentrations are achievable by i.v. administration (7, 8). Recent studies have also shown that ascorbate administration can significantly reduce tumor growth rates in mice (9–12), and in some of these studies, this is associated with the ability of ascorbate to inhibit the activity of the prosurvival transcription factor hypoxia-inducible factor-1 (HIF-1; refs. 9, 10).

HIF-1 is an important regulator of tumor growth and is involved in the successful adaptation of the tumor to its microenvironment. When activated under hypoxic stress in solid tumors, HIF-1 drives the transcription of numerous genes involved in glycolysis, angiogenesis, glucose transport, and cell life and death pathways (13, 14). It may also be upregulated under nonhypoxic conditions, resulting in aerobic glycolysis (the Warburg effect; ref. 15), and is overexpressed as an early change during carcinogenesis (16–18). High activity has been shown to promote tumor progression and resistance to chemotherapy or radiotherapy and is associated with a poor prognosis (13, 19–23).

Activation of HIF-1 is controlled by posttranslational modification on the α subunit: hydroxylation of proline residues 402 and 564 targets the protein to the proteasome for rapid degradation (24), and hydroxylation of asparagine 803 prevents transcriptional activity (25). The enzymes responsible for these reactions, prolyl-hydroxylases 1, 2, and 3 and factor inhibiting HIF (FIH; asparaginyl-hydroxylase), are collectively

called the HIF hydroxylases and belong to the family of 2-oxoglutarate dioxygenases that also hydroxylate collagen and numerous other substrates (26). Their activity is dependent on the supply of molecular oxygen, 2-oxoglutarate, and Fe^{2+}, and ascorbate functions as a cofactor, possibly by maintaining the active-site Fe in the reduced state (27). This role is highly specific to ascorbate (28, 29), which was recently shown to be structurally specific for the hydroxylase active site (30). However, despite its being a cofactor for this reaction, ascorbate has received comparatively little attention as a regulator of HIF-1.

That ascorbate deficiency compromises HIF hydroxylase activity and upregulates HIF-1 has been shown *in vitro* in both primary and cancer cells (31–33). The HIF hydroxylase K_m values for ascorbate vary between 140 and 260 μmol/L (26), indicating the intracellular concentration that would be required for optimal activity. Most tissue cells concentrate the vitamin to low millimolar levels from the plasma by active transport via sodium-dependent vitamin C transporters (SVCT) or the hexose transporters (GLUT) that transport dehydroascorbic acid competitively with glucose (34, 35), and we have shown that achieving these intracellular levels of ascorbate significantly curbs HIF-1 activation *in vitro* (32). Circulating plasma ascorbate concentrations are between 40 and 60 μmol/L (36) and access to an adequate supply from the plasma is critical, as lower levels have been shown to dramatically decrease tissue availability (37).

In a rapidly growing solid tumor with poor vasculature, delivery of ascorbate to tumor cells may be compromised, causing a deficiency state that could exacerbate HIF-1 activation, thereby enhancing tumor growth and survival. That this may indeed occur has been suggested by the demonstration in mice that ascorbate administration could suppress HIF-1–mediated tumorigenesis (9, 10). However, whether a relationship exists between ascorbate levels and HIF-1 activation in human tumors has never been established. Therefore, the aim of this study was to measure the ascorbate content of human tumor tissue and determine whether this relates to HIF-1 activity or tumor pathology.

This study was carried out using endometrial tumor tissue. This cancer is relatively common in postmenopausal women and is known to be driven by an active hypoxic response (38), with disease progression and poor prognosis being linked to HIF-1 and vascular endothelial growth factor (VEGF) levels (38–40). We have measured the ascorbate content of tissue bank samples that have patient-matched control endometrial tissue, which has enabled us to determine the relative ability of the tumor to accumulate ascorbate. We also measured HIF-1α protein levels and three prosurvival downstream targets: Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), glucose transporter 1 (GLUT-1), and VEGF.

Materials and Methods

Patients and sample preparation

The use of tissue samples donated to the Cancer Society Tissue Bank (CSTB; Christchurch) was approved by the CSTB Board. All donors gave written informed consent allowing research on their samples, and ethical approval for this study was granted by the Upper South B Regional Ethics Committee, New Zealand. Tissue was obtained from women undergoing hysterectomy for removal of endometrial tumors, and normal endometrial tissue (as part of surgical resection) was also collected, providing paired tissue samples, tumor and normal, for each case. All samples were flash-frozen within an hour of collection and stored at –80°C. A total of 51 sample pairs were analyzed and represented approximately equal numbers of International Federation of Gynecology and Obstetrics (FIGO) grade 1 (18 cases), grade 2 (15 cases), and grade 3 (18 cases) tumors (Supplementary Table S1). Higher grade represents increasing lack of tissue differentiation and infers a more aggressive tumor, where solid growth pattern is <5% in grade 1, 6% to 50% in grade 2, and >50% in grade 3 (41). Grade 3 tumors included endometrioid adenocarcinoma ($n = 5$). Serous and/or clear cell adenocarcinoma ($n = 10$) and carcinosarcoma samples ($n = 3$), which exhibit aggressive behavior, were also considered grade 3 tumors.

Frozen samples were homogenized to a fine powder in liquid nitrogen in a chilled mortar and pestle, the tissue wet weight was measured, and 200 μL of 10 mmol/L phosphate buffer (pH 7.4) were added to make a homogeneous suspension.

DNA content

The number of cells in each sample was considered an important variable in the comparison of ascorbate measurements, Western blot analyses, and VEGF assays in different tissue samples. Therefore, DNA measurement was used to standardize the normal and tumor tissue specimens for cell content. A 40-μL aliquot of the tissue homogenate suspension was diluted to 1 mL with 10 mmol/L phosphate buffer (pH 7.4) and the cells disrupted by freeze-thawing three times in dry ice/95% ethanol and sonicating for 25 seconds. Propidium iodide was added (1 mg/mL; 10 minutes at room temperature) and DNA measured by fluorescence at 544 to 590 nm, relative to a standard curve of purified calf thymus DNA. The reliability of this method was confirmed by identical loading of cell proteins in Western blots after standardizing the samples according to DNA content, shown by equal loading of the housekeeping protein β-actin (Figs. 1 and 2).

Ascorbate content

Ascorbate was extracted from the tissue homogenate by immediately adding a 1:1 volume of perchloric acid [0.54 mol/L containing 50 mmol/L diethylenetriaminepentaacetic acid (DTPA)] and spinning (10,000 rpm, 2 minutes) to pellet the protein. The supernatant was analyzed by reverse-phase high-performance liquid chromatography [mobile phase: 80 mmol/L sodium acetate (pH 4.8) with 0.54 mmol/L DTPA] with electrochemical detection as previously described (32) and a fresh standard curve of sodium-L-ascorbate prepared for each run.

Western blotting

Measurement of HIF-1α, GLUT-1, and BNIP3 protein levels was by Western blotting. Tissue homogenates were standardized to 12 μg of DNA per 100 μL of sample buffer, then boiled

Figure 1. HIF-1α protein levels in endometrial tumor and normal tissues. A, representative Western blots showing two cases of endometrial normal (N) and tumor (T) tissue pairs, with β-actin loading controls. There is little or no detection in normal tissues and strong (case 1) or weak (case 2) expression in the tumor tissues. B, boxplot of Western blot densitometry for HIF-1α in grade 1, 2, and 3 (n = 18, 15, and 18, respectively) endometrial tumor and normal tissue. P values determined by Wilcoxon signed-rank test. HIF-1α bands were normalized against a positive control and β-actin for each blot. C, immunohistochemical localization of HIF-1α in a representative endometrial tumor section showing positive staining in glandular tumor tissue (arrows) confined to the nuclei (inset; arrows). D, perinecrotic HIF-1α staining (arrows) in endometrial tumor tissue showing both nuclear and cytoplasmic localization. Necr, necrosis. Bar, 200 μm.
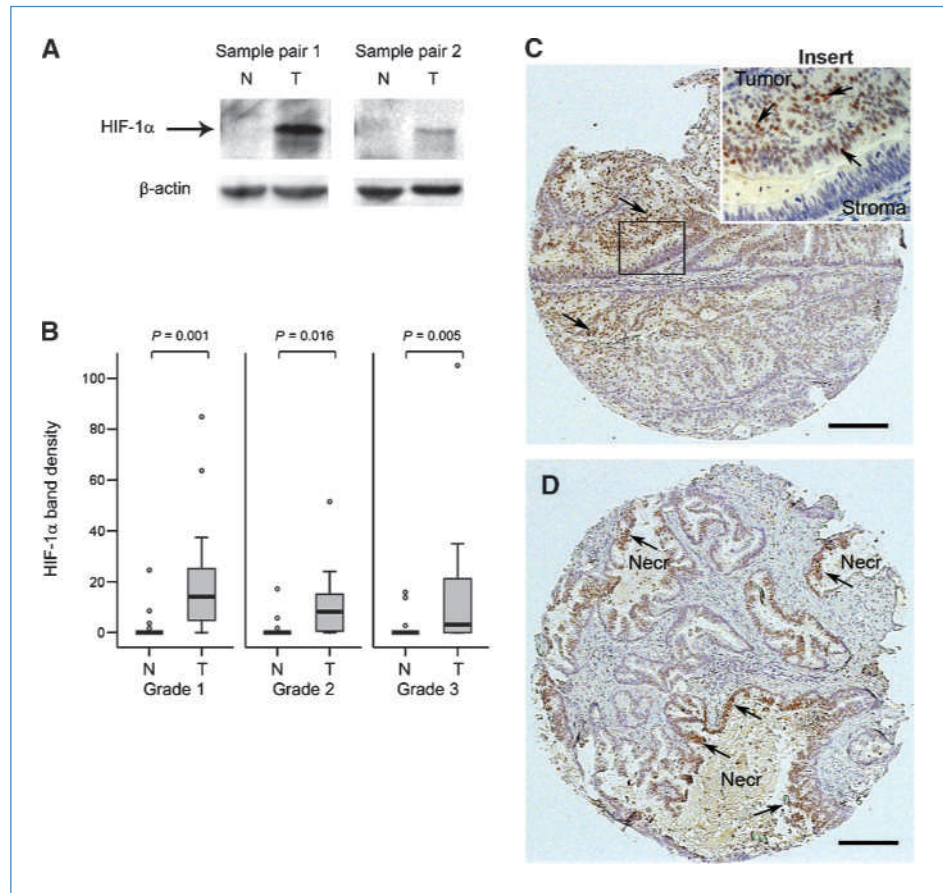
for 4 minutes before SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Each gel had the same positive control (100 μmol/L $CoCl_2$–treated A431 whole-cell lysate) to normalize the signal between blots. Membranes were blocked, incubated in primary antibody overnight at 4°C (Supplementary Table S2), washed, incubated for 1 hour at room temperature in secondary antibody conjugated with horseradish peroxidase (Supplementary Table S2), washed again, then visualized with Amersham ECL Plus Western blotting detection system (GE Healthcare) using photographic film. Each blot was reprobed for β-actin as a loading control. Bands were quantified using Quantity One software, and the protein of interest was normalized against the positive control and β-actin.

VEGF ELISA

Tissue VEGF protein levels were measured on undiluted samples prepared earlier for DNA, using a DuoSet ELISA development system (R&D Systems) according to the manufacturer's instructions.

Immunohistochemistry

A formalin-fixed, paraffin-embedded tissue microarray, containing 62 endometrial tumor 1-mm cores (33 from the tumors analyzed in this study), was obtained from the CSTB (Christchurch). Sections (3 μm) were stained with mouse anti–HIF-1α primary antibody (BD Biosciences; 1:25) and a mouse HRP-DAB

tissue staining kit (R&D Systems) according to the manufacturer's instructions. Positive control tissue from a renal cell carcinoma was treated in the same manner, and negative controls were carried out by omitting the primary antibody or replacing it with nonimmune mouse IgGκ (Sigma; 1 mg/mL). HIF-1α expression was verified independently by three individuals.

Statistical analyses

All statistical tests were performed using SPSS 13.0 for Windows, with the α level set to 0.05. All data except for tumor size were nonparametric according to the Shapiro-Wilk and Kolmogorov-Smirnov tests for normality. To test for differences between normal and tumor data, the Wilcoxon signed-rank test (two-tailed) was used. For differences between grades or ascorbate status of tumors, the Mann-Whitney (two-tailed) or Jonckheere-Terpstra (ref. 42; one-tailed for ordered data) tests were used. Correlations were determined using Spearman's correlation coefficient (two-tailed). Student's t-tests (two-tailed) were used when testing for differences in tumor size data.

Results

**Tumor tissue has higher cell density than normal tissue**

Tumor and normal tissue samples from women undergoing hysterectomy (51 cases) were obtained from the CSTB (Christchurch). The sample set contained endometrial

tumors (FIGO grades 1, 2, and 3; ref. 41), with the grade 3 tumors including 3 cases of carcinosarcoma and 10 cases of serous and/or clear cell adenocarcinoma. A single portion of the tumor and a segment of adjacent normal tissue were homogenized and analyzed. The two tissue types were standardized for cell content because normal and tumor endometrial tissues can vary considerably in their composition. We found that tumor tissue contained significantly less collagen than normal tissue (tumor/normal tissue hydroxyproline ratio was 0.20 ± 0.16; $n = 20$; $P = 0.037$), indicating a lower extracellular matrix content in tumor samples per unit of weight. That tumor tissue had a higher cell content than normal tissue was also indicated by finding significantly more DNA in tumor than in normal tissue ($130 \pm 6\%$; $P = 0.005$).

There was no difference in the cellular content between tumor grades. (Grade 1 contained 3.32 ± 0.48, grade 2 contained 3.46 ± 0.31, and grade 3 contained 3.30 ± 0.33 μg DNA/mg wet weight $\pm$ SEM.)

HIF-1α and its target genes are upregulated in tumor tissue

To determine the degree of HIF-1α protein stabilization in the endometrial tumors, HIF-1α in the tissue homogenate was measured by Western blotting. This was readily detected in 37 of 48 tumors (77%), whereas very weak expression was seen in 13 of 48 normal tissues (27%; Fig. 1A). Densitometry of the bands showed significantly higher HIF-1α protein in tumor compared with normal tissue ($P < 0.001$; Fig. 1B), with
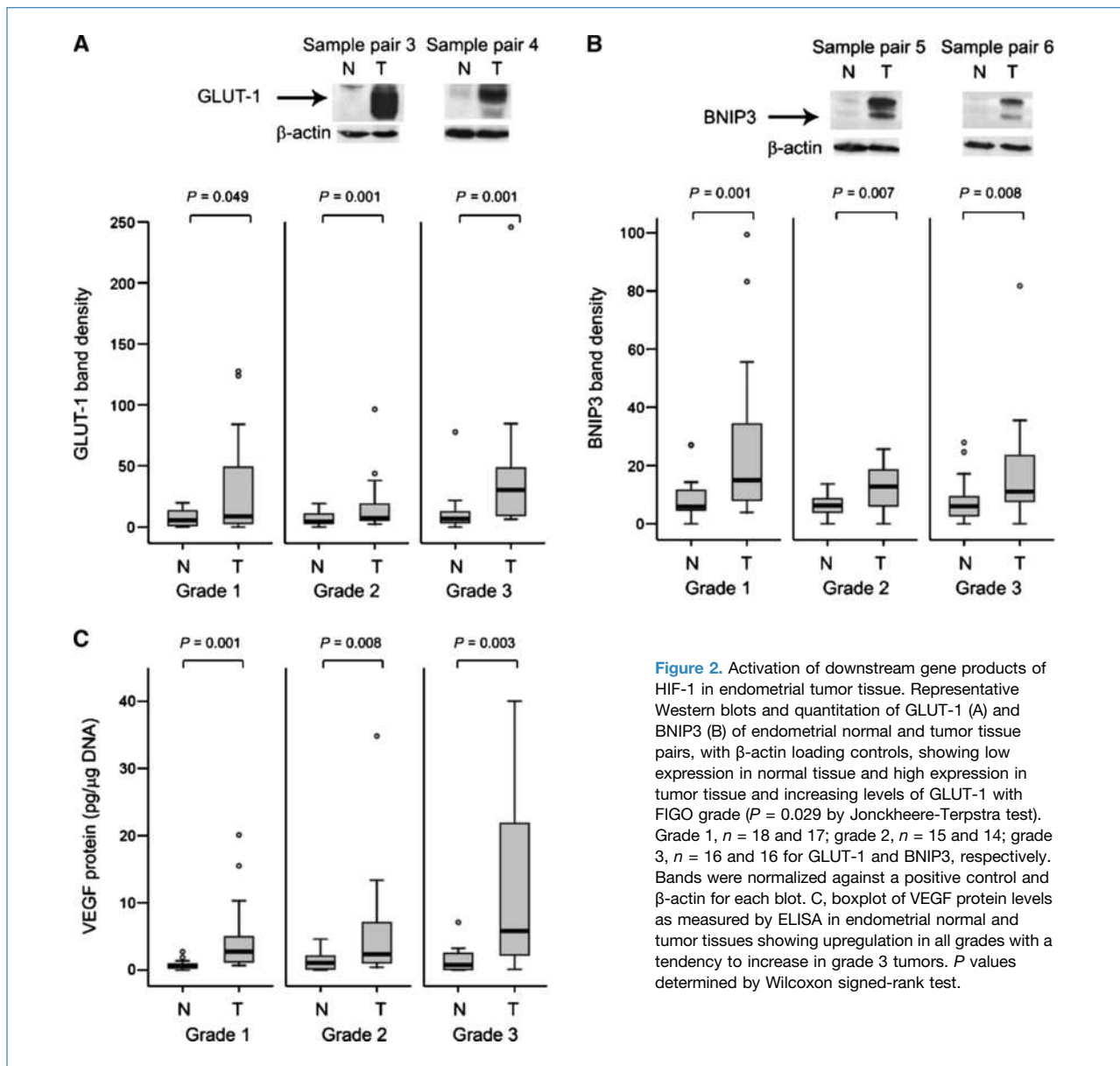
Figure 2. Activation of downstream gene products of HIF-1 in endometrial tumor tissue. Representative Western blots and quantitation of GLUT-1 (A) and BNIP3 (B) of endometrial normal and tumor tissue pairs, with β-actin loading controls, showing low expression in normal tissue and high expression in tumor tissue and increasing levels of GLUT-1 with FIGO grade ($P = 0.029$ by Jonckheere-Terpstra test). Grade 1, $n = 18$ and 17; grade 2, $n = 15$ and 14; grade 3, $n = 16$ and 16 for GLUT-1 and BNIP3, respectively. Bands were normalized against a positive control and β-actin for each blot. C, boxplot of VEGF protein levels as measured by ELISA in endometrial normal and tumor tissues showing upregulation in all grades with a tendency to increase in grade 3 tumors. P values determined by Wilcoxon signed-rank test.

Table 1. Spearman's correlations between tumor HIF-1 activity parameters, tumor size, and ascorbate content

		HIF-1α band density	GLUT-1 band density	BNIP3 band density	VEGF protein levels (pg/µg DNA)	Size of tumor (mm)	Ascorbate content (mg/100 g)
HIF-1α band density	r_s	1.000	0.302*	0.250	0.326*	0.367*	−0.026
	P	—	0.037	0.090	0.031	0.012	0.861
	n	48	48	47	44	46	48
GLUT-1 band density	r_s	0.302*	1.000	0.471[†]	0.165	0.213	−0.071
	P	0.037	—	0.001	0.283	0.150	0.627
	n	48	49	47	44	47	49
BNIP3 band density	r_s	0.250	0.471[†]	1.000	0.147	0.311*	−0.139
	P	0.090	0.001	—	0.342	0.037	0.350
	n	47	47	47	44	45	47
VEGF protein levels (pg/µg DNA)	r_s	0.326*	0.165	0.147	1.000	0.296	−0.408[†]
	P	0.031	0.283	0.342	—	0.057	0.006
	n	44	44	44	44	42	44
Size of tumor (mm)	r_s	0.367*	0.213	0.311*	0.296	1.000	−0.129
	P	0.012	0.150	0.037	0.057	—	0.376
	n	46	47	45	42	49	49
Ascorbate content (mg/100 g)	r_s	−0.026	−0.071	−0.139	−0.408[†]	−0.129	1.000
	P	0.861	0.627	0.350	0.006	0.376	—
	n	48	49	47	44	49	51

*Correlation is significant at the 0.05 level (two-tailed).
[†]Correlation is significant at the 0.01 level (two-tailed).

no difference seen between tumor grades. As previous studies have exclusively used immunohistochemistry to analyze HIF-1 activation (16, 38, 39, 43), the presence and localization of HIF-1α were confirmed in the same samples on a tissue microarray. When detected in tumors, HIF-1α was seen either throughout the entire section or in a small number of cells in the nuclei of glandular or tumor tissues (Fig. 1C). HIF-1α was also seen in surrounding areas of necrosis with both cytoplasmic and nuclear staining (Fig. 1D). Stromal and myometrial tissues were always negative (Fig. 1C and D), as were control normal endometrial sections.

To determine the transcriptional activity of HIF-1 in the tumors, protein levels of three prosurvival HIF target gene products (GLUT-1, BNIP3, and VEGF) were measured. GLUT-1, measured by Western blotting, was detected in 46 of 48 tumors (96%) and 43 of 48 normal tissues (90%), with significantly higher expression in tumor compared with normal tissue ($P < 0.001$; Fig. 2A) and increasing expression with tumor grade ($P = 0.029$). BNIP3 protein was monitored on Western blots as a 30-kDa band (44) and revealed expression in 44 of 46 tumors (96%) and 41 of 46 normal tissues (89%). BNIP3 was significantly higher in tumor compared with normal tissue ($P < 0.001$; Fig. 2B) but did not change with grade. Tissue VEGF protein, measured by ELISA, was detectable in 44 of 44 tumors (100%) and 37 of 44 normal tissues (84%). Levels were significantly elevated in tumor tissue ($P < 0.001$; Fig. 2C) and tended to increase with tumor grade, although this was not statistically significant ($P = 0.225$).

Spearman's correlations (Table 1) revealed that in the tumor tissue, HIF-1α correlated with GLUT-1 and VEGF and tended to correlate with BNIP3. GLUT-1 and BNIP3 were strongly associated with each other. Both HIF-1α and BNIP3 protein levels were associated with larger tumor size. Tumors positive for lymph/vascular invasion tended to have higher levels of both HIF-1α ($P = 0.088$) and GLUT-1 ($P = 0.068$) than tumors without lymph/vascular invasion. Taken together, these results provide evidence for the activation of HIF-1 and downstream gene expression in endometrial tumors and indicate that the HIF-1 pathway is activated in higher-grade, invasive, and larger tumors.

Ascorbate accumulation decreases with increasing tumor grade

To verify that the protocol for tissue banking did not contribute to a loss or variation in ascorbate content, a healthy mouse liver was dissected and left at room temperature for 0 to 7 hours, then stored at −80°C for 21 weeks. There was minimal loss of tissue ascorbate after up to 5 hours at room temperature [0 hours, 71.8 ± 2.67; 1 hour, 66.1 ± 2.51; 5 hours, 65.9 ± 1.77 (nmol/100 mg ± SD)] and long-term storage at −80°C had no further effect on ascorbate content, with 100% of the initial ascorbate being detected in the tissue. We also measured the stability of ascorbate in our tissue extracts and found no difference between a newly extracted sample (5.75 ± 0.15 mg/100 g) and the same extract after 4 days of storage (5.85 ± 0.01 mg/100 g). Identical levels of

ascorbate were also found when repeat measurements were made on two samples after a further 7 months of storage (11.22 versus 11.19 and 7.22 versus 6.68 mg/100 g).

To estimate the ability of the tumor tissue to accumulate ascorbate relative to adjacent normal tissue, tumor levels were compared with the matched normal tissue from the same patient. The ratio compensated for the high interindividual variation in tissue ascorbate (0.0007–1.5100 nmol ascorbate/μg DNA in normal tissue). There was a significant difference in relative ascorbate uptake between tumor grades, with the ascorbate ratio decreasing with increasing tumor grade ($P = 0.010$; Fig. 3). Grade 1 tumors had higher levels of ascorbate than paired normal tissue (although this was not significant due to sample variation), grade 2 tumors had approximately equal amounts, whereas grade 3 tumors had significantly less ascorbate than paired normal tissue ($P = 0.028$). A similar result was obtained when ascorbate was expressed as absolute levels (mg/100 g tissue), with a decreasing ratio with increasing FIGO grade (not shown). These results indicate that high-grade tumor tissue has reduced capacity to accumulate ascorbate relative to normal tissue.

Relationship between ascorbate, HIF-1 pathway activation, and tumor pathology

To determine whether ascorbate levels in the tumor tissue related to an active HIF-1 pathway and tumor phenotype, the absolute ascorbate content (mg/100 g tissue) in the tumor samples was compared with levels of HIF-1α and its target proteins, as well as with clinicopathologic features. There was considerable variation in the absolute tissue ascorbate levels: for the tumors, levels ranged from 1.02 to 32.76 mg/100 g with a median of 6.68 mg/100 g (Fig. 4A), and for the

Figure 3. Ascorbate accumulation in endometrial tumors relative to normal tissue levels. Ascorbate ratios (tumor/normal) by FIGO grade (mean ± SEM) showing increased levels in grade 1 tumors (*n* = 18), approximately even levels in grade 2 tumors (*n* = 14), and significantly reduced levels in grade 3 tumors compared with paired normal tissue (*n* = 17; *, *P* = 0.028 by Wilcoxon signed-rank test). Ascorbate accumulation decreases with increasing FIGO grade (*P* = 0.010 by Jonckheere-Terpstra test).

normal tissue, from 0.04 to 23.13 mg/100 g with a median of 4.95 mg/100 g (Fig. 4C). These levels are similar to reported uterine ascorbate levels in healthy women of 10 to 20 mg/100 g tissue (45), but our lower limit suggests that many of the samples were relatively ascorbate deficient. An ascorbate level either greater or lower than 6.7 mg/100 g (median level in tumors) was used to classify the samples as either ascorbate deficient (*n* = 26) or ascorbate replete (*n* = 25). Tumors (all grades) with high ascorbate levels all had low HIF-1 pathway activation, whereas those tumors with the highest HIF-1α or target levels were all ascorbate deficient (Fig. 4A). This association was most notable for VEGF, with ascorbate-deficient tumors having significantly higher levels than ascorbate-replete tumors ($P = 0.001$; Fig. 4A) and with there being a significant inverse correlation between VEGF protein levels and tumor ascorbate content ($P = 0.006$; Table 1). A similar trend was seen with HIF-1α protein, GLUT-1, and BNIP3 (Fig. 4A), and ascorbate-deficient tumors also tended to be larger in size (Fig. 4B).
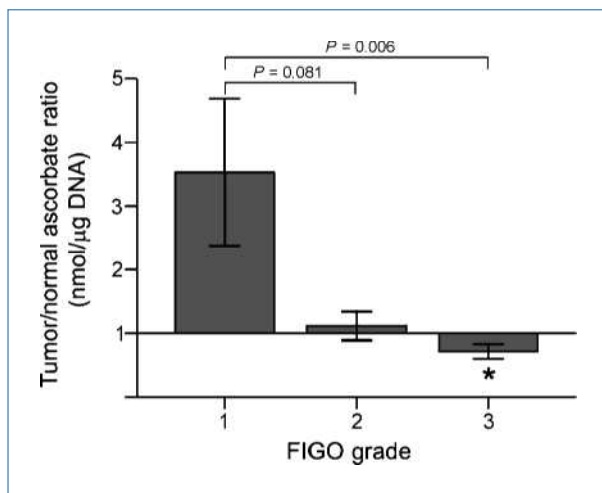
Although HIF-1α was undetectable in most normal tissue, low levels were measured in some samples (Fig. 1), and interestingly, these also occurred when ascorbate levels were low (Fig. 4C).

The results above suggested that the activation of HIF-1 pathway may correlate with low tissue ascorbate levels. To assess this, we analyzed the combined results for each tumor sample: the expression levels of HIF-1α, BNIP3, GLUT-1, and VEGF were normalized (as percent maximum expression), then added together to give each tumor a "HIF-1–related pathway protein score." This analysis suggests that low tissue ascorbate levels result in significant upregulation of the HIF-1 pathway ($P = 0.007$; Fig. 5).
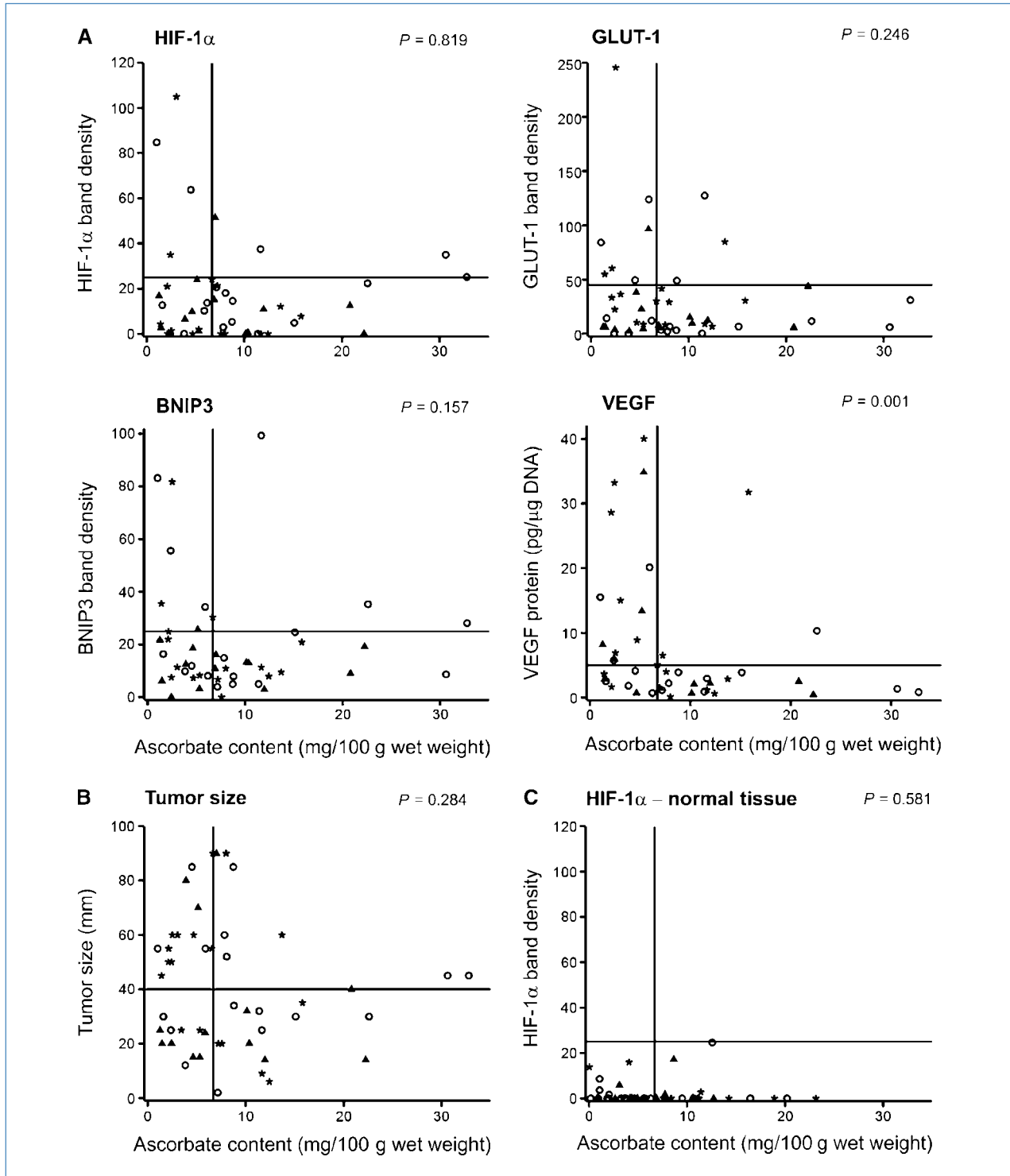
Discussion

In this study, we provide the first direct evidence for a relationship between ascorbate content, HIF-1 pathway activation, and tumor metabolism in a human tumor model. Early claims that ascorbate could retard tumor growth (1, 2) were met with skepticism in the absence of a plausible mechanism of action, but the discovery that HIF-1 is controlled by ascorbate-dependent hydroxylation has provided a possible link between ascorbate and tumor metabolism and survival. That such a link exists *in vivo* has been supported by recent animal studies in which tumor growth was inhibited by increasing ascorbate supply and was associated with downregulation of HIF-1 activity (10). However, to date, there has been no evidence to support a relationship between HIF-1, ascorbate, and tumor progression in humans.

Using human endometrial tumor tissue, we found that ascorbate accumulation decreased with increasing tumor grade and that high-grade tumors had approximately 40% less ascorbate than matched adjacent normal tissue. In addition, this is the first study in which tumor ascorbate content and markers of HIF-1 activation have been measured simultaneously. We found a significant inverse correlation between ascorbate and VEGF protein levels, and increased levels of other markers of HIF-1 activation (HIF-1α protein,

Figure 4. Relationship between tumor ascorbate content and HIF-1 activation parameters. A, scatterplots of ascorbate content (mg/100 g) in endometrial tumor tissue samples against HIF-1α protein levels (n = 48) and its downstream target gene products GLUT-1 (n = 49), BNIP3 (n = 47), and VEGF (n = 44). The median ascorbate value of 6.7 mg/100 g of tissue was used to classify samples as either ascorbate-deficient or ascorbate-replete. Ascorbate-deficient tumors had significantly higher levels of VEGF protein than ascorbate-replete tumors (P = 0.001 by Mann-Whitney test). Levels of HIF-1α, BNIP3, and GLUT-1 also tended to be increased when ascorbate levels were low. Individual tumor samples are identified by grade: circles, grade 1; triangles, grade 2; stars, grade 3. B, tumor size showed a similar distribution to markers of HIF-1 activation, with ascorbate-deficient tumors tending to be larger in size. C, normal tissue ascorbate levels were between 0.04 and 23 mg/100 g tissue. Some samples had low levels of HIF-1α protein and these were also likely to be those that had very low ascorbate levels.

GLUT-1, and BNIP3) were all associated with low ascorbate levels. There was also a tendency for ascorbate-deficient tumors to be bigger in size. When taken together, these associations showed a significant upregulation of the HIF-1 pathway and provide evidence to suggest that having inadequate tissue ascorbate levels may promote tumor progression as a result of poor control of HIF-1.

We measured the tumor ability to accumulate ascorbate by comparing levels with the adjacent normal tissue. In addition to ascorbate accumulation being compromised in grade 3 tumors, we found that HIF-1–dependent gene expression may also be elevated in these tumors, as both GLUT-1 and VEGF protein levels tended to increase with tumor grade. The combined analysis of three HIF-1–dependent targets is a strong indication of transcriptional activity and should reflect mRNA levels for these proteins, although these were not measured. Endometrial tumors are highly angiogenic and the expression of VEGF has been associated with poor outcome (40). Our finding that VEGF is increased in endometrial tumors and tends to increase with tumor grade supports these observations, and the correlation between VEGF and HIF-1α protein levels suggests that this increase is HIF-1 dependent. GLUT-1 levels also correlated with HIF-1α and BNIP3 proteins and increased with tumor 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 tumors (46).

The tendency for HIF-1–dependent gene expression to increase with tumor grade occurred despite the absence of variation in HIF-1α protein levels. This suggests that HIF-1α

Figure 5. Low tissue ascorbate is associated with increased activation of the HIF-1 pathway in endometrial cancer. The protein levels measured in Fig. 4A were each normalized (as percent maximum expression) and each tumor sample was given a combined score equal to the sum of expression of HIF-1α, BNIP3, GLUT-1, and VEGF to reflect total activation of the HIF-1 pathway for each tumor (HIF-1 pathway–related protein score). These were then compared for ascorbate-deficient ($n = 22$) and ascorbate-replete ($n = 22$) samples by Mann-Whitney test ($P = 0.007$).
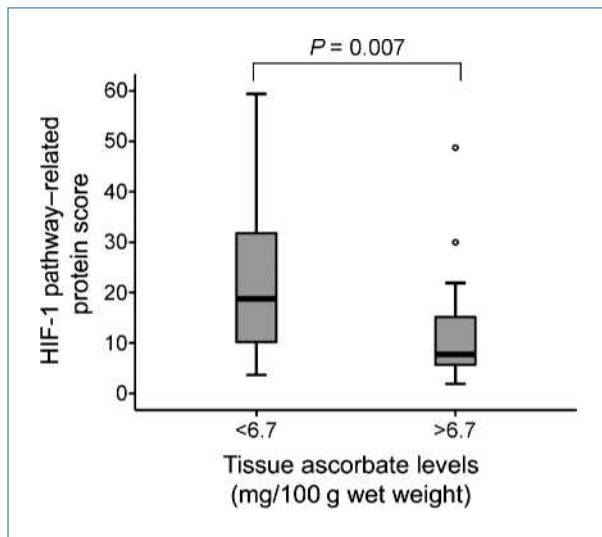
protein levels alone are not representative of its activity and may reflect the dual control of HIF-1, with asparagine hydroxylation by FIH independently controlling transcriptional activity (25, 26). Interestingly, FIH has a significantly higher K_m for ascorbate than do the prolyl-hydroxylases (26), and it is therefore likely that HIF-1–mediated gene expression would be more responsive to low levels of ascorbate than HIF-1α protein stabilization. This is reflected in the HIF-1 pathway–related protein score being higher in low-ascorbate tumors and may also explain the significant inverse correlation between VEGF protein expression and ascorbate levels, where low levels of cellular ascorbate may exacerbate the production of VEGF and promote tumor angiogenesis and growth.

The decreased ascorbate levels with increasing tumor grade and size may reflect poor accessibility of the tumor cells to the plasma supply (37). Ascorbate is delivered via the circulation and uptake is dependent on plasma levels (37) and accessibility; hence, when a tumor is poorly vascularized, ascorbate uptake could be compromised. We do not have data on the levels of SVCTs in our tissue samples, but variation in transporter expression or activity could influence ascorbate uptake. In addition, the activity of the SVCTs is reduced by as much as 70% when the extracellular pH decreases to 6.5 (35) and this could have a significant effect in the more acidic environment of the tumor (47). 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 (35).

To our knowledge, only one other study has measured ascorbate levels in tumor tissue using patient-matched normal tissue as a comparison, finding significantly reduced levels in astrocytoma compared with normal tissue (48). We found that some grade 1 tumors contained much higher levels of ascorbate than the adjacent normal tissue from the same patient. This may reflect the different tissue types, as normal, postmenopausal uterine endometrial tissue biopsies mainly contained myometrium (smooth muscle) and endometrial stroma with very few glands. Because tissues vary widely in their ability to accumulate ascorbate (49), it is possible that ascorbate would distribute differently between myometrium and glandular tissue. However, whether this occurs is unknown, but endometrioid adenocarcinoma tissue is mainly composed of glandular tissue, with grade 1 tumors most resembling this (41).

The methods used in this study have allowed for accurate 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 more precisely reflect tissue cell content (50). The use of Western blot or ELISA to measure HIF-1α and its target gene proteins also allowed for a less subjective quantification than is achievable with the more commonly used immunohistochemistry, and is more likely to represent the level of protein expression throughout the whole tumor sample. The presence and nuclear localization of HIF-1α was confirmed with immunohistochemistry, providing further evidence for its transcriptional activity.

Our results indicate that low cellular ascorbate accumulation could result in increased HIF-1 activity in human

tumors. This was predicted from *in vitro* studies that have shown that optimum intracellular levels of ascorbate can substantially curb HIF-1 activation and subsequent gene expression (31, 32). In addition, our study suggests that restoring tumor tissue ascorbate levels would limit the expression of pro-survival and angiogenic factors that promote tumor growth and poor outcome. This hypothesis could be tested in a suitable animal model that would allow for accurate monitoring of the effectiveness of delivery of ascorbate to tumor tissue.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Cameron E, Pauling L. Supplemental ascorbate in the supportive treatment of cancer: prolongation of survival times in terminal human cancer. Proc Natl Acad Sci U S A 1976;73:3685–9.
2. Cameron E, Pauling L. Supplemental ascorbate in the supportive treatment of cancer: reevaluation of prolongation of survival times in terminal human cancer. Proc Natl Acad Sci U S A 1978;75:4538–42.
3. Moertel CG, Fleming TR, Creagan ET, et al. High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy. A randomized double-blind comparison. N Engl J Med 1985;312:137–41.
4. Carosio R, Zuccari G, Orienti I, Mangraviti S, Montaldo PG. Sodium ascorbate induces apoptosis in neuroblastoma cell lines by interfering with iron uptake. Mol Cancer 2007;6:55–65.
5. Chen Q, Espey MG, Krishna MC, et al. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. Proc Natl Acad Sci U S A 2005;102:13604–9.
6. Gardiner NS, Duncan JR. Inhibition of murine melanoma growth by sodium ascorbate. J Nutr 1988;34:586–90.
7. Padayatty SJ, Sun H, Wang Y, et al. Vitamin C pharmacokinetics: implications for oral and intravenous use. Ann Intern Med 2004;140:533–7.
8. Duconge J, Miranda-Massari JR, Gonzalez MJ, et al. Pharmacokinetics of vitamin C: insights into the oral and intravenous administration of ascorbate. P R Health Sci J 2008;27:7–19.
9. Chen C, Sun J, Liu G, Chen J. Effect of small interference RNA targeting HIF-1α mediated by rAAV combined L-ascorbate on pancreatic tumors in athymic mice. Pathol Oncol Res 2009;15:109–14.
10. Gao P, Zhang H, Dinavahi R, et al. HIF-dependent antitumorigenic effects of antioxidants in vivo. Cancer Cell 2007;12:230–8.
11. Verrax J, Calderon PB. Pharmacologic concentrations of ascorbate are achieved by parenteral administration and exhibit antitumoral effects. Free Radic Biol Med 2009;47:32–40.
12. Chen Q, Espey MG, Sun AY, et al. Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. Proc Natl Acad Sci U S A 2008;105:11105–9.
13. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–32.
14. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 1994;269:23757–63.
15. Robey IF, Lien AD, Welsh SJ, Baggett BK, Gillies RJ. Hypoxia-inducible factor-1α and the glycolytic phenotype in tumors. Neoplasia 2005;7:324–30.
16. Horree N, van Diest PJ, van der Groep P, Sie-Go DMDS, Heintz APM. Hypoxia and angiogenesis in endometrioid endometrial carcinogenesis. Cell Oncol 2007;29:219–27.
17. Tanaka H, Yamamoto M, Hashimoto N, et al. Hypoxia-independent overexpression of hypoxia-inducible factor 1α as an early change in mouse hepatocarcinogenesis. Cancer Res 2006;66:11263–70.
18. Simiantonaki N, Taxeidis M, Jayasinghe C, Kurzik-Dumke U, Kirkpatrick CJ. Hypoxia-inducible factor 1α expression increases during colorectal carcinogenesis and tumor progression. BMC Cancer 2008;8:320–33.
19. Williams KJ, Telfer BA, Xenaki D, et al. Enhanced response to radiotherapy in tumors deficient in the function of hypoxia-inducible factor-1. Radiother Oncol 2005;75:89–98.
20. Liu L, Ning X, Sun L, et al. Hypoxia-inducible factor-1α contributes to hypoxia-induced chemoresistance in gastric cancer. Cancer Sci 2007;99:121–8.
21. Sermeus A, Cosse J-P, Crespin M, et al. Hypoxia induces protection against etoposide-induced apoptosis: molecular profiling of changes in gene expression and transcription factor activity. Mol Cancer 2008;7:27.
22. Yamamoto Y, Ibusuki M, Okumura Y, et al. Hypoxia-inducible factor 1α is closely linked to an aggressive phenotype in breast cancer. Breast Cancer Res Treat 2008;110:465–75.
23. Sullivan R, Pare GC, Frederiksen LJ, Semenza GL, Graham CH. Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity. Mol Cancer Ther 2008;7:1961–73.
24. Jaakkola P, Mole DR, Tian Y-M, et al. Targeting of HIF-1α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. Science 2001;292:468–72.
25. Lando D, Peet DJ, Gorman JJ, et al. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Gene Dev 2002;16:1466–71.
26. Ozer A, Bruick RK. Non-heme dioxygenases: cellular sensors and regulators jelly rolled into one? Nat Chem Biol 2007;3:144–53.
27. Koivunen P, Hirsila M, Gunzler V, Kivirikko KI, Myllyharju J. Catalytic properties of the asparagine hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. JBC 2004;279:9899–904.
28. de Jong L, Albracht SPJ, Kemp A. Prolyl 4-hydroxylase activity in relation to the oxidation state of enzyme-bound iron: the role of ascorbate in peptidyl proline hydroxylation. Biochim Biophys Acta 1982;704:326–32.
29. Myllyla R, Kuutti-Savolainen E-R, Kivirikko KI. The role of ascorbate in the prolyl hydroxylase reaction. Biochem Biophys Res Commun 1978;83:441–8.
30. Kaczmarek M, Cachau RE, Topol IA, et al. Metal ions-stimulated iron oxidation in hydroxylases facilitates stabilization of HIF-1α protein. Toxicol Sci 2009;107:394–403.
31. Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ. Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. Cancer Res 2003;63:1764–8.
32. Vissers MCM, Gunningham SP, Morrison MJ, Dachs GU, Currie MJ.

Modulation of hypoxia-inducible factor-1α in cultured primary cells by intracellular ascorbate. Free Radic Biol Med 2007;42:765–72.

33. Jones DT, Trowbridge IS, Harris AL. Effects of transferrin receptor blockade on cancer cell proliferation and hypoxia-inducible factor function and their differential regulation by ascorbate. Cancer Res 2006;66:2749–56.

34. Rumsey SC, Kwon O, Xu GW, et al. Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. JBC 1997;272: 18982–9.

35. Liang W-J, Johnson D, Jarvis SM. Vitamin C transport systems of mammalian cells. Mol Membr Biol 2001;18:87–95.

36. Evans RM, Currie L, Campbell A. The distribution of ascorbic acid between various cellular components of blood, in normal individuals, and its relation to the plasma concentration. Br J Nutr 1982;47:473–82.

37. Levine M, Conry-Cantilena C, Wang Y, et al. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. Proc Natl Acad Sci U S A 1996;93:3704–9.

38. Sivridis E, Giatromanolaki A, Gatter KC, Harris AL, Koukourakis MI. Association of hypoxia-inducible factors 1α and 2α with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. Cancer 2002;95:1055–63.

39. Giatromanolaki A, Koukourakis MI, Gatter KC, Harris AL, Sivridis E. BNIP3 expression in endometrial cancer relates to active hypoxia inducible factor 1α pathway and prognosis. J Clin Pathol 2008;61: 217–20.

40. Sivridis E. Angiogenesis and endometrial cancer. Anticancer Res 2001;21:4383–8.

41. Denny L, Hacker NF, Gori J, et al. Staging classifications and clinical practice guidelines for gynaecological cancers. Int J Gynecol Obstet 2000;70:207–312.

42. Bewick V, Cheek L, Ball J. Statistics review 10: further nonparametric methods. Crit Care 2004;8:196–9.

43. Pijnenborg JMA, Wijnakker M, Hagelstein J, Delvoux B, Groothius PG. Hypoxia contributes to development of recurrent endometrial carcinoma. Int J Gynecol Cancer 2007;17:897–904.

44. Chen G, Ray R, Dubik D, et al. The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. J Exp Med 1997;186:1975–83.

45. Menten ML, King CG. The influence of vitamin C level upon resistance to diphtheria toxin. J Nutr 1935;10:141–55.

46. Walenta S, Schroeder T, Mueller-Klieser W. Lactate in solid malignant tumors: potential basis of a metabolic classification in clinical oncology. Curr Med Chem 2004;11:2195–204.

47. Gerweck LE, Seetharaman K. Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. Cancer Res 1996;56:1194–8.

48. Landolt H, Langemann H, Probst A, Gratzl O. Levels of water-soluble antioxidants in astrocytoma and in adjacent tumor-free tissue. J Neurooncol 1994;21:127–33.

49. Hornig D. Distribution of ascorbic acid, metabolites and analogues in man and animals. Ann N Y Acad Sci 1975;258:103–18.

50. Fiszer-Szafarz B, Szafarz D. DNA and protein content as cellular biochemical parameters. A discussion with two examples: acid phosphatase and cathepsin D in rat liver and hepatoma and acid phosphatase in human breast normal tissue and adenocarcinoma. Anal Biochem 1984;138:255–8.