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Original article

Pharmacokinetic and anti-cancer properties of high dose ascorbate in solid tumours of ascorbate-dependent mice

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ABSTRACT

Despite recent evidence for an anti-tumour role for high-dose ascorbate, potential mechanisms of action are still unclear. At mM concentrations that are achieved with high-dose intravenous administration, autoxidation of ascorbate can generate cytotoxic levels of H_2O_2. Ascorbate is also a required co-factor for the hydroxylases that suppress the transcription factor hypoxia-inducible factor (HIF-1). HIF-1 supports an aggressive tumour phenotype and is associated with poor prognosis, and previous studies have shown that optimizing intracellular ascorbate levels down-regulates HIF-1 activation. In this study we have simultaneously measured ascorbate concentrations and the HIF-1 pathway activity in tumour tissue following high dose ascorbate administration, and have studied tumour growth and physiology. Gulo$^{-/-}$ mice, a model of the human ascorbate dependency condition, were implanted with syngeneic Lewis lung tumours, 1 g/kg ascorbate was administered into the peritoneum, and ascorbate concentrations were monitored in plasma, liver and tumours. Ascorbate levels peaked within 30 min, and although plasma and liver ascorbate returned to baseline within 16 h, tumour levels remained elevated for 48 h, possibly reflecting increased stability in the hypoxic tumour environment. The expression of HIF-1 and its target proteins was down-regulated with tumour ascorbate uptake. Elevated tumour ascorbate levels could be maintained with daily administration, and HIF-1 and vascular endothelial growth factor protein levels were reduced in these conditions. Increased tumour ascorbate was associated with slowed tumour growth, reduced tumour microvessel density and decreased hypoxia. Alternate day administration of ascorbate resulted in lower tumour levels and did not consistently decrease HIF-1 pathway activity. Levels of sodium-dependent vitamin C transporters 1 and 2 were not clearly associated with ascorbate accumulation by murine tumour cells *in vitro* or *in vivo*. Our results support the suppression of the hypoxic response by ascorbate as a plausible mechanism of action of its anti-tumour activity, and this may be useful in a clinical setting.

1. Introduction

Ascorbate has long been proposed to have anti-cancer activity [1–6], and is frequently administered by complementary medicine practitioners as an adjunct to cancer treatments, often as high-dose infusions [7]. In the past decade, improved understanding of the pharmacokinetics of ascorbate uptake and transport has led to renewed interest in its potential activity in cancer. Recent investigations have shown that high-dose ascorbate can affect tumour growth in mouse models [8–11], and there are currently a number of human clinical trials aiming to determine efficacy [12–16]. However, the use of ascorbate in cancer remains an area of controversy, due to a lack of robust clinical data and of a proven mechanism of action.

Ascorbate could exert a number of possible anti-tumour activities: at high local concentrations, achievable by intravenous administration, autoxidation results in significant generation of H_2O_2 that is cytotoxic to tumour cells *in vitro* [17,8,18]. High

Abbreviations: HIF-1, Hypoxia-inducible factor-1; CA-IX, Carbonic anhydrase-IX; VEGF, Vascular endothelial growth factor; HDVC, High dose vitamin C; PHH3, Phospho-histone H3; LL/2, Lewis lung carcinoma; FFPE, Formalin fixed paraffin embedded; HPLC, high performance liquid chromatography; SVCT, sodium-dependent vitamin C transporter

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concentrations of dehydroascorbate (DHA), the oxidized form of ascorbate, are proposed to lead to an energetic crisis in glycolytic cancer cells, causing cell death [11]. Ascorbate regulates the activity of the transcription factor hypoxia-inducible factor (HIF)-1 [19–21] and decreased intracellular levels up-regulate the hypoxic response [22–24]. HIF-1 drives tumour angiogenesis, modifies the extracellular pH-environment, and affects cell death and survival pathways, and its activation culminates in an aggressive cancer phenotype [25–27].

HIF-1 is controlled by proline hydroxylases (PHDs 1, 2, 3) and an asparagine hydroxylase (factor-inhibiting HIF, FIH), collectively known as the HIF hydroxylases, that together regulate the stability of the alpha subunit and transcriptional activity [28–30]. The HIF hydroxylases are iron-containing 2-oxoglutarate-dependent dioxygenases that require molecular oxygen and 2-oxoglutarate as substrates [20,31]. They also require ascorbate as a cofactor, to retain optimum activity and prevent irreversible oxidation of the active site iron [20,21]. We, and others, have shown that intracellular ascorbate availability is a major determinant in the regulation of HIF-1 activity in response to mild hypoxia, 2-oxoglutarate deprivation or metal poisoning with Co^{2+} or Ni^{2+} [22,19].

How intravenous or high-dose administration of ascorbate affects tumour growth is unknown, with the mechanism of anti-tumour activity by ascorbate not having been monitored in an *in vivo* setting. This information is required to inform human clinical study protocols. Pharmacokinetic studies in humans have demonstrated that the uptake of ascorbate following oral ingestion is tightly regulated [32,33], and that the bioavailability is increased by 70-fold when administered intravenously [7,33,14]. We have previously proposed that these supra-physiological plasma concentrations could allow ascorbate to overcome the diffusion barrier of poorly-perfused solid tumours [34], thereby boosting intracellular levels. To date, however, no study has monitored the pharmacokinetics of ascorbate uptake into tumour tissue following high dose ascorbate (high-dose vitamin C, HDVC) administration.

Uptake of ascorbate into cells varies between tissues, and is mediated by the sodium-dependent vitamin C transporter that exists as two isoforms, SVCT1 and SVCT2 [35]. The SVCTs show distinct tissue distribution, and together ensure the effective uptake and regulation of plasma and cellular ascorbate concentrations [36,35]. SVCT1 is responsible for uptake through the gut and re-absorption in the kidneys, and is thought to largely regulate plasma levels, whereas SVCT2 is concentrated in more metabolically active cells throughout the body, ensuring an adequate intracellular supply to support crucial intracellular functions [36,35]. Both SVCT1 and SVCT2 have been found in lung tissue, with SVCT1 being identified solely in the blood vessels in one study [37], and on the apical surfaces of rat lung columnar epithelial cells in another study [52], both reporting widespread distribution of SVCT2. Transporter status has not been studied in tumour tissue, and this, together with ascorbate availability and tumour vessel patency, is likely to play an important role in the anti-cancer activity of HDVC.

The present study was designed to investigate whether HDVC could affect ascorbate levels in pre-existing tumours and could impact on tumour growth, and whether increased tumour ascorbate levels affected HIF-1 activity. We have utilized the $Gulo^{-/-}$ mouse which, like humans, carries a mutation in the gulonolactone oxidase (*Gulo*) gene that encodes the terminal enzyme in the ascorbate synthesis pathway, and thus relies entirely on dietary ascorbate [38]. Using this model, we have previously reported that variable dietary ascorbate intake affects tumour ascorbate levels, HIF-1 expression and tumour growth rate in three different syngeneic tumour models, namely Lewis Lung carcinoma (LL/2), B16-F10 melanoma and CMT93 colorectal cancer [39,40]. All three tumour models showed a significant inverse association between tumour ascorbate content and HIF-1 levels. In the current study we have utilized the LL/2 model to specifically investigate the pharmacokinetics of ascorbate uptake into established tumours following HDVC, its ability to boost ascorbate levels in the tumour and in non-tumour tissue, and the effect on HIF-1 activity and subsequent tumour growth.

2. Materials and methods

2.1. Materials

Mouse Lewis lung carcinoma (LL/2), CMT-93 colorectal carcinoma and B16-F10 melanoma cells were from American Type Culture Collection, Cryosite Distribution, Australia; the control mouse mammary carcinoma cell line EO771 was kindly provided by Dr Andreas Moeller, QIMR Berghofer, Australia. Sodium L-ascorbate was from Sigma-Aldrich, St Louis, MO, USA.Pimonidazole was obtained from Hypoxyprobe™-1, Burlington, Massachusetts, USA. Antibodies against mouse HIF-1α, carbonic-anhydrase IX (CA-IX), and β-actin were from R&D Systems, Minneapolis, USA; antibodies against SVCT1 were from Aviva Systems Biology, San Diego CA, and SVCT2 from Atlas Antibodies, Stockholm, Sweden; anti-goat, anti-mouse or anti-rabbit HRP-conjugated anti-IgG were from DAKO, Australia; antibodies against phospho-histone H3 (PHH3) and CD31 were from Abcam, Melbourne, Australia.

2.2. Cell culture

Cells were cultured at 37 °C in Dulbecco's Modified Eagle Media (DMEM, Gibco Invitrogen, Carlsbad, CA, USA) with 10% foetal calf serum (FCS; Gibco Invitrogen) in a humidified CO_2-incubator, and passaged using TrypLE Express (Gibco Invitrogen). Cells at ~70% confluence were used for *in vitro* and *in vivo* experiments.

2.3. Mouse model

Animal studies were approved by the University of Otago Animal Ethics Committee (C11/13 and C1/14). United Kingdom coordinating committee on cancer research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia were strictly followed. Heterozygote C57BL6/J B6.129P2-$Gulo^{tm1Unc/Ucd}$ mice were originally purchased from the Mutant Mouse Resource Centre (University of California at Davis, US) and were fed standard mouse chow which is deficient in ascorbate. Mice bred in-house were genotyped as previously described [52]. Female $Gulo^{-/-}$ mice (6–8 weeks old) were supplemented with a maintenance dose of ascorbate in their drinking water (330 mg/L ascorbate stabilized with 10 μM EDTA) for 4 weeks prior to tumour implant, and this was continued for the duration of the experiment.

2.4. Tumour model

LL/2 cells (1×10^6) were implanted subcutaneously on the flank. Tumour volume was measured every second day using calipers (tumour volume = $\text{width}^2 \times \text{length} \times \pi/6$) and when tumours reached treatment size, the mice were injected intraperitoneally (IP) with HDVC at a dose of 1 g/kg in 0.1 mL phosphate buffered saline (PBS).

2.5. Pharmacokinetic study

When LL/2 tumours reached 400 mm^3, mice received 1 g/kg HDVC *via* a single IP injection. Mice (5 per group, n = 45) were