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Original Contribution

Pharmacokinetic modeling of ascorbate diffusion through normal and tumor tissue

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ABSTRACT

Ascorbate is delivered to cells via the vasculature, but its ability to penetrate into tissues remote from blood vessels is unknown. This is particularly relevant to solid tumors, which often contain regions with dysfunctional vasculature, with impaired oxygen and nutrient delivery, resulting in upregulation of the hypoxic response and also the likely depletion of essential plasma-derived biomolecules, such as ascorbate. In this study, we have utilized a well-established multicell-layered, three-dimensional pharmacokinetic model to measure ascorbate diffusion and transport parameters through dense tissue *in vitro*. Ascorbate was found to penetrate the tissue at a slightly lower rate than mannitol and to travel via the paracellular route. Uptake parameters into the cells were also determined. These data were fitted to the diffusion model, and simulations of ascorbate pharmacokinetics in normal tissue and in hypoxic tumor tissue were performed with varying input concentrations, ranging from normal dietary plasma levels (10–100 μM) to pharmacological levels (> 1 mM) as seen with intravenous infusion. The data and simulations demonstrate heterogeneous distribution of ascorbate in tumor tissue at physiological blood levels and provide insight into the range of plasma ascorbate concentrations and exposure times needed to saturate all regions of a tumor. The predictions suggest that supraphysiological plasma ascorbate concentrations (> 100 μM) are required to achieve effective delivery of ascorbate to poorly vascularized tumor tissue.

Introduction

Ascorbate (vitamin C) circulates through the vasculature and is actively taken up into tissue cells via the sodium-dependent vitamin C transporters (SVCTs)[2] [1]. The effective intracellular concentration varies significantly with plasma levels, but little is known of the ability of ascorbate to penetrate into the tissues and reach cells more remote from the vasculature. This is of interest in determining the effective dose of dietary ascorbate required for tissue saturation and, in particular, for those tissues with a poor blood supply. The latter scenario is particularly relevant to solid tumors that are known to contain regions of poor perfusion and hypoxia [2], caused by high intervessel distances that exceed the diffusion distance of oxygen [3], as well as poorly functional and leaky vessels causing temporary oxygen fluctuations [4]. In addition to a lack of oxygen, we hypothesize that these regions may lack other essential biomolecules delivered by the vasculature, such as ascorbate.

Plasma ascorbate levels are tightly controlled and do not normally exceed ~100 μM with dietary intake [5]. The SVCT1 tightly regulates the plasma concentration, as it has saturable transport kinetics at both the intestine and the kidney to limit absorption and reabsorption [6]. More recently, there has been increasing interest in the intravenous administration of ascorbate, which bypasses this tight control and can yield plasma levels up to 300-fold higher, with maximum levels of up to 30 mM, albeit transiently [7]. Whether these high concentrations would significantly increase delivery to inaccessible tumor tissue or affect cellular uptake is unknown.

A major function for ascorbate in vivo is as an essential cofactor for the 2-oxoglutarate-dependent dioxygenases (2-OGDDs). The 2-OGDDs require 2-oxoglutarate, molecular oxygen, ferrous iron, and ascorbate for activity and can therefore act as metabolic sensors, relaying a drop in the level of these metabolites to changes in gene expression and cellular function [8,9]. These enzymes perform hydroxylation reactions on various substrates including RNA, DNA, histones, ribosomes, prohormones, and proteins [10]. Accordingly, most cells and tissues accumulate ascorbate to millimolar

Abbreviations used: 2-OGDD, 2-oxoglutarate-dependent dioxygenase; HIF-1, hypoxia-inducible factor-1; 3D, three-dimensional; MCL, multicellular layer; SVCT, sodium-dependent vitamin C transporter

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concentrations, with very high levels being recorded in those organs with an essential functional need for the 2-OGDDs, such as the adrenals and brain [11].

A variation in intracellular ascorbate could influence the activity of numerous 2-OGDDs and has been shown to affect the hypoxia-inducible factor (HIF) hydroxylases, affecting activation of the transcription factor HIF-1 [12–15] and HIF-1-dependent tumor growth in mice [16,17]. A lack of ascorbate inhibits HIF-1 hydroxylation and promotes HIF-1 activation [13,14], thereby potentially promoting tumor progression. Tumor ascorbate levels have rarely been measured, but we have shown that both endometrial and colorectal tumors contained relatively low levels of ascorbate, and this was associated with increased activation of the HIF-1 pathway [18,19]. Of particular interest was the observation that high-grade endometrial and colorectal tumors, with a highly dedifferentiated and aggressive phenotype, had significantly reduced capacity to accumulate ascorbate compared to surrounding normal tissue [18,19]. These results indicate that despite the same available plasma concentrations, these poorly vascularized tumors cannot acquire the same cellular ascorbate levels. Given the difficulty of some tumor regions in accessing the plasma supply, it is possible that better delivery of ascorbate to tumor cells may require supraphysiological plasma levels.

To our knowledge, there are currently no data describing extravascular diffusion of ascorbate through tumor tissue and whether it is indeed likely to be limiting in hypoxic/avascular regions of a tumor. Obtaining diffusion data on ascorbate will be crucial both for understanding the need for optimal plasma levels as part of normal health maintenance and for the design of future cancer clinical intervention studies. Such information is increasingly needed to determine whether the widespread administration of ascorbate to cancer patients [20] is actually beneficial, and how.

In this study, we have employed an *in vitro* pharmacokinetic model system that utilizes linear three-dimensional (3D) in vitro multicellular layers (MCLs) to represent the extravascular compartment and emulate tumor-like tissue. The MCLs are made by growing cells on a porous Teflon support membrane [21] to form diffusion-limited structures up to several hundred micrometers in thickness, with many features in common with spheroids [22], including central hypoxia and necrosis [23]. However, MCLs have a planar structure, making them particularly amenable to drug diffusion studies within a specialized diffusion chamber [24]. This *in vitro* model was initially developed to test the pharmacokinetics and pharmacodynamics of DNA- and hypoxia-targeted anti-cancer compounds [25–27], some of which are currently undergoing clinical testing [28,29]. This model has been well-resolved to measure tissue penetration and cellular uptake, and we have now adapted this system to assess the availability of ascorbate throughout the extravascular compartment.

Using this system, we have obtained data describing ascorbate diffusion and intracellular uptake and stability, which were then used to model ascorbate pharmacokinetics and diffusion through tissue. Pharmacokinetic simulations have revealed that ascorbate is heterogeneously distributed in normal and tumor tissues at physiological plasma levels, with severe penetration problems at suboptimal plasma concentrations. The data also provide insight into the range of plasma ascorbate concentrations and exposure times needed to saturate all regions of a tumor to fully support 2-OGDD activity.

Materials and methods

Multicellular layers

HT29 human colon adenocarcinoma cells (American Type Culture Collection) were maintained in monolayers in MEM-α medium (no nucleosides), 5% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml) with weekly passaging (medium and supplements from Life Technologies, Gaithersburg, MD, USA). MCLs were grown on sterile collagen-coated Teflon support membranes in Millicell-CM inserts (Millipore Corp., Bedford, MA, USA) with a sterile polyethylene ring attached around the insert for flotation, as previously described [27]. HT29 cells were seeded at 1×10^6 cells per insert in 0.5-ml volume and floated in a large reservoir of medium (MEM-α with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin) for ~6 h at 37 °C and 5% CO_2 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.

Diffusion experiments

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 as previously described [27,30], containing 10 μM EDTA to prevent ascorbate oxidation with a final volume of 6.6 ml in each. The compartments were magnetically stirred in a 37 °C water bath and gassed with 95% N_2 and 5% CO_2 or 95% O_2 and 5% CO_2. Two radiolabeled internal standards, [^{14}C]urea (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and [^{3}H]mannitol (ICN Pharmaceuticals, Waltham, MA, USA), were added together with fresh sodium L-ascorbate (Sigma–Aldrich) to the donor compartment. The flux of [^{14}C]urea was used to determine the thickness of each MCL and [^{3}H]mannitol flux was measured as marker of paracellular diffusion and MCL integrity [31]. A schematic of the diffusion chamber experimental setup is given in Fig. 1. Samples (100 μl) of both the donor and the receiver compartments were taken using a syringe over 5 h to monitor the flux through either the bare Teflon support (i.e., in the absence of cells) or the MCL. Samples were added to 100 μl of 0.54 M perchloric acid [containing 50 mM diethylenetriaminepentaacetic acid (DTPA)]. Of this, 50 μl was taken for scintillation counting in 3 ml scintillation fluid (Emulsifier Safe; PerkinElmer, Waltham, MA, USA) on a Packard Tri-Carb liquid scintillation counter. The remaining 150 μl was immediately frozen at −80 °C for ascorbate measurement by HPLC–ECD, using fresh sodium L-ascorbate standards (concentration verified by absorbance at 245 nm), as described previously [14].

Ascorbate stability, uptake, and turnover experiments in cell monolayers

To constrain the model, parameters describing intracellular ascorbate uptake, stability, and turnover were obtained from HT29 cells in monolayer culture either under normoxic conditions or after equilibration at specified O_2 levels in a Whitley H35 HypoxyStation (Don Whitley Scientific, Shipley, West Yorkshire, UK). Cells were incubated in ascorbate-free medium [Dulbecco's modified Eagle's medium (DMEM), 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin] for ~16 h, then fresh sodium L-ascorbate was added to the culture medium to initiate the experiment. Over time the medium was sampled and cells were washed in phosphate-buffered saline, detached, and pelleted. Cell pellets and medium samples were extracted in 1:1 0.54 M perchloric acid (containing 50 mM DTPA) and H_2O. Supernatants were then analyzed for ascorbate by HPLC–ECD as described [14], with cellular values normalized to cell number (by hemocytometer) and intracellular water volume of the derivative cell line WiDr, previously measured at 2.81 pl [14].