Vitamin C modulation of H₂O₂-induced damage and iron homeostasis in human cells

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Running title: Vitamin C, DNA damage and iron homeostasis

Vitamin C modulation of H₂O₂-induced damage and iron homeostasis in human cells**Abstract**

Vitamin C (ascorbic acid, AA) is an important antioxidant in human plasma. It is clear, however, that AA has other important, non-antioxidant roles in cells. Of particular interest is its involvement in iron metabolism, since AA enhances dietary iron absorption, increases the activity of Fe²⁺-dependent cellular enzymes, promotes Fenton reactions *in vitro* and was reported to have deleterious effects in individuals with iron overload. Nevertheless, the ability of AA to modulate iron metabolism and enhance iron-dependent damage in cells, tissues and organisms has not been fully elucidated. Here we investigated the effect of AA on iron-mediated oxidative stress in normal human fibroblasts. Incubation with physiologically relevant concentrations of AA was not harmful but sensitised cells towards H₂O₂-induced, iron-dependent DNA strand breakage and cell death. We also report that AA increased the levels of intracellular catalytic iron and concomitantly modulated the expression of two well-established iron-regulated genes, ferritin and transferrin receptor. In summary, we present evidence of a novel, non-antioxidant role of AA in human cells, where it increases iron availability and enhances ROS-mediated, iron-dependent damage. We suggest that AA may exacerbate the deleterious effects of metals *in vivo* and promote normal tissue injury in situations associated with elevated ROS production.

Keywords: Ascorbic acid; DNA damage; H₂O₂; Labile iron pool; Transferrin receptor; Ferritin

Introduction

Oxidative stress is a condition that arises when the production of reactive oxygen species (ROS) overwhelms the cellular antioxidant defences. ROS are produced as a result of cell metabolism or inflammation, and can arise from exogenous sources. They are involved in normal cell processes such as cell signalling and proliferation. However, when in excess, ROS cause damage to cellular biomolecules and are implicated in the aetiology of a wide variety of diseases, including atherosclerosis, diabetes, neurodegenerative diseases, chronic inflammatory diseases, cancer and in ageing [1,2].

Iron is the most abundant transition metal in mammalian cells. Its presence is essential for the function of many cellular proteins, including those involved in respiratory electron transfer, oxygen transport, drug metabolism and DNA synthesis [3]. The toxicity of iron is a consequence of its ability to switch readily between two stable oxidation states, ferric (Fe^{3+}) and ferrous (Fe^{2+}), and can be largely explained by its participation in Fenton- and Haber-Weiss-type reactions. In these reactions, a transition metal ion reduces H_2O_2 to yield hydroxyl radical, which is one of the most potent oxidising species in biological systems, and the oxidised metal ion. Hydroxyl radicals produced in iron-mediated processes can damage DNA, causing strand breakage, depurination/depyrimidation and chemical modification of the bases or sugar, in addition to lipid peroxidation and protein modification [4].

ROS-mediated, iron-dependent injury processes in the human body are limited by efficient sequestering of free iron by high-affinity transport (transferrin, Tf) and high-capacity storage (ferritin) proteins. In healthy individuals, Tf is capable of binding all the iron present in the plasma. However, non-transferrin-bound iron (NTBI) is present in the sera of iron overloaded thalassemia patients and in other pathological conditions

associated with iron imbalance such as haemochromatosis [5,6]. Moreover, iron that enters cells bound to Tf via the transferrin receptor (TfR) remains temporarily loosely bound to proteins or low-affinity low-molecular weight chelators, before it is stored in ferritin or used in the synthesis of haemoproteins or other iron-containing enzymes. This so-called labile iron pool (LIP) is cytotoxic because of its ability to catalyse the formation of hydroxyl radicals from H_2O_2 [7,8]. Cells keep the size of the LIP under tight control by regulating the expression of ferritin and TfR. This is mediated by the binding of iron-regulatory proteins (IRPs) to iron-responsive elements (IREs) in the mRNAs for these genes. When iron is insufficient, IRPs bind to IREs in the 5' region of ferritin mRNA, blocking its translation, and to IREs in the 3' untranslated region of the TfR mRNA, increasing its stability and therefore augmenting the synthesis of the receptor. Conversely, when iron is abundant, IRPs have aconitase activity and do not bind the ferritin and TfR IREs. This allows the translation of ferritin to occur, thereby increasing the cellular capacity to safely store iron, and makes the TfR mRNA unstable, decreasing the synthesis of the receptor. Consequently, cellular iron uptake is reduced [9-11].

Vitamin C (ascorbic acid, AA) is an important antioxidant in human plasma, where it acts as a scavenger of free radicals and protects against lipid peroxidation [12]. AA is widely distributed in all tissues of the body at concentrations usually several-fold higher than in the plasma [13] and it recycles other important antioxidant molecules, such as vitamin E and glutathione [14,15]. However, the vitamin has other important, non-antioxidant biological functions [16,17], of which some are related to its ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron. AA promotes intestinal absorption of dietary non-haem Fe^{2+} iron through the divalent metal transporter 1 [18] and increases the activity of Fe^{2+} -dependent prolyl hydroxylases that participate in the synthesis of collagen and carnitine [19,20]. The ability of AA to reduce ferric to ferrous iron may,

however, have deleterious consequences. AA recycles the metal catalyst in the *in vitro* Fenton reaction, thus favouring another cycle of hydroxyl radical formation from H_2O_2 and ultimately leading to lipid, protein and DNA oxidation [1]. Although AA-mediated Fenton reactions may be controlled in the plasma and lymph of healthy individuals by efficient iron sequestration in transferrin [21,22], the redox-active NTBI found in the serum of individuals with iron overload may catalyse free radical formation in the presence of AA. AA supplements were reported to enhance cell and organ damage, and eventually cause cardiac failure, in patients with iron overload [23-25]. Additionally, the formation of hydroxyl radicals from H_2O_2 may occur intracellularly due to the presence of a pool of labile iron. It is thus possible that AA, which is present inside cells at high concentrations, may react with intracellular iron and promote intracellular Fenton reactions.

In the current study, we investigated the effect of AA on iron-mediated oxidative stress in normal human cells. To this end, we studied the effect of pre-incubation with AA or the stable vitamin C derivative AA2P on H_2O_2 -induced DNA damage and cell death in normal human diploid fibroblasts (HDFs). We have also investigated the effects of AA and AA2P on cellular iron homeostasis by measuring changes in the intracellular LIP and in the expression of cellular ferritin and TfR. We show that AA promotes intracellular Fenton-type reactions when cells are challenged with a physiologically relevant oxidant (H_2O_2). Furthermore, our results indicate that AA increases iron availability and modulates iron-regulated gene expression in HDFs.

Materials and Methods

Reagents

Minimal essential medium (MEM) with Earle's salts and non-essential amino acids, foetal bovine serum (FBS), Glutamax-I and PG SK diacetate were purchased from Invitrogen (Paisley, UK). Magnesium L-ascorbate 2-phosphate (AA2P) was obtained from Wako Pure Chemical Industries (Neuss, Germany). All other chemicals and reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Cell lines and culture conditions

GM5399 and GM5659 primary HDFs established from skin biopsies of apparently healthy donors were purchased from the NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, New Jersey, USA) and grown as a monolayer culture in Nunclon culture flasks at 37 °C in a humidified atmosphere containing 5 % CO₂. HDFs were grown in MEM with 2 mM Glutamax-I and 10 % FBS. Cells were confirmed to be free of mycoplasma contamination. All experiments were performed at passage numbers 10-17. Prior to the experiments, HDFs were incubated in growth medium until nearly confluent. All solutions were freshly prepared and sterilised by passing through a 0.22 µm membrane immediately prior to the experiments. For DNA damage experiments cells were thoroughly washed-free of any pre-treatments and incubated with 50 µM H₂O₂ prepared in medium containing 0.5 % FBS for 30 minutes on ice, in the dark. H₂O₂ solutions were freshly prepared from a 30 % stock solution.

Detection of intracellular AA

AA concentration was determined by high performance liquid chromatography with electrochemical detection (HPLC-EC) as described [26]. Intracellular AA concentration was normalised for total protein and expressed as nmol/mg protein. Protein

concentration was estimated using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK).

Single-cell gel electrophoresis (comet assay)

DNA damage was measured using the alkaline comet assay and expressed as the percentage of DNA in the tail of the comet (% tail DNA) as described [27].

Relative quantification of gene expression by real-time RT-PCR

Total RNA was extracted using TriReagent (Sigma-Aldrich), followed by DNase treatment (Turbo DNA-free kit; Ambion, Huntingdon, UK). First-strand cDNA was prepared using the StrataScript first-strand synthesis system (Stratagene, Amsterdam, Netherlands) according to the manufacturer's instructions. Primer sequences were designed with Primer 3 software [28]. The forward and reverse oligonucleotide sequences were: 5'-GCAGACTTTGCTTTCCTTGGTCAG-3' and 5'-GTCTGGCTTATATCCAACACTTCGTG-3' for Hypoxanthine phosphoribosyltransferase 1 (Hprt1); 5'-TCTGACACGTCTGCCTACCCATTCG-3' and 5'-TATGATGGTTCACTCACGGAGCTTCG-3' for TfR. The amplification product length was 83 bp for TfR and 103 bp for Hprt1. Real-time reverse transcription-PCR (RT-PCR) was performed on the MX4000 spectrofluorometric thermal cycler (Stratagene) using the SYBR Green Jumpstart™ Taq ReadyMix (Sigma-Aldrich) and 1 µl of cDNA. MgCl₂ and primer concentrations were 2.5 mM and 150 nM, respectively, for Hprt1, and 3.5 mM and 200 nM, respectively, for TfR. The amplification protocol consisted of a denaturation step at 95 °C for 4 minutes and 40 cycles of 95 °C for 30 seconds, 57 °C for 1 minute and 72 °C for 30 seconds. Serial dilutions of a cDNA sample containing the transcribed gene of interest were amplified to generate standard curves of

amplification for each target, which confirmed the linear relationship between the threshold cycle (Ct) and the log (RNA input). The quantity of TfR transcripts was estimated against the respective standard curve and normalised against the quantity of the endogenous control gene, *Hprt1*. The final gene expression value in each treated sample was subsequently determined as a ratio of the gene expression in the control sample. The amplification of a single product was verified by the existence of a single peak in the dissociation curve and further assessed by gel electrophoresis.

Measurement of intracellular chelatable iron

Relative changes in the intracellular LIP were measured using an adaptation of the method of Petrat *et al.* [29]. The method is based on quenching the fluorescence of the transition metal indicator PG SK by intracellular iron and its subsequent dequenching upon addition of a strong membrane-permeable iron chelator. After incubation with the appropriate treatment, cells grown in 96-well plates were washed with pre-warmed (37 °C) PBS and incubated with 10 µM PG SK diacetate in Earle's MEM without serum and without phenol-red for 10 minutes at 37 °C. Cells were then washed 3 times with pre-warmed MEM. Fluorescence of the metal indicator PG SK (excitation maximum 485 nm, emission maximum 520 nm) was measured just before and 15 minutes after the addition of the highly permeable chelator 2,2'-dipyridyl (2,2'-DPD, 5 mM) in a FLUOstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany) and expressed as arbitrary fluorescence units. The increase in fluorescence produced by the chelator is proportional to the size of the intracellular LIP. The size of the intracellular LIP under different treatments was compared using the equation below, where F1 and F2 stand for fluorescence before and after the addition of the chelator, respectively, as described by Darbari *et al.* [30]:

$$\text{Fractional increase in fluorescence } (\Delta F) = (F2 - F1)/F2$$

TfR immunoassay

HDFs were harvested in an enzyme-free dissociation solution (Invitrogen), centrifuged and resuspended in 100 μ l of diluent (PBS containing 1 % BSA and 0.1 % NaN₃), then incubated with 5 μ l of 200 μ g/ml mouse monoclonal anti-human CD71 (Sigma-Aldrich) or an equal concentration of isotype-matched, non-specific mouse immunoglobulin (Sigma-Aldrich) for 30 minutes. Cells were washed 3 times in diluent and then incubated with a 1:200 dilution of FITC-conjugated anti-mouse IgG goat polyclonal antibody (Abcam, Cambridge, UK) for 30 minutes. After 3 washes, cells were resuspended in diluent and kept on ice until ready to analyse. Analysis of cell-surface TfR was performed using a FACScan flow cytometer and the CellQuest software program (Becton Dickinson, Oxford, UK).

Analysis of cellular ferritin

HDFs were washed 3 times with ice-cold PBS and scraped from the culture plates in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, to which 1 mM phenylmethanesulfonyl fluoride and 1 % Igepal were added immediately prior to use). Cell lysates were centrifuged at $7500 \times g$ for 10 minutes at 4 °C and supernatants were stored at -80 °C until ready for analysis. Cellular ferritin was quantified using a commercially available ELISA (BioCheck, Burlingame, CA, USA) and corrected for total protein.

Cell viability assays

The loss of membrane integrity was routinely investigated to assess cell viability using the trypan blue dye exclusion assay. For assaying cytotoxicity, cells were harvested taking care to collect the supernatant, centrifuged and resuspended in PBS with 5 µg/ml propidium iodide. 10,000 cells were acquired in a FACScan flow cytometer and analysed using Cell Quest software (Becton Dickinson). Dead cells were identified in a FL2 *versus* FSC (forward light scatter) scatter plot by their inability to exclude propidium iodide and a decrease in cell size.

Statistical analysis

Data are expressed as mean values $\pm$ standard deviation for at least 3 independent samples. Statistical evaluation was performed using Minitab software (Minitab Ltd, Coventry, UK). Differences among multiple groups were compared by two-tailed one-way analysis of variance with post-hoc Tukey's test. Levels of cell-surface TfR were expressed as a ratio of the matched control and differences were compared by a two-tailed one-sample t-test. In all cases, statistical significance was assumed at $p < 0.05$.

Results

Intracellular accumulation of AA

HDFs accumulated AA intracellularly in a time-dependent manner (Fig. 1A). Cells incubated with AA2P, a vitamin C derivative that remains stable in culture media [31] and is hydrolysed by cellular enzymes to AA [32,33], have also accumulated AA intracellularly in a time- and dose-dependent manner (Fig. 1A and 1B).

AA modulation of DNA damage

DNA damage was measured using the alkaline comet assay. In the assay, cells are lysed and the nucleoid bodies electrophoresed under alkaline conditions (pH>13). Undamaged DNA remains supercoiled and hardly migrates during electrophoresis. However, the presence of DNA damage in the form of strand breaks or alkali-labile sites relaxes the supercoiled DNA and allows it to migrate during electrophoresis, leading to the formation of a 'tail' [34]. Cells were exposed to a physiologically relevant oxidant insult (H_2O_2) on ice to avoid enzymatic DNA strand breakage as a consequence of DNA repair or the activation of intracellular endonucleases, as well as any possible interference of peroxide degrading enzymes. As expected, H_2O_2 caused a clear increase in comet tail formation (Fig. 2A). To investigate the effect of intracellular AA on oxidative DNA damage, HDFs were pre-incubated with 100 μM AA for approximately 12 hours to allow for efficient intracellular accumulation of AA (Fig. 1A) and then exposed to 50 μM H_2O_2 . AA pre-incubation on its own did not cause any detectable DNA damage but it exacerbated H_2O_2 -induced DNA damage, as indicated by a clear increase in tail formation (Fig. 2A). Although these results were obtained with GM5399 HDFs, similar results were obtained with HDFs from a different healthy donor, GM5659, and with human breast epithelial cell lines HBL-100 and MCF-7 (data not shown).

To investigate the dose-dependence of the effect of AA on H_2O_2 -induced DNA damage, HDFs were pre-incubated with physiological to pharmacological concentrations of AA (20-500 μM) for 12 hours before exposure to H_2O_2 . Pre-incubation with AA or AA2P significantly enhanced the DNA damage caused by H_2O_2 in a dose-dependent manner (Fig. 2B). Notably, the exacerbating effect of AA was significant even when low, physiologically relevant extracellular concentrations of AA were employed (20-100 μM) and it was elicited by both AA and the stable vitamin C derivative AA2P. Whilst AA may have pro-oxidant properties when supplied to cells at high concentrations and at low

cell density, this is not the case with stable vitamin C derivatives such as AA2P [27,35]. These data suggest that the effect of AA on H₂O₂-induced DNA damage is independent from AA auto-oxidation in the extracellular medium.

H₂O₂ is poorly reactive and readily diffuses through the cell membrane. It is believed that H₂O₂ does not damage cells alone, but rather by reacting with intracellular transition metals to form much more damaging species such as the hydroxyl radical [1]. To investigate whether H₂O₂-induced DNA damage required the presence of intracellular catalytic iron, the 12 hours pre-incubation with AA was performed in the presence of a potent and specific ferric iron chelator, desferioxamine (DFO). Notably, DFO (300 µM) completely abolished H₂O₂-induced DNA damage in control and AA-loaded cells (Fig. 3A). To ensure that the effect of AA on H₂O₂-induced DNA damage was dependent on intracellular iron, rather than iron present in the medium, we performed the AA pre-incubation in the presence of an excess of *apo*-Tf (50 µg/ml). The exacerbating effect of AA on H₂O₂-induced DNA damage was not affected by the addition of exogenous *apo*-Tf (supplementary data). We could thus speculate that AA would augment H₂O₂-induced DNA damage by promoting intracellular Fenton chemistry. Experiments were therefore conducted to ascertain whether iron supplementation could further augment the exacerbating effect of AA on H₂O₂-induced DNA damage. HDFs were incubated with 185.5 µg/ml ferric ammonium citrate (FAC) for 18 hours, which promotes intracellular iron accumulation [36]. According to the information provided by the supplier, in our batch of FAC this corresponds to a concentration of Fe of 32.65 µg/ml. Cells were then thoroughly washed to remove traces of extracellular iron and incubated with 100 µM AA for 6 hours, before exposure to H₂O₂ (Fig. 3B). Without exposure to H₂O₂, none of the pre-treatments raise DNA damage above control levels. AA pre-incubation significantly increased the levels of DNA damage caused by H₂O₂. FAC on its own failed to affect

H₂O₂-induced DNA damage ($p > 0.05$), but it significantly augmented the exacerbating effect of AA ($p < 0.005$). These data support the possibility that AA exacerbates H₂O₂-induced DNA damage by promoting intracellular Fenton reactions.

We also investigated the effect of AA pre-incubation on oxidative DNA damage induced by radiation. HDFs were pre-incubated with 100 μ M AA or AA2P for 12 hours before exposure to 15 Gy X-rays. As expected, radiation caused a significant increase in tail formation (supplementary data). Radiation causes changes in the cells or tissues through which it passes by depositing energy and causing ionisation and excitation of the molecules with which it interacts. Damage to DNA occurs either by direct energy deposition in the DNA molecule or through indirect attack on the DNA molecule of hydroxyl radicals generated from water radiolysis [37]. Radiation-induced damage is therefore iron-independent, which was confirmed by the fact that it could not be prevented by pre-incubating cells with a concentration of DFO that efficiently abolished H₂O₂-induced DNA damage (data not shown). In our experiments, cells that were pre-incubated with AA or AA2P before exposure to radiation had similar levels of DNA damage to their non pre-incubated counterparts ($p > 0.05$) (supplementary data). These observations further support the view that AA promotes intracellular ROS-mediated, iron-dependent oxidative reactions.

AA modulation of cell viability

The viability of HDFs incubated with AA, AA2P, DFO or FAC at the concentrations used in the present study for 24 hours was higher than 95 %, as judged by their ability to exclude trypan blue, which shows that none of the individual treatments was cytotoxic. The effect of AA on H₂O₂-induced cytotoxicity was investigated by pre-incubating HDFs with AA before exposure to lethal or sub-lethal doses of H₂O₂ at 37 °C.

In our experimental conditions, a sub-lethal treatment consisted of 100 μM H_2O_2 for 1 hour and a lethal treatment was achieved with 200 μM H_2O_2 for 1 hour. Confluent HDFs exposed to sub-lethal oxidative stress are known to remain viable and to continue to synthesise proteins through a long-term incubation. However, cells enter a senescence-like growth arrest state in which they are unable to synthesise DNA and to re-enter the cell cycle in response to growth factors [38]. This was evident in our work by the fact that when HDFs were treated just below confluence, H_2O_2 -treated HDFs, unlike control cells, did not reach confluence within 24-48 hours in culture (data not shown). The treatment also caused a dramatic increase in the expression of p21 and Gadd45 α mRNAs and a significant G₀/G₁ cell cycle arrest (supplementary data). Despite this, H_2O_2 -treated HDFs remained viable for up to 6 days post- H_2O_2 (7.6 % dead cells; $p > 0.05$ *versus* control cells) (Fig. 4A). HDFs that were pre-incubated with 100 μM AA for 4 hours, however, were significantly sensitised to H_2O_2 (30 % dead cells; $p < 0.001$ *versus* H_2O_2 -treated cells without AA pre-incubation), as judged by the cell inability to exclude the fluorescent dye propidium iodide and by a reduction in cell size. Notably, enhanced cell death was prevented when HDFs were incubated with the iron chelator DFO for 14 hours before the 4-hour incubation with AA (6.8 % dead cells; $p > 0.05$ *versus* control cells).

AA also increased cell death caused by exposure of HDFs to a lethal oxidative challenge (82.5 % dead cells *versus* 50 % in H_2O_2 -treated cells without AA pre-incubation; $p < 0.001$) (Fig. 4B). In this case, extensive cell death was observed within 20-24 hours, with dead cells remaining attached and showing marked membrane blebs, presumably corresponding to necrotic cell death [39]. Again, H_2O_2 -induced cell death was abolished when cells were loaded with the iron-specific chelating agent DFO for 14 hours ($p > 0.05$ *versus* untreated cells).

Effect of AA on intracellular chelatable iron

The experiments described above showed that AA enhanced H₂O₂-induced DNA damage and cell death by an iron-dependent process. Whilst the majority of the intracellular iron is safely bound to storage, functional or transport proteins, a small pool of cellular iron is loosely bound to low molecular weight organic chelators and this is thought to be available for participating in the formation of highly reactive oxidant species [40]. It is thus possible to speculate that AA may promote Fenton-type oxidative damage by increasing the availability of intracellular catalytic iron. We measured relative changes in the intracellular LIP using the method of Petrat *et al.* [29] with modifications. The method is based on quenching the fluorescence of the transition metal indicator PG SK by intracellular iron and its subsequent dequenching upon addition of a strong membrane-permeable iron chelator, 2,2'-DPD. PG SK is capable of removing iron that is complexed with organic cellular iron chelators (citrate, phosphate, ATP) [41]. As depicted in Fig. 5, PG SK-loaded control cells responded to the addition of 2,2'-DPD with a discrete increase in fluorescence signal (Δ fluorescence) due to the release of iron from PG SK, which shows the existence of a detectable LIP. As expected, intracellular iron chelation with DFO reduced PG SK Δ fluorescence significantly ($p < 0.001$ *versus* control), whereas incubation with FAC significantly increased it ($p < 0.001$ *versus* control). Notably, vitamin C supplied as either AA or AA2P (100 μ M) for 12 hours caused significant increases in intracellular PG SK-chelatable iron, as illustrated by an increase in Δ fluorescence ($p < 0.001$ *versus* control).

Effects of AA on iron-regulated gene expression

Mammalian cells control intracellular iron levels tightly through the levels of proteins involved in iron uptake and storage. Ferritin is the major intracellular iron

storage protein [42], whereas the TfRs expressed at the cell surface are crucial for Tf binding and iron uptake [43]. When iron is in excess, ferritin synthesis is rapidly induced and the TfR mRNA is degraded, reducing the number of receptors in the cell-surface. So, if AA is capable of expanding the cellular LIP, we would expect to observe the corresponding effects in the expression of these two iron-regulated genes. Here we have investigated the effects of AA on ferritin and TfR expression and compared them with those of two well-established modulators of the intracellular LIP and of iron-regulated gene expression, DFO and FAC [44].

As described, regulation of TfR is determined by mRNA stability. IRPs bind to IREs when iron is insufficient and increase the half-life of TfR mRNA, whereas excessive iron has the opposite effect [45]. We measured the steady-state levels of the TfR mRNA by real-time quantitative PCR. Primers were designed for the specific amplification of a short fragment in the 3'-UTR of the TfR mRNA. The effects of iron chelation or loading on TfR mRNA expression in HDFs were assessed by incubating nearly confluent cells with DFO or FAC, respectively. As expected, HDFs responded to iron overload by decreasing the levels of TfR mRNA substantially (4-fold down-regulation, $p < 0.001$) (Fig. 6A). Conversely, iron chelation with 300 μ M DFO for 12 hours doubled the steady-state levels of TfR mRNA ($p < 0.001$). This agrees with the current understanding of the regulation of the TfR expression by the IRE/IRP system in HDFs [46,47]. Notably, incubation of HDFs with 100 μ M AA or AA2P for 12 hours caused a 2-fold down-regulation of the TfR ($p < 0.001$; Fig. 6A). The effect of AA was time-dependent, with the levels of TfR mRNA decreasing gradually during the first 6 hours of incubation and remaining significantly down-regulated until at least 24 hours from the addition of 100 μ M AA or AA2P (Fig. 6B).

The fraction of TfR expressed at the cell surface is crucial for Tf binding. Consequently, the effects of AA on the expression of functional TfR on the cell surface were also investigated. HDFs were incubated with AA, AA2P, DFO, FAC or growth medium alone for about 18 hours. Expression of the TfR at the cell-surface was measured by a flow cytometric immunoassay (Fig. 6C). As expected [46], HDFs expressed TfR on the cell-surface and iron chelation with 300 μ M DFO nearly doubled the expression of the receptor (90.9 ± 9.9 % increase, $p = 0.004$ *versus* control, $N = 3$). In contrast, iron overloading with 32.65 μ g/ml Fe supplied as FAC significantly reduced the levels of TfR by about one third (32.2 ± 9.8 % reduction, $p = 0.031$ *versus* control, $N = 3$). Notably, 100 μ M AA or AA2P significantly reduced cell-surface TfRs to a similar extent as FAC (35.7 ± 4.5 % reduction, $p = 0.006$, and 32.5 ± 3.4 % reduction, $p = 0.003$ *versus* control, respectively, $N = 3$).

The levels of cellular ferritin were measured using a commercial ELISA. HDFs responded to iron overload (FAC for 16 hours) with a dramatic increase in ferritin levels, whereas the chelation of intracellular iron pools with DFO reduced the levels of ferritin by half (Fig. 7A). Notably, incubation of HDFs with 100 μ M AA or AA2P for 16 hours increased ferritin steady-state levels by approximately 4-fold ($p < 0.001$). When HDFs were incubated with AA or AA2P for different periods of time, a time-dependent increase in ferritin steady-state levels was observed, which peaked at 6-12 hours (approximately 4-fold increase) (Fig. 7B). To further investigate the effect of AA on ferritin protein levels, HDFs were incubated with AA for 16 hours in the presence or absence of protein synthesis and transcription inhibitors (Fig. 7C). The stimulatory effect of AA on ferritin expression was inhibited by co-incubation with the protein synthesis inhibitor cycloheximide. This observation indicates that AA increases the steady-state levels of ferritin by increasing protein synthesis, rather than by inhibiting its degradation.

HDFs incubated with the transcription inhibitor actinomycin D had increased baseline levels of ferritin, as previously described [48]. Nevertheless, actinomycin D did not abrogate the stimulatory effect of AA on ferritin steady-state levels (3.9-fold increase), which indicates that vitamin C increases ferritin synthesis by a post-transcriptional mechanism such as the IRE/IRP system.

Discussion

Using the alkaline comet assay, we have shown that AA exacerbated H_2O_2 -induced DNA strand breakage in HDFs. The induction of DNA strand breaks by exposure of human cells to H_2O_2 corresponds to a mechanism of oxidative DNA damage [49] and is well correlated with multiple DNA base lesions [50], so we would expect AA to enhance the formation of these lesions too. Other authors have recently reported an enhancing effect of AA on H_2O_2 -induced formation of 5-hydroxy-2'-deoxycytidine, a product of hydroxyl radical-induced oxidation of 2'-deoxycytidine [51]. As in the present work, the effect was enhanced when cells were pre-incubated with FeSO_4 and diminished by pre-incubation with DFO, suggesting that the lesion could be formed by Fenton-type reactions. The work of Riviere *et al.* [51] with Jurkat cells and our work with HDFs and human breast epithelial cell lines indicate that AA sensitises different human cell types for H_2O_2 -induced DNA damage. Whilst DNA strand breaks and 5-hydroxy-2'-deoxycytidine are not mutagenic lesions, we can speculate that, in the presence of H_2O_2 , AA would equally favour the formation of mutagenic lesions such as 8-hydroxyguanine.

Our data suggest that AA exacerbates H_2O_2 -induced DNA damage and cell injury by increasing the availability of catalytically active iron. Notably, we have observed an increase in the LIP in the absence of added exogenous iron, which suggests that AA may mobilize endogenous iron by reducing ferric to ferrous iron and assisting in its diffusion

from sites of iron storage, such as cellular ferritin, into the LIP [52]. The changes in the expression of ferritin and TfR reported herein further suggest that AA affects iron homeostasis in human cells by increasing the levels of intracellular bioavailable iron. Our data are in agreement with the observation that guinea pigs fed an AA-free diet have low serum iron levels, as well as reduced ferritin protein levels and increased hepatic TfR mRNA in the liver [53]. Our hypothesis is also supported by the scarce data available from human trials. Khumalo *et al.* [54] reported that oral AA supplements caused a small but highly significant decrease in serum TfR concentration in individuals from rural African families with a history of traditional, high-iron content beer consumption. A small but highly significant decrease in serum TfR was also observed in chronic haemodialysis patients given intravenous AA supplementation [55]. It is thus possible that AA may affect intracellular iron metabolism *in vivo*.

Potential mechanisms of AA-mediated oxidative damage

Different mechanisms have been described by which iron may enhance oxidative cell damage. A possible mechanism involves diffusion of H₂O₂ into the nucleus, where it would react with metal ions bound to DNA, generating highly reactive hydroxyl radicals. These radicals are very short-lived, so they would attack the DNA molecule at the vicinity of the site of formation, causing site-specific damage [56]. AA could aggravate site-specific oxidative damage by recycling the metal catalyst, which would undergo repeated cycles of reduction and oxidation.

Alternatively, AA could enhance oxidative damage by facilitating iron mobilization from the sites of sequestration within the cell. DFO protects cells from H₂O₂-induced DNA damage [57,58] and cytotoxicity [59] by chelating iron within endosomal vesicles [60,61], it was hypothesised that these compartments contain the

pool of cellular redox-active labile iron required for H₂O₂-induced cell damage. In fact, iron is released inside endosomes following receptor-mediated uptake of Tf-TfR complexes and inside lysosomes as a consequence of ferritin autophagy. The acidic conditions and the reducing environment of these compartments would favour the maintenance of at least a proportion of the iron in the ferrous state. Under oxidative stress conditions this labile ferrous iron may cause lipid peroxidation [62], destabilising the lysosomal membrane and diffusing into the nucleus, where it can take part in Fenton reactions. Whilst DFO could protect cells against H₂O₂-induced cell damage by keeping lysosomal iron in a non-reactive form [58,63], it is possible that AA, at low pH, would promote endosomal/lysosomal membrane destabilization and the concomitant release of low molecular weight iron chelates into the cytosol.

Potential role of AA in oxidative cell injury in vivo

A study by Anderson *et al.* [64] has previously shown that AA oral supplementation significantly increased chromosome aberrations induced by *ex vivo* bleomycin challenge of lymphocytes. As the ability of bleomycin to damage DNA depends on the presence of certain transition metal ions (like iron) within the DNA-bleomycin complex [65], it was suggested that AA could enhance DNA damage by modulating the iron status *in vivo*. However, the relevance of the pro-oxidant effect of AA *in vivo* remains elusive [reviewed by 17].

In the current work we show that AA affects cellular iron homeostasis in HDFs and may accelerate oxidative damage caused by exogenous sources of ROS. Large amounts of superoxide and H₂O₂ are produced at sites of inflammation [1] and iron is also released during inflammation, possibly due to the ability of superoxide to mobilise Fe²⁺ from ferritin [66]. By increasing the availability of catalytic iron, AA may

exacerbate the formation of hydroxyl radical in inflammatory states. This hypothesis is supported by a human supplementation study where AA augmented the levels of bleomycin-detectable iron in the serum and the levels of lipid hydroperoxide during induced acute inflammation [67]. Ultimately, AA may accelerate the iron-dependent, oxidative stress-mediated destruction of normal tissues, a deleterious consequence of inflammatory reactions commonly observed in rheumatoid arthritis and other chronic inflammatory conditions [68,69]. So, whilst AA supplements are currently amongst the most popular vitamin and mineral supplements, our findings suggest that AA supplementation would likely be more detrimental than beneficial in pathological conditions associated with iron overload and inflammation.

It is important to remember, however, that this study compared the effects of ascorbate depletion vs. repletion, which may be an extreme example as the former is rarely encountered in humans. Likewise, it is worth noting that, whilst the present study shows that AA modulates iron homeostasis in HDFs, it is not clear whether this is simply explained by an increase in the cellular LIP and/or the stimulation of Fenton reactions, or whether other factors are involved. The possibility that AA may affect iron homeostasis (e.g. expression of iron-regulatory genes) independently of the intracellular LIP should be further investigated.

Summary

This study demonstrated that AA alters cellular iron homeostasis. We have shown that AA expands the intracellular catalytic iron pool of HDFs, which is further supported by the observed concomitant increase in cellular ferritin and down-regulation of the TfR. Therefore, we propose that, under normal conditions, AA increases iron bioavailability. Notably, AA pre-incubation enhanced iron-dependent DNA damage formation and cell

killing when HDFs were exposed to a physiologically relevant oxidant insult, H₂O₂. We have shown that, under oxidative stress, AA promotes intracellular Fenton-type free radical formation reactions. AA could enhance iron mobilisation from the sites of sequestration within the cell into the cytosol and nucleus, thereby enhancing oxidative damage. In addition, AA could enhance H₂O₂-induced oxidative damage by reducing DNA-bound iron, thus favouring site-specific attack on DNA. We propose that AA may promote normal tissue injury in situations associated with elevated ROS production, such as during chronic inflammation.

Our findings help elucidating the mechanisms by which the vitamin influences iron availability in human cells. Furthermore, the data show that AA promotes Fenton reactions in human cells and suggest that the vitamin has the potential to exacerbate the deleterious effects of metals *in vivo*.

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Abbreviations: 2,2'-DPD, 2,2'-dipyridyl; AA, ascorbic acid; AA2P, ascorbic acid 2-phosphate; DFO, desferrioxamine; FBS, foetal bovine serum; HDF, human diploid fibroblast; IRE, iron-responsive element; IRP, iron-regulatory protein; LIP, labile iron

pool; MEM, minimal essential medium; NTBI, non-transferrin-bound iron; ROS, reactive oxygen species; Tf, transferrin; TfR, transferrin receptor.

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Fig. 1. Intracellular accumulation of AA in HDFs. Intracellular AA concentration was determined by HPLC-EC and normalized for total protein. A, HDFs were incubated with 100 μ M AA (•) or AA2P (□) for 0, 2, 6, 12 or 24 hours. Results are the mean $\pm$ S.D. from 3 separate determinations. B, HDFs were incubated with 0, 20, 100 or 500 μ M AA2P for 12 hours. Results are the mean $\pm$ S.D. from 3 separate determinations.

Fig. 2. Effect of AA pre-incubation on H₂O₂-induced DNA damage in HDFs. A, HDFs were pre-incubated with 100 μ M AA (right panels) or growth medium alone (left panels) for 12 hours. Subsequently, cells were exposed to 50 μ M H₂O₂ (bottom panels) or medium alone (top panels) and subjected to alkaline comet assay analysis as described in Materials and Methods. B, HDFs were pre-incubated with different concentrations of either AA or AA2P for 12 hours before exposure to 50 μ M H₂O₂. DNA damage was determined with the alkaline comet assay. Results are the mean $\pm$ S.D. from 3 separate determinations. * $p < 0.05$; *** $p < 0.001$ versus H₂O₂ treatment without AA or AA2P pre-incubation.

Fig. 3. Effect of iron chelation or supplementation on H₂O₂-induced DNA damage in HDFs. DNA damage was determined with the alkaline comet assay. A, Cells were incubated with 100 μ M AA or medium for 12 hours in the presence or absence of DFO before exposure to 50 μ M H₂O₂. Results are the mean $\pm$ S.D. from 3 separate determinations. ** $p = 0.01$ versus H₂O₂ treatment without AA pre-incubation. B, HDFs were incubated with 32.65 μ g/ml Fe supplied as ferric ammonium citrate (FAC) for 18 hours. Cells were then thoroughly washed to remove traces of extracellular iron and incubated with 100 μ M AA for 6 hours, before exposure to 50 μ M H₂O₂. Results are the mean $\pm$ S.D. from 3 separate determinations. ##, $p < 0.005$ versus AA pre-incubated,

H₂O₂-treated cells; ***, $p < 0.001$ *versus* H₂O₂ treatment without pre-incubation; +++, $p < 0.001$ *versus* FAC pre-incubated, H₂O₂-treated cells.

Fig. 4. Effect of AA on H₂O₂-induced cell death. Dead cells were identified by their inability to exclude the fluorescent dye propidium iodide (x axis) and by a reduction in cell size (y axis). A, HDFs were either left untreated or exposed to 100 μ M H₂O₂ in low-serum medium at 37 °C for 1 hour. In some cases, cells were pre-incubated with AA for 4 hours before exposure to H₂O₂ or loaded with 300 μ M DFO for 14 hours before the 4-hour incubation with AA. Cells were washed 3 times with PBS between treatments. Cell viability was estimated at 6 days post-H₂O₂. Results are the mean $\pm$ S.D. from 4 separate determinations. ***, $p < 0.001$ *versus* H₂O₂-treated cells without AA or DFO pre-incubation; NS¹, $p > 0.05$ *versus* control cells; NS², $P > 0.05$ *versus* H₂O₂-treated cells without AA or DFO pre-incubation. B, HDFs were either left untreated or exposed to 200 μ M H₂O₂ in low-serum medium at 37 °C for 1 hour. In some cases, cells were pre-incubated with AA for 4 hours before exposure to H₂O₂ or loaded with 300 μ M DFO for 14 hours before the 4-hour incubation with AA or for 18 hours before exposure to H₂O₂. Cells were washed 3 times with PBS between treatments. Cell viability was estimated at 20-24 hours post-H₂O₂. Results are the mean $\pm$ S.D. from 4 to 11 separate determinations. ***, $p < 0.001$ *versus* H₂O₂-treated cells without AA pre-incubation; NS, $p > 0.05$ *versus* control cells.

Fig. 5. Effect of AA on the intracellular chelatable iron pool of HDFs. Cells were incubated in 96-well plates with either 300 μ M DFO or 100 μ M AA or AA2P for 12 hours, or with 32.65 μ g/ml Fe supplied as FAC for 15 hours. Cells were then incubated with 10 μ M PG SK diacetate for 10 minutes at 37 °C. Fluorescence was measured just

before and 15 minutes after the addition of the permeable chelator 2,2'-DPD, as described in Materials and Methods. The increase in fluorescence produced by the chelator (Δ fluorescence) is proportional to the size of the intracellular LIP. Results are the mean $\pm$ S.D. of multiple replicate wells pooled from 4 independent experiments. ***, $p < 0.001$ *versus* control.

Fig. 6. Effect of AA on TfR mRNA and protein expression in HDFs. A, HDFs were incubated with either 32.65 μ g/ml Fe supplied as FAC, 300 μ M DFO, 100 μ M AA or AA2P or medium alone for 12 hours. Total RNA was extracted and the steady-state levels of the TfR mRNA were measured by real-time quantitative PCR. Results are the mean $\pm$ S.D. from 6 separate determinations. ***, $p < 0.001$ *versus* control. B, HDFs were incubated with 100 μ M AA or AA2P or medium alone for 2, 6, 12 and 24 hours. Total RNA was extracted and the steady-state levels of the TfR mRNA were measured by real-time quantitative PCR. Results are the mean $\pm$ S.D. from 3 separate determinations. ***, $p < 0.001$ *versus* the time-matched control (C). C, HDFs were incubated with 100 μ M AA or growth medium alone (control) for 18 hours. HDFs were then sequentially incubated with monoclonal anti-human CD71 and FITC-anti-mouse IgG antibodies. The amount of TfR antigenic sites on the cell surface was expressed as arbitrary FL1 fluorescence units. Results are representative of 3 separate determinations.

Fig. 7. Effect of AA on cellular ferritin. Cellular ferritin levels were determined with a commercially available ELISA and normalised for total protein. A, HDFs were incubated with 32.65 μ g/ml Fe supplied as FAC, 300 μ M DFO, 100 μ M AA or AA2P, or growth medium alone (control) for 16 hours. Results are the mean $\pm$ S.D. from 3 to 6 separate determinations. ***, $p < 0.001$ *versus* control; NS, not significant *versus* control. B,

HDFs were incubated with 100 μ M AA (white bars) or AA2P (grey bars) for 0, 2, 6, 12 or 24 hours. Results are the mean $\pm$ S.D. from 3 separate determinations. ***, $p < 0.001$ *versus* control; NS, not significant *versus* control. C, HDFs were incubated with 100 μ M AA for 16 hours in the presence or absence of 3 μ g/ml cycloheximide (CHX) or 1 μ g/ml actinomycin D (ActD). Results are the mean $\pm$ S.D. from 3 separate determinations. ***, $p < 0.001$ *versus* control; NS, not significant *versus* control.

Figure1

Figure 1

Figure 2

Figure 3

Figure4

Figure 4

A.

B.

Figure 5

Figure 6

A.

B.

C.

Figure7

Figure 7

A.

B.

C.

Supplementary Material

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