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Can vitamin C induce nucleotide excision repair? Support from *in vitro* evidence

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Intracellular vitamin C acts to protect cells against oxidative stress by intercepting reactive oxygen species (ROS) and minimising DNA damage. However, rapid increases in intracellular vitamin C may induce ROS with subsequent DNA damage priming DNA repair processes. Herein, we examine the potential of vitamin C and the derivative ascorbate-2-phosphate (2-AP) to induce a nucleotide excision repair (NER) response to DNA damage in a model of peripheral blood mononuclear cells. Exposure of cells to elevated levels of vitamin C induced ROS activity, resulting in increased levels of deoxycytidine glyoxal (gdC) and 8-oxo-2′-deoxyguanosine (8-oxodG) adducts in DNA; a stress response was also induced by 2-AP, but was delayed in comparison to vitamin C. Evidence of gdC repair was also apparent. Measurement of cyclobutane thymine–thymine dimers (T < >T) in DNA and culture supernatant were included as a positive marker for NER activity; this was evidenced by a reduction in DNA and increases in culture supernatant levels of T < >T for vitamin C-treated cells. Genomics analysis fully supported these findings confirming that 2-AP, in particular, induced genes associated with stress response, cell cycle arrest, DNA repair and apoptosis, and additionally provided evidence for the involvement of vitamin C in the mobilisation of intracellular catalytic Fe.

Vitamin C: DNA damage: Genomics: Nucleotide excision repair

Vitamin C is regarded as the most important water-soluble antioxidant in human plasma and mammalian cells, which has mechanisms to recycle and accumulate the vitamin against a concentration gradient. This strongly suggests that the vitamin might have important intracellular functions, and there is increasing evidence to suggest vitamin C as regulator of reduction and oxidation (redox) reactions in the cell[1].

At physiological concentrations (60–100 μmol/l), vitamin C acts as an antioxidant, scavenging free radicals in plasma and preventing oxidative damage to cells from reactive oxygen species (ROS)[2]. However, at much higher pharmacological concentrations (0·3–20 mmol/l), vitamin C exhibits pro-oxidant activity, leading to oxidative damage to DNA; this is dependent on the presence of transition metals such as Cu and Fe through which hydroxyl radicals are formed[3]. The pro-oxidant properties of vitamin C, potentially via cellular modification or cell signalling by ROS or reactive carbonyl species (RCS), may modulate gene expression. Indeed, vitamin C has been shown to modulate gene expression of *in vitro* AP-1 and fra-1[4], Raf-1 and extracellular signal-regulated kinase pathway[5] and p73 and Mut L homologue-1[6], and *in vivo* hOOG1 in chronic haemodialysis subjects[7] and intracellular adhesion molecule-1 (ICAM-1)[8] in healthy subjects. Several studies have also shown a pro-oxidant action for vitamin C through its induction of cell cycle arrest[9,10], apoptosis via ROS signalling[11] and sister chromatid exchanges[12]. There appears to be a conflict between the antioxidant, transient pro-oxidant activity via ROS/RCS and irreversible pro-oxidant action of vitamin C. The effect of supplemented vitamin C, whether at a physiological level or *in vitro*, on the above may also be dependent on both vitamin C dose and the redox status of the target cell.

The bioactive nature of vitamin C has been very much redefined in recent years in a number of *in vitro* studies. Vitamin C generally undergoes two-by-one electron oxidation to dehydroascorbate[13]. Reports on the effect of vitamin C *in vitro* show it to have dose-dependent damaging end points, especially to DNA. ROS have been implicated as the modifying elements following incubation of native DNA with vitamin C[14]. This is thought to arise principally from superoxide anion and hydroxyl radicals via metal-dependent reactions involving hydrogen peroxide. Indirect products of ROS activity act upon DNA, and vitamin C can generate RCS such as glyoxal and malondialdehyde[15–17], which can adduct to DNA and proteins potentially affecting DNA synthesis and cellular function. ROS and RCS are also important

Abbreviations: 2-AP, ascorbate-2-phosphate; CCRF-HSB-2, cultured human acute lymphoblastic leukaemic cell line; gdC, deoxycytidine glyoxal; 8-oxodG, 8-oxo-2′-deoxyguanosine; NER, nucleotide excision repair; RCS, reactive carbonyl species; ROS, reactive oxygen species; SVCT, sodium-dependent transporters.

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cell signalling molecules generated via phosphorylation of c-Jun N-terminal kinase, p38 mitogen-activated protein kinase and c-Jun[18–20].

The antioxidant or reducing properties and acidic nature of vitamin C arise from its two enolic hydrogen atoms, which derivatives of ascorbate such as ascorbate-2-phosphate (2-AP) do not possess. Although of no physiological relevance, 2-AP is able to increase intracellular vitamin C levels and affect gene expression. However, it does not undergo oxidation to DHA and therefore acts as an appropriate control for vitamin C because it will enter the cell without being oxidised first and thus more accurately represents normal uptake of vitamin C.

Induction of repair processes by vitamin C *in vivo* may arise from changes in the redox environment of the cell; for instance, the cellular GSH:GSSG and/or dehydroascorbic acid: ascorbic acid ratio. Furthermore, the mechanism of vitamin C transport into the cell may influence the redox status, as indicated in Fig. 1. Dietary vitamin C can be transported into the cell via sodium-dependent transporters (SVCT)[21]. SVCT1 has a higher affinity, but lower transport capacity for vitamin C than SVCT2. In human subjects, SVCT1 is expressed predominantly in epithelial cells, including intestine, kidney and liver, and has been called the 'bulk' transporter of vitamin C. In contrast, SVCT2 is found in metabolically active and specialised cells such as those in the brain, eye and placenta, and has been proposed to maintain intracellular levels of vitamin C vital for neuronal function[22]. The oxidised product of vitamin C, DHA, is transported into the cell via GLUT, more specifically GLUT 1 and GLUT 3[23]. Thioredoxin reductase[24] and glutaredoxin[25] can reduce DHA back to ascorbate by a process that has been shown to be GSH-dependent and reported to induce ROS formation[26,27]. ROS production is inherent to aerobic metabolism. It is becoming increasingly clear that ROS are an integral part of cellular function and defence. ROS are important cell-signalling molecules and may reflect the redox environment of the cell. Vitamin C and glutathione essentially cooperate to maintain a stable redox environment[28,29]. It is feasible to assume that cells that intrinsically possess low vitamin C, such as cultured cells[30], may benefit from supplementation. However, increasing intracellular vitamin C may in effect cause, through Fenton chemistry, the production of free radical species with consequent oxidative stress that may be transient or otherwise[31,32].

Herein, we examine the potential of vitamin C and the derivative 2-AP to induce a nucleotide excision repair (NER) -adaptive response to DNA damage in a cultured human acute lymphoblastic leukaemic cell line (CCRF-HSB-2). This cell line is a model for peripheral blood mononuclear cells that cannot be maintained in long-term culture. NER removes bulky adducts in DNA, producing a short segment of single-stranded DNA that includes the adduct. We postulate that a rapid accumulation of intracellular vitamin C, but not 2-AP, by cultured (vitamin C-depleted) cells may expose them to increased oxidative stress and, furthermore, that the resulting transient pro-oxidant effect in elevating ROS and subsequent deoxycytidine glyoxal adduct (gdC) and 8-oxo-2′-deoxyguanosine (8-oxodG) DNA adduct levels 'primes' NER processes.

Experimental methods

Unless stated otherwise, all materials were purchased form Sigma-Aldrich Company Ltd (Gillingham, Dorset, UK) or Fisher Scientific (Loughborough, UK).

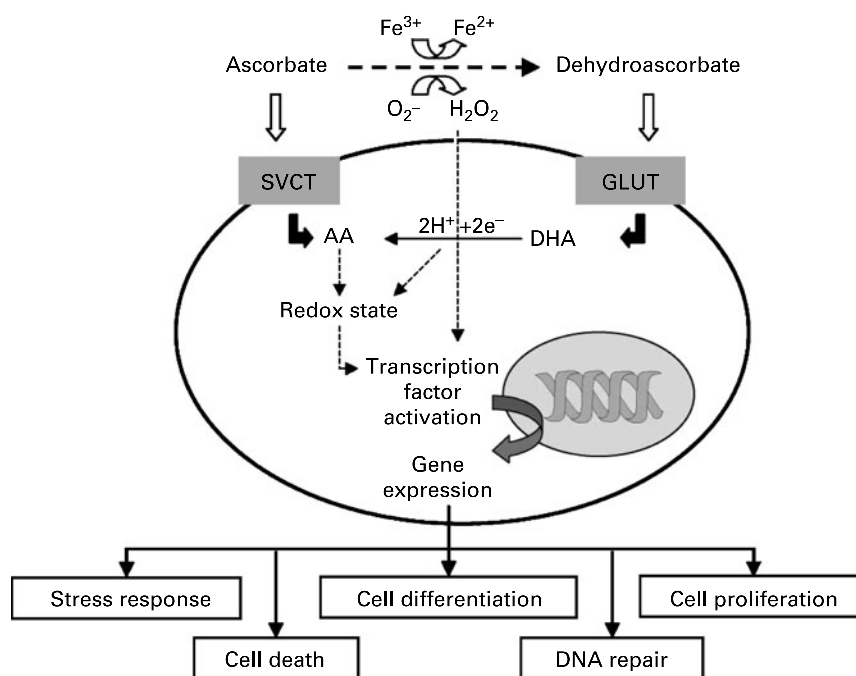

Fig. 1. Proposed mechanism of action of vitamin C in mammalian cells (from Duarte & Lunec[1]). SVCT, sodium-dependent vitamin C transporter; AA, ascorbic acid; DHA, dehydroascorbic acid.

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Cell culture

CCRF-HSB-2 cell lines (T-lymphoblastoid line obtained from the peripheral blood of an 11-year-old Caucasian male with acute lymphoblastoid leukaemia, which has been passaged eight times through newborn Syrian Hamsters) were purchased from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK). Complete medium consisted of RPMI-1640 (without additional glutamine) containing 200 mmol/l glutamax (L-alanyl-L-glutamine) and 10 % fetal calf serum. Cells were grown to confluence, 5×10^5 cells/ml and split to a final concentration of 3×10^5 cells/ml. Cells were maintained at 37°C in 5 % CO_2 incubator.

Cell viability was assessed using trypan blue as follows: CCRF-HSB-2 cells (10 μl suspended cells) were incubated with an equal volume of trypan blue solution (20 mg/10 ml) for 15 min. A cell count was performed using a haemocytometer, and the percentage of trypan blue-stained cells (% dead) was calculated from the total cell count.

Incubation of cultured human acute lymphoblastic leukaemic cell line cells with vitamin C or ascorbate-2-phosphate

Before treatment, CCRF-HSB-2 cells were serum-starved for 48 h in growth medium containing 2 % fetal calf serum, causing cell cycle arrest in order to synchronise growth phase. This was a necessary step for evaluating gene expression. Medium was then replenished with fresh medium containing 10 % fetal calf serum and 150 μmol/l (final concentration) vitamin C or 2-AP, each flask containing approximately 15×10^6 cells in 30 ml media. Cells were incubated for 0, 2, 8 or 24 h. Cells replenished with normal growth medium without added vitamin C were used as control. Following incubation, CCRF-HSB-2 cells were harvested by centrifugation at room temperature for 5 min at 1500 ***g***, and cells and supernatant collected for subsequent vitamin C, DNA damage and genomics analysis as described later.

Determination of supernatant and intracellular vitamin C concentration by HPLC

Following harvesting, CCRF-HSB-2 cells and cell culture supernatant were immediately mixed with an equal volume of 10 % (v/v) metaphosphoric acid and stored at −80°C for determination of vitamin C concentration. Samples were separated on a Luna 5 μm C18 (2) HPLC column (150 × 4·60 mm; Phenomenex, UK) with eluent comprising 15 mmol/l sodium phosphate buffer containing 9 % methanol (pH 6·0). Detection was achieved using UV detection at 254 nm. Linearity of standards was achieved for concentrations up to 100 μmol/l vitamin C with sample intra- and inter-assay CV % of <10 %. Supernatant and intracellular vitamin C concentration was calculated from a known standard curve; supernatant concentrations were expressed as μmol/l and intracellular concentrations in terms of sample protein content (μmol/mg protein) as a reference value.

DNA extraction

Following harvesting of CCRF-HSB-2 cells, DNA was extracted using the European Standards Committee on Oxidative DNA Damage recommended procedure[33].

Assessment of DNA damage by ELISA

Competitive ELISA was performed to assess DNA damage in CCRF-HSB-2 cells exposed to vitamin C or 2-AP. The method used was as follows, with all standards and samples being assayed in triplicate: 50 μl/well of double- or single-stranded calf-thymus DNA (50 μg/ml) were bound to a 96-well ELISA plate by incubation in a humidified environment at 37°C for 1 h. The plate was washed thrice with PBS (0·01 mol/l, pH 7·4), and excess binding sites were blocked by the addition of 150 μl/well 4 % (w/v) dried skimmed milk in PBS (4 % milk in PBS) for 1 h at 37°C in a humidified environment. The plate was again washed with PBS as earlier. For DNA and cell culture supernatant analysis, antigen-specific primary antibody (25 μl) was mixed with CCRF-HSB-2 DNA (25 μl of 50 μg/ml solution) or cell supernatant (25 μl) and incubated in the wells of the ELISA plate for 1 h at 37°C. The plate was again washed thrice with PBS containing 0·05 % (v/v) Tween 20 (Sigma), and 50 μl/well of secondary antibody conjugate (peroxidase-labelled anti-mouse immunoglobulins at a 1:2000 dilution in 4 % milk in PBS) were applied. Following incubation for 1 h at 37°C, the plate was washed thrice with PBS/Tween 20, and 50 μl/well of substrate solution (0·5 mg/ml *ortho*-phenylenediamine in 0·05 mol/l phosphate–citrate, pH 5·0, containing 0·03 % w/v sodium perborate) were added. The plate was incubated for 15 min at room temperature and the reaction was stopped using 25 μl/well 3 mol/l H_2SO_4. The resulting absorbance was read at 492 nm using an ELISA plate reader.

Deoxycytidine glyoxal adducts. Analysis was performed as described earlier, utilising a primary ‘in-house’ monoclonal antibody (F3/9) against the deoxycytidine adduct, purified for IgM antibodies from cell supernatants[34,35]. The purified antibody (376 μg/ml) was used at a final dilution of 1:500 in 4 % milk/PBS, equivalent to 0·75 μg/ml. Glyoxal-modified dsDNA was used as the solid-phase antigen (50 μg/ml) and standards composed of oligomers (20-mer) of deoxycytidine (produced in-house), which had been treated with glyoxal. DNA damage was measured as x μg/ml oligomer gdC and expressed as a fold change relative to untreated control samples following subtraction of baseline values.

8-Oxo-2′-deoxyguanosine adducts. Analysis was performed as described previously, utilising a commercially available monoclonal antibody against 8-oxodG (antibody N45.1, Japan Institute for the Control of Aging, Shizuoka, Japan). Analysis of 8-oxodG in cell culture supernatants was used as a measure of DNA repair, with single-stranded methylene blue/white light-modified DNA utilised as the solid-phase antigen with primary antibody (N45.1) and secondary peroxidase-labelled antibody conjugate used at a dilution of 1:500. A commercially available kit (Japan Institute for the Control of Aging) incorporating antibody N45.1 was used for the analysis of 8-oxodG in DNA isolated from CCRF-HSB-2 cells. Levels of 8-oxodG were expressed as a fold change relative to untreated control samples following subtraction of baseline values.

Thymine–thymine dimer (T < >T) adducts. T < >T adducts were measured as an indicator of non-oxidative radical-induced DNA damage in CCRF-HSB-2 cells or, as a measure of DNA repair, in cell culture supernatants using the general competitive ELISA method described previously,

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with UVC-modified ssDNA (200 mJ/cm^2) as the solid-phase antigen (50 μg/ml). A commercially available monoclonal antibody (antibody KTM53) against thymine–thymine dimer (Kamiya Biomedical Company, Seattle, WA, USA) was used as the primary antibody at a final dilution of 1:2000 in 4 % milk/PBS. Levels of T < >T were expressed as a fold change relative to untreated control samples following subtraction of baseline values.

Immunocytochemical analysis of deoxycytidine glyoxal adduct formation

Following incubation of CCRF-HSB-2 cells with vitamin C or 2-AP, medium was removed by aspiration and cells were fixed with 100 % ice-cold ethanol. Excess ethanol was discarded and cells were allowed to dry at room temperature. Monoclonal antibody against gdC adduct (F3/9 diluted 1:500 in PBS) was added (200 μl/chamber) and incubated for 1 h at 37°C. Following two washes with PBS, a secondary fluorescein isothiocyanate-labelled mouse immunoglobulin, 200 μl/chamber (1:100 diluted in PBS), was added and incubated for a further hour as described earlier, protected from exposure to light. The slides were washed twice with PBS and counterstained with 4′-6-diamidino-2-phenylindole (1 μg/ml) for 5 min. Following a final PBS wash, slides were mounted using Vectashield fluorescence mounting media and analysed using a Zeiss Axioskop fluorescence microscope; excitation and emission wavelengths of 490 and 520 nm, respectively, accompanied by Openlab software with three-dimensional imaging facility (Improvision, Coventry, UK).

Genomics analysis

RNA extraction. Total RNA was extracted with 1 ml TRIzol reagent (Invitrogen, Paisley, UK) using the protocol provided by the manufacturer. RNA concentration was determined by spectrometry, and the RNA quality was assessed using the Agilent bioanalyzer (Agilent Technologies, West Lothian, UK).

cRNA synthesis. A Roche (Welwyn Garden City, Hertfordshire, UK) microarray reagent kit was used as per the manufacturer's instructions with a double amplification method consisting of two cycles of cDNA synthesis and two cycles of IVT (*in vitro* transcription) and cRNA amplification.

Probe array preparation. Fifteen micrograms of biotin-labelled cRNA were fragmented and the quality assessed using the Agilent bioanalyzer (Agilent Technologies). Hybridisation cocktail (300 μl) was prepared using 15 μg fragmented cRNA, 5 μl control oligoB2 (Affymetrix Inc., Santa Clara, CA, USA), 15 μl 20X eukaryotic hybridisation control kit (Affymetrix), 3 μl Herring Sperm DNA (Promega, Southampton, UK), 3 μl acetylated BSA Invitrogen) and 150 μl 2X hybridisation buffer (Affymetrix protocol). Hybridisation cocktail was heated at 99°C for 5 min, while the probe arrays (human genome U133 plus 2, Affymetrix) were filled with 1X hybridisation buffer and incubated at 45°C for 10 min. Probes were treated with hybridisation cocktail and incubated for 16 h at 45°C. Washing and staining of the probe arrays was performed as per Affymetrix's protocol, and arrays were scanned using the Affymetrix GeneArray Laser Scanner.

Microarrays' data analysis. Transcription profiles of the CCRF-HSB-2 cells exposed to vitamin C or 2-AP were generated using the human GeneChip U133 plus 2·0 arrays (single array representing 14·5 K well-characterised human genes used to explore human biology and disease processes). Affymetrix Microarray Suite version 5.0 was used for image analysis. Data analysis of the CEL image files (stores the results of the intensity calculations on the pixel values of the DAT file) generated was carried out using D chip data-mining software, which includes comparison of fold changes to a range of housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase. In addition, Affymetrix web-based NetAffx was used to link study results to comprehensive online databases and resources. Comparisons were made between transcription profiles obtained from CCRF-HSB-2 cells treated with control media, which were used as baseline, and CCRF-HSB-2 cells treated with 150 μmol/l vitamin C and 2-AP, using the analysis software dChip version 2005. Gene induction or suppression was considered significant if the change in hybridisation intensity was greater than twofold, and signal intensity differences between arrays were 50 (arbitrary units) or greater and were selected for further evaluation. The selected genes were categorised according to their molecular function in relation to toxicity, and included genes involved in apoptosis, immunity, signalling, oxidation/reduction reactions, stress response and transcription, DNA repair and cell cycle arrest.

Statistical analysis

Statistical analysis was carried out to assess the significance of any differences in levels of intracellular vitamin C and DNA damage between control, vitamin C and 2-AP-treated cells. Significance for all samples was calculated through the application of non-parametric unpaired *t* tests using GraphPad Prism 2 software (Sigma-Aldrich Company Ltd).

Results

Intracellular vitamin C concentration

Following exposure of CCRF-HSB-2 cells to 150 μmol/l vitamin C, intracellular levels of vitamin C were seen to increase significantly relative to baseline after 8 h incubation (1·15 (SEM 0·04) μmol/l vitamin C, $P=0·011$) and had returned to baseline levels following 24 h incubation (0·69 (SEM 0·21) μmol/l vitamin C). In contrast, following supplementation of CCRF-HSB-2 cells with 150 μmol/l 2-AP, intracellular levels of vitamin C were seen to decrease from those at baseline (1·03 (SEM 0·01) μmol/l vitamin C) after 8 h incubation (0·48 (SEM 0·15) μmol/l vitamin C), and although levels were elevated from baseline following 24 h incubation, this was not significant (1·09 (SEM 0·05) μmol/l vitamin C). Data were described but not shown.

Analysis of DNA damage

Deoxycytidine glyoxal adduct. As shown in Fig. 2(a) following exposure to vitamin C, intracellular levels of gdC were

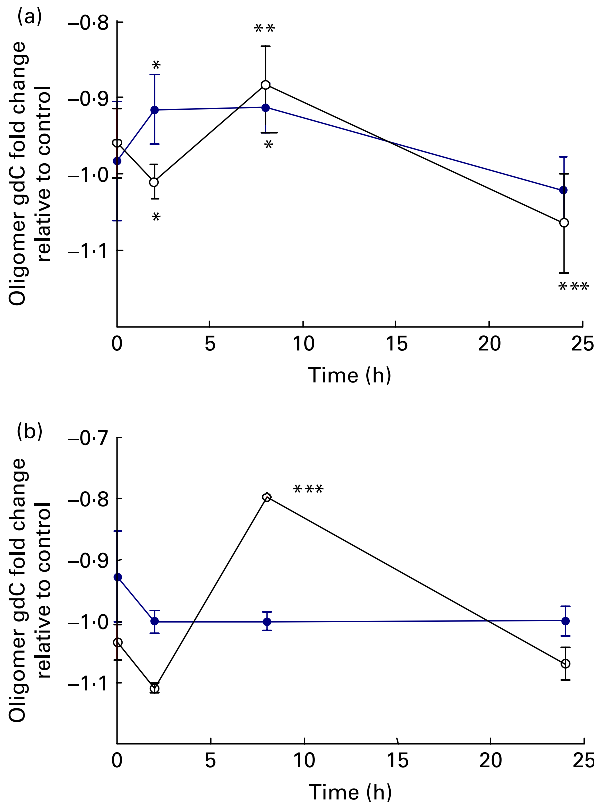

Fig. 2. Measurement of deoxycytidine glyoxal (gdC) adduct in DNA extracted from cultured human acute lymphoblastic leukaemic cell line cells (a) or in cell culture supernatant (b) following incubation with 150 μmol/l vitamin C or 150 μmol/l ascorbate-2-phosphate (2-AP) for 0–24 h. Analysis was carried out by ELISA using oligomer gdC standards (μg/ml). Values represent fold changes with respect to untreated control (50 and 45 μg/ml for DNA and supernatant, respectively) and represent the mean values and standard deviations of triplicate determinations of three individual experiments. For vitamin C (DNA), $P=0.0392$ and 0·0246 at 2 and 8 h, respectively. For 2-AP (DNA), $P=0.0122$, 0·004 and 0·0011 at 2, 8 and 24 h, respectively; for 2-AP (supernatant) $P<0.0001$ at 8 h. ○, 2-AP; ●, vitamin C. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

significantly increased from those at baseline after 2 and 8 hours incubation (mean fold change of −0·916 (SEM 0·016) and −0·913 (SEM 0·011); $P=0.0392$ and 0·0246 at 2 and 8 h, respectively). Following 24 h incubation, gdC levels in DNA returned to baseline levels (−1·021 (SEM 0·015) and −0·983 (SEM 0·026) at 24 h and baseline, respectively). Following exposure to 150 μmol/l 2-AP, DNA gdC levels were significantly reduced from those at baseline after 2 h incubation (−1·010 (SEM 0·011) and −0·959 (SEM 0·018) for 2 h and baseline, respectively; $P=0.0122$ at 2 h). However, levels were significantly raised from those at baseline following 8 h incubation with 150 μmol/l 2-AP (−0·883 (SEM 0·019); $P=0.004$) with levels at 24 h being significantly reduced from those at baseline (−1·064 (SEM 0·023), $P=0.0011$).

Fig. 2(b) shows the change in levels of gdC measured in cell culture supernatant following incubation of CCRF-HSB-2 cells with 150 μmol/l vitamin C or 2-AP. Exposure of cells to vitamin C stimulated a decrease (non-significant) in supernatant gdC levels following 2 h incubation; gdC remained at this reduced level for up to 24 h (−1·039 (SEM 0·078)). Incubation of CCRF-HSB-2 cells with 150 μmol/l 2-AP resulted in an initial decrease in gdC levels from those at baseline, 2 h after exposure. However, following 8 h exposure, levels in supernatant were significantly raised from baseline (mean fold change of −0·797 (SEM 0·017) and −1·034 (SEM 0·088 for 8 h and baseline, respectively; $P<0.0001$ at 8 h).

8-Oxo-2′-deoxyguanosine adduct. Following exposure to vitamin C, levels of 8-oxodG in DNA were significantly reduced from those at baseline after 8 h incubation (mean fold change of −1·412 (SEM 0·007) and −1·059 (SEM 0·02) at 8 h and baseline, respectively; $P<0.0001$ at 8 h). Levels of 8-oxodG in DNA at 2 and 24 h incubation were comparable to those measured at baseline (−1·023 (SEM 0·05) and −1·036 (SEM 0·074) at 2 and 24 h, respectively). Following exposure to 150 μmol/l 2-AP, levels of 8-oxodG in DNA were initially raised after 2 h incubation from those at baseline (−0·978 (SEM 0·034) and −1·060 (SEM 0·02) at 2 h and baseline, respectively). However, levels remained at approximately those of baseline following 8 and 24 h incubation. Data were described but not shown.

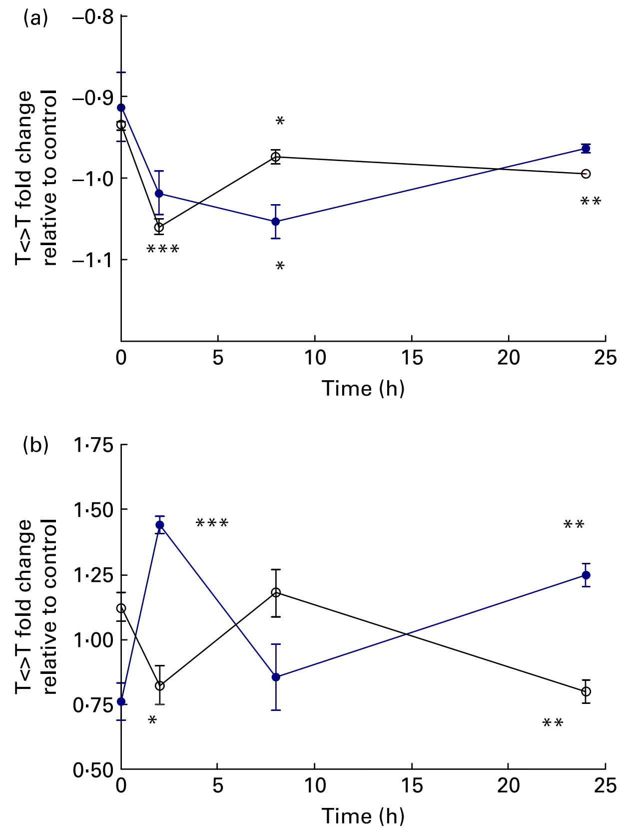

Fig. 3. Measurement of T<>T adduct in DNA extracted from cultured human acute lymphoblastic leukaemic cell line cells (a) or in cell culture supernatant (b) following incubation with 150 μmol/l vitamin C or 150 μmol/l ascorbate-2-phosphate (2-AP) for 0–24 h. Analysis was carried out by ELISA. Values represent fold changes with respect to untreated control (28 and 18 μg/ml UVC DNA for DNA and supernatant, respectively) following subtraction of baseline values, and represent the mean values and standard deviations of triplicate determinations of three individual experiments. For vitamin C (DNA, $P=0.039$ at 8 h; for vitamin C (supernatant), $P=0.001$ and 0·0045 at 2 and 24 h, respectively. For 2-AP (DNA), $P=0.0003$, 0·0216 and 0·0016 at 2, 8 and 24 h, respectively; for 2-AP (supernatant) $P=0.03$ and 0·0084 at 2 and 24 h, respectively. ○, 2-AP; ●, vitamin C. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

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Exposure of CCRF-HSB-2 cells to vitamin C resulted in a decrease in supernatant 8-oxodG levels following 2 and 8 h incubation. Although levels were seen to be increased after 24 h exposure to vitamin C (mean fold change of 1·920 (SD 0·159)), the increase was not found to be statistically significant in comparison to baseline levels (1·145 (SD 0·460)). Incubation of CCRF-HSB-2 cells with 150 μmol/l 2-AP resulted in an increase in supernatant 8-oxodG levels after 24 h incubation, in comparison with baseline levels (0·966 (SD 0·471) and 0·570 (SD 0·430) for 24 h and baseline, respectively); however, this was not found to be statistically significant. Data were described but not shown.

Cyclobutane thymine–thymine dimer (T < >T). As shown in Fig. 3(a) following exposure to vitamin C, levels of T < >T in DNA were reduced from baseline levels at 2 and 8 h (mean fold change of − 1·018(SD 0·045) and − 1·053 (SD 0·036) for 2 and 8 h, respectively) reaching significance at 8 h incubation (P=0·039). Following 24 h incubation with vitamin C, levels of T < >T in DNA had increased from those at 8 h but had not returned to baseline measurements (− 0·963 (SD 0·009) and − 0·912 (SD 0·072) for 24 h and baseline, respectively). Following exposure to 150 μmol/l 2-AP, levels of T < >T in DNA were significantly reduced from those at baseline following 2 h incubation (− 1·060 (SD 0·015) and − 0·934 (SD 0·012) at 2 h and baseline, respectively; P=0·0003 at 2 h). Although T < >T levels in DNA rose after 8 and 24 h exposure, levels remained significantly below baseline (− 0·974 (SD 0·015) and − 0·995 (SD 0·008); P=0·0216 and 0·0016 for 8 and 24 h, respectively).

Fig. 3(b) shows that exposure of CCRF-HSB-2 cells to vitamin C for 2 h resulted in a statistically significant increase from baseline in levels of supernatant T < >T (mean fold change of 1·439 (SD 0·056) and 0·761 (SD 0·125) for 2 h and baseline, respectively; P=0·001 at 2 h), and remained statistically significantly higher than baseline after 24 h exposure (1·247 (SD 0·076); P=0·0045). Incubation of CCRF-HSB-2 cells with 150 μmol/l 2-AP resulted in an initial significant decrease in supernatant T < >T levels from those at baseline, following 2 h incubation (0·824 (SD 0·127) and 1·126 (SD 0·097) for 2 h and baseline, respectively; P=0·0304 at 2 h). Although levels of T < >T appeared to return to baseline following 8 h incubation, supernatant levels were significantly lower than baseline levels following 24 h incubation (0·801 (SD 0·064); P=0·0084).

Immunocytochemical analysis of intracellular deoxycytidine glyoxal adduct formation

Changes in intracellular gdC levels assessed by immunocytochemistry are shown in Fig. 4. An anti-gdC monoclonal antibody, F3/9, was used in conjunction with an fluorescein isothiocyanate-labelled secondary antibody conjugate (green staining) to confirm that gdC levels were greatly increased from baseline (0 h), following 2 h incubation with 150 μmol/l vitamin C and after 8 h incubation with 150 μmol/l 2-AP. Specific antibody staining was seen to be reduced to baseline levels by 24 h incubation. CCRF-HSB-2 cells were also counterstained with 4′-6-diamidino-2-phenylindole as a nuclear stain (red staining). The merging of fluorescein isothiocyanate- and 4′-6-diamidino-2-phenylindole-stained images (merged images), in conjunction with three-dimensional analysis (not shown), allows for localisation of specific antibody staining within cells. This procedure confirmed that gdC was associated with the nucleus of the CCRF-HSB-2 cells.

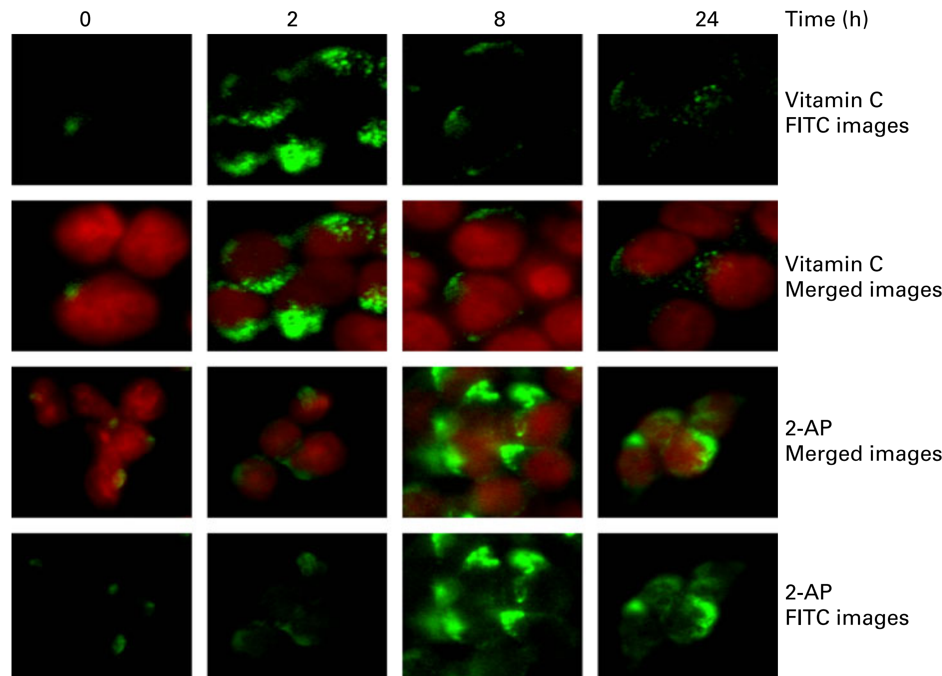

Fig. 4. Immunocytochemical localisation of deoxycytidine glyoxal (gdC) lesions in cultured human acute lymphoblastic leukaemic cell line cells exposed to 150 μmol/l vitamin C or 150 μmol/l ascorbate-2-phosphate (2-AP) for 24 h. DNA damage detected using monoclonal antibody F3/9 and visualised using fluorescein isothiocyanate(FIFC)-labelled secondary antibodies (light grey/green staining). The cells were also counterstained with 4′-6-diamidino-2-phenylindole nuclear stain (dark grey/red staining) and merged images allow detection of specific antibody binding associated with cell nucleus.

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Genomics analysis

Exposure of cells to vitamin C resulted in fifty genes being significantly induced or suppressed following 8 h incubation. In contrast, exposure of CCRF-HSB-2 cells to 2-AP resulted in 1200 significant gene expression changes. The induced genes were categorised according to their molecular function as detailed below: there were no significant differences in gene expression between 2-AP and vitamin C after 2 h of incubation, but 8 h of incubation produced substantial changes particularly in 2-AP-treated cells.

Acute phase stress response genes. As shown in Table 1 following 8 h exposure to vitamin C, there was no appreciable difference in the acute phase stress response genes compared to that of the control media. In contrast, incubation of CCRF-HSB-2 cells with 2-AP for 8 h resulted in a significant induction among these candidate genes.

Cell cycle arrest and DNA repair. As shown in Table 2, following 8 h exposure to vitamin C, there was no appreciable induction of genes associated with cell cycle arrest and DNA repair compared to that of the control media. In contrast,

Table 1. Induction of acute-phase stress response genes* following exposure of cultured human acute lymphoblastic leukaemic cell line cells to control (C8C), 150 μmol/l vitamin C (V8C; ascorbic acid) and 150 μmol/l ascorbate-2-phosphate (AP8C) for 8 h

Gene	C8C signal	V8C signal	Fold change	AP8C signal	Fold change
Chemokine (C motif) ligand 1/ligand 2	13·01	13·6	1·04	76·33	5·87
Chemokine (C–C motif) receptor 1	12·94	20·77	1·61	34·18	2·64
Chemokine (C–C motif) receptor 8	496·8	545·04	1·1	192·44	−2·58
Chemokine (C–X–C motif) receptor 4	1744·46	1585·99	−1·1	695·01	−2·51
Cytochrome b-561 domain containing 2	122·96	135·03	1·1	253·52	2·06
Cytochrome c oxidase subunit IV isoform 1	33·38	31·97	−1·04	67·75	2·03
Cytochrome P450, family 1, subfamily A, pp1	18·58	26·48	1·43	49·71	2·68
Cytochrome P450, family 2, subfamily E, pp2	118·32	114·04	−1·04	257·49	2·18
Cytochrome P450, family 2, subfamily U, pp1	28·81	31·54	1·09	64·92	2·25
Cytochrome P450, family 26, subfamily A, pp1	25·76	23·05	−1·12	74·6	2·9
Cytochrome P450, family 27, subfamily B, pp1	22·25	15·56	−1·43	60·49	2·72
Cytochrome P450, family 3, subfamily A, pp5	75·91	76·82	1·01	32·65	−2·32
Cytochrome P450, family 4, subfamily V, pp2	36·14	40·9	1·13	76·36	2·11
Cytochrome P450, family 51, subfamily A, pp1	575·55	557·84	−1·03	124·5	−4·62
Cytokine-induced protein 29 kDa	36·99	43·82	1·18	91·19	2·46
Cytokine-like nuclear factor n-pac	14·85	10·81	−1·37	38·81	2·61
Heat shock 105 kDa/110 kDa protein 1	36·42	60·65	1·67	122·45	3·36
Heat shock 60 kDa protein 1 (chaperonin)	110·78	114·1	1·03	46·57	−2·38
Heat shock 60 kDa protein 1 (chaperonin)	21·6	24·92	1·15	71·69	3·32
Heat shock 70 kDa protein 14	78·06	85·87	1·1	160·89	2·06
Heat shock 70 kDa protein 1B	355·2	430·41	1·21	147·08	−2·41
Heat shock 70 kDa protein 5	33·59	43·13	1·28	73·5	2·19
Heat shock 70 kDa protein 6 (HSP70B')	40·91	39	−1·05	18·34	−2·23
Haem oxygenase (decycling) 2	28·41	22·17	−1·28	75·31	2·65
Hypoxia-inducible protein 2	1187·66	1118·82	−1·06	179·39	−6·62
Immediate early response 3	241·02	235·06	−1·03	584·3	2·42
Interferon-induced protein 44	27·88	17·32	−1·61	70·37	2·52
Interferon-induced protein with tetratricopeptide repeats 3	20·37	43·28	2·12	42·19	2·07
Interferon-related developmental regulator 2	150·25	173·96	1·16	403·97	2·69
IL 17D	37·94	37·23	−1·02	78·32	2·06
IL 18 binding protein	24·87	23·39	−1·06	86·39	3·47
IL 21 receptor	65·56	82·25	1·25	9·25	−7·09
IL 22	6·03	3·74	−1·62	29·71	4·92
IL 9 receptor	948·07	990·23	1·04	466·04	−2·03
IL enhancer binding factor 3, 90 kDa	128·81	156·64	1·22	292·54	2·27
IL-1 receptor-associated kinase 4	104·3	93·95	−1·11	49·39	−2·11
Early growth response 1	44·66	100·26	2·25	219·43	4·91
Mitogen-activated protein kinase 3	309·98	245·2	−1·26	137·29	−2·26
Mitogen-activated protein kinase 8	136·46	148·97	1·09	59·74	−2·28
Mitogen-activated protein kinase kinase 2	34·13	69·54	2·04	14·79	−2·31
Mitogen-activated protein kinase kinase kinase 1	89·16	107·91	1·21	43·52	−2·05
Mitogen-activated protein kinase kinase kinase 15	91·39	99·76	1·09	199·04	2·18
Transferrin receptor (p90, CD71)	460·46	382·45	−1·2	198·5	−2·32
Metallothionein 1E (functional)	81·94	94·7	1·16	193·96	2·37
Metallothionein 1F (functional)	116·41	112·62	−1·03	240·18	2·06
Metallothionein 1G	8·79	1·42	−6·19	34·74	3·95
Metallothionein 1H	126·91	118·31	−1·07	308·4	2·43
Metallothionein 1K	29·06	31·56	1·09	58·78	2·02
Metallothionein 1X	141·61	147·31	1·04	344·43	2·43
Metallothionein 2A	481·72	447·53	−1·08	1143·37	2·37
NADPH oxidase 1	26·62	27·72	1·04	69·66	2·62
Peroxisomal biogenesis factor 7	23·47	37·26	1·59	52·72	2·25
Peroxisome biogenesis factor 10	86·8	87·02	1	179·72	2·07

* Mitogenic recruitment, inflammation signalling, anti-oxidation, stress response and xenobiotic metabolism.

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Table 2. Induction of cell cycle arrest and DNA repair genes following exposure of cultured human acute lymphoblastic leukaemic cell line cells to control (C8C), 150 μmol/l vitamin C (V8C; ascorbic acid) and 150 μmol/l ascorbate-2-phosphate (AP8C) for 8 h

Gene	C8C signal	V8C signal	Fold change	AP8C signal	Fold change
Cell division cycle 25A	22·91	27·07	1·18	57·23	2·5
Cell division cycle 25C	243·42	249·76	1·03	111·37	−2·19
Cell division cycle 2-like 6 (CDK8-like)	161·6	185·19	1·15	54·19	−2·98
CHK1 checkpoint homologue (*S. pombe*)	48·72	44·49	−1·1	15·2	−3·21
Cyclin B1 interacting protein 1	1114·79	1070·98	−1·04	465·66	−2·39
Cyclin E1	83·07	90·55	1·09	210·48	2·53
Cyclin E2	41·07	46·73	1·14	112	2·73
Cyclin G1	2678·07	2828·71	1·06	1099·26	−2·44
Cyclin G2	303·03	304·95	1·01	104·33	−2·9
Cyclin G2	578·51	632·06	1·09	247·45	−2·34
Cyclin M2	38·02	24·26	−1·57	17·24	−2·21
Cyclin-dependent kinase 5	15·26	17·3	1·13	60·2	3·95
Cyclin-dependent kinase 6	78·05	86·5	1·11	168·33	2·16
BRCA2 and CDKN1A interacting protein	106·58	89·41	−1·19	246·88	2·32
Breast cancer metastasis-suppressor 1	28·12	40·44	1·44	64·97	2·31
Ataxia telangiectasia mutated	306·27	416·28	1·36	47·57	−6·44
Damage-specific DNA-binding protein 2	518·16	513·59	−1·01	215·43	−2·41
DNA-damage-inducible transcript 3	234·09	190·52	−1·23	60·12	−3·89
DNA damage-inducible transcript 4	3794·6	3413·71	−1·11	1889·51	−2·01
Tumour protein p53-inducible nuclear protein 1	740·08	758·54	1·02	105·35	−7·02
Tumour protein p53-inducible protein 3	80·04	101·04	1·26	209·82	2·62
X-ray repair complementing defective repair in Chinese hamster cells 5	29·3	22·77	−1·29	89·38	3·05
Excision repair cross-complementing rodent repair deficiency	32·27	30·93	−1·04	80·37	2·49
mutS homologue 5 (*E. coli*)	206·96	175·68	−1·18	93·12	−2·22
RAD18 homologue (*S. cerevisiae*)	51·95	59·87	1·15	118·06	2·27
RAD21 homologue (*S. pombe*)	1618·35	1444·14	−1·12	761·14	−2·13
RAD50 homologue (*S. cerevisiae*)	25·67	35	1·36	53·18	2·07

incubation of CCRF-HSB-2 cells with 2-AP showed a significant induction among these candidate genes.

Apoptosis. As shown in Table 3 following exposure to vitamin C for 8 h, there was no apparent induction of genes associated with apoptosis. In contrast, incubation of CCRF-HSB-2 cells with 2-AP showed a significant induction among apoptotic genes.

Discussion

Intracellular vitamin C acts to protect the cell against oxidative stress by intercepting ROS such as superoxide anion and hydroxyl radicals, and subsequently minimise DNA damage. The intracellular level of vitamin C before an oxidative insult could therefore have a considerable effect upon the cells ability to resist that oxidative insult. It is well known that cultured cells inherently possess lowered antioxidant levels[36]; as a direct consequence, increases in intracellular vitamin C over a short period of time may have the potential to induce ROS. Such an increase in ROS may depend upon the mechanism of vitamin C transport into the cell, and therefore it is feasible that the transport of vitamin C may induce more ROS activity than 2-AP.

In general, it appeared that vitamin C supplementation was associated with a greater increase in intracellular levels of vitamin C in comparison to the vitamin C derivative, 2-AP. The pro-oxidant activity of vitamin C on gdC DNA damage in CCRF-HSB-2 cells was shifted to 8 h for 2-AP compared with 2 h for vitamin C. This could indicate that 2-AP has a slower uptake by cells than vitamin C, probably via the

Table 3. Induction of genes associated with apoptosis following exposure of cultured human acute lymphoblastic leukaemic cell line cells to control (C8C), 150 μmol/l vitamin C (V8C; ascorbic acid) and 150 μmol/l ascorbate-2-phosphate (AP8C) for 8 h

Gene	C8C signal	V8C signal	Fold change	AP8C signal	Fold change
Apoptosis inhibitor 5	50·56	71·98	1·42	131·26	2·6
Apoptosis, caspase activation inhibitor	64·52	76·67	1·19	181·23	2·81
Apoptosis-related protein PNAS-1	504·64	506·57	1	247·09	−2·04
B-cell CLL/lymphoma 3	353·6	303·36	−1·17	121·79	−2·9
B-cell CLL/lymphoma 6 (zinc finger protein 51)	154·53	160·67	1·04	28·54	−5·41
BCL2 binding component 3/BCL2 binding component 3	147·18	164·32	1·12	35·89	−4·1
BCL2/adenovirus E1B 19 kDa interacting protein 3	509·7	495·62	−1·03	175·91	−2·9
BCL2/adenovirus E1B 19 kDa interacting protein 3-like	726·32	675·31	−1·08	356·91	−2·04
BCL2-antagonist of cell death	54·4	77·13	1·42	122·9	2·26
BCL2-associated athanogene	434·32	508·37	1·17	883·35	2·03
BCL2-associated transcription factor 1	111	130·19	1·17	289·16	2·6
BH3 interacting domain death agonist	132·06	150·46	1·14	299·03	2·26
Caspase 1, apoptosis-related cysteine protease	46·89	66·42	1·42	104·43	2·23

sodium channel or that the measurable response to 2-AP is slower. The use of an oxidisable form of vitamin C for *in vitro* experiments has been considered controversial, as it is well established that entry into the cell partially occurs through uptake of the oxidised form of vitamin C, dehydroascorbic acid (DHA), by the GLUT receptor; this represents an artificial route of uptake of vitamin C into cells in comparison to that occurring *in vivo*. 2-AP has been established as an alternative non-oxidisable form of vitamin C for use in culture systems. 2-AP can increase intracellular vitamin C levels through uptake via SVCT1 and affect gene expression; however, the rate at which it does this is likely to differ from vitamin C entering as DHA through the GLUT receptor. In addition, as cells in culture readily become depleted in vitamin C[30], this effect should be superimposed on a timeline of slow uptake of 2-AP and faster uptake of vitamin C.

It should also be noted that the RCS glyoxal is an additional oxidative breakdown product of vitamin C with the potential to impact on gdC DNA levels. Furthermore, evidence for gdC removal through repair processes within the cell was reflected in increased cell culture supernatant levels of excluded gdC for both vitamin C and 2-AP-treated cells. Levels of the DNA hydroxylation product, 8-oxodG, were increased in vitamin C-treated CCRF-HSB-2 cells, but evidence of its repair in cell culture supernatant was less pronounced. Minimal effects upon 8-oxodG DNA generation and repair were suggested following 2-AP treatment. The use of 2-AP in the present study also suggested that DNA damage was not a result of autoxidation; DNA damage occurred whether vitamin C went into the cell (through GLUT receptors as DHA) or 2-AP. The significant down regulation of the transferrin receptor ($-2{\cdot}32$-fold change) noted following incubation of CCRF-HSB-2 cells with 2-AP is consistent with vitamin C having a role in the mobilisation of intracellular catalytic Fe. These results support those described by Duarte & Jones[37] who described similar findings following incubation of human diploid fibroblasts with physiologically relevant concentrations (100 μmol/l) of ascorbic acid.

These results suggest that ROS activity may be a prerequisite to induction of DNA repair through an induction by DNA damage. Measurement of levels of T < >T in DNA and cell culture supernatants were included in the present study as a positive marker for NER activity. In support of this, reduction in DNA levels for T < >T was sustained up to 8 h following vitamin C treatment in comparison to 2-AP. This was further reflected in T < >T supernatant levels for vitamin C relative to 2-AP-treated CCRF-HSB-2 cells. The results obtained indicate that vitamin C may play a positive role in excising T < >T from DNA even in the absence of NER induction through UV irradiation. In addition, Lunec *et al.*[38] demonstrated vitamin C to activate AP-1 binding to DNA and the subsequent induction of 8-oxodG repair. NER has also been postulated to be involved in the removal of 8-oxodG following the minimal detection of this lesion in a XPA cell line relative to normal lymphoblastoid cells following vitamin C treatment[38].

In the present study, vitamin C showed short-lived gene expression induction in keeping with promoting a transient positive adaptive response. However, 2-AP, at the same dose, induced genes associated with stress response, cell cycle arrest, DNA repair and apoptosis, indicating that 2-AP induced DNA damage and significant cell toxicity. This may be a feature of 2-AP metabolism, half-life in the cell and poor absorption and transport across the cell. It is also possible that the cause of the increased DNA damage and consistent gene expression changes associated with oxidative stress arise through an increased rate of oxidation induced within the cell related to an increased mobilisation of Fe (Fe^{2+}). The gene expression results reported here support such an interpretation, with the reduction in transferrin receptor gene expression acting as a feedback mechanism to prevent additional Fe entering the cell. Evidence in support of the present findings has been recently reported in a study investigating the effects of vitamin C and 2-AP on gene expression profiles in human skin cells[39].

A recent study reporting proteomics analysis of CCRF-HSB-2 cells exposed to vitamin C confirmed its potential to elicit a redox shift within the cells, and an ability to modulate the T cell proteome through altering expression of proteins associated with signalling, carbohydrate metabolism, apoptosis, transcription and immune function[1]. The expression of phosphatidylinositol transfer protein (responsible for intracellular signalling) was found to be increased following exposure of CCRF-HSB-2 cells to vitamin C[40]. The results reported by Grant *et al.*, and those reported in the present study support the hypothesis that rapid changes in intracellular vitamin C concentration result in a transient pro-oxidant effect, which subsequently 'primes' NER-like processes. This hypothesis is now being investigated further in an *in vivo* ascorbic acid intervention study.

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The authors state that there are no conflicts of interest.

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