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The Effects of Ascorbic Acid and Iron Co-Supplementation on the Proliferation of 3T3 Fibroblasts

CLIFFORD S. COLLIS, MIN YANG, SUSAN J. PEACH, ANTHONY T. DIPLOCK and CATHERINE RICE-EVANS

Free Radical Research Group, Division of Biochemistry and Molecular Biology, UMDS-Guy's Hospital, London SE1 9RT

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Exposure of 3T3 fibroblasts to Fe^{II} reveals a concentration-dependent inhibition of cell proliferation compared to control cells, the apparent threshold for this iron-mediated effect being 5 μM Fe^{II}. The inhibition of cell proliferation was accompanied by an enhancement of total malondialdehyde (MDA) levels (as detected directly by hplc) in the cells at higher iron concentrations. The co-supplementation of Fe^{II} with varying concentrations of ascorbic acid over the range 5 μM to 240 μM had no significant effect on the threshold for iron toxicity or lipid peroxidation. These results show that there is neither a significant exacerbation of the pro-oxidant effect of Fe^{II} nor any protective effect of ascorbate when cultures of 3T3 mouse fibroblasts are exposed to co-supplementation regimes of iron with ascorbic acid.

Key words: fibroblasts, ascorbic acid, iron, malondialdehyde, free radicals, antioxidant

INTRODUCTION

Vitamin C has been claimed to have many antioxidant properties *in vitro*[1–3] and has been described as 'the most effective aqueous phase antioxidant in human plasma'.[4] However, experiments have shown that in some *in vitro* systems vitamin C can have strong pro-oxidant properties particularly in the presence of transition metals; for example, iron/ascorbate mixtures readily produce hydroxyl radicals.[5]

The ability of iron to exist in two redox states has given this transition metal a critical status in living systems. In cells and tissues, iron is usually sequestered with enzymes and proteins and its availability tightly regulated.[6] However, iron non-specifically bound to low-molecular-weight ligands is potentially toxic, capable of catalysing the generation of free radicals.[6] There are, however, situations in which the iron status can change, either locally, as, for example, in ischaemic tissue, or systemically as with idiopathic haemochromatosis or transfusional iron overload. In these circumstances, abnormal levels of iron may promote toxicity.

One potential mechanism by which iron can be

Correspondence to C.S. Collis

released from the storage protein, ferritin, is through the action of reducing agents such as superoxide radical or ascorbic acid. In view of the dual pro-oxidant/antioxidant behaviour of ascorbic acid, it is not known to what extent additional supplementation with ascorbic acid in iron-loaded systems might alter the potential for iron-mediated free radical reactions and, in particular, whether and under what conditions such supplementation might display pro-oxidant or protective effects.

The objective of these experiments was to investigate the effects and toxicity of various concentrations of Fe^{II} on the growth of fibroblasts and the effects on iron toxicity of co-supplementation with various amounts of ascorbic acid.

MATERIALS AND METHODS

Tissue Culture and Cell Techniques

Mouse 3T3 fibroblasts were cultured in Dulbecco's Modified Eagles Medium supplemented with 10% foetal calf serum, 50 IU/ml penicillin and 50 mg/ml streptomycin. They were incubated at 37°C in humid air gassed with 5% CO_2. Under these conditions the cells became confluent in 72–96 h. The iron (as ferrous sulphate, Sigma Chemicals) and ascorbic acid (L-ascorbic acid, Sigma Chemicals) supplements were prepared as concentrated stock solutions in ultrapure water immediately before use. Aliquots of these solutions were added to the culture medium 3 h after seeding to allow time for cell adhesion. The volume of aliquot added was adjusted to bring the final concentration of the supplement in the medium to the required final level and was less than 1% of the total volume of the medium in each replicate. The supplements added were in addition to the iron contained in Dulbecco's Modified Eagles Medium (0.1 mg/l as $Fe(NO_3)_3 . 9H_2O$), as well as the total iron (1.98 μg/ml) and vitamin C (14 μg/ml) contained in foetal calf serum (Life Technologies, Private Communication).

Cells were counted on a Coulter Counter Model Dn and protein assessed by the method of Bradford.[7]

In the initial experiments the cells were harvested at 24 h intervals and growth curves constructed. Since confluence was usually reached in 72–96 h, in later experiments the cells were harvested only after this longer period. In each experiment 4–8 replicates for each treatment were prepared and each experiment was repeated 2–4 times. The data presented is from one typical experiment of the series unless otherwise stated.

Total Iron

Total iron was measured by inductively coupled plasma optical emission spectrometry following digestion of the samples with nitric acid using a low flow microconcentric nebuliser with a conventional Scott-type double pass spray chamber (Jobin Yvon 24).

Transferrin Iron

The iron-saturation status of the transferrin was determined using 6M-urea-polyacrylamide gel electrophoresis (6M-urea-PAGE).[8] Tissue culture medium samples were treated with rivanol, before and after the addition of excess iron nitrilotriacetate (FeNTA) to saturate any iron-free sites on the transferrin. Samples were analysed by 6M-urea-PAGE, alongside samples of rivanol-treated human serum transferrin (+/− FeNTA) and samples of 'pure' iron-free bovine transferrin (Sigma) which had been loaded with increasing amounts of iron.

Ascorbic Acid

When cells were harvested, samples were diluted 1:1 in 10% metaphosphoric acid, centrifuged and the supernatant stored at −70°C. Ascorbic acid was measured using a modified hplc assay,[9] using a Lichrosorb amino acid phase column (250×4.8 mm, Hichrom U.K.) and UV detection at 254 nm. The mobile phase consisted of 83.9% v/v

acetonitrile, 16% v/v potassium dihydrogen phosphate (15 mM) and 0.1% v/v glacial acetic acid at a flow rate of 2.0 ml/min. Ascorbic acid-containing samples were injected using a Rheodyne syringe loading injector with a loop volume of 20 µl.

Malondialdehyde

Total MDA (free and bound) was measured applying a modified hplc assay,[10] using a Spherisorb amino acid phase column (250×4.8 mm, Hichrom U.K.). The mobile phase consisted of 60% acetonitrile and 40% tris buffer (0.03 M, pH 7.0) at a flow rate of 1.0 ml/min. MDA containing samples were injected using a Rheodyne syringe loading injector with a loop volume of 20 µl. MDA was detected and quantified by reference to standards at 267 nm using a Varian UV-50 variable wavelength detector. The MDA standard solutions were prepared by hydrolysis of 1,1,3,3-tetra-ethoxypropane and the concentration confirmed by uv. spectroscopy at 245 mm (ε=13,700). Bound MDA was released by a modification of the alkaline hydrolysis system.[11]

Statistics

Data were compared using a Students t test, p values <0.05 were considered significant.

RESULTS

Iron Supplementation and Fibroblast Proliferation

The approach was to determine the time-dependency of the effects of supplemental Fe^{II} (as ferrous sulphate) on the growth of 3T3 fibroblasts. The supplements added were in addition to the pre-existing levels in the medium and serum, as defined in the Materials and Methods section. Initially the fibroblasts were cultured and harvested at 24 h intervals over a period of 96 h in the medium alone, or medium supplemented with iron alone (20 µM) (Figure 1). Fe^{II} at this concentration slowed the proliferation of the fibroblasts, as manifested in the population level of cells containing this level of iron (being 45% of the control cells).

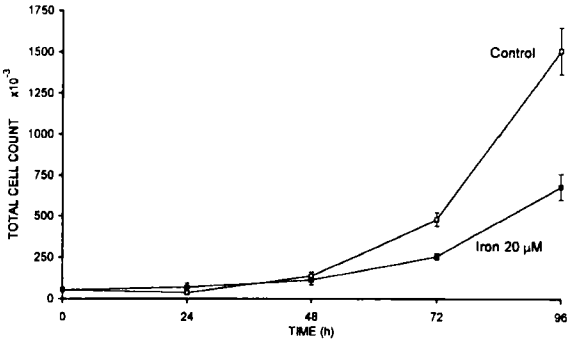

FIGURE 1 The effects of Fe^{II} (20 µM) on the growth of 3T3 mouse fibroblasts. Mean and s.d. shown from 5 replicates of a typical experiment.

Other experiments were performed to compare the effects of different concentrations of Fe^{II} with the view to establishing the threshold level for iron-mediated effects on cell growth. The cells were allowed to grow for 96 h before harvesting (Figure 2). Lower concentrations of Fe^{II} (5 µM) had no significant effect on cell growth compared with control cells. At higher concentrations, however, there was a concentration-dependent inhibition of fibroblast growth when expressed both in terms of cell number and cell protein, with increasing levels of iron having a plateau of cell death at 50 µM.

Ascorbic Acid Supplementation and Fibroblast Proliferation

Supplementation of the growth medium with varying concentrations of ascorbic alone had no significance on fibroblast growth (Figure 3).

Ascorbic acid status

Analysis of cellular ascorbic acid levels demonstrated that cells supplemented with ascorbic acid

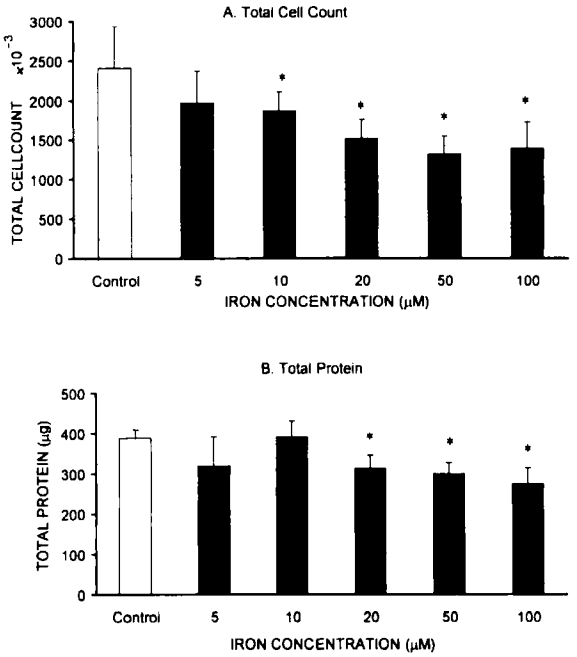

FIGURE 2 The effects of varying concentrations of Fe^{II} on 3T3 fibroblast growth after 96 h expressed as; A. Total cell count and B. Total Protein Mean and s.d. shown from 5 replicates of a typical experiment. *p<0.05 significantly different *vs* Control, Students t test.

contained a significantly higher level of ascorbic acid compared with the non-supplemented control ($p<0.01$) but the uptake was not proportional to the added concentration (Figure 4).

Iron with Ascorbic Acid Co-Supplementation

Ascorbic acid did not display a deleterious effect on cell growth when added exogenously to the growth medium in the presence of added 5 μM iron, in the molar ratios of 1:1, 1:8, 1:16, 1:48 excess of ascorbate over iron (Figure 5). Further experiments were carried out to observe whether ascorbic acid supplementation at a high and a low molar ratio to iron (20 μM) displayed any significant enhancement or inhibition of proliferation compared to the effects of iron alone. No attenuation or exacerbation of the growth inhibitory effects of 20 μM Fe was observed with the molar ratios 1:0.5 and 1:12 (Figure 6).

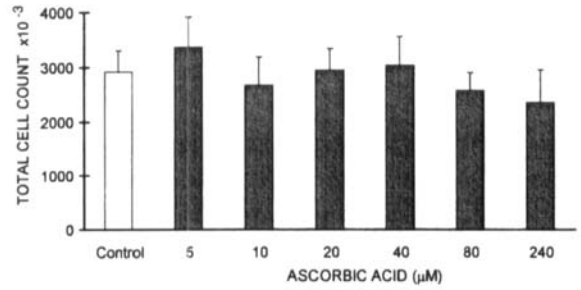

FIGURE 3 The effects of varying concentrations of ascorbic acid on 3T3 fibroblast growth after 96 h. Mean and s.d. shown from 5 replicates of a typical experiment.

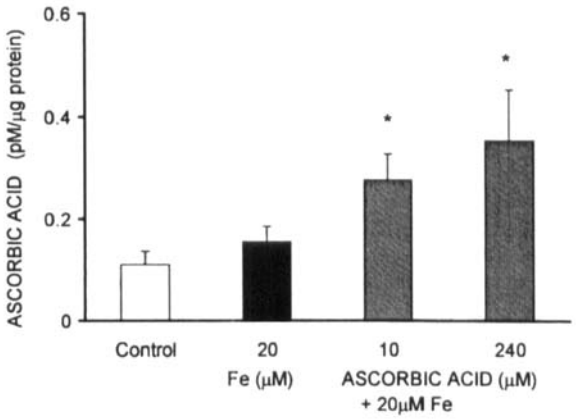

FIGURE 4 The effects of varying concentrations of ascorbic acid on 3T3 fibroblast ascorbic acid content after 72 h. Mean and s.d. shown from 8–10 replicates from 3 experiments. *p<0.05 significantly different *vs* Control, Students t test.

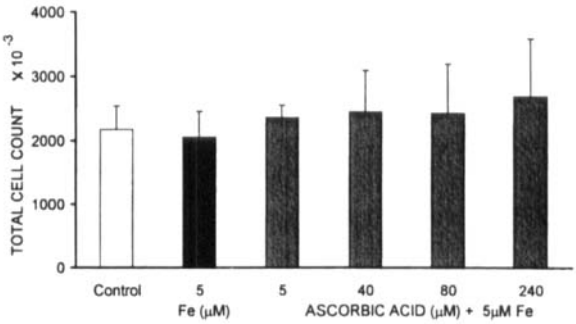

FIGURE 5 The effects of varying concentrations of ascorbic acid co-supplementation with 5 μM Fe^{II} on 3T3 fibroblast growth after 96 h Mean and s.d. shown from 5 replicates of a typical experiment.

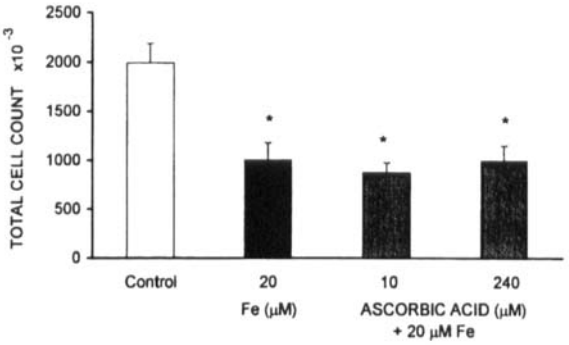

FIGURE 6 The effects of high (240 μM) and low (10 μM) concentrations of ascorbic acid co-supplementation with 20 μM Fe^{II} on 3T3 fibroblast growth after 96 h Mean and s.d. shown from 5 replicates of a typical experiment. *p<0.05 significantly different *vs* Control, Students t test.

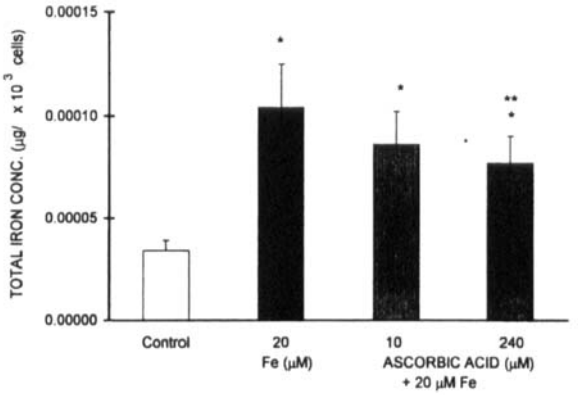

FIGURE 7 Total iron levels of mouse 3T3 fibroblasts grown in the presence of high (240 μM) and low (10 μM) concentrations of ascorbic acid co-supplemented with 20 μM Fe^{II} after 96 h. Mean and s.d. shown from 8 replicates from 2 experiments. *p<0.05 significantly different *vs* Control & ** *vs* 20 μM Fe, Students t test.

Iron Status

Iron levels and transferrin

Gel electrophoresis was carried out to separate apotransferrin, the two forms of monoferric transferrin and diferric transferrin. Samples analysed included the tissue culture medium alone, medium in which cells had been grown in varying concentrations of iron alone and iron in combination with vitamin C and the cells themselves. This analysis revealed that the transferrin in the medium alone was already fully saturated with diferric transferrin and the levels of iron and ascorbic acid used, 20 μM and 80 μM respectively, did not alter this saturation status. The cell samples studied contained insufficient transferrin to be detectable by the urea-PAGE analysis.

Total iron levels

The total iron levels of the fibroblasts were analysed by atomic absorption spectroscopy. Cells grown in medium supplemented with 20 μM iron alone and with ascorbic acid supplementation all showed a significant uptake of iron (Figure 7). The level of iron in the cells decreased significantly ($p<0.05$) with ascorbic acid supplementation at the 240 μM level compared with cells supplemented with iron alone.

Lipid Peroxidation Products (Malondialdehyde, MDA)

The MDA levels measured in the fibroblasts were significantly increased in the presence of increasing concentrations of iron (Figure 8A). This was not affected by the presence of high or low levels of added ascorbic acid (10 or 240 μM) (Figure 8B).

DISCUSSION

The results of these experiments reveal a concentration-dependent effect of Fe^{II} on 3T3 fibroblasts as manifested in a slower rate of cell proliferation compared to control cells. Varying the concentration of added iron revealed that, under these conditions, the cells in culture can accommodate up to 5 μM supplemental iron as judged by the lack of influence on cell growth and on markers of oxidative damage; furthermore, addition of varying concentrations of ascorbic acid over the range 10 to 240 μM had no significant modulatory effect. In those cell cultures exposed to iron, MDA levels were significantly higher suggesting that these effects are mediated by free radicals.

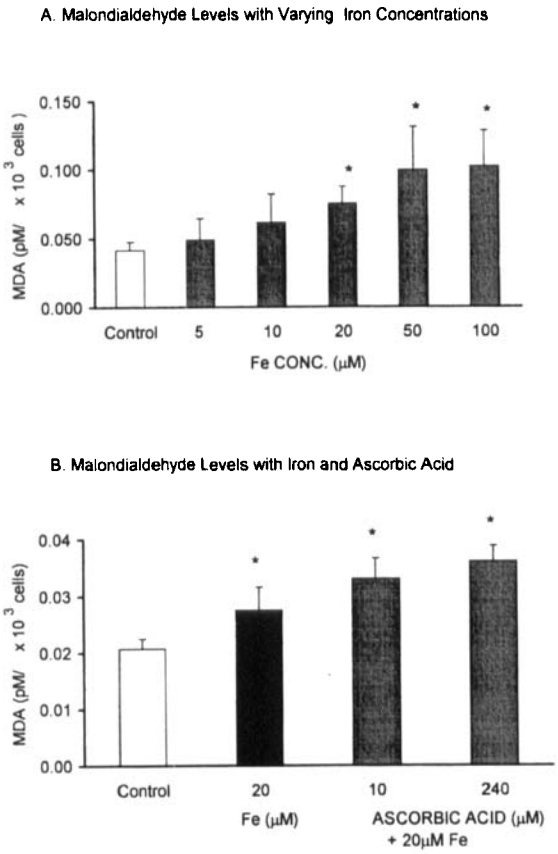

FIGURE 8 *Malondialdehyde (MDA) levels of mouse 3T3 fibroblasts grown in the presence of A. increasing concentrations of Fe^{II} and B. high (240 μM) and low (10 μM) concentrations of ascorbic acid co-supplementation with 20 μM Fe^{II} after 96 h. Mean and s.d. shown from 8 replicates from 2 experiments. *$p<0.05$ significantly different vs Control, Students t test.*

The effects and fate of ascorbic acid alone and in combination with iron in the culture medium are complex, and there are conflicting views on the stimulatory and inhibitory actions on cell proliferation. Ascorbic acid is contained in small quantities in foetal calf serum.[12] In culture medium alone, without cells and in the presence of transition metals, ascorbic acid is capable of autoxidation[13] and has been reported as decreasing in concentration by about 10% every 2 h[14,15] or completely after 10 h.[16] However, in the presence of cells, approximately 10% of the initial concentration of ascorbate remained after 24 h incubation in the case of HL-60 cells[17] and 20% with 3T6 cells.[12] One possible explanation may relate to the nature of ascorbic acid uptake. In human skin fibroblasts exposed to 500 μM added ascorbic acid for 1 h, uptake of ascorbic acid has been shown to be dependent on cell density[18] with proportionally less accumulation (2.04 ± 2.6 pmol/μg cell protein; mean ± s.d.) in high density cultures (182.1 ± 5.6 μg cell protein/well) compared to the content (204 ± 0.036 pmol/μg cell protein) of low density cultures (54.3 ± μg cell protein/well). A similar density-dependent response was also seen in cells exposed to a tenfold lower level of ascorbic acid. However, when comparing the two groups of cells exposed to high and low concentrations of ascorbic acid, the tenfold increase in external ascorbic acid concentration resulted in only a twofold increase in intracellular concentration. These observations might suggest that in longer term cultures the levels of ascorbic acid in the culture medium may initially drop principally because of the autoxidation of ascorbate and, to a lesser extent, by cellular uptake. It should be noted that some cells, eg activated neutrophils, will preferentially take up dehydroascorbic acid.[19]

Zheng and Zheng[20] investigated the effects of different concentrations of Fe^{II}/vitamin C on the human fibroblast 2BS line. They used combinations of Fe^{II}/vitamin C molar ratios 1:25 at iron levels 5, 10 and 20 μM and reported increasing levels of MDA with time and concentration; a reduced rate of proliferation compared to the control was also described with the exception of the 20 μM Fe^{II}/500 μM vitamin C combination. Their interpretation was that a certain level of lipid peroxidation products could increase the life span, decrease the subculture interval and increase DNA synthesis. Concentrations above or below this specific level of Fe^{II}/ascorbate did not have the same effect.

In the experiments reported here, ascorbic acid alone in concentrations ranging from 5–240 μM had no significant effect on cell proliferation. Ascorbic acid (50 μg/ml, 286 μM) has been reported as having a small stimulatory effect on

3T6 fibroblast proliferation (8% ± 4)[21] after 7 days. Contrariwise, ascorbic acid has been reported to inhibit cell adhesion[21] and display toxicity at concentrations above 0.3 mM by inducing hydrogen peroxide production.[22] The highest concentration applied in the studies described here was 240 μM and, consistent with the foregoing, no toxic effects were observed.

It is of interest to note that while the transferrin in the culture medium to which our cells were exposed was found to be saturated with iron, enhanced supplemental iron levels up to 5 μM posed no adverse effects on cell proliferation nor on cell membrane oxidation under these conditions. It might be anticipated from the known chemistry of iron/ascorbate/oxygen systems that co-supplementation of iron with ascorbate in these cell cultures might promote pro-oxidant effects at these levels. This was not found to be the case. Furthermore, the responses of the increased levels of iron which promoted cell membrane oxidation and decreased cell growth were neither exacerbated nor attenuated by co-supplementation with ascorbic acid. A recent report[23] has suggested that co-supplementation of iron with vitamin C in humans may have toxic effects through hydroxyl radical-mediated damage. This is not supported by the results of the cultured fibroblast model described here.

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