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On the Cytoprotective Role of Ferritin in Macrophages and its Ability to Enhance Lysosomal Stability

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Macrophages have a great capacity to take up (e.g. by endocytosis and phagocytosis) exogenous sources of iron which could potentially become cytotoxic, particularly following the intralysosomal formation of low-molecular weight, redox active iron, and under conditions of oxidative stress. Following autophagocytosis of endogenous ferritin/apoferritin, these compounds may serve as chelators of such lysosomal iron and counteract the occurrence of iron-mediated intralysosomal oxidative reactions. Such redox-reactions have been shown to lead to destabilisation of lysosomal membranes and result in leakage of damaging lysosomal contents to the cytosol. In this study we have shown: (i) human monocyte-derived macrophages to accumulate ferritin in response to iron exposure; (ii) iron to destabilise macrophage secondary lysosomes when the cells are exposed to H_2O_2; and (iii) endocytosed apoferritin to act as a stabiliser of the acidic vacuolar compartment of iron-loaded macrophages. While the endogenous ferritin accumulation which was induced by iron exposure was not sufficient to protect cells from the damaging effects of H_2O_2, exogenously added apoferritin, as well as the potent iron chelator desferrioxamine, afforded significant protection. It is suggested that intralysosomal formation of haemosiderin, from partially degraded ferritin, is a protective strategy to suppress intralysosomal iron-catalysed redox reactions. However, under conditions of severe macrophage lysosomal iron-overload, induction of ferritin synthesis is not enough to completely prevent the enhanced cytotoxic effects of H_2O_2.

Abbreviations: EDTA, disodium ethylene diamine tetra-acetic acid; FCS, foetal calf serum; FeAC, ferric ammonium citrate; $HO^{\cdot}$, hydroxyl radical; LDH, lactic dehydrogenase; MDM, monocyte-derived macrophages; $O_2^{\cdot-}$, superoxide radical; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline

INTRODUCTION

Macrophages play a key role in iron metabolism *in vivo*.[1] In addition to the role of the macrophage in peripheral iron homeostasis (viz. erythrocyte turnover), this cell type has a high capacity to acquire iron from a variety of external sources. Thus, macrophages can take up iron in the form of immune complexes,[2] low molecular weight chelates,[3] simple salts and oxides,[4] heme, transferrin, as well as from senescent or oxidatively

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damaged erythrocytes.[5,6] The precise mechanisms of uptake are not known in all cases. However, it is clear that the physical nature of the iron which is acquired dictates the route of uptake and can have a bearing on its subsequent intracellular location. For example, precipitated iron in the form of hydroxylated iron-phosphate complexes (formed by addition of $FeCl_3$ to phosphate-containing solutions) and iron-dextran are targeted to the lysosomal compartment.[6,7] Given time, cytoplasmic iron, stored as ferritin, could also be directed to lysosomes via normal autopha-gocytic activity.

Most cell types can efficiently limit their intracellular iron content via down-regulation of transferrin receptor expression.[8] However, in stark contrast, iron up-regulates transferrin receptor expression in human macrophages.[5] In addition, the uptake of iron-chelates by human macrophages is further induced by pre-exposure of the cells to iron.[3] These facts imply that macrophages may serve a useful role as a repository for excess extracellular iron. Indeed, it has been shown that iron sequestration by macrophages decreases the potential for damaging extracellular free radical formation.[9] While the high capacity of macrophages to scavenge extracellular iron may be beneficial to the surrounding tissue, little attention has been paid to the effect of iron-loading on macrophage survival, particularly under conditions of oxidative stress which may be encountered during inflammation.

We have previously shown that macrophages which have been loaded with iron within their acidic vacuolar apparatus become sensitised to oxidative damage,[10,11] a phenomenon which has also been demonstrated in other cell types.[7] This cytotoxicity involves iron-catalysed reactions which result in lysosomal membrane damage.[12,13] Chelation of macrophage lysosomal iron with desferrioxamine preserves lysosomal membrane integrity and diminishes the cytotoxicity of exogenously added H_2O_2.[13] Other recent studies have shown that apoferritin, when exogenously added to endothelial cells, is taken up and affords protection against H_2O_2-mediated cytotoxicity.[14] It was suggested that both the ferroxidase activity and iron-sequestering capacity of ferritin limits the availability of cellular Fe^{2+} and thereby limits the potential for H_2O_2-mediated oxidative reactions (eg. via Fenton-type chemistry), consistent with previous reports, e.g..[15] Exactly where in the cell ferritin/apoferritin is exerting its cytoprotective effects has, however, not been studied. The presence of iron-loaded macrophages in clinical conditions with an inflammatory component, such as atherosclerosis[16] and secondary haemochromatosis,[17] highlights the need for a greater understanding of the possible cytoprotective effects of ferritin in this cell type.

In the present studies we examine the efficacy of endogenous (Fe-stimulated) ferritin synthesis as a cytoprotective strategy for human macrophages (exposed to both soluble and insoluble forms of Fe) and subsequently given a bolus dose of H_2O_2. In a separate series of studies we then use the J774 macrophage cell line, a model system we have previously characterised in some detail,[6,12,13,16,18] to show that exogenously added apoferritin exerts at least part of its cytoprotective effect by stabilising lysosomal membranes.

MATERIALS AND METHODS

Reagents

RPMI 1640 medium and foetal calf serum (FCS) were from GIBCO (Paisley, UK). H_2O_2 was from Aldrich-Chemie (Steinheim, Germany), desferrioxamine mesylate (Desferal) from Ciba-Geigy (Basel, Switzerland), apoferritin (horse spleen) from Sigma (St Louis, MO, USA), and ferritin (human liver) from Chemicon (Temecula, CA, USA). Citric acid and Na-cacodylate were from Merck (Darmstadt, Germany). Ammonium sulphide and hydroquinone were from BDH (Poole, UK). Epon-812 and Ag-lactate were form Fluka AG (Buchs, Switzerland) and gluteraldehyde from Bio Rad (Cambridge, MA, USA). Crystalline gum arabic was a gift from Dr Gorm Danscher (Institute of

Anatomy, University of Arhus, Denmark). Other reagents were of the highest quality available and obtained from standard laboratory suppliers.

Isolation and Culture of MDM and J774 Cells

Human monocytes were isolated from buffy coats of donor blood as previously described in detail.[6] Briefly, peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque (Flow, Rickmansworth, UK) according to the method of Böyum.[19] Aliquots of the PBMC were stained using May-Grünwald-Giemsa's stain and found to contain approximately 10 to 20% monocytes with the remainder primarily lymphocytes with varying degrees of platelet contamination. Neutrophils were not detected in any of the PBMC preparations used. PBMC were dispensed in 22 mm diameter tissue culture wells (Costar, Cambridge, MA, USA) to yield a final concentration of 1×10^6 monocytes per well. After rinsing to remove non-adherent cells, monocytes were cultured for up to 8 days in RPMI 1640 supplemented with 15% (v/v) FCS, 2 mM glutamine, 100U/ml penicillin G and 100µg/ml streptomycin with media changes every 48h. It has been previously established by morphological and immunochemical criterion that human monocytes cultured under the present conditions yield differentiated macrophages (>95%) after 6 days *in vitro*.[20] The murine macrophage-like J774.A1 cell line was cultured in the same medium as above except that 10% (v/v) FCS was used. J774 cells were subcultivated every 7 days and prior to use in experiments were plated at 1.8×10^5 cells per 35 mm diameter dish (Costar) and incubated for a further 24h. All cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Where iron was added to macrophages, either $FeCl_3$ or ferric ammonium citrate (Fe^{3+}/ammonium citrate ratio 0.16/1, w/w) was first prepared as a 100 X stock in H_2O, filter sterilised (0.2 µm), and added to the media as indicated. Iron solutions were prepared freshly on the day of each experiment.

Degradation of Hydrogen Peroxide

MDM cultured for 6 days were rinsed twice in Dulbecco's Phosphate Buffered Saline (PBS) and exposed to H_2O_2 in PBS (1 ml/22 mm well) at 37°C. 10 µl aliquots were taken from cell supernatants and the H_2O_2 concentration was assayed using parahydroxyphenylacetic acid (pHPA) as a probe.[12,21] In the presence of horse radish peroxidase, H_2O_2 oxidises pHPA to its fluorescent dimer. Fluorescence intensity was measured at $\lambda\ ex_{315nm}$ and $\lambda\ em_{410nm}$ using an RF-540 fluorescence spectrophotometer (Shimadzu) connected to a DR-3 data recorder.

Determination of Lysosomal Stability using Acridine-Orange

J774 cells, cultured on glass coverslips, were stained with 2 ml acridine orange-solution (5 µg/ml in complete growth medium) for 15 min at 37°C, rapidly rinsed in PBS at 37°C and then exposed to H_2O_2 in PBS at 37°C for various periods of time. Following a rinse in PBS at 37°C the coverslips were mounted in a drop of PBS on excavated microculture slides. The intensity of red, granular fluorescence (indicating lysosomes with preserved proton gradients) from 100 cells/coverslip were simultaneously measured by static cytofluorometry using an MPV III (Leitz, Wetzlar, Germany) microscope photometer equipped with an H2 filter cube and an extra 630nm barrier filter (Leitz, Wetzlar) connected to an ABC 800 computer as described previously.[22]

Measurement of Cellular Ferritin by ELISA

Cells grown in 22 mm diameter wells were washed 3 times with PBS at 37°C and lysed in 0.5 ml of Lysis Buffer (1% Triton X-100, 0.5% Nonidet P40, 0.15 M NaCl, 10 mM Tris-HCl pH 7.2, 5 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride). The cell lysate was then diluted in PBS containing 0.1% (v/v) Tween 20 (PBST) and 0.5% (w/v) bovine serum albumin (BSA) and

assayed for ferritin content using a double antibody sandwich ELISA. 96 well microtitre plates (NUNC maxisorp, Kamstrup, Denmark) were coated with 100 µl rabbit anti-human ferritin polyclonal antibody (DAKO, Glostrup, Denmark) diluted 1/700 in 0.01 M phosphate buffer, 0.145 M NaCl, pH 7.2 and incubated at 4°C for 18h. After washing with PBST, plates were blocked with 200 µl PBST 0.5% BSA for 30 min at 22°C. Cell lysate samples and human liver ferritin were then added to the plates in a volume of 100 µl. The range of the ferritin standards was from 2 to 200 ng/ml. After incubation of samples and standards for 2h at 22°C, the plates were washed with PBST and incubated for 1h with peroxidase-conjugated rabbit anti-human ferritin polyclonal antibody (DAKO) diluted 1/10 000 in PBST. Finally the plates were washed in PBST and 100 µl 3,3',5,5'-tetra-methylbenzidine (TMB, DAKO) was used as a peroxidase substrate. After acidification, theabsorbance of the reaction product was measured at 450 nm using an Anthos HT automated plate reader (Laboratorie design AB, Lidingö, Sweden).

Demonstration of Lysosomal Iron by Autometallography and Transmission Electron Microscopy

Low-molecular-weight-iron was demonstrated by electron microscopy while using a modified cytochemical sulphide-silver technique.[23] Although this technique does not discriminate between different transition metals, since Fe is by far the most abundant intracellular metal under the conditions employed here, it is a useful tool to illustrate Fe localisation at the subcellular level. J774 cells (5×10^5 cells/dish), either with or without prior exposure to $FeCl_3$, were fixed at 22°C in 2% (w/v) glutaraldehyde in 0.1 M Na-cacodylate-HCl buffer, pH 7.2, with 0.1 M sucrose for 3h. Following a short rinse in double-distilled water, the cells were sulphidated in 1% (v/v) ammonium sulphide in 70% (v/v) ethanol, pH 9, at 22°C for 15 min. The cultures were then rinsed with distilled water and developed in a physical, colloid protected, photographic-type developer directly in the dishes at 26°C for 30 to 40 min (110 mg Ag-lactate and 850 mg hydroquinone were separately prepared in 15 mldouble distilled water, mixed with 60 ml 25% gum arabic and 10 ml Na-citrate buffer, pH 3.8). Controls were: (i) developed without previous sulphidation and, (ii) sulphidated but not developed. After developing, the cells were quickly rinsed with distilled water, dehydrated in a gradient series of ethanol solutions, and embedded in Epon-812. Thin sections were stained with lead citrate and examined and photographed in a Jeol 2000-EX electron microscope (Tokyo, Japan) at 100 kV.

Demonstration of Lysosomal (Apo)Ferritin by Immunofluorescence

The intracellular distribution of ingested apoferritin was demonstrated by immunofluorescence microscopy. J774 cells grown on glass coverslips were fixed for 20 min at 4°C in 4% paraformaldehyde in PBS, rinsed in PBS and exposed to 0.1% saponin and 5% FCS in PBS for 20 min at room temperature. The coverslips were then placed in a humidifier, 30 µl of the same rabbit anti-human ferritin polyclonal antibody as used for the ELISA study (1:100 in PBS containing 0.1% saponin and 5% FCS) was added to each coverslip and the cells were incubated at 4°C overnight, rinsed for 2×5 min in PBS with 0.1% saponin and 5% FCS, and incubated for 60 min at room temperature with 30 µl anti-rabbit IgG Texas Red conjugate (1:200 in PBS with 0.1% saponin and 5% FCS). Following a rinse in PBS and distilled water, the coverslips were mounted in Gelvatol. Finally they were examined andphotographed in a Nikon photomicroscope using green exciting light and a red barrier filter (G-1B, DM 580 Nikon filter cube).

Determination of Cell Viability

Cell viability was determined using the delayed trypan blue dye exclusion test as previously

described,[13] after the cells were returned to ordinary culture conditions for 5 h. In each culture the number of stained (non-viable) and unstained (viable) cells were counted, using an inverted microscope, in ≥5 random fields of vision at low magnification (×250). Cell viability was also assessed by measurement of lactic dehydrogenase (LDH) activities in both the incubation media and cellular pellet (homogenised in 0.1% Triton X-100). LDH activities were determined at 37°C by measuring the rate of NADH oxidation (monitored spectrophotometrically at 340 nm) in the presence of pyruvate.[24]

Determination of Total Cellular Protein and Iron

Cells were lysed in lysis buffer (as above). The protein content was determined using the bicinchoninic acid method (Sigma, cat.# TPRO562) with BSA as a standard. Total cellular iron was determined by atomic absorption spectrophotometry (Z-8270 Polarised Zeeman, Hitachi).

Statistical Analysis

Statistical significance was determined by using the 2-tailed Student's *t*-test. A P value <0.05 was considered significant.

RESULTS

Changes in Ferritin Levels in Human Monocyte/Macrophages During Differentiation

The ferritin content of human monocyte/macrophages which were not exposed to exogenously added Fe (ie. other than that present in growth medium) was first assessed over periods of eight days. Total cell protein levels decreased after the first day in culture, then increased to approximately 150–250 μg protein (per 10^6 monocytes seeded) after 1 week (Fig. 1). Cellular ferritin levels sharply increased during the first 24h of culture (Fig. 1). After this initial increase, ferritin levels decreased (when expressed relative to total protein) and plateaued after 4 days (Fig. 1). The same biphasic pattern of change in cellular ferritin content was observed using cells isolated from two different donors (compare Fig. 1A to Fig. 1B).

In order to define H_2O_2 exposure conditions for subsequent MDM cytotoxicity studies, the ability of day 6 MDM to metabolise H_2O_2 was examined. MDM removed 0.5–1.0 mM concentrations of H_2O_2 from their surrounding medium (ie. 500–1000 nmol/ml/10^6 cells) in approximately 1 h (Fig. 2). The rate of H_2O_2 loss was strongly dependent on the concentration of H_2O_2 present (see inset Fig. 2). The decrease in the rate of H_2O_2 removal which occurred as time progressed was therefore not likely to be due to the loss of intracellular reducing equivalents but rather to substrate limitation. Approximately 85–90% of MDM remained viable after exposure to 1 mM H_2O_2 (see below). Thus human MDM had a greater capacity to degrade (and were more resistant to the cytotoxic effects of) extracellular H_2O_2 when compared with J774 cells; we have previously shown that the latter can remove up to 500 nmol H_2O_2/ml/10^p cells after 1 h but can not withstand doses of 500μM or more.[13,18] Based on the present studies we chose to expose human MDM to higher H_2O_2 concentrations than would normally be used to induce cytotoxicity in J774 cells.

Iron-Induced Increase in MDM Ferritin Content and its Effect on H_2O_2-Induced Cytotoxicity

Since we have previously shown that an increase in macrophage iron content decreases their resistance to oxidative stress,[10,11] and others have suggested that cellular ferritin levels may effectively chelate excess intracellular iron and thereby act as a cytoprotective agent,[14] we measured the extent of iron-induced changes in ferritin levels in MDM

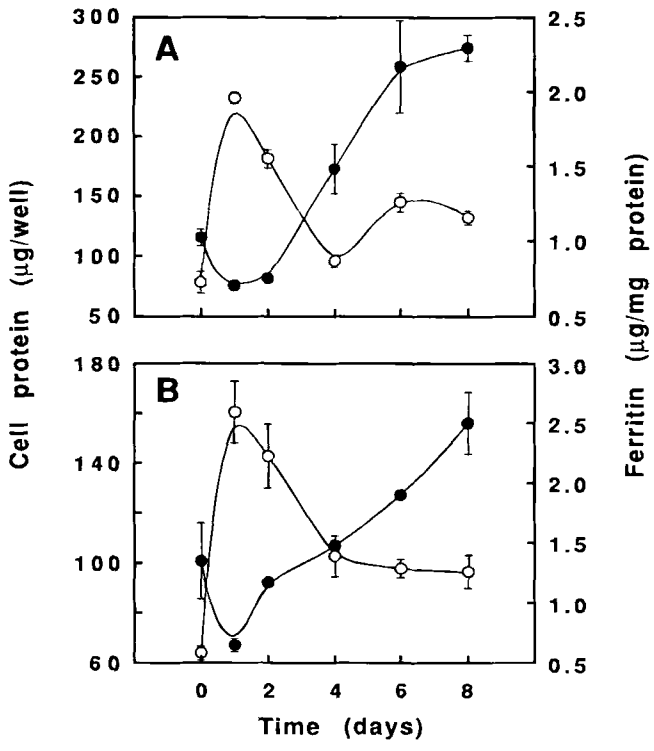

FIGURE 1 Changes in cellular protein and ferritin levels during differentiation of human monocyte/macrophages. Human monocytes were cultured in RPMI 1640 medium in the presence of 15% FCS for up to 8 days. At the times indicated, cells were washed with PBS at 37°C and lysed in "lysis buffer". Aliquots of the cell lysate were then analysed for total protein (closed circles) and ferritin (open circles). Data are the means of duplicates with range shown by the error bars. Two independent experiments were performed using monocytes isolated from different donors (A and B).

as well as their susceptibility to H_2O_2-induced cytotoxicity. We chose to focus on 4-day-old MDM as baseline ferritin levels were more stable at and beyond this time compared with earlier time points (Fig. 1). The data presented in Table I show the accumulation of intracellular iron in MDM cultured in the presence of ferric ammonium citrate (FeAC, 100 μg/ml, ie. approximately 100 μM with respect to Fe), $FeCl_3$ (50 μM) or growth medium alone for 4 days. The total iron levels present in MDM exposed to either source of iron were similar after approximately 4 days in culture (Table I). FeAC initially (in the first 48 h) induced a more rapid rise in iron levels compared to $FeCl_3$ (Garner, Roberg and Brunk unpublished data).

Both $FeCl_3$ and FeAC increased MDM ferritin levels substantially (Table I). The data presented in Table I show that after 4 days *in vitro*, there was approximately twice the amount of ferritin present in cells treated with FeAC compared to those treated with $FeCl_3$. While the greater degree of ferritin induction observed with FeAC was observed using monocytes isolated from unrelated donors, there was a considerable variation between the donors (Table I). Using data derived from Table I, ferritin was not likely to be saturated by iron (ie. 4500 iron atoms/ferritin molecule) under any of the culture conditions tested. The differing effect of these sources of iron on ferritin levels in day 4 MDM provided a model system to assess the efficacy of ferritin as a cytoprotective protein under conditions of H_2O_2-induced oxidative stress.

Iron loading alone did not affect cell viability (Fig. 3). MDM which were not treated with iron but which were given a bolus dose of 1 mM H_2O_2,

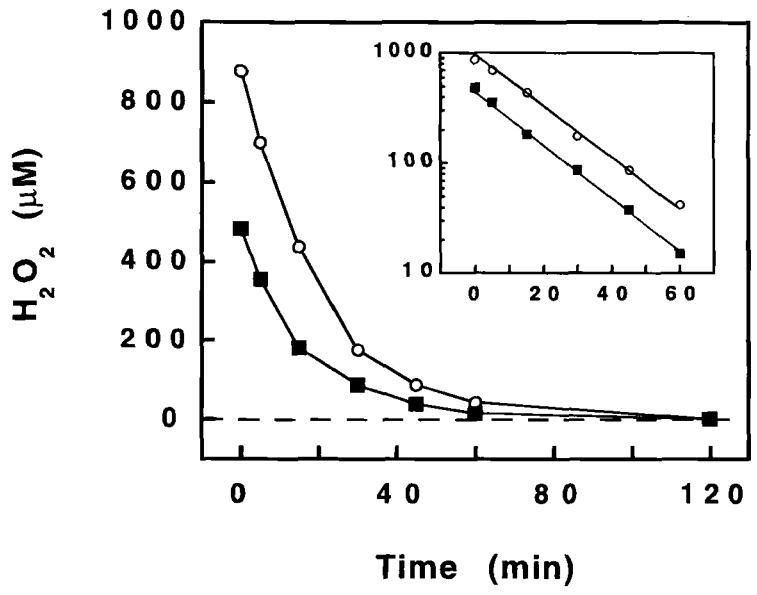

FIGURE 2 Degradation of H_2O_2 by human MDM. Human monocytes were cultured, and matured into macrophages, for 6 days. The cells were then washed with PBS at 37°C and incubated at 37°C in PBS containing either 1000 (circles) or 500 μM (squares) H_2O_2 for up to 2h. At the times indicated, a 10μl aliquot of the cell supernatant was removed and analysed for H_2O_2 content. The inset shows the same data with log transformed H_2O_2 concentrations on the vertical abscissa. H_2O_2 incubated in parallel culture dishes in the absence of MDM was not detectably degraded within 2h. Data represent the mean of duplicate values from one experiment representative of 2 independent experiments using monocytes from different donors. The range of the duplicate samples was less than 14% for all values, error bars are obscured by the symbols in all points shown. The inter-experimental variation did not exceed 6.5%.

suffered only a 10–15% loss of viability as assessed by the delayed trypan blue assay (Fig. 3). MDM which were treated with either $FeCl_3$ or FeAC were more sensitive to H_2O_2-induced cytotoxicity than control cells (Fig. 3) despite having an increased ferritin content and a lower intracellular iron to ferritin molar ratio (using data derived from Table I). While it is possible that the higher ferritin levels kept the cytotoxic effects of H_2O_2 at only modest levels, it appeared that the cellular iron content was a more important predictor of the cell's sensitivity to H_2O_2-induced damage than the ratio of cellular iron to ferritin.

TABLE I Characteristics of day 4 MDM cultured in the presence or absence of Fe

	Control		$FeCl_3$		FeAC	
	Ferritin	Fe	Ferritin	Fe	Ferritin	Fe
Donor						
1	1.48	0.38	6.45	0.65	12.5	0.68
	(0.18)	(0.02)	(0.32)	(0.23)	(0.78)	(0.01)
2	0.90	0.24	2.64	0.43	6.10	0.51
	(0.04)	(0.01)	(0.15)	(0.05)	(0.18)	(0.01)

Human monocytes were cultured for 4 days in RPMI 1640 medium with 15% FCS alone (Control), or with the addition of ferric chloride ($FeCl_3$, 50 μM) or ferric ammonium citrate (FeAC, 100 μM). The cells were then washed with PBS at 37°C lysed in "lysis buffer" and analysed for ferritin and total Fe by ELISA and atomic absorption spectroscopy respectively as described in the Methods section. Values are μg/mg cell protein and are means of duplicates with range in parentheses from two unrelated donors.

Exogenously Added Apoferritin Protects Macrophages from H_2O_2-Induced Cytotoxicity, and Increases Lysosomal Membrane Stability

A previous study has shown that when endothelial cells were supplied with apoferritin, their resistance to H_2O_2-induced cytotoxicity was significantly increased.[14] We also investigated the possibility that apoferritin supplementation could protect macrophages (and iron-loaded macrophages) from H_2O_2-induced cytotoxicity. For these studies, the macrophage-like J774 cell

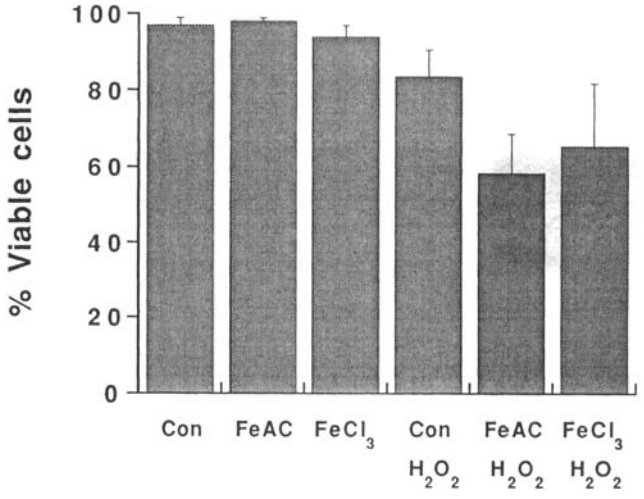

FIGURE 3 Chronic exposure of human monocyte/macrophages to iron increases their susceptibility to H_2O_2-induced cytotoxicity. Human monocytes were cultured for 4 days in the presence of 15% FCS alone (Con), or with the addition of ferric ammonium citrate (100μg/ml, FeAC) or ferric chloride (50μM, $FeCl_3$). The cells were then washed with PBS at 37°C and incubated at 37°C in PBS containing 1 mM H_2O_2 for 1h. After exposure to H_2O_2, the cells were returned to culture medium containing 15% FCS for a further 5h before staining for viability using trypan blue. Data are means of 2 independent experiments each performed in duplicate (5 to 10 determinations from each duplicate well for each condition). Error bars show range. The loss of viability induced by H_2O_2 treatment was statistically significant in both experiments ($P < 0.05$) as was the enhanced cytotoxic effect of culturing MDM in the presence of either FeAC or $FeCl_3$ ($P < 0.05$).

line was used. We have previously studied this cell line in detail and characterised its sensitivity to H_2O_2-induced cytotoxicity at a cellular and subcellular level.[12,13] Since these previous studies suggested an important role for lysosomal iron in H_2O_2-induced cytotoxicity, we focussed on $FeCl_3$ as an iron source. This is taken up by the cell as hydroxylated-iron-phosphate complexes which are initially directed to the lysosomal compartment.[16]

When J774 macrophages were treated with $FeCl_3$ (50 μM) for 24h followed by a 1h "chase" in culture medium (without iron added), a considerably enhanced lysosomal distribution of iron was demonstrated (Fig. 4). Figure 4 also demonstrates a slight increase in cytosolic iron, presumably due to iron which is newly incorporated into ferritin, and an absence of iron deposits associated with the plasma membrane or nucleus. J774 cells which were similarly treated with FeAC (50 μM, 24h) did not contain increased deposits of lysosomal iron but did exhibit slightly increased diffuse cytosolic positivity compared to the non-iron-supplemented controls (data not shown).

When J774 cells were given a bolus dose of 200 μM H_2O_2, a 40% loss of viability resulted (Fig. 5). This loss in viability tended to be less in cells that were pre-incubated with horse spleen apoferritin and was almost totally reversed by pre-treatment of the cells with the iron-chelator desferrioxamine (Fig. 5). The inhibition of cytotoxicity which was observed with desferrioxamine confirms our previous findings.[13] H_2O_2-induced cytotoxicity was significantly increased in cells that were iron-loaded (Fig. 5). When the cells were initially supplied with apoferritin or desferrioxamine and then iron-loaded, their susceptibility to subsequent H_2O_2-induced cytotoxicity was diminished (Fig. 5). Treatment of cells with BSA rather than apoferritin did not alter the susceptibility of iron-loaded cells to H_2O_2-induced cytotoxicity (data not shown). Pre-treatment of J774 cells with these agents did not affect their ability to load with iron as shown by the sulphide-silver technique (data not shown).

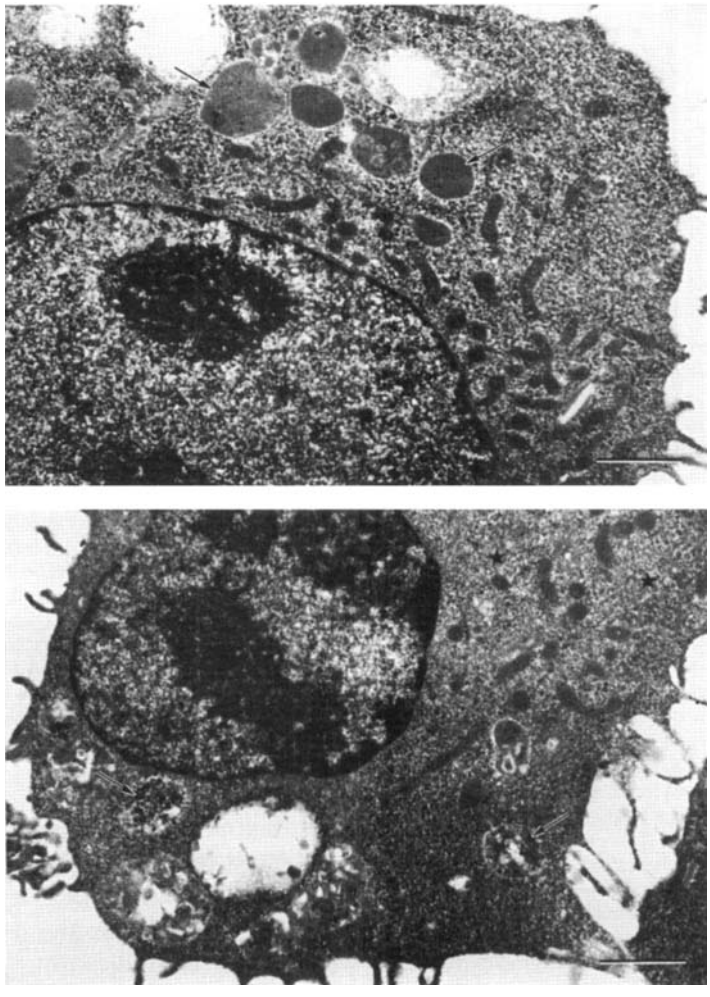

FIGURE 4 Cytochemical demonstration of iron in J774 macrophages exposed to culture medium ±$FeCl_3$. J774 macrophages were cultured for 24 h in medium alone **(A)** or in medium containing 50μM, $FeCl_3$ **(B)**. At the end of this period, the cells were washed with PBS at 37°C, and returned to ordinary culture medium for 1 h at 37°C before fixation in gluteraldehyde, exposure to ammonium sulphide, and physical development for the demonstration of heavy metals (iron) as described in the Methods section. A few secondary lysosomes with occasional silver precipitates, indicating Fe-sulphides, are arrowed in "A". Note the absence of reaction product within the nucleus, mitochondria and the cytosol, indicating that iron which is stably bound within metalloproteins is not reactive. In "B" two secondary lysosomes containing reaction product are arrowed, and areas with cytosolic reaction product (most likely ferritin with some newly incorporated, and therefore still-reactive, iron) are labelled with stars. Note the absence of reaction product along the plasma membrane. Bar = 500 nm.

We previously showed that desferrioxamine exerts at least part of its cytoprotective effects against H_2O_2-induced damage by chelating low-molecular-weight iron in the lysosome, and thus enhancing lysosomal membrane stability by preventing iron-catalyzed (Fenton-type) oxidative reactions.[13] In view of the cytoprotective effects observed with apoferritin supplementation in the present studies, we considered the possibility that apoferritin was also taken up by the cells and directed to the lysosomal compartment where it could also chelate low-molecular-weight iron. J774 cells which were treated with apoferritin for 16 h showed a dramatic increase in intracellular, immunodetectable (apo)ferritin which had a distinctly lysosomal pattern of distribution (Fig. 6).

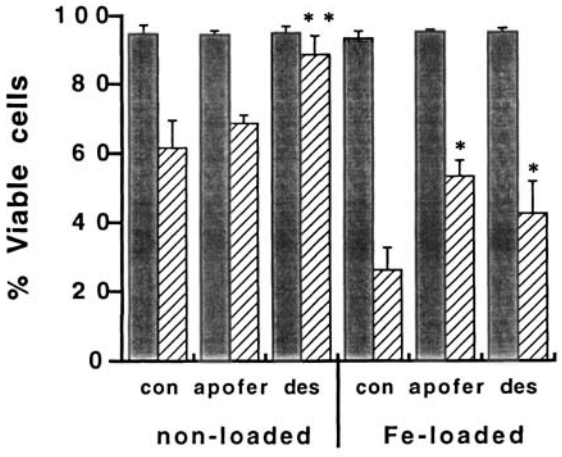

FIGURE 5 Cytoprotective effect of apoferritin and desferrioxamine in Fe-loaded macrophages exposed to H_2O_2. J774 macrophages were cultured for 24h after subcultivation in the presence of 10% FCS. The cells were then cultured for a further 16h period in medium alone (con), or in the additional presence of horse spleen apoferritin (2mg/ml, apofer), or for the last hour of the 16h period in the presence of desferrioxamine (1mM, des). The cells were then washed and treated with medium alone (non-loaded) or medium containing ferric chloride (50μM, Fe-loaded) for 1h. After a further wash in PBS, the cells were incubated in the absence (solid bars) or presence (hatched bars) of 200 μM H_2O_2 in PBS for 60 min. The cells were briefly rinsed with PBS at 37°C and incubated in medium alone for a further 5h period. Cell viability was then assessed by measuring LDH activity in the cell supernatants. The amount of total cellular LDH activity was assessed after lysing cells in 0.1% triton X100. Asterisks indicate significant differences between the apoferritin or desferrioxamine treated cells with control conditions in both the non-loaded and Fe-loaded groups exposed to H_2O_2. Data represent the means and S.E. of 3 experiments each performed in triplicate. *, $P < 0.05$; **, $P < 0.01$

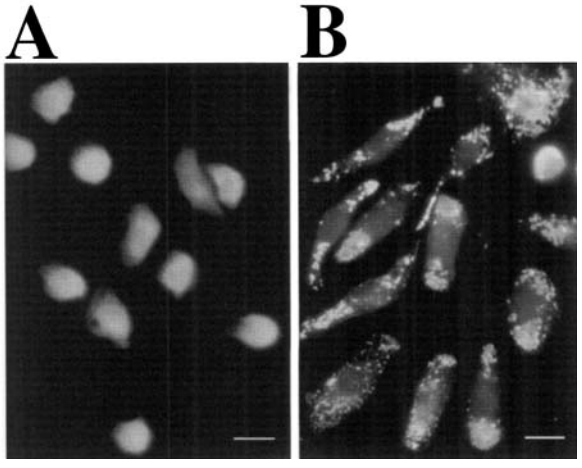

FIGURE 6 Immunofluorescence demonstration of lysosomal (apo)ferritin in apoferritin-loaded macrophages. J774 macrophages were cultured for 24h after subcultivation in the presence of 10% FCS. They were then cultured for a further 16h period under ordinary conditions **(A)**, or in the additional presence of horse spleen apoferritin (2mg/ml, **B**). The cells were then treated with rabbit anti-human ferritin polyclonal antibody followed by anti-rabbit IgG Texas Red conjugate as described in "Methods" before examination under a fluorescence microscope.Bar = 20 μm.

The fact that the protein was recognised by the antibody employed, implies that at least a proportion of the protein remained intact. The diffuse positive staining in both the control and apoferritin-treated cells is due to the presence of cytosolic ferritin which is normally present in many cell types.

We next assessed lysosomal stability in iron-loaded J774 cells that had been exposed to a bolus dose of H_2O_2 and which were also pre-treated with apoferritin. In the iron-loaded cells, lysosomal damage was significantly increased during exposure to H_2O_2 compared to non-loaded control cells which were also given H_2O_2 (Fig. 7). Pre-treatment of the cells with apoferritin significantly reduced the lysosomal damage observed in the iron-loaded cells (Fig. 7). Note that at incubation times greater than 1h after acridine-orange staining, some fluorescence was also lost from the control cells (exposed to H_2O_2 only). This is most likely due to the active exocytosis (characteristic of macrophages) of lysosomal acridine-orange after prolonged incubation, and thus indicates that the technique is more accurate at the earlier times studied for this cell type. Taken together, the results suggest that, like desferrioxamine, exogenously added apoferritin may reduce the cytotoxic effect of H_2O_2 by chelating intralysosomal iron, thus suppressing Fenton-type reactions, and ultimately preserving lysosomal membrane integrity.

DISCUSSION

Ferritin, due to its high capacity to chelate iron and convert it to the ferric state, could serve an impor-

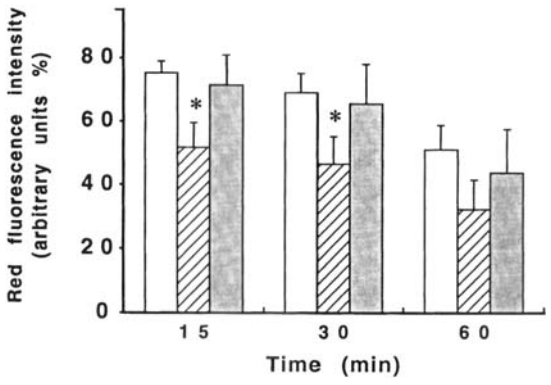

FIGURE 7 Apoferritin stabilises lysosomal membranes in Fe-loaded macrophages. J774 macrophages were cultured and treated with medium only (open bars), $FeCl_3$ (cross hatched bars) or $FeCl_3$ with apoferritin (solid bars) as described in the legend to Fig. 5. Cells were then stained with acridine orange for 15 min at 37°C, rapidly rinsed in PBS at 37°C and exposed to 200 μM H_2O_2 in PBS at 37°C for the times indicated. Intensity of red fluorescence was measured by static cytofluorometry. An asterisk indicates significant differences ($P < 0.05$) compared to the "medium only" condition at each time point. Data represent the means and S.E. of 4 separate experiments.

tant role as a cytoprotective protein. [15] In this way iron would presumably not be as readily available for participation in Fenton-type reactions[25] and would thus be limited in its capacity to catalyze oxidative reactions in the presence of H_2O_2. However, ferritin-derived iron may also be a potential *catalyst* for oxidative reactions (ie. leading to $HO^{\bullet}$ formation) when cells are exposed to cytotoxic amounts of H_2O_2.[26] Furthermore, the reductive release of a very small amount of iron from horse spleen ferritin (using $O_2^{\bullet -}$ as a reductant) has been demonstrated.[27] The latter work suggested that it was only the iron which was newly-incorporated into the core of the ferritin molecule, ie. that which is available for chelation by desferrioxamine and is also released after reduction with $O_2^{\bullet -}$, which was available to participate in Fenton reactions.[27] It was therefore not clear to what extent ferritin could be cytoprotective under conditions of iron overload. Since macrophages are able to scavenge iron from a variety of sources,[2–6] are present at inflammatory sites *in vivo* where they and other cells produce H_2O_2,[28] and finally, have been identified as iron-loaded in pathological conditions in humans,[16,17] it seemed important to know what degree of cytoprotection is afforded by increased ferritin levels in this cell type under conditions of iron-overload.

The present studies showed that during the first two days of monocyte differentiation, ferritin levels increased (on a per cell protein basis) then decreased and remained stable beyond four days. The ratio of iron to ferritin was lower in differentiated MDM compared to monocytes. This change in ferritin levels with differentiation is similar to the changes which occur in the ability of monocyte/macrophages to produce H_2O_2[28] and, therefore, could be considered to be beneficial to the cell if the relative amounts of iron to ferritin were predictive of the cytotoxicity of H_2O_2.[14] The high capacity of MDM to degrade H_2O_2 is also an important cytoprotective mechanism. The present studies showed that MDM could withstand doses of H_2O_2 up to 1mM with only a 10–15% loss of viability. However, H_2O_2 degradation was clearly not sufficient to protect the cells when they were iron-loaded. The kinetic studies indicated that the H_2O_2-degrading mechanism of MDM was not easily saturated, consistent with a role for catalase.[29]

When MDM were exposed to $FeCl_3$ or FeAC for up to 8 days, their resistance to H_2O_2 cytotoxicity was significantly reduced. Since we have previously shown that increasing the "chelatable pool" of intracellular Fe in J774 macrophages does not alter their capacity to degrade H_2O_2[30]

and others have shown that Fe-loading of human MDM does not affect catalase or glutathione peroxidase levels[9] we favour the hypothesis that the Fe is sensitising the cell to H_2O_2-induced toxicity by increasing the extent of free radical production within the lysosomal compartment.[13] The sensitisation observed in the present studies occurred despite large increases in the amounts of cellular ferritin present; both in absolute terms and in relation to cellular iron content. While the degree of sensitisation may have been limited by both the cell's ability to degrade H_2O_2 and by the induction of ferritin synthesis, the data indicate that the increase in MDM ferritin levels which occurs as a result of macrophage iron uptake was not sufficient to completely protect the cell from H_2O_2-induced damage. Using a different experimental system, endothelial cell ferritin content was shown to be positively correlated with cytoprotection against hemin-H_2O_2 induced damage.[14] Several differences between these studies may explain why ferritin induction was protective in the endothelial cells but not in the MDM; eg. cell type and iron source. Another important difference between these studies is that in the endothelial cell study the cytoprotective effects of ferritin were demonstrated by pulsing cells with hemin (to induce ferritin synthesis) then incubating the cells for a further 15h before sensitisation and exposure to H_2O_2.[14] A pulse-induction experimental approach is difficult to interpret using the macrophage model as when iron-containing media are removed from macrophages *in vitro*, they have a demonstrated capacity to divest themselves of iron; most likely in the form of ferritin.[6,20,31]

In contrast to the lack of effect of iron-induced ferritin induction, exogenously added apoferritin did protect J774 macrophages from H_2O_2-induced damage in the present studies. The protective effect afforded by apoferritin was similar to that observed by pre-treating macrophages with desferrioxamine. Previous studies from this group strongly suggest that desferrioxamine added to culture medium is endocytosed, transported to lysosomes, and thereby inhibits H_2O_2-induced cytotoxicity in J774 macrophages by chelating a lysosomal pool of low-molecular-weight iron.[13,32] That lysosomal iron is sufficient to mediate the cytotoxic effects of H_2O_2 was also demonstrated by earlier studies which showed that cells loaded with iron-dextran (and therefore with iron localised within lysosomes only) were sensitised to H_2O_2-induced damage.[7] We were therefore prompted to investigate the ability of apoferritin to stabilise lysosomes of iron-loaded macrophages during H_2O_2 exposure. For this purpose we used the lysosomotropic weak base, acridine orange.[12] Acridine orange relocalisation indicates the loss of the proton gradient over the lysosomal membrane and is a sensitive assay for lysosomal membrane stability. We have previously shown that a decrease in red, granular acridine orange-induced fluorescence in J774 cells following exposure to H_2O_2 is correlated with: a loss of retention of lysosomal lucifer yellow or neutral red stains, leakage of cathepsin D from lysosomes to the cytosol, plasma membrane blebbing, and loss of cell viability.[13]

Treatment of macrophages with apoferritin prior to iron-loading resulted in the appearance of immunologically detectable (and therefore presumably not severely degraded) (apo)ferritin which was concomitant with significantly reduced H_2O_2-induced relocalisation of acridine orange. Apoferritin thus appeared to be cytoprotective by virtue of its ability to stabilise the lysosomal membrane, most likely by preventing iron-catalyzed oxidative damage to membrane lipids and/or proteins. The lack of protection afforded by apoferritin supplementation of non-iron-loaded macrophages could suggest that the normal amount of lysosomal iron in these cells is already complexed to a maximal degree in ferritin and haemosiderin.

Our results could also explain why endothelial cells are more resistant to H_2O_2-induced damage after exposure to apoferritin.[14] It is noteworthy that in the latter studies, treatment of endothelial cells with apoferritin led to a coarse granular dis-

tribution of intracellular ferritin (ie. a lysosomal pattern of distribution). The physiological relevance of apoferritin uptake by cells (most likely by fluid phase endocytosis in extrahepatic tissue) under oxidative stress *in vivo* is uncertain at present. However, it should be noted that small amounts of ferritin do circulate in plasma and while about 50% of the mass of Fe-saturated ferritin can be attributed to Fe, serum ferritin has a relatively low Fe content with only about 2–7% of the mass due to Fe.[33] It may be that circulating ferritin could also play a cytoprotective role if it were taken up by Fe-loaded macrophages. Studies which demonstrate the presence of extracellular apoferritin (or ferritin with low a Fe/protein ratio) at sites of inflammation, or iron-overload, would help to clarify these issues.

The increased sensitivity of iron-loaded macrophages to H_2O_2-induced damage could be particularly important in pathological conditions where a chronic inflammatory response is accompanied by the presence of iron-loaded macrophages. One example of such a condition is atherosclerosis. We have recently shown that macrophages in human atherosclerotic tissue contain lysosomal deposits of iron and cytosolic ferritin.[16] Other recent studies have shown that nitric oxide is a potent inhibitor of catalase and can therefore increase cellular sensitivity to H_2O_2-induced damage.[34] Since it is likely that endothelial-derived nitric oxide exists in the subendothelial layers of arteries, iron-loaded macrophages may be especially sensitive to oxidant mediated cytotoxicity in the intima, and this may contribute to the formation of a necrotic core of cellular debris and lipid as the disease progresses.

In summary, the present studies indicate that iron-induced ferritin synthesis in human MDM is not sufficient to completely protect the iron-loaded cell from the cytotoxic effects of H_2O_2. Furthermore, the relative amounts of cellular iron to ferritin do not predict the sensitivity of the cell to damage induced by H_2O_2. We also demonstrate that apoferritin protects iron-loaded macrophages from H_2O_2-induced cytotoxicity, at least partially, by stabilising lysosomal membranes.

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