Relocalized redox-active lysosomal iron is an important mediator of oxidative-stress-induced DNA damage

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Oxidative damage to nuclear DNA is known to involve site-specific Fenton-type chemistry catalysed by redox-active iron or copper in the immediate vicinity of DNA. However, the presence of transition metals in the nucleus has not been shown convincingly. Recently, it was proposed that a major part of the cellular pool of loose iron is confined within the acidic vacuolar compartment [Yu, Persson, Eaton and Brunk (2003) *Free Radical Biol. Med.* **34**, 1243–1252; Persson, Yu, Tirosh, Eaton and Brunk (2003) *Free Radical Biol. Med.* **34**, 1295–1305]. Consequently, rupture of secondary lysosomes, as well as subsequent relocation of labile iron to the nucleus, could be an important intermediary step in the generation of oxidative damage to DNA. To test this concept we employed the potent iron chelator DFO (desferrioxamine) conjugated with starch to form an HMM-DFO (high-molecular-mass DFO complex). The HMM-DFO complex will enter cells only via fluid-phase endocytosis and remain within the acidic vacuolar compartment, thereby chelating redox-active

iron exclusively inside the endosomal/lysosomal compartment. Both free DFO and HMM-DFO equally protected lysosomal-membrane integrity against H₂O₂-induced oxidative disruption. More importantly, both forms of DFO prevented H₂O₂-induced strand breaks in nuclear DNA, including telomeres. To exclude the possibility that lysosomal hydrolases, rather than iron, caused the observed DNA damage, limited lysosomal rupture was induced using the lysosomotropic detergent *O*-methyl-serine dodecylamine hydrochloride; subsequently, hardly any DNA damage was found. These observations suggest that rapid oxidative damage to cellular DNA is minimal in the absence of redox-active iron and that oxidant-mediated DNA damage, observed in normal cells, is mainly derived from intralysosomal iron translocated to the nucleus after lysosomal rupture.

Key words: DNA damage, hydrogen peroxide, lysosomal iron, lysosome, oxidative stress, telomere.

INTRODUCTION

It is generally believed that most DNA mutations that are known to induce malignancies, and also other diseases, are a consequence of cellular oxidative stress. The chemical reactions that bring about such mutations are based on the formation of the highly reactive and short-lived hydroxyl radical (HO•) in close proximity to DNA [1–7].

Normally, ROS (reactive oxygen species), such as superoxide (O₂•[−]) and H₂O₂, are continuously generated in amounts that are adequately compensated by cellular antioxidant systems [1–4,8]. However, under a variety of conditions, ROS may be overproduced. Under such circumstances, oxidative stress may overwhelm intrinsic defence mechanisms [1,3,9]. Among the different ROS, H₂O₂ plays a crucial role since it diffuses freely across cellular membranes and undergoes homolytic cleavage when interacting with transition metals, such as iron or copper (Fenton chemistry). Hydroxyl radicals are then formed [1–4].

Since Fenton-type reactions engage H₂O₂ and transition metals in site-specific reactions, resulting in hydroxylation or other DNA modifications, it is widely assumed that such metals in redox-active form are originally bound to nDNA (nuclear DNA) or released at the site of the reaction due to the influence of H₂O₂ on metal-containing nuclear enzymes [2–4,10,11]. However, this is by no means certain.

It has been suggested that a major part of the cellular pool of low-molecular-mass, labile and reactive iron exists within

lysosomes [12–15], which are responsible for the degradation of all organelles and most long-lived proteins. Recently, using ESR techniques in combination with lysosomotropic iron chelators or by inhibiting intralysosomal proteolysis by increasing lysosomal pH, it was found that most, if not all, of the cellular ‘loose’ iron originates in the acidic vacuolar compartment [16,17]. Thus we hypothesized that the relocation of lysosomal iron might be involved in oxidative-stress-dependent DNA damage. It has been repeatedly shown that lysosomes are particularly vulnerable to oxidative stress and may burst due to intralysosomal Fenton-type reactions with ensuing peroxidative-membrane destabilization [18–20]. Such a rupture, if moderate and associated with the release of a modest amount of hydrolytic enzymes, induces co-ordinated apoptotic cell death, whereas a more extensive rupture is followed by severe, unregulated degradation and necrosis [20–23]. As explained earlier, lysosomal rupture would release not only lytic enzymes into the cytosol, but also iron, which might relocate to the nucleus and cause site-specific induction of hydroxyl radicals if the oxidative stress situation is sustained.

Support for the importance of intralysosomal iron in oxidant-dependent cellular damage includes the observation that the iron chelator DFO (desferrioxamine), which binds all six co-ordination sites of iron and thereby makes it inert [24], will protect cells against oxidant challenge [16–20]. Being of hydrophilic character and with a molecular mass of > 0.6 kDa, DFO is taken up predominantly via endocytosis and localizes almost exclusively within the lysosomal compartment, where it seems to remain

Abbreviations used: AO, Acridine Orange; DFO, desferrioxamine; FADU, fluorescence-detected alkaline DNA unwinding; HMM-DFO, high-molecular-mass DFO; MSDH, *O*-methyl-serine dodecylamine hydrochloride; nDNA, nuclear DNA; ROS, reactive oxygen species.

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[19,25–28]. Hence, it is probable that the protection against oxidative cell killing afforded by DFO derives from the control of reactivity of the intralysosomal iron pool.

To test this hypothesis rigorously, we examined the protective capacity of HMM-DFO (high-molecular-mass DFO complex, molecular mass, ~ 75 kDa) coupled with starch. Neutral molecules of this mass are taken up via fluid-phase endocytosis, and the modified (hydroxyethyl) starch backbone remains undegraded within the lysosomal compartment. We found that both low- and high-molecular-mass DFOs stabilize lysosomes against oxidative stress, and protect DNA equally well. This suggests that DFO largely functions to chelate endosomal and/or lysosomal redox-active iron and the latter is a critical effector of oxidative-stress-induced DNA damage. This conclusion is further substantiated by the finding that the rupture of lysosomes caused by a lysosomotropic detergent, MSDH (*O*-methyl-serine dodecylamine hydrochloride) [21], showed hardly any early DNA damage, excluding the possibility that a combination of lysosomal hydrolases is responsible for the early DNA damage observed after oxidative stress.

EXPERIMENTAL

Chemicals

RPMI 1640, L-glutamine, penicillin, streptomycin, H_2O_2 , PBS, SYBR[®] Green I, S1 nuclease and DFO mesylate were obtained from Sigma–Aldrich (Poole, Dorset, U.K.). Foetal bovine serum was purchased from Labtech (Ringmer, U.K.). HMM-DFO was a gift from Biomedical Frontiers (Minneapolis, MN, U.S.A.). HMM-DFO has a molecular mass of approx. 75 kDa and the solution obtained was 40 mM with respect to DFO. AO (Acridine Orange) hydrochloride hydrate was obtained from Fluka (Buchs, Switzerland) and proteinase K and *HinfI* from Roche (Lewes, East Sussex, U.K.). Low-melting agarose and pulsed-field agarose were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.) and fluorescein and tetramethylrhodamine double-labelled dextran (70 kDa) were from Molecular Probes (Eugene, OR, U.S.A.). MSDH was a gift from Dr Gene Dubowchick (Pharmaceutical Research Institute, Bristol-Myers Squibb, Wallingford, CT, U.S.A.).

Cell culture and exposure to DFO, HMM-DFO, MSDH and H_2O_2

Acute lymphoblastic T cells 1301 (Dako, Copenhagen, Denmark) were grown in suspension in RPMI 1640, supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37 °C in humidified air containing 5 % CO_2 . Cells were subcultured twice a week.

Optimal concentrations and exposure times for DFO- and HMM-DFO-induced protection against oxidative stress were established in preliminary experiments. In the final experiments, cells were exposed (under otherwise standard culture conditions) to fresh and complete medium with or without 1–3 mM DFO or HMM-DFO (the latter expressed in DFO equivalents) for up to 6 h.

After a brief wash with PBS, cells were oxidatively stressed for 30 min at 37 °C by exposure to a bolus dose of 50 μM H_2O_2 in PBS (5×10^5 cells/5 ml of PBS). Note that, under these conditions, the H_2O_2 concentration decreases quickly and is almost depleted after 30 min [18,19,23]. Cells degraded H_2O_2 equally well irrespective of pretreatment with or without DFO or HMM-DFO (results not shown). Immediately after exposure to H_2O_2 , cells were assayed for DNA damage or returned to standard culture

conditions and further analysed for DNA damage/repair and lysosomal stability after various periods of time.

In control experiments, designed to exclude DNA damage due to release of lysosomal hydrolytic enzymes, untreated cells or cells pretreated for 2 h with 3 mM DFO were exposed for 30 min, under otherwise standard conditions, to the lysosomotropic detergent MSDH (75 and 100 μM), as described previously [21]. As a result of MSDH exposure, lysosomes ruptured extensively.

Assay of DNA damage

DNA damage was assessed using a semi-automated FADU (fluorescence-detected alkaline DNA unwinding) assay developed recently [29], modified from the assay described by Birnboim and Jevcak [30]. The method detects single- and double-strand breaks by their effect on the rate of DNA denaturation in alkali, monitored by the fluorescence intensity of an intercalating dye. Briefly, cells from each sample were lysed in 0.25 M *meso*-inositol, 1 mM MgCl_2 and 10 mM $\text{Na}_2\text{PO}_4/\text{NaH}_2\text{PO}_4$ (pH 7.2); subsequently, 70 μl (7×10^4 cells) was transferred on to a custom-made 96-well plate and 70 μl of denaturation buffer [9 M urea/10 mM NaOH/25 mM CDTA (*trans*-1,2-diaminocyclohexane-*N,N,N'*, *N'*-tetra-acetic acid)/0.1 % SDS] and 70 μl of alkali solution (200 mM NaOH, 40 % denaturation buffer) was added. All additions were performed at 4 °C and each sample was analysed by 8–10 replicate experiments. Partial alkaline DNA unwinding, starting from DNA ends and strand breaks, was activated under controlled alkaline conditions for 40 min at 30 °C and it was stopped by the addition of 140 μl of neutralization solution (1 M glucose/15 mM 2-mercaptoethanol). The remaining double-stranded DNA was then stained by adding 470 μl of the intercalating dye SYBR[®] Green (diluted 1:25 000 in 13 mM NaOH) per well and incubating for 7 min at ambient temperature. Fluorescence was measured using a fluorescence reader Spectrafluor Plus (Tecan, Crailsheim, Germany) at $\lambda_{\text{ex}} = 492$ nm and $\lambda_{\text{em}} = 520$ nm. The fluorescence intensity was inversely correlated to the number of DNA strand breaks present at the time of lysis.

Measurement of telomere-specific single-strand breaks

Frequency of single-strand breaks in telomeres was measured by S1 nuclease assay as described in [31]. Briefly, DNA was isolated by standard methods for pulsed-field gel electrophoresis. Cell pellets were embedded in low-melting agarose plugs and digested with proteinase K. The DNA was digested until completion with *HinfI* (60 units/plug) at 37 °C. Plugs were treated with S1 nuclease for 5 h at 1 or 2 units/ μg DNA in a buffer containing 33 mM sodium acetate, 50 mM NaCl and 1 mM ZnCl_2 (pH 4.5) before electrophoresis [32,33]. Single-stranded sites are cut by S1 nuclease, and the accumulation of such sites in DNA results in a progressive shift of the average hybridization signal to lower molecular masses with increasing S1 nuclease concentration. Plugs were analysed on a 1 % agarose gel by pulsed-field gel electrophoresis (CHEF-DRIII-System; Bio-Rad Laboratories) at 6 V/cm for 15 h with a switching time of 2–10 s in $0.5 \times \text{TBE}$ (4.5 mM Tris/4.5 mM borate/0.1 mM EDTA, pH 8.0). The gel was dried for 1 h on a gel dryer 583 (Bio-Rad Laboratories) and hybridized overnight with the telomeric probe $(\text{TTAGGG})_4$, which was end-labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Signals were visualized using a PhosphorImager (Storm 820; Molecular Dynamics, Sunnyvale, CA, U.S.A.; Amersham Biosciences, Piscataway, NJ, U.S.A.). Mean fragment length per lane was calculated as weighted mean of the absorbance value, as described in [34], using AIDA densitometry software (Raytek, Sheffield,

Figure 1 Accumulation of neutral high-molecular-mass compounds in lysosomes of 1301 cells

Cells were incubated with dextran (molecular mass of 70 kDa) double-labelled with fluorescein and tetramethylrhodamine. After 3 h under standard culture conditions, strong red (rhodamine) and weak green (fluorescein) punctuated cytoplasmic fluorescence was found, indicating the accumulation of the labelled dextran in cytoplasmic organelles of low pH. BF, phase-contrast bright field image.

U.K.). Frequency of S1-sensitive sites (n_x) per Mb of DNA for an S1 nuclease concentration of x units/ μ g of DNA was calculated using the relation

$$n_x = \frac{L_0 - L_x}{L_0 L_x} \quad (1)$$

where L_0 is the mean fragment length of DNA (in Mb) without S1 nuclease treatment and L_x the mean fragment length at a nuclease concentration of x units.

Assay of lysosomal-membrane stability

AO, which is a metachromatic fluorophore (red at high concentrations and green at low concentrations) and a lysosomotropic base ($pK_a = 10.3$), is retained in its charged form (AOH^+) by proton trapping mainly inside the acidic vacuolar compartment, preferentially in secondary lysosomes (pH 4–5). Cells in complete medium were preloaded with AO (5 μ g/ml for 15 min, followed by two washes with PBS to diminish cytosolic AOH^+ , leaving almost all AO retained in the acidic vacuolar compartment as AOH^+). The cells then show intense granular (lysosomal) red fluorescence with faint green cytosol and nuclei. On such AO-loaded cells, flow cytometry was used for monitoring early lysosomal rupture and the resulting AO relocation, with ensuing increased green cytoplasmic and nuclear fluorescence due to oxidative stress (the AO relocation method [17–19,21,35]). In other experiments, cells were stained with AO (as described above) either 5 h after the end of oxidative stress and return of the cells to standard culture conditions (the AO uptake method [17–19,21,35,36]) or immediately after treatment with MSDH. Using the AO uptake method, cells with a decreased number of intact AO-accumulating lysosomes ('pale' cells) were detected by their diminished red fluorescence. Green (FL1 channel) and red (FL3 channel) fluorescence were recorded in logarithmic scale, using a PAS flow cytometer (Partec, Münster, Germany) equipped with a 488 nm exciting argon laser and appropriate barrier filters. FlowMax software (Partec) was used for image acquisition and analysis.

Endocytosis assay

After incubation of 1301 cells for 3 h in standard medium containing 1 mg/ml fluorescein and tetramethylrhodamine double-labelled dextran (70 kDa), uptake into the cells was assessed

using a Nikon Eclipse E1000 fluorescence microscope (Nikon, Kanagawa, Japan) connected to a DVC-1312C digital video camera (DVC, West Austin, TX, U.S.A.).

Statistical analysis

Results are given as means $\pm$ S.E.M. Statistical comparisons were made using ANOVA. Pairwise multiple comparisons were made by the Tukey HSD (honestly significantly different) test ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

RESULTS

DFO protects cells from oxidant-mediated damage to both lysosomes and DNA. It is well proven that DFO is taken up into cells via endocytosis [19,25–28] and thus compartmentalized in the lysosomes. It is possible that chelation of the redox-active iron present in lysosomes and prevention of lysosomal-membrane breakdown are major pathways to DFO-mediated protection of DNA. However, it might also be possible that a fraction of the DFO is released from lysosomes, traverses the nuclear membrane and protects DNA from oxidative attack by chelating the iron already present in the immediate vicinity of DNA.

To discriminate between these possibilities, we examined the protection of cells by HMM-DFO. This complex, with a molecular mass of 75 kDa, is much less likely to be released from lysosomes and to reach the nucleus. In fact, fluorescence-labelled dextran with a similar molecular mass shows a distinct cytoplasmic granular staining pattern without any nuclear staining even after > 3 h incubation, indicative of stable lysosomal localization (Figure 1).

We next examined the protection of lysosomes by DFO and HMM-DFO against oxidative damage. Incubation of 1301 cells with 50 μ M H_2O_2 for 30 min induced significant lysosomal membrane rupture as illustrated by an approx. 2.5-fold increase in green AO fluorescence, indicating relocation of AO from lysosomes into the cytoplasm (Figure 2A). Most of this relocation was blocked by exposure to DFO for 2–3 h at concentrations between 1 and 3 mM. HMM-DFO was as effective as DFO at 2 and 3 mM, although 1 mM HMM-DFO still did not offer full protection (Figure 2A). We also examined the long-term stability of lysosomal membranes after oxidative challenge. 1301 cells were pre-incubated with 3 mM DFO or HMM-DFO, treated with different concentrations of H_2O_2 for 30 min and the integrity of the lysosomal membrane was measured for 5 h after the treatment by assessing the red (lysosomal) fluorescence intensity per cell

Figure 2 Preservation of lysosomal membranes from oxidative stress by endocytosed DFO and HMM-DFO

(A) 1301 cells were left unprotected or were pre-incubated for 3 h at the indicated concentrations with either DFO or HMM-DFO (HMM), preloaded with AO and then exposed to a bolus dose of 50 μM H₂O₂ for 30 min. Green fluorescence intensity was then measured and expressed as a percentage of that of the unprotected, unexposed cells. Results are means ± S.E.M., *n* = 5. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significant differences compared with unprotected, H₂O₂-exposed cells. (B) Cells were left unprotected or pre-incubated for 3 h with 3 mM DFO or HMM-DFO (HMM), exposed to H₂O₂ as above at the indicated concentrations and returned to standard conditions for another 5 h. AO uptake by intact lysosomes was then measured and expressed as red fluorescence intensity as a percentage of that of the unprotected, unexposed cells. Other cells were exposed to MSDH for 30 min to rupture lysosomes independent of oxidative stress, and AO uptake was measured immediately thereafter. Results are means ± S.E.M., *n* = 3–5. Statistical comparisons for H₂O₂-exposed cells, pre-incubated cells and MSDH-treated cells were made separately. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significant differences compared with unexposed cells. Differences between DFO and HMM-DFO were not significant.

after AO loading (Figure 2B). H₂O₂ treatment decreased red fluorescence intensity in a concentration-dependent manner. This decrease was completely blocked by either DFO or HMM-DFO. We conclude that HMM-DFO, at least at ≥ 2 mM, is as efficient as DFO in protecting lysosomal membranes of 1301 cells.

To measure DNA damage, we used a robotized version of the FADU assay, which detects DNA single- and double-strand breaks based on their acceleration of alkaline DNA denaturation [29,30]. A 50 μM concentration of H₂O₂ produced an amount of strand breaks that nearly saturated the assay when it was performed im-

Figure 3 Protection against oxidative-stress-induced DNA strand breaks by DFO and HMM-DFO

(A) 1301 cells were left unprotected or were pre-incubated for 3 h with either DFO or HMM-DFO (HMM) at indicated concentrations and then exposed to a bolus dose of 50 μM H₂O₂ for 30 min. Nuclear strand breaks were then immediately assessed by a robotized FADU assay as percentage of SYBR® Green fluorescence in unprotected, unexposed cells. After the oxidative stress period, some cells were returned to standard culture conditions for 4 h before DNA assay (50 + 4 h). Other cells were exposed to MSDH for 30 min to obtain lysosomal rupture in the absence of oxidative stress. Statistical comparisons for pre-incubated cells, repair and MSDH-exposed cells were made separately. Results are means ± S.E.M., *n* = 3 or 4. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significant differences compared with unprotected, H₂O₂-exposed cells. (B) Cells were left unprotected or were pre-incubated with 3 mM DFO or HMM-DFO (HMM) for the indicated periods of time and then exposed for 30 min to a bolus dose of 50 μM H₂O₂. DNA strand breaks were assayed as in (A). Results are means ± S.E.M., *n* = 4 or 5. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significant differences compared with unprotected, H₂O₂-exposed cells.

mediately after oxidative stress. However, if cells were returned to normal culture conditions for a few hours, almost all DNA damage was found to be repaired (Figure 3A). This efficient nDNA repair suggests that the observed nDNA damage was not due to the activity of released lysosomal hydrolytic enzymes (if so, nDNA damage would be expected to increase with time). Pretreatment with DFO had a highly significant concentration-dependent protective effect and 3 mM DFO essentially abolished any H₂O₂-induced DNA damage (Figure 3A). Interestingly, HMM-DFO also showed a significant concentration-dependent protection of DNA against H₂O₂-induced strand breaks, although the efficiency of protection was lower (Figure 3A). This

lower efficiency could be partly compensated for by longer pre-incubation times (Figure 3B).

As reported previously [21], MSDH (75 and 100 μ M; 30 min) ruptures lysosomes. MSDH was as efficient as H_2O_2 in inhibiting the uptake of lysosomal AO (Figure 2B). By itself, such rupture caused hardly any significant nDNA damage (Figure 3A), whereas the ensuing exposure to 50 μ M H_2O_2 resulted in DNA damage as extensive as that caused by H_2O_2 alone (Figure 3 and results not shown). This shows (in agreement with the repair findings reported above) that oxidative-stress-induced nDNA damage is not due to the activity of released lysosomal hydrolases, but rather to relocation of lysosomal iron.

Telomeres are particularly vulnerable to oxidative stress [32, 37], and they play an important role as triggers for stress responses and senescent cell-cycle arrest [38]. Therefore we wanted to know whether DFO and HMM-DFO would protect not only total nDNA but also telomeres against oxidative-stress-induced single-strand breaks. Single-strand breaks were converted into double-strand breaks by treatment with low concentrations of S1 nuclease, and the size of the resulting telomeric fragments was measured by pulsed-field gel electrophoresis and hybridization to a telomeric probe (Figure 4A). Frequencies of strand breaks in telomeric DNA were calculated (Figure 4B). Our results show a significant protection of telomeric DNA against H_2O_2 -induced single-strand breaks by both DFO and HMM-DFO, although protection by lower concentrations of HMM-DFO was significantly less efficient compared with DFO.

Taken together, our results show that HMM-DFO conveys significant protection for both total nDNA and telomeric DNA against H_2O_2 -induced strand breaks. However, it protects DNA less efficiently when compared with DFO.

DISCUSSION

DNA damage after oxidative stress is a well-recognized phenomenon in which redox-active transition metals are supposed to play an important role [6,11,39–44]. However, the exact mechanisms underlying the participation of such metals in the observed hydroxylation reactions are not well characterized. The current assumption is that during an oxidative stress situation, H_2O_2 diffuses throughout the cell and interacts with transition metals previously bound to nDNA. The extremely reactive hydroxyl radical ($HO^\bullet$) would then be generated, and site-specific DNA damage would follow [1–4]. There are indications, however, that the first interaction between H_2O_2 and 'loose' transition metals (mainly iron), resulting in Fenton-type chemistry, probably takes place in the acidic endosomal/lysosomal compartment and only indirectly results in damage to nDNA [16,17,28].

Lysosomes contain a comparatively high concentration of redox-active iron, which is mainly generated from the degradation of iron-containing proteins [12,16–20]. The low pH value and the reducing milieu of that compartment further facilitates iron-catalysed oxidative reactions [45,46]. Previous experimental evidence indicates that the interaction between H_2O_2 and iron results in reparative autophagocytosis or cell death, either by apoptosis or necrosis depending on the severity of the lysosomal rupture. Moderate lysosomal rupture results in apoptosis, whereas necrosis follows a more extensive rupture [20–22]. Apart from release of lysosomal enzymes to the cytosol, partial rupture of the acidic vacuolar compartment would also relocate lysosomal redox-active loose iron to the cytosol and the nucleus.

In the present study, we have specifically chelated endosomal/lysosomal iron in non-redox-active form and then examined the effects of H_2O_2 -mediated oxidative stress on the incidence of DNA

Figure 4 Protection against oxidative-stress-induced telomeric strand breaks by DFO and HMM-DFO

(A) Southern-blot analysis of telomeric DNA. Cells were left unprotected (CON) or were pre-incubated for 3 h with either DFO or HMM-DFO (HMW) at the indicated concentrations and then exposed to 50 μ M H_2O_2 for 30 min. For each experiment, DNA was treated with 0, 1 or 2 units of S1 nuclease. Mean telomere lengths are indicated by white bars. (B) Frequencies of single-strand breaks in telomeres (per Mb) were calculated as indicated in the Experimental section for both concentrations of S1 nuclease using the mean telomere lengths of four experiments. Results are means $\pm$ S.E.M. Statistical comparisons for both S1 nuclease concentrations were made separately. * P < 0.05, ** P < 0.01, *** P < 0.001, significant differences compared with unprotected, H_2O_2 -exposed cells.

damage. DFO is a rather specific iron chelator, which has been shown previously to accumulate intralysosomally after fluid-phase endocytosis [19,26,27]. We also used a high-molecular-mass variety of DFO (molecular mass of approx. 75 kDa) obtained by conjugating DFO with hydroxyethyl starch. This ensures that only cellular uptake via fluid-phase endocytosis would be possible. Since the starch backbone is not degradable by

lysosomal enzymes, this approach, moreover, ensures that DFO could not leave the acidic vacuolar compartment and, consequently, the effects would be due to chelation of endosomal/lysosomal iron only.

In short-term experiments, DFO has been shown previously to prevent lysosomal rupture and the resulting apoptosis/necrosis during oxidative stress [17–20]. In the present study, protection of lysosomes against oxidative-stress-induced rupture was observed with both DFO and HMM-DFO. Although both forms of DFO prevented lysosomal rupture equally well, HMM-DFO was slightly less efficient than DFO in the protection of total DNA, including telomeres, against H₂O₂-induced strand breaks. This difference cannot be due to a slower endocytotic uptake or a more rapid exocytosis, since lysosomes were equally well protected after 3 h pre-incubation with either compound, whereas strand break formation was not completely abolished even after 6 h pre-incubation with HMM-DFO.

The most obvious interpretation of our results is that, under non-stressed conditions, transition metals are absent from the nucleus or present in negligible amounts. The fact that most of the H₂O₂-initiated DNA damage can be blocked by chelating iron exclusively within the endosomal/lysosomal compartment strongly suggests that relocation of loose iron from this compartment is a major prerequisite for such damage. The possibility that released lysosomal hydrolases were responsible for the observed DNA damage was excluded by rupturing lysosomes with a lysosomotropic detergent, MSDH [21]. In this case, DNA damage only occurred if oxidative stress was superimposed on the lysosomal rupture and such damage could be prevented by pretreatment of the cells with DFO. Moreover, if cells were returned to normal culture for a few hours after oxidative stress, almost all the DNA damage was repaired; this would not have happened if the released lysosomal enzymes were responsible for the DNA damage.

Results of the present study, together with other recently published findings [16,17,28], allow us to suggest that a major part of cellular redox-active 'labile or loose' iron resides within the acidic vacuolar compartment from where it is transported into the cytosol. Labile cytosolic iron originates either from imported transferrin or is a result of autophagocytotic degradation of iron-containing metalloproteins, such as ferritin or cytochromes [16–20]. Loose cytosolic iron would then represent only a small fraction of the pool of labile iron under transport from the acidic vacuolar compartment to storage in ferritin or for use in the synthesis of iron-containing metalloproteins. As part of this hypothesis, it is envisaged that iron cannot be released and utilized in the cytosol directly from ferritin and that ferritin needs to be autophagocytosed and degraded before iron is liberated and transported into the cytosol for renewed storage in ferritin or incorporation in iron-containing proteins. In summary, results of the present study clearly indicate that a major part of cellular redox-active iron is located in the acidic vacuolar compartment and, secondary to lysosomal rupture, is a key mediator of H₂O₂-induced DNA damage.

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