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MODIFICATION OF THE *IN VITRO* CYTOTOXICITY OF HYDROGEN PEROXIDE BY IRON COMPLEXES

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The effect of a range of iron chelates on the cytotoxicity of H_2O_2 was studied on a mammalian epithelial cell line. Iron complexes which were internalised enhanced the cytotoxicity of H_2O_2 measured by delayed thymidine incorporation. Iron complexed to 8-hydroxyquinoline (Fe/8-HQ) potentiated the cytotoxicity of 50 μM by 38% and Fe/dextran by 23%. Pre-exposure of cells to Fe/dextran at 4°C did not result in any potentiation of H_2O_2-induced cytotoxicity which we ascribe to failure of the Fe/dextran to be endocytosed at low temperature. Iron complexes which are slowly taken up or remain extracellular protected the cells from H_2O_2-induced cytotoxicity. Thus, Fe/EDTA inhibited the cytotoxicity of 50 μM H_2O_2 by 33%; Fe/ADP by 80% and Fe/ATP by 88%, suggesting mutual extracellular detoxification.

KEY WORDS: Cytotoxicity, H_2O_2, iron complexes, internalisation, reduction.

INTRODUCTION

The extensive interest in H_2O_2-induced cytotoxicity over the past 10 years has developed as a result of the similarity of DNA lesions produced by H_2O_2 and those resulting from ionising radiation,[1] although the comparative yields differ considerably.[2] Numerous studies have provided evidence showing that HO· radicals generated by the Fenton reaction are the most probable cause of this type of damage.[3–7]

Although it is likely that cell death is a consequence of irreversible damage to DNA, the type of damage responsible for cell killing is still unclear. There exist a number of possible cellular targets of oxidative attack that may result in cell death including membrane disruption due to lipid peroxidation[8] or damage to crucial membrane proteins.[9] Nevertheless it is widely considered that DNA is the major target. DNA single strand breaks, have been implicated previously[10] but this has been contradicted by other results showing that no correlation exists between single strand breaks in DNA and cell killing at low concentrations of H_2O_2.[11,12] It is possible that other lesions, possibly including DNA double strand breaks are involved. DNA double strand breaks have been produced following exposure of cells to H_2O_2 in at least two studies[13,14] although the correspondence with cell death is unclear.

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Sensitisation of cells to the effects of H_2O_2 by L-histidine have resulted in double-strand breaks and increased cytotoxicity.[15]

Recently both Cu^+ and Fe^{2+} have been shown to cause specific mutations which are inhibited by radical scavengers. Evidence has been published of an association between cytosine (C) to tyrosine (T) base transitions and the presence of copper tightly bound to DNA.[16] Support for site-specific damage to DNA by H_2O_2 and chelated iron has also been recently provided,[17] and other studies have demonstrated that H_2O_2-induced cytotoxicity depends on the interaction with DNA.[18] The probable involvement of Fenton chemistry in the production of damage to DNA implicates DNA-associated transition metals.

We have recently shown that several externally added iron complexes either do not enter cells or become localised in different compartments of the cell. It was shown in these studies that iron complexed to low molecular weight hydrophilic metal chelators such as EDTA, DTPA and charged ligands such as ADP and ATP, remained extracellular, whereas when bound to the low molecular weight lipophilic agent 8-hydroxyqinoline it entered the cells by diffusion and was distributed widely within them. By contrast, the high molecular weight complex Fe/dextran was endocytosed and the iron retained in phagosomes.[19] In the present paper we investigate the effects of this differential metal localisation on H_2O_2-induced toxicity.

MATERIALS AND METHODS

Chemicals

Ferrous sulphate and ferric chloride were obtained from Sigma Chemical Co. The ligands EDTA (ethylenediamine-tetraacetic acid; free acid) ATP (adenosine 5′-triphosphate, disodium salt, equine muscle), ADP (adenosine 5′-diphosphate) and 8-HQ (8-hydroxyquinoline, free base) were obtained from Sigma Chemical Co.

Batches of iron dextrans were kindly provided by Fisons Pharmaceuticals Ltd. Phosphate buffered saline (PBS) was prepared by dissolving 8 g NaCl, 0.2 g KCl, 0.132 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgCl_2 \cdot H_2O$, 1.15 g $Na_2HPO_4 \cdot 2H_2O$ and 0.2 g KH_2PO_4 in 1 L of distilled water and the pH adjusted to 7.4.

Minimum essential medium, Eagle (modified) with Earle's salts was obtained from Flow Laboratories. This medium was supplemented with 20 mM N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES, Flow Labs) and 10% foetal bovine serum (Imperial Labs), 0.4 mM L-glutamine, penicillin (100 u/ml) and streptomycin (100 μg/ml). This is referred to as growth medium.

A stock solution of tritiated thymidine (^{3}H-TdR, [methyl-^{3}H]-thymidine, specific activity 5 mCi per mmol) was obtained from Amersham International Plc.

Preparation of iron complexes

The iron complexes were prepared by mixing solutions of iron salts and ligands in which the concentrations were adjusted to give molar ratios of iron to ligand as indicated in each experiment. All complexes were prepared immediately prior to the experiment and the pH adjusted to 7.4 by addition of HCl or NaOH as appropriate

before making up to volume at 10 × the final concentration with buffer. The solutions were shielded from light to avoid the possibility of photon-induced reduction.

Batch BM324S of the iron dextrans was used in all experiments. This standard preparation contained 5% iron bound to 20% dextran (i.e. a molar ratio of 1:4) and was diluted as appropriate with PBS.

H_2O_2 solution

H_2O_2 was obtained from Aldrich. The solution is 30% w/v which corresponds to a concentration of 8.8 M. Appropriate dilutions were made in single distilled water to give a 1 mM stock solution which was kept refrigerated for a maximum of 1 hour prior to use.

Cells

A mammalian epithelial cell line (CNCM I221) was used for all experiments. Cells between passage 21 and 30 were used. Cells were grown in culture medium either in polystyrene flasks (Falcon flasks, Scientific Supplies Ltd.) or multiwell plastic trays, each well being of 1.5 cm diameter (1.77 cm^2 growth area). Subcultivation was carried out weekly by trypsinisation as described previously.[20]

Cytotoxicity Assays

Plating efficiency. 0.1 ml of 5×10^4 c/ml of washed cells were placed into polypropylene tubes with 1.9 ml of PBS containing 5 μM freshly prepared Fe(II)/EDTA (1:1). These tubes contained either 0, 5, 10 or 20 μM H_2O_2. The suspensions were left on a Denley Spiramix rolling machine for 1 hour at 37°C. The plating efficiency assay was carried out as described elsewhere.[20]

In another series of experiments 5×10^4 c/ml of attached cells were pre-incubated with Fe/dextran (equivalent to 100 μM Fe) for 2 hours in PBS. The cells were washed and trypsinised and resuspended in growth medium for a few minutes. Following washing and resuspension in fresh PBS, 0.1 ml was removed and transferred to polypropylene tubes containing 1.9 ml of H_2O_2 in PBS in final concentrations of 30 or 50 μM. After 1 hour incubation at 37°C the cells were washed and processed for plating efficiency.

Delayed ^{3}H-TdR incorporation. In these studies we have used a delayed [^{3}H]-thymidine incorporation assay as an index of toxicity which correlates well with results from plating efficiency.[21] We refer to this indicator of reproductive viability as the survival index (S.I.). The S.I. of cells incubated either in the presence of iron complexes and H_2O_2 or pre-incubated with an iron complex prior to exposure to H_2O_2 was investigated. Incubation in H_2O_2 was carried out for 1 hour at 37°C or 4°C prior to washing, incubation in growth medium for 24 hours and processing for the ^{3}H-TdR incorporation assay.

The protocols are outlined in Schemes 1 and 2. In the case of iron dextran pre-incubations were carried out at different temperatures as shown in Scheme 2.

All P values were estimated using the Student's T-test.

CELLS ATTACHED IN MULTIWELLS ~1.5 × 10^5 CELLS IN 1 ML GROWTH MEDIUM
↓
WASH 2× IN PBS
↓
1 ML PBS pH 7.40
CONTAINING Fe/COMPLEX (100 μM:500 μM)
ADD 50 μM H_2O_2
INCUBATE FOR 1 HOUR AT 37°C
↓
WASH 2× IN PBS
↓
1 ML GROWTH MEDIUM 24 HOURS 37°C
↓
ADD 1 μCi ^{3}H-TdR 30 MINS
↓
WASH; FIX; DIGEST
↓
SCINTILLATION COUNTER

SCHEME 1

CELLS ATTACHED IN MULTIWELLS ~1.5 × 10^5 CELLS IN 1 ML GROWTH MEDIUM
↓
WASH 2× IN PBS
37°C ← ADD 0.9 ML PBS pH 7.40 → 4°C
↓ INCUBATE ↓
2 HRS +/− 0.1 ML Fe/COMPLEX 2 HRS
WASH 37°C — WASH 4°C — PBS — WASH 37°C — WASH 4°C
↓ ↓ ↓ ↓
1 HR 37°C — 1 HR 4°C — INCUBATE +/− 50 μM H_2O_2 — 1 HR 37°C — 1 HR 4°C
→ WASH PBS ←
↓
INCUBATE IN GROWTH MEDIUM 24 HRS 37°C
↓
^{3}H-TdR INCORPORATION
1 μCi/ML 30 MINS
↓
WASH; FIX; DIGEST
↓
SCINTILLATION COUNTER

SCHEME 2

RESULTS

Exposure to H_2O_2 in the presence of iron complexes

As previously shown[19] cells exposed to Fe(III) complexed to low molecular weight hydrophilic ligands, such as Fe(III)/ATP, Fe(III)/ADP or Fe(III)/EDTA are unaffected, whereas reduced iron is potentially toxic. This toxic effect of Fe(II) is

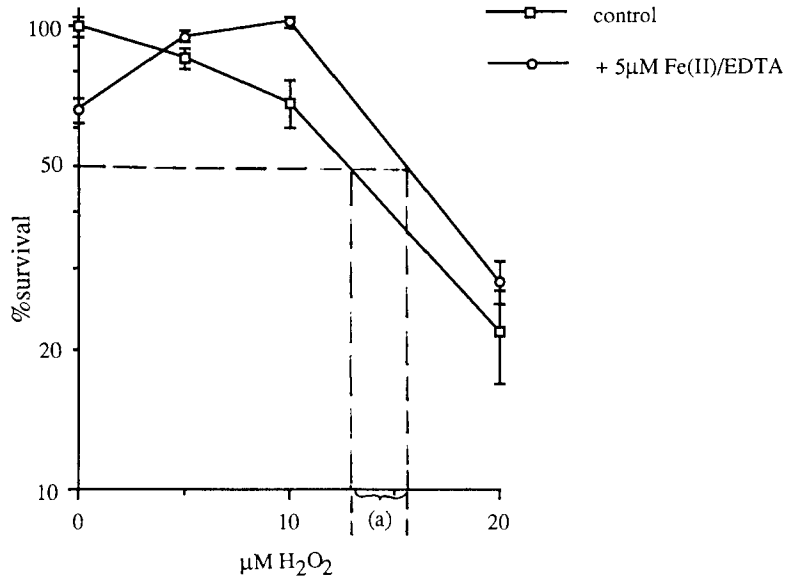

FIGURE 1 Effect of simultaneous exposure of cells to Fe(II)/EDTA and H_2O_2on cell survival. 5×10^4 cells/ml in suspension were exposed to H_2O_2 in the presence or absence of 5 μM Fe(II)/EDTA (1:1) and H_2O_2 for 1 hour at 37°C prior to processing for plating efficiency. The results are the means of triplicate estimations and are expressed as % survival. Vertical bars represent standard deviations. The difference in LD_{50} is indicated as (a) and corresponds to approximately 2.5 μM H_2O_2.

illustrated in Figure 1 in which the plating efficiency of cells exposed to 5 μM Fe(II)/EDTA is reduced by 35%.

Under the conditions of our assay hydrogen peroxide exerts a dose-dependent toxicity on cells. Low concentrations (less than 10 μM) of H_2O_2 completely protected cells exposed to 5 μM Fe(II)/EDTA. Above 10 μM H_2O_2 a reduced toxicity in the presence of Fe(II)/EDTA was found compared to the toxicity observed with H_2O_2 alone (Figure 1). Since it has been previously shown[16] that iron complexed to EDTA enters cells at a slow rate, we interpret the mutual detoxification of Fe(II) and H_2O_2 to be the result of their extracellular interaction (i.e. oxidation of the Fe(II) and degradation of H_2O_2) which effectively reduces their concentration in the system. The effect observed shows an approximately 3 μM increase in the LD_{50} in the presence of the Fe(II)/EDTA. We attribute the lack of 1:1 stoichiometry to the autoxidation of Fe(II) in this complex, which has a half-life of the order of 1.2 minutes under the conditions employed for these experiments.[18]

Exposure to H_2O_2 in the presence of iron complexes with differing characteristics with regard to uptake by cells was monitored by the survival index using the delayed thymidine incorporation method.[21] The results are shown in Table I. In all cases the effect of adding both the iron complex and H_2O_2 to the cells was compared to the effect observed with H_2O_2 alone. The data shown in Table I indicate that the survival of cells exposed to H_2O_2 is increased in the presence of Fe(II)/ATP, Fe(II)/ADP and Fe(II)/EDTA but diminished in cultures exposed to Fe/dextran or Fe(II)/8-HQ. To illustrate the protective effect of the nucleotide and EDTA complexes we have calculated the differences between the sum of the individual toxicities of H_2O_2 and

TABLE I
Cytotoxic effects of simultaneous exposure of cells to iron complexes and hydrogen peroxide

Agent 1 Fe:5 LIG	H_2O_2 (50 μM)	% Survival index ±S.D.	
—	−	100 ± 8	(a)
—	+	38 ± 9	
Fe(II)/ATP	−	74 ± 23	n.s.
,,	+	100 ± 14	(a)[1]
Fe(II)/ADP	−	72 ± 25	(a)
,,	+	90 ± 19	(a)[1]
Fe(II)/EDTA	−	81 ± 10	(b)
,,	+	52 ± 10	n.s.[1]
Fe/dex	−	79 ± 11	(a)
,,	+	15 ± 10	(a)[1]
Fe(II)/8-HQ	−	18 ± 1	(a)
,,	+	0.5 ± 0	(a)

1.5×10^5 cells/ml were treated according to Scheme 1 of the Materials and Methods section
* 100μM Fe; 500μM Ligand, except Fe/dextran (100μM Fe: 400μM Dextran). The results are the means of quadruplicate experiments.
(a) $P < 0.01$.
[1]Compared to treatment with H_2O_2 alone.

the iron complex and their combined effect. This calculation which was designed to reveal synergistic effects maximises the apparent protection afforded by these complexes. By this criterion Fe(II)/ATP, Fe(II)/ADP and Fe(II)/EDTA protected the cells by 88%, 80% and 33% respectively. These differences may partly be due to differences in their cellular uptake and also the result of the greater stability to autoxidation of the reduced iron in the nucleotide complexes; the comparative half-lives of Fe(II) chelated by ATP and ADP under the conditions employed being approximately 72 and 19 minutes respectively.[18] We have previously shown that the ligands employed in this study exhibit toxic rather than protective effects with the exception of 8-HQ.[19]

Both Fe(II)/8-HQ and iron dextran were toxic to the cells, particularly the 8-HQ complex, which reduced the survival index by 82% in the absence of H_2O_2. Although 8-HQ exerts a slight toxic effect by itself, the major toxicity is due to the presence of iron. This may be due to the rapid rate of autoxidation of the iron in this complex ($t_{1/2}$ = 1.6 minutes)[19] which could generate cytotoxic levels of $O_2^{\cdot -}$. In the presence of 50 μM H_2O_2 this toxicity was enhanced by a further 97% indicating that a substantial proportion of the intracellular iron was in the reduced state. Iron dextran was slightly toxic in the absence of H_2O_2 but increased the toxicity due to 50 μM H_2O_2 by 81%.

To establish the extent to which the uptake of these complexes influenced the effects observed, experiments were carried out in which cells were pre-incubated with the relevant iron complex and subsequently exposed to H_2O_2 (see Scheme 2).

TABLE II
Effect of pre-incubation of cells with Fe/ATP on the H_2O_2-induced cytotoxicity

Fe(III)/ATP*	H_2O_2 (50 μM)	% Survival index ±S.D.	
−	−	100 + 20	(a)
−	+	47 + 16	(a) n.s.
+	−	100 + 14	(a)
+	+	38 + 5	(a)

1.5×10^5 cells/ml were treated according to Scheme 2 of the Materials and Methods section.
* 100μM $FeCl_3$: 500μM ATP
(a) $P < 0.01$.
The results represent the means of quadruplicate estimations.

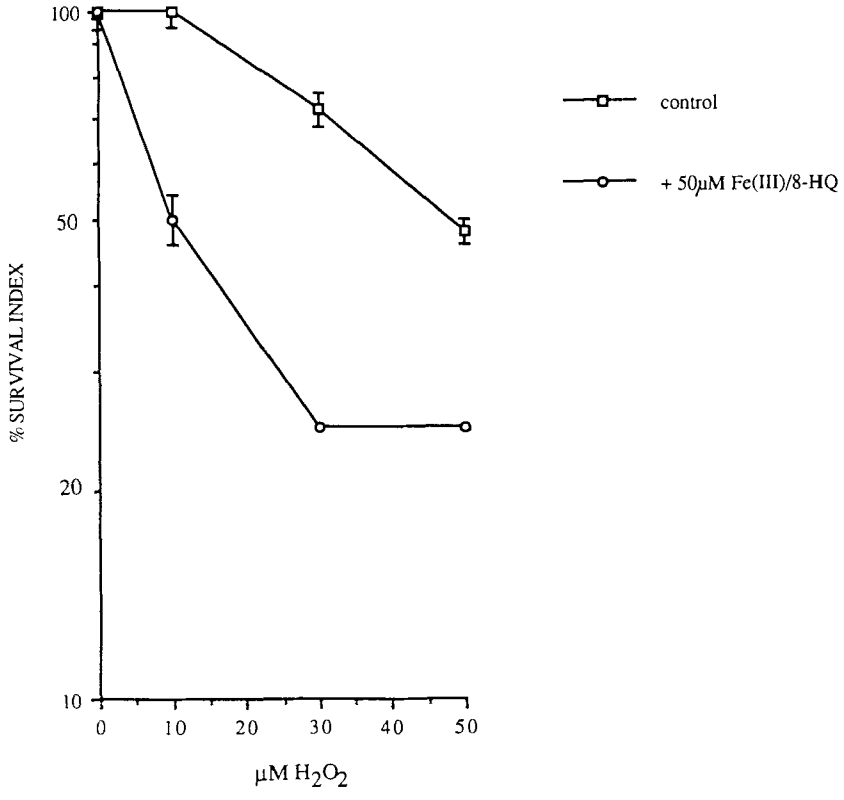

FIGURE 2 Effect of pre-incubation of cells with Fe/8-HQ on the H_2O_2 cytotoxicity. 1.5×10^5 cells/ml were pre-incubated with 50 μM Fe(III)/100 μM 8-hydroxyquinoline at 37°C followed by exposure to H_2O_2 at 37°C according to Scheme 2 of the Materials and Methods section. The results are the means of quadruplicate estimations. Vertical bars represent standard deviations. The data for cells exposed to this iron complex are normalized to take account of the complex in the absence of H_2O_2 exposure.

Pre-incubation of cells with iron complexes followed by exposure to H_2O_2

Pre-incubation for 2 hours with Fe(III)/ATP was without effect on cells, nor did it modify the cytotoxicity of subsequent exposure to 50 μM H_2O_2 (Table II). Similar results were obtained with the other low molecular weight hydrophilic agents (results not shown). Pre-incubation of cells for 2 hours with a non-toxic concentration of Fe(III)/8-HQ on the other hand enhanced the cytotoxic effect of H_2O_2 considerably (Figure 2). Taking into account the toxic effect of Fe(III)/8-HQ alone, the percentage enhancement observed in the presence of 50 μM H_2O_2 was 23%. These results indicate that the redox state of the iron in the starting material is not the deciding factor in determining the cytotoxic effect.

In the case of the high molecular weight hydrophilic complex, Fe/dextran, a similar enhancement of the cytotoxic action of H_2O_2 was observed (Figure 3). Since the iron in Fe/dextran is predominantly in the oxidised form it also suggests that the redox state of the starting material does not determine whether or not the complex

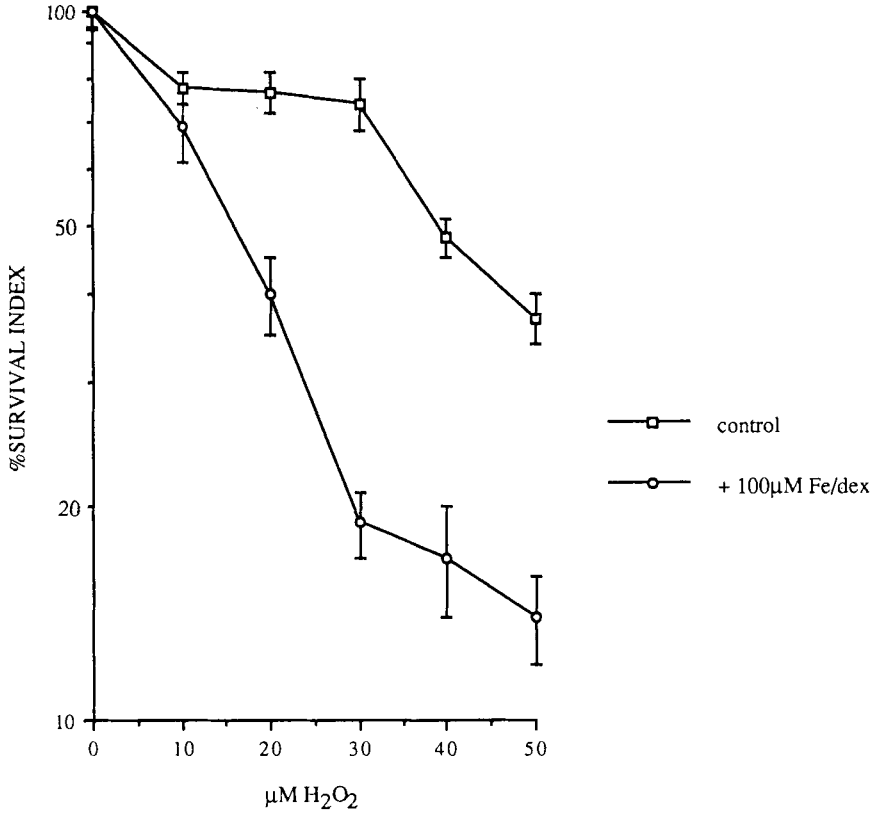

FIGURE 3 Effect of pre-incubation of cells with Fe/dextran on the H_2O_2 cytotoxicity measured by plating efficiency. 1.5×10^5 cells/ml were pre-incubated with 100 μM Fe/dextran followed by exposure to H_2O_2 according to Scheme 2 of the Materials and Methods section. The results shown are the means of quadruplicate estimations. Vertical bars represent standard deviations. The survival index data for cells exposed to the complex are normalized to allow for the toxicity of iron dextran alone.

will enhance the H_2O_2-induced cytotoxicity. The enhancement of the cytotoxicity of 50 μM H_2O_2 by Fe/dextran, taking the toxic effect of Fe/dextran alone into account, was also 23% and this compares with the enhancement of the H_2O_2 toxicity which resulted from simultaneous exposure of cells to the two agents (see Table I).

Effect of temperature

We have previously shown that the cytotoxicity of H_2O_2 is a temperature-dependent process.[20] The enhancement of the cytotoxicity by Fe/dextran is inhibited if cells are pre-incubated with Fe/dextran at 37°C, followed by exposure to H_2O_2 at 4°C (Table III). Although there is a slight increase in the toxicity, this is not significant compared to the toxic effect of Fe/dextran alone. No enhancement occurred when the cells were pre-incubated with Fe/dextran at 4°C, but exposed to H_2O_2 at 37°C, consistent with the inhibition of endocytosis of Fe/dextran.[19] The cells were completely protected by exposure to both agents at 4°C.

TABLE III
Effect of temperature on pre-incubation of cells with Fe/dextran on the H_2O_2-induced cytotoxicity measured by 3H-TdR-incorporation

Fe/dex*	Temperature of pre-incubation (°C)	H_2O_2 (50 μM)	Temperature of incubation (°C)	% Survival index ±S.D.		
–	37	–	37	100 ± 17	(a)	
–	,,	+	,,	46 ± 14		(b)
+	,,	–	,,	72 ± 24	(b)	
+	,,	+	,,	18 ± 10		
–	4	–	,,	100 ± 20	(a)	
–	,,	+	,,	42 ± 7		n.s.
+	,,	–	,,	100 ± 9	(a)	
+	,,	+	,,	44 + 9		
–	37	–	4	100 ± 5	n.s.	
–	,,	+	,,	104 ± 18		(b)
+	,,	–	,,	84 ± 10	n.s.	
+	,,	+	,,	74 ± 6		
–	4	–	4	100 ± 18	n.s.	
–	,,	+	,,	90 ± 5		n.s.
+	,,	–	,,	102 ± 18	n.s.	
+	,,	+	,,	108 ± 17		

1.5×10^5 cells/ml were treated according to Scheme 2 of the Materials and Methods section.
* Batch BM324S (100μM Fe: 400μM dextran).
(a) $P < 0.01$ (b) $P < 0.05$.
The results are the means of quadruplicate estimations.

DISCUSSION

Our studies show that exposure to iron complexes may modify the cytotoxic effect of H_2O_2 on cells in culture. The effect was either one of enhancement or protection depending on the nature of the complex. We have previously shown that the nature of the ligand to which iron is bound strongly influences the equilibrium redox state of the metal as well as the uptake by cells and the location of the complex within the cell. Non-internalised iron is non-toxic but internalised iron is cytotoxic.[19] There is considerable evidence that the cytotoxicity of H_2O_2 is a transition-metal dependent process.[4,22–24,20] The present study is consistent with a cytotoxic mechanism involving the Fenton reaction. All the ligands employed in this study permit the chelated metal to undergo redox reactions and autoxidation of Fe(II) may have contributed to some of the effects observed. However, in those cases where both the iron complex and H_2O_2 were simultaneously present, the predominant reaction is oxidation of iron by H_2O_2: at pH 7.4 the rate constant of Fe(II)/EDTA + O_2 is $10^2\ M^{-1}\ s^{-1}$ at pH 7.4 (ref. 25) and the rate constant for Fe(II)/EDTA + H_2O_2 is $2 \times 10^4\ M^{-1}\ s^{-1}$ (refs. 25, 26). Thus, the reaction with H_2O_2 is two orders of magnitude more rapid than autoxidation of Fe(II) under the conditions studied.

In those cases where both the iron complex and H_2O_2 were present simultaneously during the incubation period, cells were protected from damage by H_2O_2 when Fe(II) was bound to EDTA, ADP or ATP. Previously we have shown that these complexes are either very slowly taken up or remain extracellular. This suggests that any redox reactions involving the added iron take place predominantly outside the cell and therefore reduce the effective H_2O_2 concentration to which the cells are exposed. Extracellular production of HO· does not damage cells.[27]

By contrast both Fe/8-HQ and Fe/dextran potentiated the cytotoxic effect of H_2O_2. These complexes enter cells by different mechanisms: (i) Fe/8-HQ probably by passive diffusion and (ii) Fe/dextran by active endocytosis.[19] The degree of enhancement of the cytotoxicity by Fe/8-HQ was approximately two times greater than for an equivalent amount of iron internalised as Fe/dextran, indicating that the localisation of the iron complex within the cell influences the cytotoxic effect.

From the results shown by the present study two basic requirements for the potentiation by iron complexes of cytotoxic damage by H_2O_2 emerge: (i) the metal must be internalised and (ii) the metal must be in a reduced state. The latter criterion becomes evident from the results shown in Table III. Since the uptake of Fe/dextran involves endocytosis it was inhibited at low temperature and did not result in enhancement of the cytotoxicity of H_2O_2. However, when cells did internalise Fe/dextran during pre-incubation at 37°C, but were subsequently exposed to H_2O_2 at 4°C, there was no potentiation of the H_2O_2 action. Exposure of cells to low concentrations of H_2O_2 at temperatures of 4°C or lower does not result in any cellular damage.[28,20,20] We suggest that this is because the process of metabolic reduction of iron is inhibited under these conditions. Consistent with the requirement for internalisation are the results with Fe/ATP, which by contrast, did not modify the effects of H_2O_2 when cells were pre-incubated with this complex (see Table II).

In the presence of increasing concentrations of H_2O_2 the enhancement of the cytotoxic effect is much greater with Fe(III)/8-HQ than with Fe/dextran (compare Figures 2 and 3). In both cases the complex is internalised and the starting material is the oxidised form of iron. The measure of cytotoxicity is based on DNA replication in both the assays applied indicating that the genome is the ultimate target. The

general intracellular distribution of Fe/8-HQ suggests that reaction with H_2O_2 yielding HO· radicals would be likely to occur in closer proximity to DNA than the equivalent reaction with Fe/dextran. The iron derived from Fe/dextran is concentrated in cytosolic vesicles[19] and the mechanism of damage may therefore not involve direct interaction with DNA. Instead the production of HO· radicals within the lysosomes may result in lysosomal membrane damage (e.g. by lipid peroxidation) with subsequent release of hydrolytic enzymes. Generation of free radicals within lysosomes by photosensitisation has been shown to release hydrolases from lysosomes[30] and lipid peroxidation has been implicated to be a possible direct mechanism of cell death following exposure to H_2O_2.[31]

In the case both of Fe/8-HQ and Fe/dextran the redox state of the starting material was the oxidised form of iron suggesting that for intracellular damage to occur in the presence of H_2O_2 conditions must exist for intracellular reduction of iron. A possible mechanism of intracellular metal reduction may involve ascorbate as we have already demonstrated.[20] Other possibilities include reduced nucleotides produced by the glycolytic pathway or the citric acid cycle or alternatively superoxide produced by the mitochondrial electron transport system. The extent to which these may actively contribute to the reduction of internalized transition metals is currently under investigation.

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