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EFFECTS OF IRON CHELATES ON THE TRANSFERRIN-FREE CULTURE OF RAT DERMAL FIBROBLASTS THROUGH ACTIVE OXYGEN GENERATION

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SUMMARY

Effects of nonchelating and chelating agents at 10 m*M* on the serum-free culture of rat dermal fibroblasts were investigated. A strong iron-chelating agent, iminodiacetic acid (IDA), and a weak one, dihydroxyethylglycine (DHEG), decreased iron permeation into preconfluent fibroblasts. A weak iron-chelating agent, glycylglycine (GG), a nonchelating agent, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and human apotransferrin (10 μg/ml) increased the permeation with time. Iron may be essential for survival of fibroblasts because subconfluent fibroblasts exposed to 100 μ*M* $FeSO_4$ in combination with transferrin, HEPES, or GG significantly decreased to release lactate dehydrogenase into the medium. Superoxide dismutase and dimethyl sulfoxide blocked the enzyme release, suggesting that superoxide and hydroxyl radical induce cellular damage but hydrogen peroxide (H_2O_2) generated by superoxide dismutation does not. GG significantly reduced H_2O_2 cytotoxicity. DHEG acted as a potent promoter of the iron-stimulated cellular damage if ascorbate or H_2O_2 was added to the medium. $FeSO_4$ and $FeCl_3$ (50 to 100 μ*M*) individually combined with IDA maximally promoted fibroblast proliferation. Ascorbate increased formation of thiobarbituric acid-reactive substances from deoxyribose in the medium supplemented with $FeSO_4$ and either IDA or DHEG. Conversely, ascorbate decreased the formation in the medium with $FeSO_4$ and with or without other agents. Fibroblast proliferation may thus be stimulated through the active oxygen generation mediated by a redox-cycling between Fe^{3+} and Fe^{2+}, which are dissolved in the medium at a high concentration, rather than through delivery of iron into the cells.

Key words: cytotoxicity; fibroblast proliferation; hydrogen peroxide; iron delivery.

INTRODUCTION

It has been reported that iron is an essential trace element for growth, development, and survival of living organisms through metabolism controlled by various iron-loading proteins as well as iron-associated enzymes. Aerobic cells need oxygen for maintaining a variety of cellular functions. Nonetheless, active oxygen species derived from oxygen metabolites including hydroxyl radical (OH·), superoxide anion radical (O_2^-), and hydrogen peroxide (H_2O_2) are known to be cytotoxic (5,10,11). These species are usually generated in aerobic living organisms (10,11). The formation of $OH^·$ from O_2^- and H_2O_2 is catalyzed by some transition metals such as iron and copper (3,4,16).

The toxicity most frequently appears not only in the degradation process of biomolecules such as carbohydrates, amino acids, nucleic acids, and lipids at the cellular level but also in the alternation process at the tissue, organ, and individual levels (7,19,41). These noxious phenomena are presumably mediated by active oxygen species. In addition, continuous oxidative stress much affects aging by reducing the inherent antioxidant defense system (18,36).

Some chelating agents at 5- to 10-m*M* levels greatly promote proliferation of mouse-to-mouse hybridoma cells in serum-free medium when much higher concentrations of $FeSO_4$ or $FeCl_3$ (greater than 2 μ*M*) are present (44). These chelating agents include iminodiacetic acid (IDA), glycylglycine (GG), dihydroxyethylglycine (DHEG), ethylenediamine-*N,N*-dipropionic acid, hydroxyethylimindiacetic acid, and nitrilotripropionic acid. All of them preferentially combine with Fe^{3+} and Fe^{2+} more firmly than with Ca^{2+} and Mg^{2+}. Accordingly, these compounds may have potential as iron-chelating agents.

The aims of our study were to investigate the ability of chelating agents such as IDA, GG, and DHEG in the presence of iron to damage diploid fibroblasts, which are thought to be more sensitive to the oxidative stress than hybridoma cells, and effects on cell proliferation. A nonchelating agent *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), which has been traditionally used as a buffer component (27,37), and iron-binding protein transferrin (Tf) (1,30) were used for comparison.

Serum contains a variety of antioxidative molecules including proteins and vitamins (13,40,42). For this reason, cultured cells must be protected from the cytotoxicity mediated with active oxygen species. The protection is needed particularly when the cells are in contact with iron in the absence of serum. We had already developed serum-free NYSF-404 medium for culturing mouse-to-mouse hybridoma cells (44). NYSF medium may be inadequate for use in an optimal fibroblast culture even if the medium is further supplemented with epidermal growth factor (EGF). Nonetheless, use of NYSF medium would at least facilitate the study when the effects of iron chelates on fibroblasts are examined.

MATERIALS AND METHODS

Materials. RPMI 1640 medium, Eagle's minimum essential medium (MEM), and Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) as a dry powder were purchased from Nissui Pharmaceutical Co., Tokyo, Japan. DHEG, IDA, and dimethyl sulfoxide (DMSO) were from Dojindo Laboratories Co., Kumamoto, Japan. Bovine erythrocyte superoxide dismutase (SOD), bovine serum albumin, 2-deoxy-D-ribose, GG, human apotransferrin (apoTf), D-mannitol, sodium salt of pyruvic acid, 1,1,3,3-tetraethoxypropane, and 2-thiobarbituric acid (TBA) were from Sigma Chemical Co., St. Louis, MO. $^{59}FeCl_3$ (specific activity 3 to 20 mCi/mg iron) was from Amersham International, Amersham Place, UK. EGF, isolated from mouse submaxillary gland, was from Toyobo Co., Osaka, Japan. Fetal bovine serum (FBS) was from Flow Laboratories, North Ryde, Australia. A diagnostic determination kit for serum lactate dehydrogenase (LDH) was from Kyokuto Pharmaceutical Industries Co., Tokyo, Japan. Other chemicals including L-ascorbic acid, $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, and polyoxyethylene (20) sorbitan monolaurate (Tween 20) were from Wako Pure Chemical Industries Co., Osaka, Japan. A reagent grade of deionized water was purified from distilled water with Milli-Q water purification apparatus (Millipore Corp., Bedford, MA).

Serum-free media. A double concentrate of NYSF-404 medium (44) composed of a 1:1 mixture of RPMI 1640 medium and MEM was prepared on one day before assay; however, DHEG, holoTf, and pyruvate were omitted from the original medium formulation. The concentrated medium was supplemented with 20 μg of EGF per liter. $FeCl_3 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ were individually dissolved in deionized water and 1 m*M* HCl, respectively, to make a 100-fold final concentration on the day of assay. On the basis of the data of previous experiments, the concentration of HEPES, IDA, GG, and DHEG used in the present study was established as 10 m*M*. Twenty microjmoles of these agents were prepared by adjusting pH to 7.4 with NaOH. The iron solutions were diluted 50 times with either the agent solutions or deionized water. The mixtures were combined with an equal volume of the concentrated medium to make the actual medium. Agent withdrawal medium was divided into two parts. One was supplemented with 10 mg of human apoTf per liter. The serum-free media were thus supplemented with various concentrations of iron (0 to 100 μ*M*, unless otherwise stated) and with or without a constant concentration of agents (10 m*M* each) including apoTf.

Iron content of the original medium NYSF-404 formulated without DHEG and holoTf was quantified with a multielement sequential inductively coupled plasma emission spectrometer (model SPS 1200A; Seiko Instruments Inc., Tokyo, Japan). The iron level was taken as 0.055 ppm (corresponding to 0.96 μ*M* Fe).

Fibroblasts and their maintenance. Skin fragments were aseptically excised from a male Wistar strain rat weighing about 100 g and minced into approximately 1-mm^3 pieces. The pieces were placed on 6-cm petri dishes and cultured in MEM supplemented with 10% FBS (FBS-containing medium) in a humidified atmosphere containing 5% CO_2 at 37° C. Fibroblasts were detached by tryptic digestion and successfully subcultured at a 1:3 split ratio when confluence was achieved. The culture was fed twice weekly. Fibroblasts were routinely used between Passages 3 and 6 for each experiment.

OH^- measurement. Generation of OH^- was assessed by measuring the formation of TBA-reactive substances according to the colorimetric method of Aruoma and Halliwell (2). Briefly, 1 ml of various media were individually mixed with an equal volume of potassium phosphate buffer (PPB; 14 m*M* K_2HPO_4, 6 m*M* KH_2PO_4, 10 m*M* NaCl; pH 7.4). Reaction was initiated by addition of 40 μl of 200 m*M* deoxyribose dissolved in PPB. The reaction mixtures were left for 30 min at 37° C in a CO_2 incubator. After the incubation, the mixtures were combined with a series of 1 ml of 1% TBA dissolved in 50 m*M* NaOH and 1 ml glacial acetic acid. The final mixtures were boiled at 95° C for 15 min in a water bath and cooled to room temperature. They were centrifuged and insoluble substances were removed. The yield of TBA-reactive substances in clarified reaction mixtures was given in terms of absorbance at 532 nm (OD_{532}) from which blank values produced by the clarified reaction mixtures without deoxyribose were subtracted.

^{59}Fe permeation assay. Fibroblasts were grown to preconfluence (50 to 70% of confluence) in 2-cm^2 (16-mm diameter) wells of Falcon 24-well culture plates (Becton Dickinson & Co., Lincoln Park, NJ) with 0.5 ml of FBS-containing medium per well. Fibroblasts were washed twice with 1 ml of PBS per well and once with an equal volume of the serum-free medium without iron and any agent. All cultures were incubated for 1 h at 37° C in a CO_2 incubator. The cultures were shifted into various serum-free media that were supplemented with 1 μCi of $^{59}FeCl_3$ per ml (roughly estimated as 5.4 μ*M* Fe on a mCi/mg iron basis) substituted for nonisotopic iron. Incubation was continued for the indicated period. Fibroblasts were washed three times with 1 ml of chilled PBS per well. Whole cellular contents were lysed by incubation in 1 ml of 0.5 *M* NaOH per well for 30 min at 37° C. The radioactivity of an aliquot (0.5 ml) of the cell lysates was counted to determine the amount of ^{59}Fe permeating into fibroblasts. The permeation data were expressed as a percentage of the radioactivity to the total ^{59}Fe activity added to the culture.

LDH release assay. Measurement of the release of a cytosolic enzyme LDH into incubation medium is based on the reaction in which LDH catalyzes NAD-dependent conversion of lactate to pyruvate. The validity of the LDH measurement had been preliminarily examined by monitoring LDH activity of FBS to which the following substances were individually added: each agent (10 m*M*), SOD (10 μg/ml), mannitol (10 m*M*), DMSO (10 m*M*), pyruvate (10 m*M*), ascorbate (1 m*M*), H_2O_2 (100 μ*M*), and $FeSO_4$ (100 μ*M*). These substances at the indicated final concentrations decreased the serum LDH activity; however, the decrease did not exceed 5% of the native enzyme activity. The results thereby confirmed availability of the kit.

After fibroblasts were grown to subconfluence (80 to 90% of confluence) in 2-cm^2 wells distributed with 0.5 ml of FBS-containing medium per well, they were washed twice with 1 ml of PBS per well and once with an equal volume of the serum-free medium without iron and any agent. The cultures were shifted into various serum-free media. When the effect of antioxidants or active oxygen species on LDH release was analyzed, the cultures were loaded with SOD (10 μg/ml), mannitol (10 m*M*), DMSO (10 m*M*), pyruvate (10 m*M*), ascorbate (1 m*M*), or H_2O_2 (up to 100 μ*M*). Incubation was continued for another 24 h at 37° C in a CO_2 incubator. Culture supernatant fluids were collected and depleted of cell debris by centrifugation. An aliquot (100 μl) of the clarified supernatant fluids was assessed for extracellular LDH activity. After withdrawal of the spent medium, the cell layers were washed twice with 1 ml of PBS per well and once with an equal volume of the media that had the same formulation as those used for the 24-h incubation. Fibroblasts were disrupted by being incubated further for 30 min at 37° C in the presence of 0.1% Tween 20. An aliquot (100 μl) of the cell lysates was assessed for intracellular LDH activity. The LDH release data were expressed as the ratio of the extracellular LDH activity to the sum of the extracellular activity plus the intracellular activity in each culture well.

Lipid peroxidation assay. Fibroblasts were grown to subconfluence in Falcon 25-cm^2 culture flasks distributed with 5 ml of FBS-containing medium per flask. Fibroblasts were washed twice with 10 ml of PBS per flask and once with an equal volume of the serum-free medium without iron and any agent. The cultures were shifted into various serum-free media. Incubation was continued for another 24 h at 37° C in a CO_2 incubator. The cell layers were washed three times with 10 ml of chilled PBS per flask. Fibroblasts were harvested with a rubber scraper, suspended in 2.5 ml of PBS per flask, and disrupted by three cycles of freezing and thawing. Malondialdehyde (MDA), a product of lipid peroxidation (9), in the disrupted cell suspensions was quantified. In brief, an aliquot (1 ml) of the suspensions was combined with a series of 1 ml of 1% TBA dissolved in 50 m*M* NaOH and 1 ml glacial acetic acid. The mixtures were boiled at 95° C for 15 min in a water bath and cooled to room temperature. Absorbance of the chromogen developing was measured at 532 nm. The measurement was calibrated with a tetraethoxypropane solution prepared in 10 m*M* HCl (20). Protein content in the cell lysates was also measured according to the method of Lowry et al. (25) with bovine serum albumin as a standard. The extent of lipid peroxidation was universalized as μ*M* MDA per mg protein in each culture flask.

Cell proliferation assay. Fibroblasts were harvested from the stock culture in FBS-containing medium by tryptic digestion and washed in the serum-free medium without iron and any agent. They were plated at 1×10^4 cells/cm^2 onto 2-cm^2 wells containing 0.5 ml of various serum-free media per well. When the effect of antioxidants or active oxygen species on fibroblast proliferation was analyzed, the cultures were loaded with SOD (10 μg/ml), DMSO (10 m*M*), pyruvate (5 m*M*), or H_2O_2 (20 μ*M*). Fibroblasts were cultured for 4 d at 37° C in a CO_2 incubator. After culture, the cells were detached by tryptic digestion. The number of cells was then counted by a hemacytometer. The assay was also done by culturing fibroblasts in FBS-containing medium.

Statistics. Replicate determination data were summarized as the mean ± one standard deviation (SD). Student's *t*-test for unpaired determinations was used to analyze statistical differences. The criterion of significance was set at $P < 0.05$.

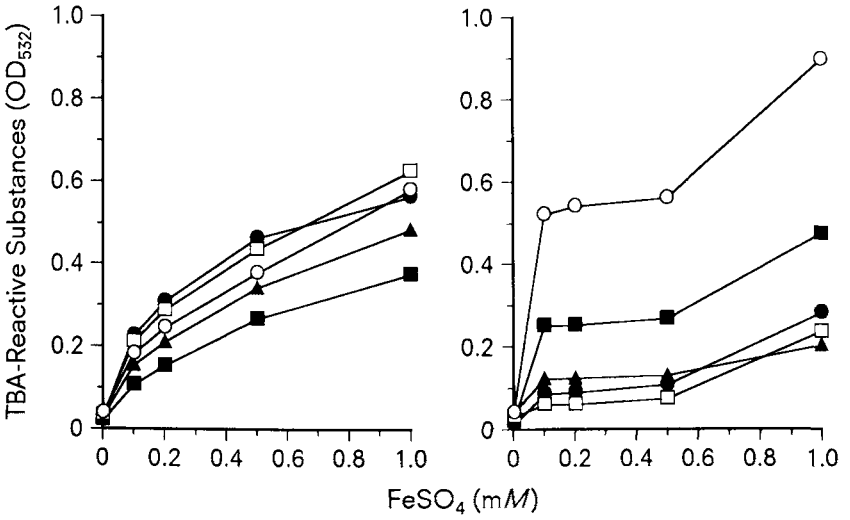

FIG. 1. Dose effect of ferrous iron on the formation of TBA-reactive substances in various media and changes in reactivity when ascorbate was added to the media. *Left:* various concentrations of $FeSO_4$ (0, 0.1, 0.2, 0.5, and 1 mM) were assessed for their ability to stimulate the formation of TBA-reactive substances in the media supplemented with or without the indicated agents (▲, agent withdrawal; □, HEPES; ●, GG; ○, IDA; ■, DHEG). *Right:* similar determinations were made by adding 1 mM ascorbate to the media. Data points represent the average of duplicate determinations.

RESULTS

Ferrous iron-stimulated formation of TBA-reactive substances in various serum-free media. The effect of $FeSO_4$ (0, 0.1, 0.2, 0.5, and 1 mM) in combination with or without agents on the formation of TBA-reactive substances in the media was analyzed first. $FeSO_4$ stimulated the formation of TBA-reactive substances irrespective of the presence or absence of any agent (Fig. 1, *left*). The response depended on the concentration of $FeSO_4$ up to at least 1 mM. Agents such as HEPES, GG, and IDA augmented the formation of the substances stimulated with $FeSO_4$ compared to the agent withdrawal control. DHEG was found to slightly depress the response.

An additional set of experiments was conducted to analyze how agents affected the formation of TBA-reactive substances stimulated with $FeSO_4$ when 1 mM ascorbate was added to the media. Ascorbate greatly changed the TBA response (Fig. 1, *right*). The yield of TBA-reactive substances increased with the concentrations of $FeSO_4$ up to 0.1 mM, leveled off between 0.1 and 0.5 mM, and then resumed increasing at more than 0.5 mM in either the presence or absence of any agent. The reaction profile could be clearly divided into two groups. One included the agent withdrawal control, GG, and HEPES where ascorbate greatly inhibited the formation of the substances. Another included IDA and DHEG where ascorbate augmented formation of the substances, particularly in the presence of 0.1 to 0.2 mM $FeSO_4$.

Permeation of ^{59}Fe into preconfluent fibroblasts incubated in the serum-free media. Permeation of ^{59}Fe during various incubation periods (3, 8, and 24 h) was assessed on preconfluent fibroblasts. After 3 h of incubation in the agent withdrawal medium or in the medium supplemented with apoTf, HEPES, or GG, 4 to 6% of Fe permeated into fibroblasts (Fig. 2). Permeation increased with time, reaching 10 to 12% of total ^{59}Fe after 24 h of incubation. In comparison, iron permeation was comparatively less in the presence of IDA or DHEG. Only 3% of ^{59}Fe permeated into fibroblasts. Permeation did not increase even after fibroblasts were incubated for 24 h.

Ferrous iron-stimulated LDH release from subconfluent fibroblasts. To analyze the effect of $FeSO_4$ (0, 20, 50, and 100 μM) in combination with or without agents on the destruction of cell membranes of subconfluent fibroblasts, the LDH release assay was performed. The level of LDH release did not change in the agent withdrawal medium or in the medium supplemented with IDA although $FeSO_4$ increased to 100 μM (Fig. 3). The release level did not change even in the presence of $FeSO_4$ up to 50 μM but significantly decreased in the presence of 100 μM $FeSO_4$ when fibroblasts were incubated in the medium supplemented with apoTf, HEPES, or GG. In comparison, LDH release greatly increased in the medium with DHEG as the concentration of $FeSO_4$ increased.

Colloidal aggregates formed when $FeSO_4$ or $FeCl_3$ at the final concentrations of more than 20 μM was mixed with deionized water or each of the stock solutions containing agents except IDA and then diluted with the concentrated serum-free medium. Cytotoxicity was assessed before and after aggregates were removed by centrifugation to analyze effect of iron aggregates on cellular damage. Removal of aggregates from such serum-free media did not essentially affect LDH release (data not shown).

Effect of antioxidants on the ferrous iron-stimulated LDH release. To clarify whether $FeSO_4$ (100 μM) in combination with or without agents induced oxidative cellular damage, antioxidants such as SOD (10 μg/ml), mannitol (10 mM), DMSO (10 mM), pyruvate (10 mM), and ascorbate (1 mM) were assessed for their effect on LDH release from subconfluent fibroblasts. Unless iron was provided, fibroblasts spontaneously released 15 to 20% of total intracellular LDH in various media irrespective of either the presence or absence of agents (Fig. 4). Exposure of fibroblasts to SOD or DMSO in the presence of $FeSO_4$ decreased LDH release to below the spontaneous release levels achieved under the conditions without iron and antioxidants. This

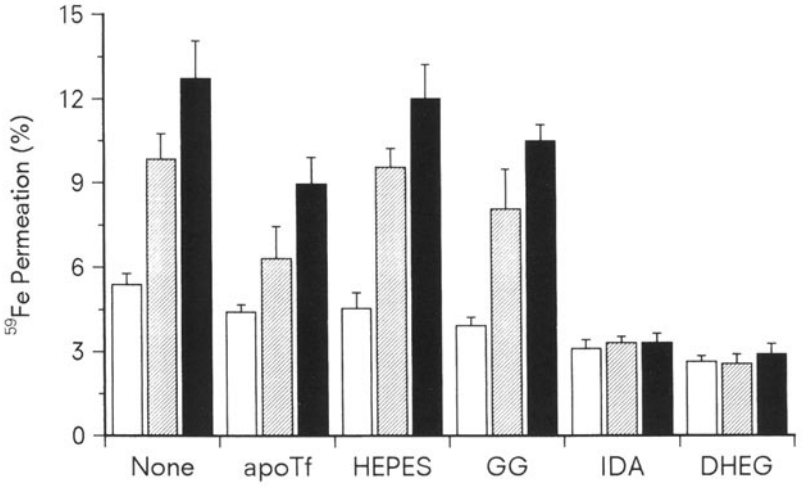

FIG. 2. ^{59}Fe permeation into preconfluent fibroblasts was assessed in the media supplemented with 1 μCi/ml $^{59}FeCl_3$ instead of nonisotopic iron and with or without the indicated agents. The cells were incubated for 3 h (*open columns*), 8 h (*hatched columns*), or 24 h (*closed columns*). *Columns* and *error bars* denote the mean percentage and SD, respectively, for four culture wells.

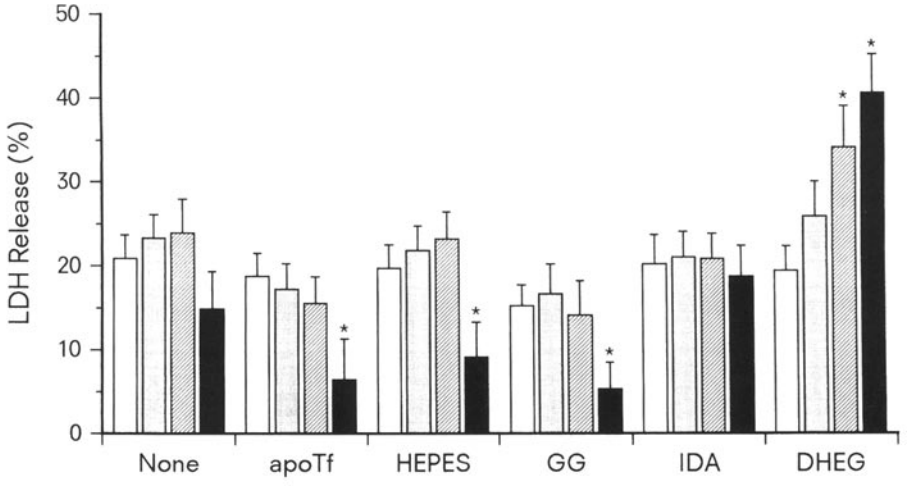

FIG. 3. Augmentation and inhibition of LDH release from subconfluent fibroblasts by ferrous iron. LDH release was assessed by incubating fibroblasts in the media supplemented with various concentrations of $FeSO_4$ (*open columns*, 0 μ*M*; *shaded columns*, 20 μ*M*; *hatched columns*, 50 μ*M*; *closed columns*, 100 μ*M*) and with or without the indicated agents. *Columns* and *error bars* denote the mean percentage and SD, respectively, for four culture wells. A significant influence of $FeSO_4$ on LDH release is indicated with an *asterisk*.

occurred whether or not agents were present. Pyruvate and mannitol were ineffective in decreasing enzyme release from fibroblasts. Ascorbate was also ineffective if fibroblasts were incubated in the absence of *DHEG*. A combination of $FeSO_4$ with *DHEG* augmented LDH release to 90% of the total enzyme activity. LDH release from fibroblasts exposed to antioxidants in the presence of 20 μ*M* $FeSO_4$ was at the same level as was achieved in the presence of 100 μ*M* $FeSO_4$ (data not shown).

Cytotoxicity of exogenously added H_2O_2. LDH release from subconfluent fibroblasts exposed to various concentrations of H_2O_2 (0, 20, 50, and 100 μ*M*) in various media supplemented with 100 μ*M* $FeSO_4$ was monitored. As shown in Fig. 5, 20 μ*M* H_2O_2 tended to decrease LDH release from fibroblasts incubated in the media without IDA or DHEG. By increasing the concentration of H_2O_2 to more than 50 μ*M*, the extent of LDH release increased when fibroblasts were incubated in the media irrespective of either the presence or absence of agents. Interestingly, however, the increase in H_2O_2-dependent LDH release only in the presence of GG was the smallest of all incubation conditions. This tendency was apparent when fibroblasts were exposed to 100 μ*M* H_2O_2. On the contrary, H_2O_2-dependent LDH release was mostly promoted with DHEG. The H_2O_2-dependent dose-response described above similarly occurred in the presence of 20 μ*M* $FeSO_4$ (data not shown).

Ferrous iron-stimulated lipid peroxidation. Decomposition of cellular lipids was analyzed by incubating subconfluent fibroblasts in the media supplemented with various concentrations of $FeSO_4$ (0, 20, and 100 μ*M*). Twenty micromoles of $FeSO_4$ with or without agents except DHEG did not significantly stimulate lipid peroxidation (Fig.

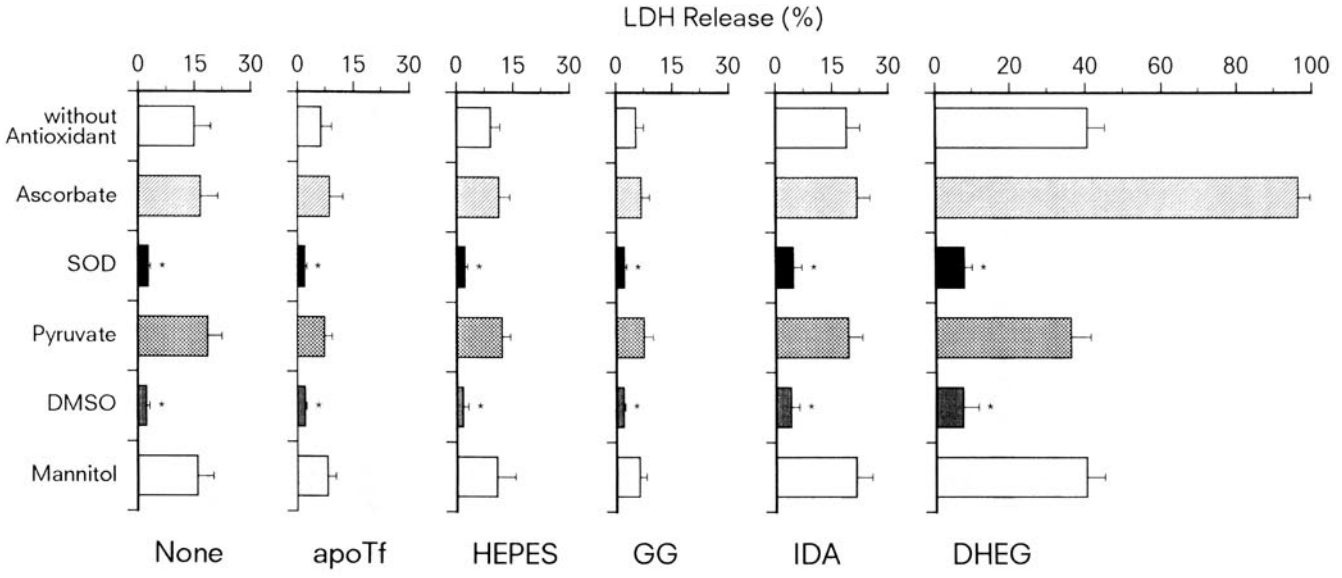

FIG. 4. Influence of antioxidants on LDH release from subconfluent fibroblasts incubated with ferrous iron. LDH release was assessed by exposing fibroblasts to various antioxidants in the media supplemented with 100 μM $FeSO_4$ and with or without the indicated agents. *Columns* and *error bars* denote the mean percentage and SD, respectively, for four culture wells. An *asterisk* indicates significant inhibition of LDH release by antioxidants. *Shaded ranges* along x axes represent the mean percentage ± SD achieved under the conditions with the corresponding agents but without iron and antioxidants.

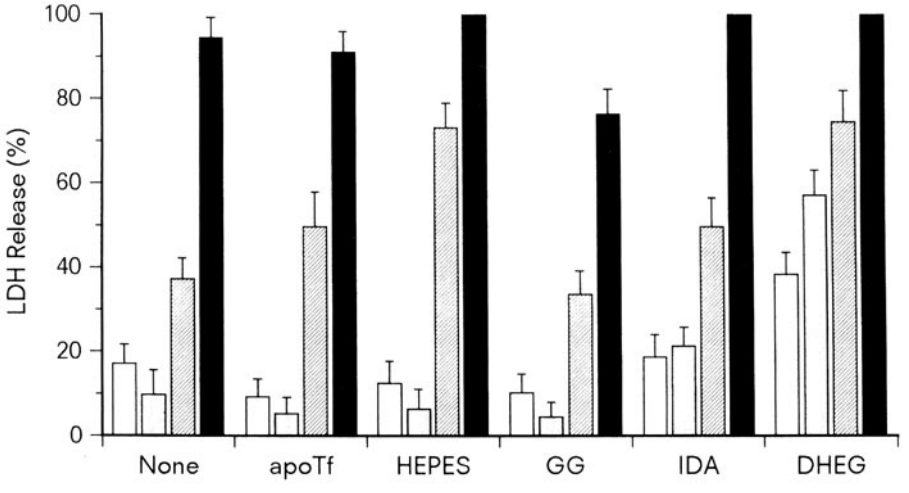

FIG. 5. H_2O_2-dependent LDH release from subconfluent fibroblasts incubated with ferrous iron. LDH release was assessed by exposing fibroblasts to various concentrations of H_2O_2 (*open columns*, 0 μM; *shaded columns*, 20 μM; *hatched columns*, 50 μM; *closed columns*, 100 μM) in the media supplemented with 100 μM $FeSO_4$ and with or without the indicated agents. *Columns* and *error bars* denote the mean percentage and SD, respectively, for four culture wells.

6). By increasing the concentration of $FeSO_4$ up to 100 μM, peroxidation increased two to three times. On the other hand, peroxidation increased 10-fold when the concentration of $FeSO_4$ was increased up to 100 μM in the presence of DHEG.

Removal of iron aggregates by centrifugation from the media supplemented with 100 μM $FeSO_4$ and with or without iron aggregate-inducible agents except DHEG decreased lipid peroxidation to levels comparable to those achieved under conditions without iron. The level of cellular lipid peroxidation remained noticeably elevated even after the removal of iron aggregates from the medium supplemented with 100 μM $FeSO_4$ and DHEG. Lipid peroxidation did not decrease when fibroblasts were incubated in the clarified supernatant fluid of the medium supplemented with 100 μM $FeSO_4$ and an aggregate-noninducible agent IDA.

Iron-stimulated fibroblast proliferation. Fibroblasts proliferated 1.5 times the initial inoculum cell density when cultured in the media supplemented with only chelating agents but without iron (Fig. 7). On the other hand, the apparent proliferation-stimulating effect of

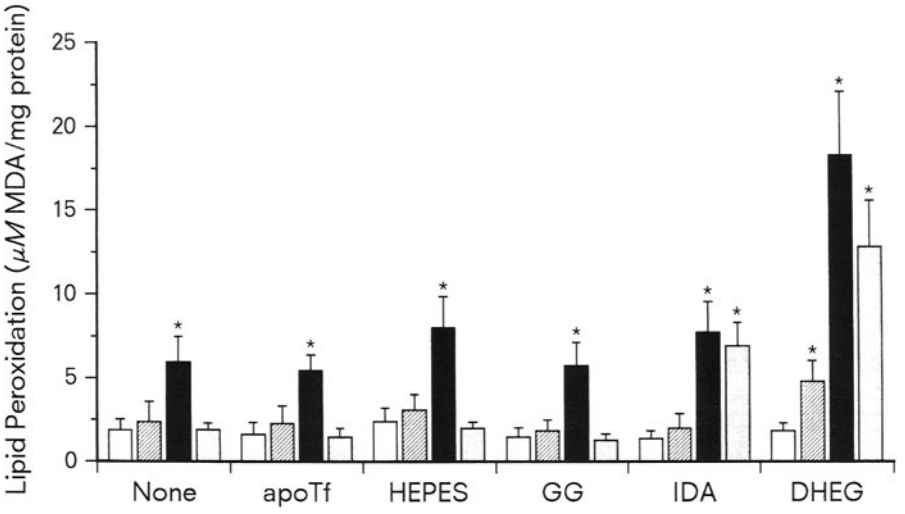

FIG. 6. Augmentation of lipid peroxidation by ferrous iron. Lipid peroxidation was assessed by incubating fibroblasts in the media supplemented with various concentrations of $FeSO_4$ (*open columns*, 0 μ*M*; *hatched columns*, 20 μ*M*; *closed columns*, 100 μ*M*) and with or without the indicated agents. The $FeSO_4$-supplemented media were clarified by centrifugation (*shaded columns*). *Columns* and *error bars* denote the mean percentage and SD, respectively, for four culture flasks. The lipid peroxidation-stimulating effect of iron was significant where indicated with an *asterisk*.

iron was noted at a concentration of 20 μ*M* and occurred irrespective of either the presence or absence of agents. Fifty micromoles of $FeSO_4$ at which colloidal aggregates formed in the media supplemented without IDA inhibited fibroblasts from attaching to the culture substratum and surviving. This inhibition was virtually due to the presence of iron aggregates (Table 1). The yield of iron aggregates derived from $FeSO_4$ was larger than that derived from $FeCl_3$ so that the toxic effect of $FeSO_4$ was greater than that of $FeCl_3$ under the iron aggregate-inducible culture conditions. A hundred micromoles of either species of iron in combination with an aggregate-noninducible agent IDA still provided the great proliferation stimulus for fibroblasts (data not shown).

Effect of exogenous H_2O_2 on fibroblast proliferation. Effect of exogenously added H_2O_2 on fibroblast proliferation was analyzed. The concentrations of $FeSO_4$ at 20 μ*M* and H_2O_2 at 20 μ*M* were chosen because both concentrations were found not so toxic to subconfluent fibroblasts (Fig. 3 and Fig. 5, respectively). Table 2 shows that H_2O_2 was very toxic to growing fibroblasts in the agent withdrawal medium or in the medium supplemented with apoTf, HEPES, or DHEG. In comparison, H_2O_2 was not toxic to growing fibroblasts cultured in the presence of GG or IDA. Addition of 5 m*M* pyruvate abolished the toxic effect of H_2O_2 almost completely.

Effect of antioxidants on fibroblast proliferation. The final step of this report was to evaluate effect of antioxidants such as SOD and DMSO, which could eliminate LDH release from subconfluent fibroblasts as shown in Fig. 4, on the proliferation of fibroblasts. Proliferation was assessed by exposing fibroblasts to these antioxidants in the media at the injurious concentration of $FeSO_4$ (50 μ*M*) shown in Fig. 7. Fibroblasts tended to adhere and spread on the culture substratum when SOD or DMSO were added to the media. No effect of these antioxidants was observed if IDA was present (Table 3).

DISCUSSION

Inspection with a $^{59}FeCl_3$ probe verified that iron permeation into preconfluent fibroblasts was not associated with iron-chelating ability of agents. A preliminary experiment confirmed that ^{59}Fe remained in a supernatant fraction of the serum-free medium supplemented with IDA 100 and 14 times, at least, more than in the supernatant fractions of the media supplemented with GG and DHEG, respectively, if 100 μ*M* $FeCl_3$ was present (data not shown). This demonstrates that the iron-chelating activity of IDA is strong. Unexpectedly, however, the most strong iron-chelating agent, IDA, used in the present study was found to have very poor ability to allow iron to permeate into preconfluent fibroblasts. A similar phenomenon has been recently reported: transport of iron into human intestinal Caco-2 cells is greatly reduced if iron is combined with a membrane-permeable chelating agent, 1,2-dimethyl-3 hydroxypyridin-4-one (17). Even in the absence of chelating agents, ferric iron could permeate into fibroblasts with time. These results postulate that chelating agents do not always act as an iron carrier in the serum-free culture of dermal fibroblasts.

Our results clearly show that the different toxic effects of iron appeared depending on not only confluence of fibroblasts but also species of chelating agents added to the culture medium. Subconfluent fibroblasts were not damaged by $FeSO_4$ at a concentration as high as 100 μ*M*, as proven by the LDH release assay in which fibroblasts were incubated for 24 h in the serum-free media, unless DHEG was added. Astonishingly, 100 μ*M* $FeSO_4$ significantly decreased the extent of cellular damage when subconfluent fibroblasts were incubated in the medium supplemented with apoTf, HEPES, or GG. A similar effect of $FeSO_4$ was found in the absence of any agent although the effect was not significant. We cannot explain why the damage was depressed even in the presence of 100 μ*M* $FeSO_4$. Probably, (sub)confluent fibroblasts require an adequate amount of iron for survival. That subconfluent fibroblasts incubated in the medium supplemented with 100 μ*M* $FeSO_4$ and IDA did not decrease to release LDH may be due to the poor ability of IDA to support iron permeation into fibroblasts. In contrast to these incubation conditions, LDH release from subconfluent fibroblasts incubated in the medium with DHEG, which had also poorly supported iron perme-

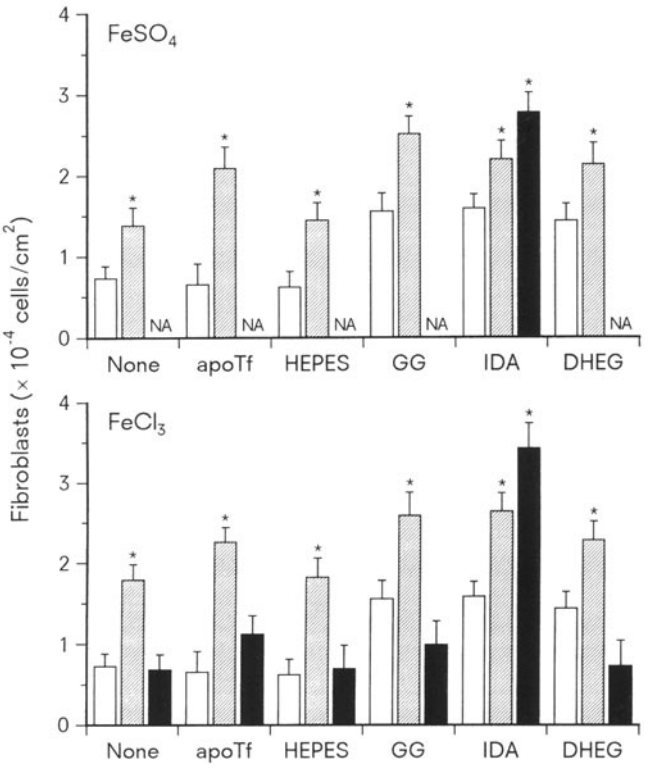

FIG. 7. Augmentation and inhibition of fibroblast proliferation by iron. Proliferation was assessed by culturing fibroblasts (1×10^4 cells/cm^2) in media supplemented with various concentrations of $FeSO_4$ or $FeCl_3$ (*open columns*, 0 μM; *hatched columns*, 20 μM; *closed columns*, 50 μM) with or without the indicated agents. *Columns* and *error bars* denote the mean percentage and SD, respectively, for four culture wells. The proliferation-promoting effect of iron was significant where indicated with an *asterisk*. NA: not adherent (fibroblasts did not adhere to the culture substratum and failed to proliferate). The culture in FBS-containing medium yielded (4.73 ± 0.54) $\times 10^4$ cells/cm^2.

TABLE 1

INFLUENCE OF COLLOIDAL AGGREGATES ON FIBROBLAST PROLIFERATION

Agents[a]	Without iron	Clarification of the media[b]	
		No	Yes
None	0.73 ± 0.15[c]	NA[d]	0.79 ± 0.26
apoTf	0.65 ± 0.26	NA	0.93 ± 0.22
HEPES	0.62 ± 0.19	NA	0.84 ± 0.29
GG	1.55 ± 0.23	NA	1.78 ± 0.36
IDA	1.58 ± 0.18	2.77 ± 0.27	2.51 ± 0.48
DHEG	1.43 ± 0.21	NA	1.25 ± 0.32

[a]The media were supplemented with or without 50 μM $FeSO_4$ and with or without the indicated agents.

[b]The media supplemented with $FeSO_4$ were divided into two parts. One was clarified by centrifugation to remove iron aggregates. Another was not centrifuged.

[c]Values express the mean cell number ± SD for four culture wells.

[d]NA; not adherent. Fibroblasts did not adhere to the culture substratum. Accordingly, fibroblasts failed to proliferate.

TABLE 2

EFFECT OF H_2O_2 AND PYRUVATE ON FIBROBLAST PROLIFERATION

Agents[a]	Additives			
	None	H_2O_2	Pyruvate	H_2O_2 + pyruvate
None	1.38 ± 0.28[b]	NA[c]	1.76 ± 0.13	1.62 ± 0.19
apoTf	2.09 ± 0.27	NA	2.31 ± 0.20	2.11 ± 0.25
HEPES	1.44 ± 0.22	NA	2.26 ± 0.24	1.72 ± 0.21
GG	2.51 ± 0.21	2.25 ± 0.21	2.93 ± 0.27	2.74 ± 0.32
IDA	2.77 ± 0.25	1.08 ± 0.12	2.61 ± 0.31	2.16 ± 0.26
DHEG	2.13 ± 0.27	NA	2.02 ± 0.22	1.93 ± 0.15

[a]The media were supplemented with 20 μM $FeSO_4$ and with or without the indicated agents. Fibroblasts were plated at 1×10^4 cells/cm^2, loaded with or without the indicated additives (20 μM H_2O_2 and 5 mM pyruvate), and cultured for 4 d in these media.

[b]Values express the mean cell number ± SD for four culture wells.

[c]NA: not adherent. Fibroblasts did not adhere to the culture substratum. Accordingly, fibroblasts failed to proliferate.

TABLE 3

FIBROBLAST SURVIVAL WHEN ANTIOXIDANTS ARE ADDED TO THE SERUM-FREE MEDIA SUPPLEMENTED WITH THE INJURIOUS CONCENTRATION OF $FeSO_4$

Agents[a]	Antioxidants		
	None	SOD	DMSO
None	NA[b]	AD[c]	1.72 ± 0.19[d]
apoTf	NA	AD	1.21 ± 0.23
HEPES	NA	AD	0.92 ± 0.14
GG	NA	AD	1.36 ± 0.20
IDA	2.04 ± 0.31	2.19 ± 0.25	2.33 ± 0.28
DHEG	NA	AD	1.58 ± 0.21

[a]The media were supplemented with 50 μM $FeSO_4$ and with or without the indicated agents. Fibroblasts were plated at 1×10^4 cells/cm^2, loaded with or without 10 μg/ml SOD or 10 mM DMSO, and cultured for 4 d in these media.

[b]NA: not adherent. Fibroblasts did not adhere to the culture substratum. Accordingly, fibroblasts failed to proliferate.

[c]AD: adherent. Fibroblasts adhered to the culture substratum; however, the number of cells could not be determined, owing to low cell density.

[d]Values express the mean cell number ± SD for four culture wells.

ation, increased with the concentration of $FeSO_4$. This suggests that DHEG affects fibroblast culture by different mechanisms.

The deleterious effect of iron on lipid peroxidation of plasma membranes has been clarified. The literature reports that extreme peroxidation of unsaturated lipids breaks down membrane integrity (14.19.33). Some chelating agents including ethylenediaminetetraacetic acid (EDTA) are known to inhibit induction of iron-mediated lipid peroxidation (8.22.23). $FeSO_4$ concentrations as high as 100 μM induced the decomposition of lipids of fibroblasts. The LDH release assay, however, showed that such high concentration of $FeSO_4$ did not induce destruction of membranes unless the medium was supplemented with DHEG, as just mentioned.

OH is thought to be a major causative species responsible for LDH release from subconfluent fibroblasts into incubation medium because DMSO as a scavenger of OH· (39) blocked enzyme release almost completely. Failure of mannitol as a scavenger of OH· (29) to

decrease LDH release was probably due to its low membrane permeability (17,35) or low reactivity with OH^-, or both. Failure of ascorbate to decrease LDH release suggests that ascorbate did not merely act as a scavenger of O_2^- in the (sub)confluent fibroblast culture. SOD was found to block LDH release to a similar extent as DMSO. It is noteworthy that SOD increases the level of a membrane-permeable oxidant, H_2O_2, through O_2^- dismutation (10,15). If the destruction of membranes was caused by H_2O_2, pyruvate known as a scavenger of H_2O_2 (32) would eliminate H_2O_2 cytotoxicity. In contrast to above assumption, pyruvate did not decrease LDH release. *It is therefore suggested that H_2O_2 generated through O_2^- dismutation* is not a major causative species inducing the destruction of membranes. The experiment dealing with the effect of H_2O_2 on subconfluent fibroblast culture revealed that H_2O_2 at 20 μM did not raise the level of LDH release unless DHEG was added to the medium. Particularly, subconfluent fibroblasts were more resistant to the cytotoxicity of H_2O_2 at 20 to 100 μM if GG was added. Although the precise mechanism(s) by which GG can control cytotoxicity is not defined at present, we do not rule out the possibility that (sub)confluent fibroblasts are stimulated to produce stress proteins to oxidants such as H_2O_2 (21,26,34).

There is an exception to emphasize: $FeSO_4$ up to 100 μM is not toxic to subconfluent fibroblasts, as mentioned earlier. A combination of $FeSO_4$ and DHEG greatly increased cellular alterations such as the breakdown and destruction of membranes to a greater extent than did $FeSO_4$ alone. Williams et al. (43) reported that DHEG can cross cell membranes to sweep out excess intracellular iron. Kukielka and Cederbaum (24) have recently reported that iron mobilized from ferritin by chelating agents such as EDTA and ATP has the potential to catalyze the formation of active oxygen species if an appropriate *in vivo* reducing agent is present. These observations by others may partially resolve the question of why subconfluent fibroblasts exposed to ascorbate were greatly damaged in the presence of both $FeSO_4$ and DHEG. Fibroblast damage induced with $FeSO_4$ in the presence of DHEG but in the absence of an exogenous reducing agent may also be influenced by *in vivo* reducing agents generated by fibroblast metabolism.

A permeable amount of iron (approximately 1 μM) as a contaminant dissolved in the medium did not stimulate fibroblast proliferation. Meanwhile, fibroblasts were stimulated to proliferate if they were cultured in the medium supplemented with any chelating agent but without exogenous iron. It is unclear why chelating agents such as GG, IDA, and DHEG stimulate fibroblast proliferation. A possible explanation is that chelating agents adsorb trace elements, which contaminate the medium and are toxic to fibroblasts.

Comparatively higher concentrations of $FeSO_4$ and $FeCl_3$ (20 μM or more) promptly stimulated fibroblast proliferation only when IDA was added to the medium. Such a requirement for iron may be due to the poor ability of IDA to support iron permeation into fibroblasts. IDA strongly induced the decomposition of membrane lipids in concentrations of $FeSO_4$ as high as 100 μM but such decomposition may not bring on the subsequent destruction of membranes. It is therefore likely that iron-mediated lipid decomposition occurs at extracellular sites on cell membranes owing to the poor iron permeation in the presence of IDA. Murrel et al. (31) reported that human fibroblasts are stimulated to proliferate in response to low concentrations of active oxygen species such as O_2^- and H_2O_2. We also cannot exclude the possibility that the proliferation stimulus is provided by active oxygen species, which were generated in the medium at a level less than that which could destroy cell membranes.

Our data show that IDA is the strongest promoter of the formation of ferrous iron-stimulated, ascorbate-dependent, TBA-reactive substances from deoxyribose. It has been established that the formation of TBA-reactive substances from deoxyribose is induced with OH^- (15). Once $FeSO_4$ was mixed with culture medium, Fe^{2+} was oxidized with time. The elevated level of OH^- generation by adding ascorbate to the medium supplemented with $FeSO_4$ and IDA is therefore thought to result from reduction of Fe^{3+}, which is the oxidized form of $FeSO_4$. Probably, IDA does not inhibit the cellular redox-cycling between Fe^{2+} and Fe^{3+}. In short, IDA does not impair the redox activity of iron that is essential for the living cells.

Fibroblasts were apparently inhibited from proliferating by saturated colloidal aggregates (38) that formed when iron was combined with or without agents except IDA at physiological pH values and were subsequently diluted in the serum-free medium or when iron was directly diluted in the medium. Removal of the aggregates from the media greatly reduced the deoxyribose-dependent TBA response (data not shown). Although iron aggregates are thought to be nontoxic to subconfluent fibroblasts because of a lack of ability to raise the level of LDH release, they raised the level of lipid peroxidation. Furthermore, SOD and DMSO enabled fibroblasts to adhere to the culture substratum and to survive even in the presence of iron aggregates. These results suggest that iron aggregates act as a generator of active oxygen species toxic to growing fibroblasts.

Although Tf is believed to act as an antioxidant (2,13), it is reported that Tf and its fragments have the potential to injure cultured cells by generating active oxygen species depending on its iron-saturating status (6,12,28). Concentrations of $FeSO_4$ and $FeCl_3$ greater than 20 μM are estimated to be too high for 10 μg apoTf per ml (corresponding to roughly 0.125 μM) to trap iron because apoTf binds ferric iron at a 1:2 molecular ratio in the presence of HCO_3^- (1). In the fibroblast culture, 10 mg of human apoTf per liter did not serve as an antioxidant. This was clearly shown when growing fibroblasts were exposed to 20 μM H_2O_2.

In conclusion, a potent stimulus of fibroblast proliferation in serum-free medium was provided by the extracellular high concentration of solubilized iron without eliminating the redox-cycling activity of iron ions. Tf may have little ability to provide such proliferation stimulus.

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REFERENCES

1. Aasa, R.; Aisen, P. An electron paramagnetic resonance study of the iron and copper complexes of transferrin. J. Biol. Chem. 243:2399–2404; 1968.
2. Aruoma, O. I.; Halliwell, B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Are lactoferrin and transferrin promoters of hydroxyl-radical generation? Biochem. J. 241:273–278; 1987.
3. Aruoma, O. I.; Halliwell, B.; Gajewski, E., et al. Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. Biochem. J. 273:601–604; 1991.

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4. Aust, S. D.; Morehouse, L. A.; Thomas, C. E. The role of transition metals in oxygen radical reactions. J. Free Radic. Biol. Med. 1:3–25; 1985.
5. Balin, A. K.; Goodman, D. B. P.; Rasmussen, H., et al. The effect of oxygen and vitamin E on the lifespan of human diploid cells in vitro. J. Cell Biol. 74:58–67; 1977.
6. Brieland, J. K.; Clarke, S. J.; Karmiol, S., et al. Transferrin: a potential source of iron for oxygen free radical-mediated endothelial cell injury. Arch. Biochem. Biophys. 294:265–270; 1992.
7. Breimer, L. H. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. Mol. Carcinog. 3:188–197; 1990.
8. Chance, B.; Sies, H.; Boveris, A. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59:527–605; 1979.
9. Esterbauer, H.; Cheeseman, K. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol. 86:407–421; 1990.
10. Fridovich, I. Superoxide dismutases. Annu. Rev. Biochem. 44:147–159; 1975.
11. Fridovich, I. The biology of oxygen radicals. Science 201:875–880; 1978.
12. Fujimoto, S.; Kawakami, N.; Ohara, A. Nonenzymatic glycation of transferrin: decrease of iron-binding capacity and increase of oxygen radical production. Biol. Pharm. Bull. 18:396–400; 1995.
13. Gutteridge, J. M. C.; Quinlan, G. J. Antioxidant protection against organic and inorganic oxygen radicals by normal human plasma: the important primary role for iron-binding and iron-oxidising proteins. Biochim. Biophys. Acta 1156:144–150; 1993.
14. Gutteridge, J. M. C.; Richmond, R.; Halliwell, B. Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and lipid peroxidation by desferrioxamine. Biochem. J. 184:469–472; 1979.
15. Halliwell, B.; Gutteridge, J. M. C. Formation of thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. FEBS Lett. 128:347–352; 1981.
16. Halliwell, B.; Gutteridge, J. M. C. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219:1–14; 1984.
17. Hamilton, K. O.; Stallibrass, L.; Hassan, I., et al. The transport of two iron chelators, desferrioxamine B and L1, across Caco-2 monolayers. Br. J. Haematol. 86:851–857; 1994.
18. Harman, D. The aging process. Proc. Natl. Acad. Sci. USA 78:7124–7128; 1981.
19. Horton, A. A.; Fairhurst, S. Lipid peroxidation and mechanisms of toxicity. Crit. Rev. Toxicol. 18:27–79; 1987.
20. Kann, H. E., Jr.; Mengel, C. E.; Clancy, W. T., et al. Effects of in vivo hyperoxia on erythrocytes. VI. Hemolysis occurring after exposure to oxygen under high pressure. J. Lab. Clin. Med. 70:150–157; 1967.
21. Keyse, S. M.; Tyrrell, R. M. Heme oxygenase is the major 32-KDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc. Natl. Acad. Sci. USA 86:99–103; 1989.
22. Knight, J. A.; Blaylock, R. C.; Searles, D. A. Lipid peroxidation in platelet concentrates: effects of irradiation and metal chelators. Ann. Clin. Lab. Sci. 23:333–339; 1993.
23. Kozlov, A. B.; Ostrachovitch, E. A.; Afanas'ev, I. B. Mechanism of inhibitory effects of chelating drugs on lipid peroxidation in rat brain homogenates. Biochem. Pharmacol. 47:795–799; 1994.
24. Kukielka, E.; Cederbaum, A. I. Ferritin stimulation of hydroxyl radical production by rat liver nuclei. Arch. Biochem. Biophys. 308:70–77; 1994.
25. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L., et al. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
26. Lu, D.; Maulik, N.; Moraru, I. I., et al. Molecular adaptation of vascular endothelial cells to oxidative stress. Am. J. Physiol. 264:C715–C722; 1993.
27. Medzon, E. L.; Gedies, A. Substitution of 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) for bicarbonate in protein-free animal cell culture medium: application to vaccinia virus quantitation and fluorogenic acetylesterase assay in living LM cells. Can. J. Microbiol. 17:651–653; 1971.
28. Miller, R. A.; Britigan, B. E. Protease-cleaved iron-transferrin augments oxidant-mediated endothelial cell injury via hydroxyl radical formation. J. Clin. Invest. 95:2491–2500; 1995.
29. Morrison, A. R.; Winokur, T. S.; Brown, W. A. Inhibition of soybean lipoxygenase by mannitol. Biochem. Biophys. Res. Commun. 108:1757–1762; 1982.
30. Morgan, E. H. Studies on the mechanism of iron release from transferrin. Biochim. Biophys. Acta 580:312–326; 1979.
31. Murrell, G.; Francis, N.; Bromley, L. Modulation of fibroblast proliferation by oxygen free radicals. Biochem. J. 265:659–665; 1990.
32. O'Donnell-Tormey, J.; Nathan, C. F.; Lanks, K., et al. Secretion of pyruvate. An antioxidant defense of mammalian cells. J. Exp. Med. 165:500–514; 1987.
33. Pacifici, R. E.; Davies, K. J. A. Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited. Gerontology 37:166–180; 1991.
34. Pantopoulos, K.; Hentze, M. W. Rapid responses to oxidative stress mediated by iron regulatory protein. EMBO J. 14:2917–2924; 1995.
35. Pasantes-Morales, H.; Murray, R. A.; Sanchez-Olea, R., et al. Regulatory volume decrease in cultured astrocytes. II. Permeability pathway to amino acids and polyols. Am. J. Physiol. 266:C172–C178; 1994.
36. Sawada, M.; Carlson, J. C. Changes in superoxide radical and lipid peroxide formation in the brain, heart and liver during the lifetime of the rat. Mech. Ageing Dev. 41:125–137; 1987.
37. Shipman, C., Jr. Evaluation of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as a tissue culture buffer. Proc. Soc. Exp. Biol. Med. 130:305–310; 1969.
38. Spiro, T. G.; Allerton, S. E.; Renner, J., et al. The hydrolytic polymerization of iron(III). J. Am. Chem. Soc. 88:2721–2726; 1966.
39. Steiner, M. G.; Babbs, C. F. Hydroxyl radical generation by postischemic rat kidney slices in vitro. Free Radic. Biol. Med. 9:67–77; 1990.
40. Stocker, R.; Glazer, A. N.; Ames, B. N. Antioxidant activity of albumin-bound bilirubin. Proc. Natl. Acad. Sci. USA 84:5918–5922; 1987.
41. Trush, M. A.; Kensler, T. W. An overview of the relationship between oxidative stress and chemical carcinogenesis. Free Radic. Biol. Med. 10:201–209; 1991.
42. Wayner, D.; Burton, G.; Ingold, K., et al. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochem. Biophys. Acta 924:408–419; 1987.
43. Williams, A.; Pugh, A.; Hoy, T. G., et al. Screening for iron chelating drugs. In: Saltman, P.; Hegenauer, J., ed. The biochemistry and physiology of iron. Amsterdam: Elsevier North Holland, Inc.; 1982:199–204.
44. Yabe, N.; Kato, M.; Matsuya, Y., et al. Role of iron chelators in growth-promoting effect on mouse hybridoma cells in a chemically defined medium. In Vitro Cell. Dev. Biol. 23:815–820; 1987.