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DOES MANGANESE PROTECT CULTURED HUMAN SKIN FIBROBLASTS AGAINST OXIDATIVE INJURY BY UVA, DITHRANOL AND HYDROGEN PEROXIDE?

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Reactive oxygen species (ROS) are involved in the mechanism of photoaging and carcinogenesis. Skin is endowed with antioxidant enzymes including superoxide dismutases (SOD): cytosolic copper zinc SOD and mitochondrial manganese SOD. The aim of our study was to estimate the protective effect of manganese against oxidative injury on cultured human skin fibroblasts. Dithranol, hydrogen peroxide and UV-A radiation (375 nm) were employed as oxidative stressors. The supply of manganese chloride produced an increase in cellular content of this element up to 24 fold without concomitant elevation of MnSOD activity. Nevertheless, manganese protects cells against two of the three ROS generating systems assessed, namely hydrogen peroxyde and UV-A. This protective effect depends on the concentration of manganese in the medium, 0.1 mM and 0.2 mM protect against UVA cytotoxicity, only 0.2 mM protects against H_2O_2 cytotoxicity.

KEY WORDS: antioxidant, Manganese superoxide dismutase, free radicals, cytotoxicity

Abbreviations: Mn, Manganese; ROS, Reactive Oxygen Species; SOD, Superoxide dismutase; HSBc, Healthy Skin Biopsied cells; UV-A, Ultraviolet radiations in the A region; Tris, Tris(hydroxymethyl)-aminomethane; DTPA, Diethylenetriamine pentaacetic acid; PBS, Phosphate buffered saline; FCS, Fetal calf serum.

INTRODUCTION

There is growing evidence that oxygen free radicals are implicated in numerous processes such as carcinogenesis, photoaging or inflammation[1–3]. These highly reactive species are likely to directly or indirectly damage nucleic acids, proteins or membrane phospholipids.

Numerous constitutive protective systems serve to defend humans against oxidative stress, and some of them employ trace elements: catalase, seleno-dependent glutathione peroxidase, copper zinc superoxide dismutase (CuZnSOD) or manganese superoxide dismutase (MnSOD). MnSOD plays an essential antioxidant role as it is located in the mitochondria of eucaryotic cells, where oxidative phosphorylation produces oxygen

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free radicals. Manganese is the metal at the active site of this enzyme, which catalyses dismutation of superoxide anions to hydrogen peroxide.

Moreover MnSOD belongs to the stress proteins which are believed to protect cells against the agents responsible for their induction[4], and some stress proteins are induced by low intensity UV-A irradiation[5].

Lastly, in the midst of mineral or non enzymic organic complexes, Mn ion is likely to catalyse the dismutation of superoxide[6,7] or the disproportionation of hydrogen peroxide[8,9].

These observations suggest that manganese can exhibit antioxidant properties in cells. The aim of our work was thus to determine whether or not Mn^{2+} supplied in the medium of cultured human skin fibroblasts provides protection against the following oxidative injuries: hydrogen peroxide, a diffusible molecule able to generate hydroxyl radicals by the Fenton reaction in the presence of transition metals[10]; UV-A radiation, generating reactive oxygen species during photochemical reactions[11]; and dithranol (anthralin; 1,8-dihydroxy-9-anthrone), a dermatological therapeutic molecule used against psoriasis, which generates intracellular superoxide anions[12].

MATERIALS AND METHODS

Chemicals

L-glutamine, sodium bicarbonate 7.5% and Puck's saline A were purchased from Gibco (Grand Island, USA). RPMI medium, fetal calf serum FCS, penicillin, streptomycin, kanamycin, trypsin were purchased from Boehringer (Mannheim, Germany), fungizone from Squibb (Princetown, USA). Hydrogen peroxide and Manganese chloride ($MnCl_2$) were from Prolabo (Paris, France), diethylenetriamine pentaacetic acid (DTPA), cacodylic acid and dithranol from Sigma Chemical Co. (Saint Louis, Mo, USA), and Tris from Merck (Darmstadt, Germany).

Cell Culture

Human fibroblasts obtained from healthy skin biopsies were used between the fifth and the fifteenth passage. Culture medium RPMI 1640 with $NaHCO_3$, penicillin 180 000 UI/l, streptomycin 180 mg/l, kanamycin 56 mg/l, fungizone 0.9 mg/l, L-glutamine 1.8 mM, was added with 10% FCS. Cells were incubated at 37°C in a 5% CO_2-enriched atmosphere (Forma Scientific incubator). Culture flasks and petri dishes were from Falcon. For trace elements determination or enzyme assays, subconfluent cells were trypsinized in 75 cm^2 flasks, washed 3 times by 5 ml of isotonic, 400 mM, trace element-free Tris-HCl buffer pH 7.30, and then ground in a potter tissue homogenizer in 5 ml of hypotonic Tris-HCl buffer (isotonic buffer diluted in 1/20). After 10 minutes of centrifugation at 4000 rpm, the lysate was assayed for the activities of the metalloenzymes or stored at –20°C until trace element determination. To determine cytotoxicity, cells were seeded in 35 mm culture dishes (130 000 cells/dish) until near confluency, achieved after 4 days.

Manganese Chloride Supply

The RPMI medium with 10% FCS was added with $MnCl_2$ (filtered through a 0.2 μM pore membrane) in variable concentration. Cells were supplied for 48 hours with fresh

medium added or not with manganese. The manganese concentrations assayed were those surrounding one tenth of the TD50 (dose leading to a 50% cytotoxicity) determined as explained below. Final manganese concentrations in the media were determined by electrothermal atomic absorption spectrophotometry (Perkin Elmer model 560 fitted with AGA 500 furnace, AS40 autosampler, and equipped with a deuterium background correction). Samples were stored at −18°C until analysis, and then injected under a 50 μl volume in a pyrolytically coated graphite tube. Standards were prepared in Triton X 100 (Prolabo, Paris, France) using tritisol (Merck, Darmstadt, Germany).

Intracellular Trace Elements Assay

Intrafibroblastic manganese and iron concentrations were determined by electrothermal atomic absorption spectrophotometry, using the centrifuged cells lysate supernatant. Their level was normalized to cell protein content.

SOD Assay

Total SOD, MnSOD and CuZnSOD were determined using the pyrogallol assay following the procedure described by Marklund[13], based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD, photometrically read at $\lambda = 420$ nm. Briefly, 150 μl of the sample were added with 1.8 ml of Tris(50 mM)-DTPA(1 mM)-cacodylic acid buffer pH 8.3 and with pyrogallol 10 mM in order to induce an absorbance change of 0.020 in absence of SOD. The amount of SOD inhibiting the reaction rate by 50% in the given assay conditions is defined as one SOD unit. The specific CuZnSOD inhibition by KCN (60 μl of KCN 54 mM added to 300 μl of lysate) allows the MnSOD determination in the same conditions. Each sample was assayed twice, and results were expressed as SOD units and normalized to the cell protein content.

Cytotoxicity Determination

Cytotoxicity was measured using the adhesion-proliferation method. This technique has been compared with the MTT method to make sure that it can be used to evaluate oxidative injury[10]. After stress, cells were washed twice with Puck's saline, trypsinized and transferred on new dishes. Fresh medium was added, and the cells placed in an incubator for 18 hours. The culture dishes were then rinsed vigorously with isotonic saline to remove non adherent cells. Total cell protein was determined according to the procedure described by Shopsis and Mackay[14]. Two dishes of each of the 3 strains were tested, and each dish was run in duplicate.

Results are expressed as the cytotoxicity % evaluated with the formula 1-(Ts/Tc) with Tc = total protein in control dishes and Ts = total protein in stressed dishes.

Oxidative Stress Application

Hydrogen peroxide: sub-confluent cells were rinsed twice with PBS and then left for 30 minutes in the dark in 2 ml of H_2O_2 diluted in PBS, without added $MnCl_2$. The final H_2O_2 concentrations were varying from 0 to 25 mM. Control fibroblasts were kept in PBS under the same environmental conditions.

Dithranol: dithranol was solubilized in ethanol. Dilutions were done in PBS so that

final solutions contained $1/40^{th}$ of ethanol and 0 to 10 μM of dithranol. Immediately, obtained solutions were applied for 30 minutes on the sub-confluent cells, in the dark, in a 2 ml volume. Control fibroblasts were kept under the same environmental conditions with 2 ml of PBS-ethanol (39/1; V/V).

UV-A irradiation: Cells were irradiated with an UVASUN 2000 apparatus (Mutzhas, Munich, Germany) whose spectrum ranges from 340 to 420 nm with a maximum intensity at 375 nm. The energy the cells effectively received (0 to 10 J/cm^2) was controlled with a compensated Kipp and Zonen Thermopile coupled to a digital voltmeter. Sub-confluent cells were washed twice with 2 ml of PBS, placed in 1 ml of PBS and irradiated at a 20 cm distance from the source for varying times. Control fibroblasts were sham-irradiated in 1 ml of PBS for the same time.

Evaluation of the Manganese Effect Against Oxidative Injury:

The effect of two $MnCl_2$ concentrations (0.1 and 0.2 mM) was assessed against each oxidative stress. Hydrogen peroxide, dithranol and UV-A were used at the following concentrations: H_2O_2 2.5 mM; dithranol 2.5 μM; UV-A radiation 7 Joules/cm^2. Manganese was supplied during the 48 hours before stress application, and during the 18 hours following trypsinization. Manganese was not added to the cells during the periods of stress.

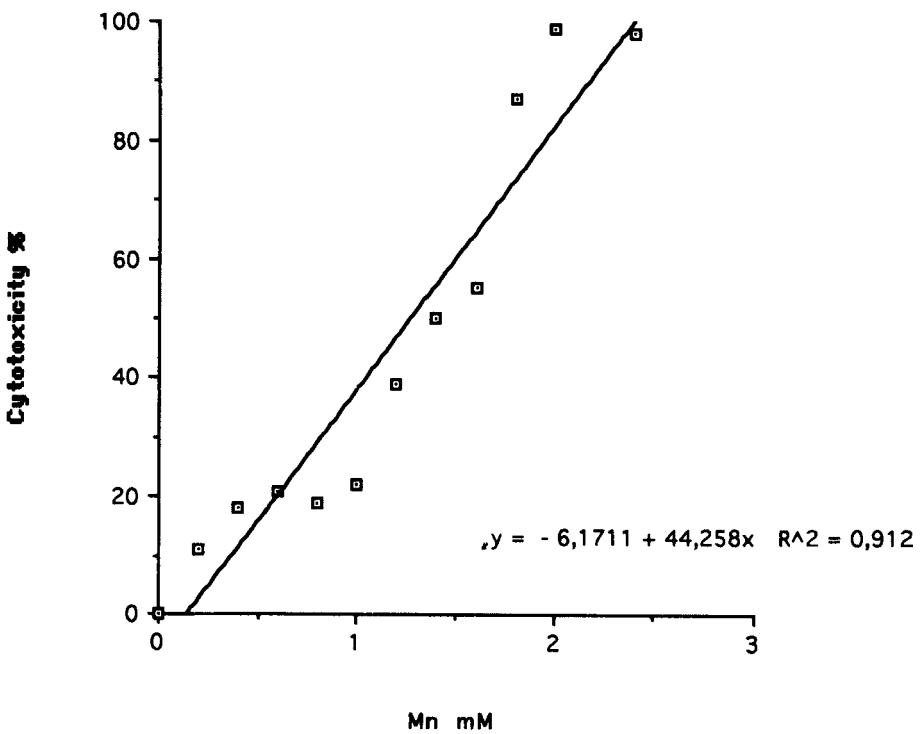

FIGURE 1 $MnCl_2$ toxicity on HSB fibroblasts. Adhesion and proliferation capacity was determined by measuring total protein content using the method of Shopsis and Mackay[14].

Statistics

The data have been analysed by a Mann-Whitney U-test.

RESULTS

$MnCl_2$ Toxicity Measurement for Determination of the Supply Concentrations

The toxicity of $MnCl_2$ supplied with growing concentrations is presented in Figure 1. The toxic 50 dose, measured after 48 h, is 1.3 mM for Healthy Skin Biopsied fibroblasts (HSBc). For later experiments we chose a concentration area surrounding one tenth of the TD_{50}, that is to say varying from 0 to 0.3 mM. In the standard medium with 10% FCS, the manganese concentration is $0.085 \pm 0.015\,\mu M$.

Intracellular Manganese Penetration

Intracellular manganese concentrations after 48 hours, expressed as micrograms per protein gram, are presented in Figure 2. Intracellular manganese grows with the MnCl2 supply, up to 24 fold (from 7 to 169 $\mu g/g$) after 48 h with the 0.2 mM concentration.

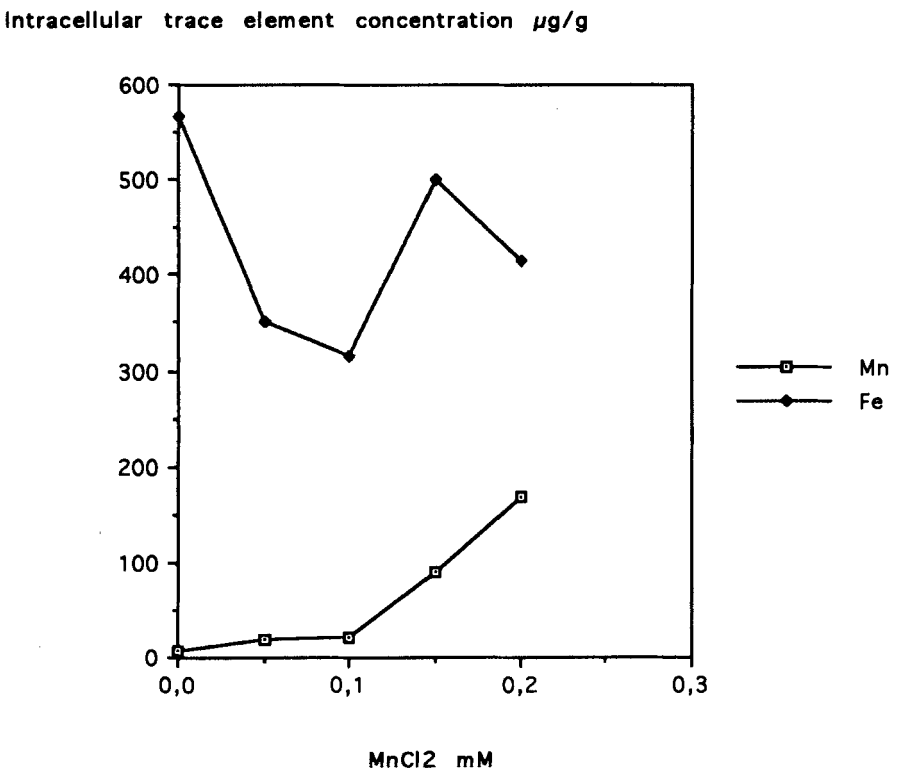

FIGURE 2 Intracellular manganese and iron concentration following a 48 h manganese supply. Trace element determination was done by electrothermal atomic absorption spectrophotometry.

Intracellular Iron Variability

To check if there is an interaction between iron and manganese intracellular levels, we determined the intracellular iron concentration under the manganese supply (Figure 2). These results don't allow us to conclude that the manganese supply is responsible for a variation in intracellular iron concentrations in these cells.

SOD Activity

SOD activities (MnSOD and CuZnSOD) according to the manganese supply in the medium are shown in Figure 3. Neither total SOD, CuZnSOD, nor MnSOD is influenced by the $MnCl_2$ supply.

Cytotoxicity of the Different ROS Generating Systems

The cytotoxicity of the three oxidative stressors was determined to establish the doses used for the study of the manganese potential protective effect on the HSBc. The cytotoxicity according to the stress intensity is shown in Figure 4a to 4c.

Evaluation of the Manganese Cytoprotective Effect Against Oxidative Injury

H_2O_2: results are presented in Figure 5 (mean ± SD, n = 3). The protection provided by manganese against cytotoxic effect of H_2O_2 is statistically significant for the $MnCl_2$ concentration 0.2 mM.

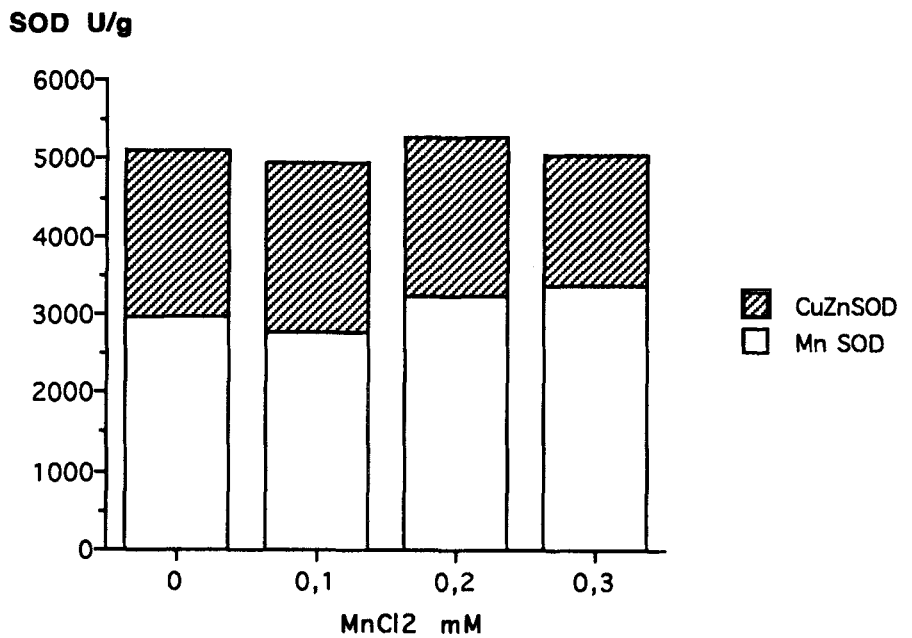

FIGURE 3 Influence of the manganese supply on the SOD activities, determined using the method of Marklund[13]. Each point has been run in duplicate and is the average of two separate experiments.

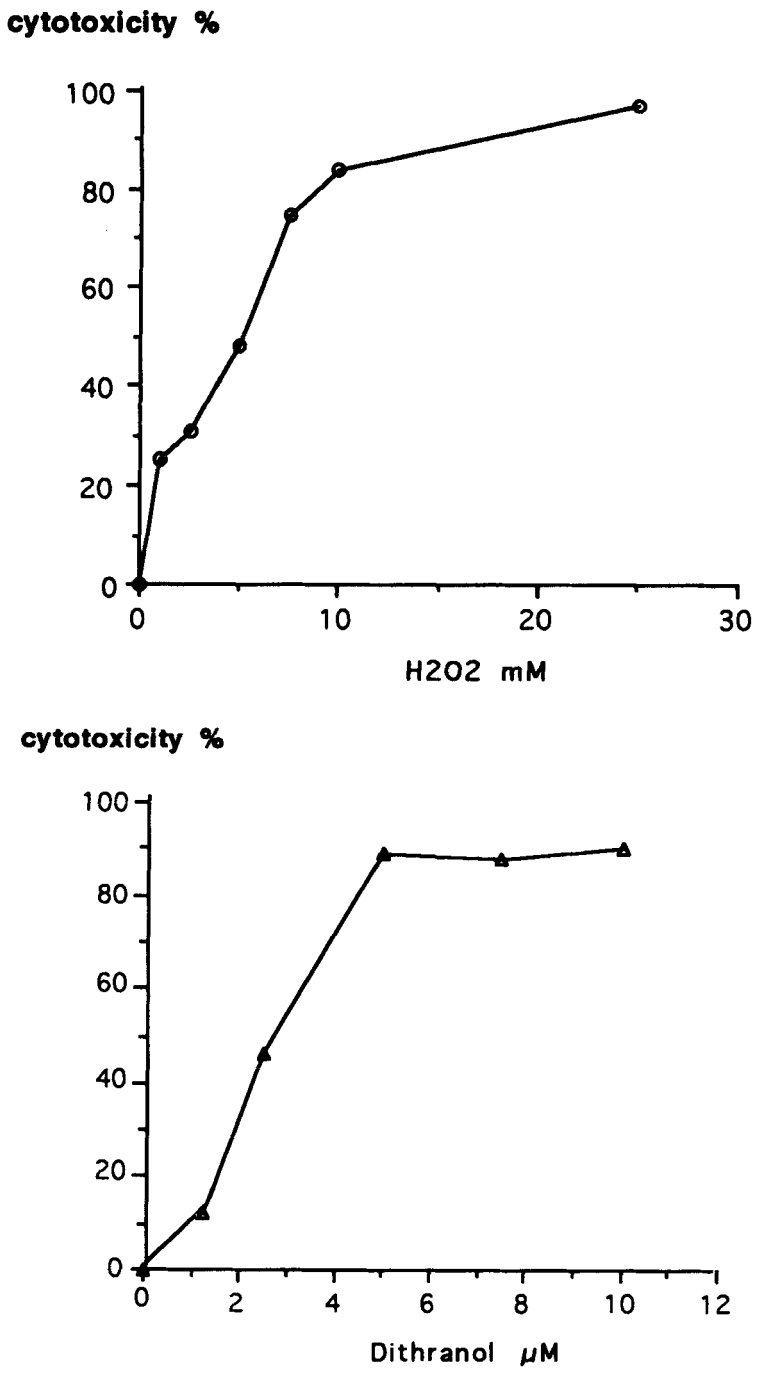

FIGURE 4 Cytotoxicity of increasing doses of the three stressors, hydrogen peroxide (a), dithranol (b) and UVA radiation (c), on HSB fibroblasts, as determined by the adhesion-proliferation method using the total measurement of protein content by the method of Shopsis and Mackay[14].

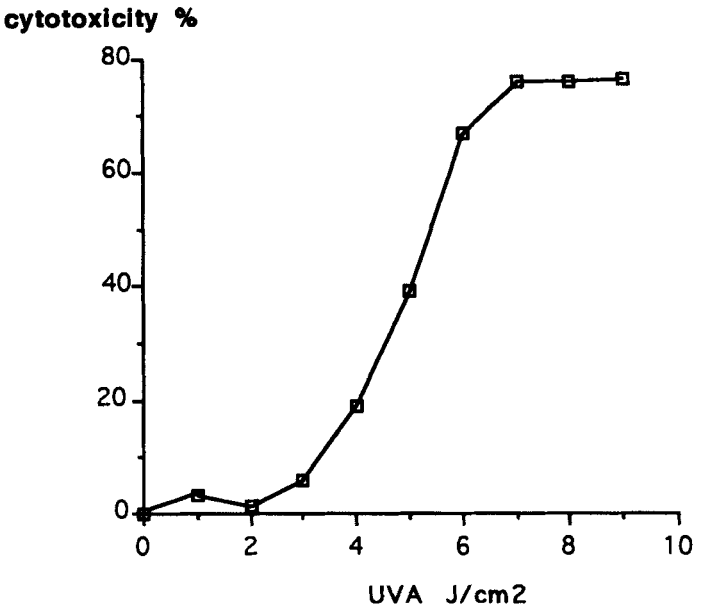

FIGURE 4 *continued*

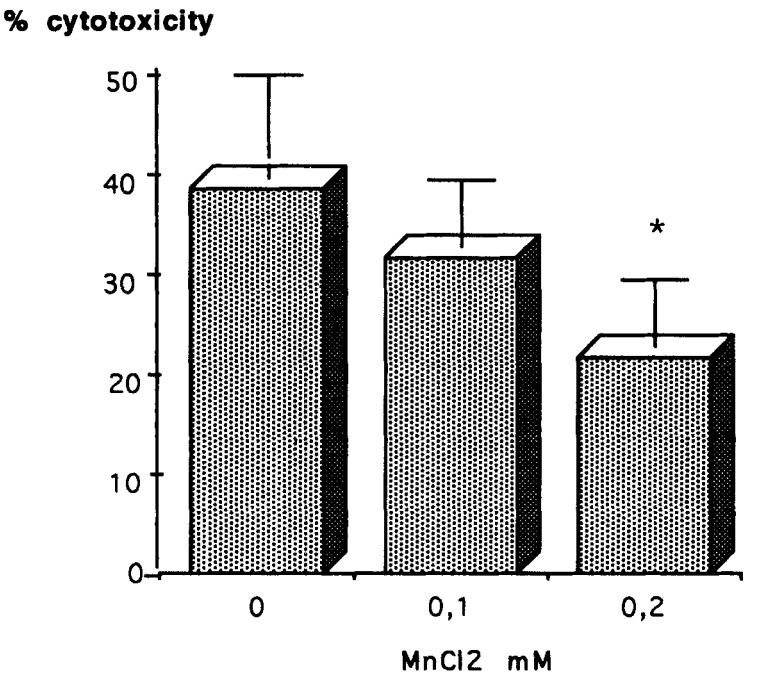

FIGURE 5 Protective effect of Mn against hydrogen peroxide cytotoxicity on HSBc. H_2O_2 concentration was 2.5 mM. Proliferation capacity was determined by measuring total protein by the method of Shopsis and Mackay[14]. Values represent mean ± SD of three separate experiments. * $p = 0.05$, $MnCl_2$ 0.2 mM treated and H_2O_2 exposed cells versus H_2O_2 exposed cells.

Dithranol: the effect of both concentrations of $MnCl_2$ 0.1 and 0.2 mM was assayed twice on each of the 3 tested strains. Results are shown in fig 6. No statistically significant difference is shown in the cytotoxicity of dithranol in manganese treated versus untreated cells.

UV-A: the effect of manganese against UV-A (7 J/cm2) is shown in fig 7. The UV-A cytotoxicity is significantly reduced for both 0.1 mM and 0.2 mM $MnCl_2$ treated cells. The protection provided by manganese is dose-dependent.

DISCUSSION

Among the mechanisms leading to photoaging and carcinogenesis, reactive oxygen species seem to have a significant role. They are involved in UV radiation deleterious effects, directly and through inflammatory processes (free radicals, cytokines). Furthermore, they are implicated in the cytotoxicity of many xenobiotics, of which

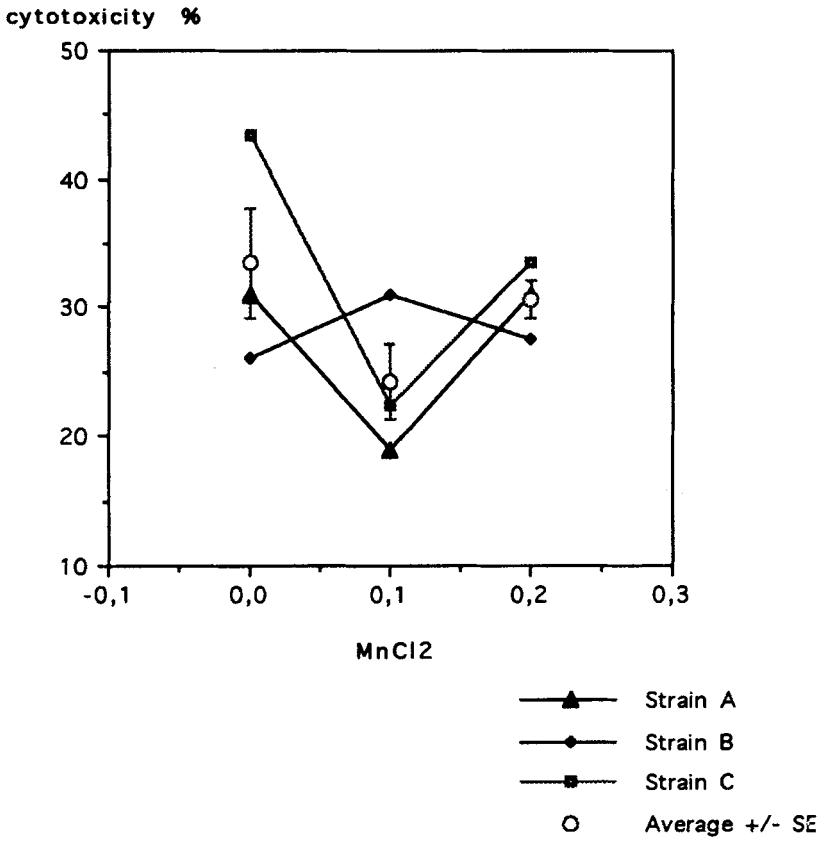

FIGURE 6 Effect of Mn supply against dithranol cytotoxicity on HSBc. Dithranol concentration was 2.5 μM. The result of each strain is shown by closed symbols, open symbols represent mean ± SE. Proliferation capacity was determined by measuring total protein by the method of Shopsis and Mackay[14].

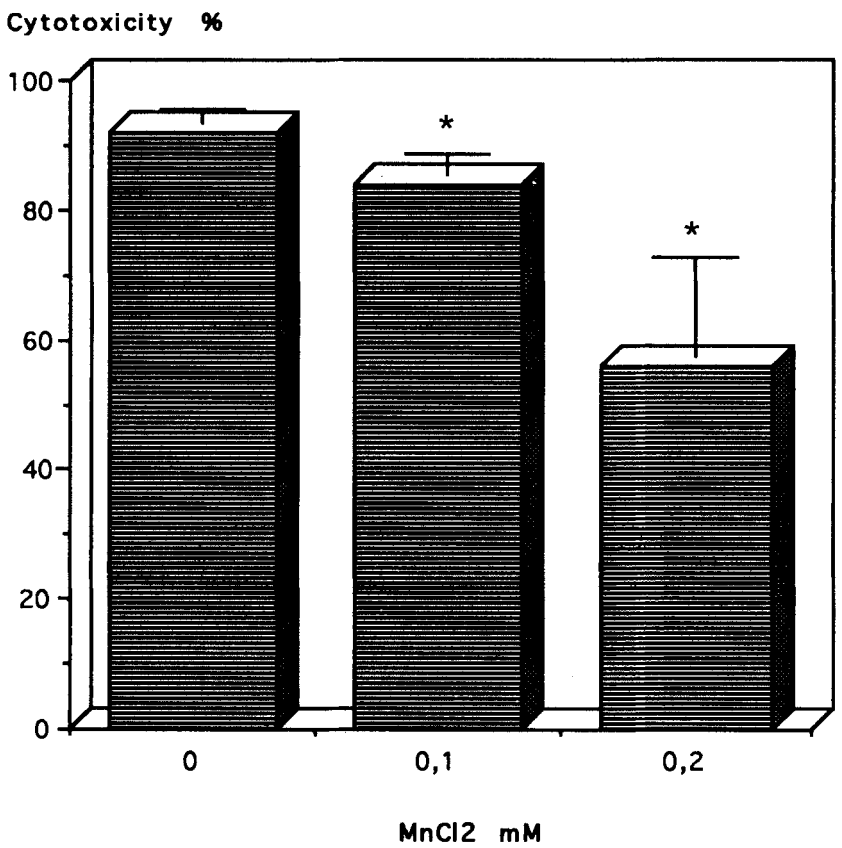

FIGURE 7 Effect of manganese supply against UVA (7 J/cm^2) on HBSc. Proliferation capacity was determined by measuring total protein by the method of Shopsis and Mackay[14]. Values represent mean ± SD of three separate experiments. * p = 0.05, $MnCl_2$ treated and UVA irradiated cells versus UVA irradiated cells.

dithranol[12], a widely used antipsoriasis drug with tumor-promoting and skin-irritating properties. Against free-radical attack, cells are equiped with ROS degrading endogenous systems, non enzymatic (vitamins, glutathione) and enzymatic (SOD, catalase, peroxidases).

The antioxidant role of manganese in different forms (mineral, organic, enzymatic) is the focus of active research in the therapeutic field[15–18]. Recent works have dealt with the antioxidant role of manganese *in vitro* and *in vivo*, but studies concerning eucaryotic cells are rare. Moreover, to our knowledge, none of them describes the link between manganese supply and MnSOD activity in cultured human fibroblasts. Nevertheless, it is interesting to note that manganese can also exhibit oxidative properties: Aust *et al.* have pointed out that manganese belongs to the metals undergoing redox reactions and participating in the promotion of autooxidative and enzymatic peroxidation of polyunsatured fatty acids[19]; Snyder *et al.* have shown manganese-induced DNA strand breaks, but only above elevated doses[20].

Our study shows that manganese supply raised the intracellular amount of this trace

element. Cytotoxicity appears for low concentrations and is maximum above 2 mM. Like the other trace elements, manganese has thus to be supplied to the cells in an optimal range of concentration.

After a 48 h supply of manganese chloride at a 0.1 mM and sometimes 0.2 mM concentration in the medium, protection against two ROS generating systems appears. These systems, hydrogen peroxide and UVA radiations, are connected to the different physiopathological ROS production and their cytotoxicity through ROS has been previously established in the literature[10,11]. This cytoprotection indicates that manganese can limit the deleterious effects of ROS. This observation agrees with Varani's results, that show the protective effect of manganese against H_2O_2 on cultured endothelial cells and *in vivo* on rats[21].

Mitochondria are the first target of dithranol toxicity. This compound generates mostly intracellular superoxide, and this effect is partially inhibited by SOD addition[12]. In our study, the protection against dithranol is not statistically significant, contrarily to the protection against hydrogen peroxide or UVA; this observation supports the hypothesis that manganese is acting independently from MnSOD to protect the cells, for MnSOD would have been expected to prevent mitochondria-initiated cytotoxicity.

Different mechanisms can explain the cytoprotective effect of manganese against oxidative injury. In order to study the effect of the manganese that cells accumulated during the 48 hours supply against ROS generated in the period of stress, cells are not in contact with manganese during the oxidative stress application. One explanation for manganese cytoprotection may deal with the well-known antagonism between manganese and iron existing at the intracellular transport level in vivo[22–23]. Iron is implicated in Fenton reaction, by which hydrogen peroxide is able to generate hydroxyl radicals, and a decrease in the intracellular concentration of iron could be expected to decrease cellular damage due to this highly reactive species. Our data do not indicate a clear intervention of the manganese supply on the intracellular amount of iron, nor a participation of iron in the protection provided by manganese. Further experiments in this field are in progress.

Another explanation of the protection provided by manganese supply implies the MnSOD activity modulation by manganese. We couldn't show an increase in MnSOD activity following $MnCl_2$ supply. Other authors' studies have shown a manganese-induced increase in MnSOD activity, but never in MnSOD mRNA or immunoreactive protein. Indeed it is an increase in MnSOD activity that was shown in E. Coli[24]. It is proposed in this study that Mn doesn't affect MnSOD induction. However, procaryotic cells exhibit a different regulation of MnSOD, where iron is strongly implicated at a transcriptional level. In a study based on Saccharomyces cerevisiae[25], manganese provided in the culture medium up to very high concentrations increases manganese intracellular concentration and MnSOD activity, but this activity is shown to be due to manganese complexes. After human oral supplementation with manganese *in vivo*, Davis showed an increase in MnSOD activity in lymphocytes[23].

On the other hand, manganese deprivation in animals leads to a decrease in MnSOD activity. Recently it has been proposed that manganese deficiency could be directly responsible for the decrease of MnSOD activity, by a pretranscriptionnal regulation mechanism and not solely because of a lack of the metal at the enzyme active site[26].

In our study, manganese protective effect seems to be independent from MnSOD activity, but the relationship between manganese and MnSOD mRNA is the object of current work. Antioxidant metalloenzymes are under different modulations by the amount of their active site metal; for example, glutathione peroxidase synthesis requires

selenium because of a co-translational mechanism for the incorporation of selenium into the enzyme[27].

Although manganese doesn't seem to induce MnSOD, the observed cytoprotective effect against H_2O_2 or UV-A radiation can be explained by the participation of manganese in reactions which limit the extent of oxygen radical reactions. Mn-polyphosphates or Mn-pyrophosphates have SOD-like activity[6,7], manganese in the presence of bicarbonate ions exhibits catalase-like properties[8] and Mn-bicarbonate-amino acid complexes have SOD-like and catalase-like activities[9]. We thus postulate that the intracellular formation of this kind of complex is the mechanism responsible for the antioxidant effect of manganese.

We have shown in this work a protective effect of manganese on cultured human fibroblasts submitted to two different systems generating reactive oxygen species, H_2O_2 and UV-A radiation. Additional experimentation will clarify the mechanism of the manganese antioxidant efficacy, which seems to be independent from a MnSOD activity elevation. Studying thoroughly the interaction between iron and manganese on cells, and following the MnSOD expression instead of its activity, will be the next steps of our work.

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