Stable Overexpression of Manganese Superoxide Dismutase in Mitochondria Identifies Hydrogen Peroxide as a Major Oxidant in the AP-1-mediated Induction of Matrix-degrading Metalloprotease-1*

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Reactive oxygen species (ROS) are important second messengers for the induction of several genes in a variety of physiological and pathological conditions. Here we addressed the question of whether isolated, unbalanced overexpression of the antioxidant enzyme manganese superoxide dismutase (Mn-SOD) may modulate signal transduction cascades, finally leading to connective tissue degradation, a hallmark in carcinogenesis and aging. Therefore, we generated stably Mn-SOD-overexpressing fibroblasts with an up to 4.6-fold increase in Mn-SOD activity. The Mn-SOD-overexpressing cells revealed specific resistance to the superoxide anion (O2·−)-generating agent paraquat, whereas no resistance to UVA-generated oxidative stress was found. Treatment of the Mn-SOD-overexpressing cells with various ROS-generating systems resulted (due to the enhanced dismutation of superoxide anion to hydrogen peroxide) in an up to 9.5-fold increase in matrix-degrading metalloprotease-1 (MMP-1) mRNA levels. A similar increase in MMP-1 mRNA was also seen when the intracellular H2O2 concentration was increased by the inhibition of different H2O2-detoxifying pathways. Furthermore, prooxidant conditions led to a strong induction of c-jun and c-fos mRNA levels resulting in a 4-fold higher transactivation of the transcription factor AP-1 in the Mn-SOD-overexpressing cells. Collectively, we have found that enhanced Mn-SOD activity, via an unbalanced H2O2 overproduction and detoxification, induces MMP-1 mRNA levels, and this effect is at least partly mediated by the DNA recognition sequence AP-1.

Although reactive oxygen species are part of normal regulatory circuits, imbalance or loss of cellular redox homeostasis results in oxidative stress (1, 2), causing severe damage of cellular components. Apart from permanent genetic changes involving protooncogenes and tumor suppressor genes, reactive oxygen species (ROS)1 activate cytoplasmatic signal transduction pathways that are related to growth, differentiation, senescence, and tissue degradation. Therefore, ROS have been implicated to play a causal role in cancer, aging, and other degenerative diseases like arteriosclerosis, osteoarthritis, and impaired wound healing. These pathological states share unique features and are all characterized by a dysregulated localized (as is the case for cancer, invasion, and metastasis) or diffuse connective tissue breakdown due to enhanced activity of various matrix-degrading metalloproteases (3–10). The family of matrix-degrading metalloproteases now comprises at least 19 members with partly distinct, partly overlapping substrate specificities for different extracellular matrix proteins of the connective tissue. Due to promoter similarities, a variety of matrix-degrading metalloproteases (MMPs) like the interstitial collagenase (MMP-1) (11) and stromelysin-1 (MMP-3) (12) have been shown to be similarly regulated in different experimental settings. Accordingly, MMP-1 and MMP-3 have been found to be induced upon UVA and UVB irradiation (13–15). The promoter of MMP-1 carries five AP-1 sites, and that of MMP-3 carries a single AP-1 site (12), which are transactivated by binding of the newly synthesized and heterodimerized Fos and Jun, which constitute the AP-1 transcription factor (15, 16). Research on the regulation of the synthesis and activity of transcription factors by endogenous and environmental stimuli like ROS is a matter of increasing interest and relevance (17–19), since it may provide ultimate clues for mechanisms underlying connective tissue degradation in pathological states. In fact, ROS have been shown to transactivate transcription factors like NF-κB and AP-1 in carcinoma cell lines of epithelial origin (20, 21). Large efforts have been made to better define the involvement of distinct ROS in degenerative conditions to identify enzymes and molecules that can scavenge oxygen radicals for their potential in the prevention and therapy of these disorders.

To protect against oxidant injury, aerobic cells have evolved a multilayered interdependent antioxidant system that includes enzymatic and nonenzymatic components. The individual antioxidant enzymes are located in specific subcellular sites and reveal distinct substrate specificity. Among these, manganese superoxide dismutase (Mn-SOD) has been the subject of particular interest because it is located in the mitochondria and represents the first line of defense against superoxide radicals produced as byproduct of oxidative phosphorylation, upon UV

peroxide; MMP, matrix-degrading metalloprotease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; O2·−, superoxide anion; DFO, desferrioxamine; bp, base pair(s); PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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1 The abbreviations used are: ROS, reactive oxygen species; ATZ, aminotriazole; BSO, buthionine sulfoximine; CAT, chloramphenicol acetyltransferase; SOD, superoxide dismutase; Cu,Zn-SOD, copper, zinc SOD; Mn-SOD, manganese superoxide dismutase; H2O2, hydrogen peroxide; MMP, matrix-degrading metalloprotease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; O2·−, superoxide anion; DFO, desferrioxamine; bp, base pair(s); PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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6 irradiation and during immunological and nonimmunological inflammatory processes. Mn-SOD can be induced by its substrate, the superoxide anion itself, and appears to be involved in processes like tumor suppression and cellular differentiation (22–26). Superoxide anions are dismutated by Mn-SOD to hydrogen peroxide, which is subsequently detoxified by catalase present in peroxisomes or by glutathione peroxidase present in mitochondria and the cytosol. Isolated overexpression or deficiencies of superoxide dismutases as seen for the copper, zinc superoxide dismutase (Cu,Zn-SOD) in Down's syndrome (trisomy 21) and amyotrophic lateral sclerosis is associated with premature aging, neurodegeneration, and death (27, 28). Strong interindividual differences in the spontaneous activity and inducibility of Mn-SOD have been suggested to confer differences in the individual susceptibility for the development of skin cancer and metastasis (26).

Fibroblasts stably overexpressing Mn-SOD with a defined capacity for the removal of superoxide anions and concomitant accumulation of hydrogen peroxide were generated as well suited tools (1) to evaluate the protective role of increased Mn-SOD activity in terms of resistance to different oxidant injuries and (2) to further dissect the role of distinct reactive oxygen species overproduced at defined subcellular sites in signaling mechanisms underlying connective tissue degradation. Stably transfected cell clones with an up to 4.6-fold overexpression of Mn-SOD revealed specific resistance to superoxide anion-induced cytotoxicity, while increased production of hydrogen peroxide, due to enhanced dismutation of superoxide anion, resulted in a dramatic induction of MMP-1. This hydrogen peroxide-dependent 9.5-fold induction of MMP-1 was found to be at least in part due to enhanced c-fos and c-jun transcription and subsequently enhanced transactivation of AP-1. Thus, isolated overexpression or stimulation of Mn-SOD without coordinate increase in interdependent antioxidant enzymes working in the same detoxification pathway such as catalase and glutathione peroxidase results in an intracellular increase in distinct ROS, which activate signal transduction pathways regulating the expression of transcription factors and effector genes related to connective tissue degradation.

**Experimental Procedures**

**Reagents—**Buthionine sulfoximine (BSO), an indirect inhibitor of glutathione peroxidase (29); aminotriazole (ATZ), an inhibitor of catalase (30); the iron chelator desferrioxamine (DFO) (31); and the redox cycling agent paraquat (32) were obtained from Sigma (Deisenhofen, Germany). The MMP-1 probe used was a 920-base pair (bp) fragment of human collagenase cDNA (33), the probe for c-fos and c-jun originally was a 1400-bp HindIII/BamHI fragment of the cDNA clone hcf-1 (34), and the probe for c- fos was an 800-bp BglII/NcoI fragment originally inserted in pUC 18 (35). A 24-mer oligonucleotide (5'-ACG GTA TCT GCT ATG CCG TPT CGT CTA GAC-3') (36) for the 18 S rRNA was synthesized (Amersham Pharmacia Biotech, Freiburg, Germany).

**Cell Cultures—**The human skin fibroblast cell line 1306 was obtained from ECACC (Salisbury, United Kingdom; ECACC no. 90011887). Cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Egggenstein, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), glutamine (2 mM), penicillin (400 units/ml), and streptomycin (50 mg/ml) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were passaged at a 1:3 dilution every 3 days. Recombinant cells were selected in neomycin (G 418) (Life Technologies) at 150 μg/ml, a concentration that completely inhibited the growth of nontransfected cells.

**Expression Vector for Human Mn-SOD and Cell Transfection—**The 1.0-kilobase pair human Mn-SOD cDNA fragment flanked by the EcoRI restriction site was introduced into the expression vector pcDNA3 (Invitrogen, San Diego, CA). The human liver Mn-SOD cDNA (37) (generously provided by Dr. Jonathan Wispe of the Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH) encodes the entire 198-amin acid proprotein and includes 95 bp of the 5'-untranslated region and 216 bp of 3'-untranslated sequences. The used expression vector contains the human cytomegalovirus major immediate early promoter/enhancer region and a neomycin resistance marker for the selection of stable transfectants in the presence of G418. The cytomegalovirus promoter is enhanced by the SV40 promoter. Subconfluent cultures of the fibroblast cells 1306 were transfected with the Mn-SOD expression vector (20 μg) by calcium-phosphate precipitation (38) or by the transfection reagent pcDNA3 (Life Technologies). Both methods gave similar results. For selection of stable transfectants, G418 (Life Technologies) was added to the cells 24 h after transfection. Individual neomycin-resistant cell clones were screened by measuring Mn-SOD activity (Ref. 2; see below). Control cells were transfected with the vector pcDNA3 alone and maintained under identical conditions.

**Assays for Determination of the Activity of Different Antioxidant Enzymes—**All assays were performed with cells in a logarithmic growth phase. The activity of SOD was detected by the nitro blue tetrazolium reduction method according to Beauchamp and Fridovich (39). The inhibition by SOD of nitro blue tetrazolium in the aerobic xanthine/xanthine oxidase system was followed at 560 nm. One unit of SOD corresponds to 50% inhibition of nitro blue tetrazolium reduction. Mn-SOD activity was determined from Cu,Zn-SOD by its resistance to 5 mM cyanide. Catalase activity was measured by monitoring the disappearance of H₂O₂ at 240 nm in the presence of cellular lysates (40). Selenium-dependent glutathione peroxidase was assayed using GSH and t-butylhydroperoxide as substrate and monitoring GSSG production through NADPH oxidation by glutathione reductase (41). The phosphate-dependent glutathione peroxidase activity was similarly determined except that phosphatidylcholine hydroperoxide instead of t-butylhydroperoxide was used (42). Protein content was determined using Coomassie Blue with albumin as the standard (43).

**Light Source and UV Irradiation—**The cells were irradiated at a distance of 40 cm using a high intensity halogen metal halide UVA source (UVASUN®3000 equipped with the UVASUN® safety filters) emitting wavelengths in the 340–450-nm range (Mutzhas, Munich, Germany) (44). The spectral distribution of the UVASUN®3000 source was determined with a Beckman UV 5270 spectrophotometer. The incident dose at the surface of the cells was 66 milliwatts/s. Dose rates were monitored with a combined UVA/UVB ultravioletometer (Centra-UV dosimeter; Osrn, Munich, Germany) (45). During irradiation, cells were incubated in phosphate-buffered saline (PBS) and maintained at 37 °C in a thermostatically controlled water bath. Following irradiation, PBS was replaced by fresh medium with 10% fetal calf serum, and the cells were incubated for various periods of time.

**Cytotoxicity Assay—**The viability of the transfectants was monitored 24 h after treatment with paraquat, UVA irradiation, or incubation with BSO or DFO, 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was used for the quantification of living metabolically active cells. Mitochondrial dehydrogenases metabolize MTT to a purple formazan dye, measured photometrically between 550 and 600 nm (46). Cytotoxicity was calculated as the percentage of formazan formation in cells treated under various conditions and chemical agents compared with mock-treated cells. We intended to avoid interference of cytotoxicity assays with the cells. Therefore, DFO was used at a nontoxic concentration of 10 μM, ATZ was used at 100 μM, and BSO was used at 10 μM.

**Determination of Hydrogen Peroxide Concentrations—**The cellular release of hydrogen peroxide (H₂O₂) into serum-free and dye-free media was assayed using a scopoletin fluorescence assay. In this assay, H₂O₂ oxidizes up to 300 nm scopoletin to a nonfluorescent state in a reaction catalyzed by 1 unit/ml horseradish peroxidase (47). The decrease in fluorescence was measured using a Perkin-Elmer luminescence spectrometer at an excitation wavelength of 340 nm. Emission was measured at 460 nm. Plots were standardized with defined concentrations of H₂O₂. The specificity of the generation and release of H₂O₂ was tested by the addition of catalase (4000 units/ml).

**Electron Immunocytochemistry—**For specimen fixation and embedding, cells were fixed in situ for 1 h in Carson Millonig's fixative (4% formaldehyde in 0.16 M monobasic sodium phosphate buffer, pH 7.2). Prior to embedding in medium grade LR White (Electron Microscopy Science, Ft. Washington, PA), samples were partially dehydrated in 70% ethanol with subsequent immersion and infiltration in undiluted LR White resin. All dehydration and infiltration steps with LR White were carried out over 24 h. LR White resin polymerization was thermally induced in sealed gelatin capsules at 45 °C for 48 h in the absence of any accelerator. Ultrathin sections (70–80 nm) were cut on a Sorvall MT2-B ultramicrotome with a Diatome knife and subsequently transferred to 300-nm nickel grids for immunolabeling experiments.

**Postembedding Immunocytochemistry—**For postembedding immuno-gold procedure, sections were first incubated with 2% bovine serum...
albumin, 0.2% Tween 20, and 0.06% sodium azide in Tris-buffered saline (TBS; 0.05 M Tris base with 0.9% NaCl, pH 7.6) for 30–60 min at 4 °C to block nonspecific antibody binding and then with rabbit polyclonal antihuman kidney Mn-SOD in TBS containing 1% bovine serum albumin for 18 h at 4 °C. After briefly washing the sections in TBS buffer (1:5 dilution of block buffer) and then in TBS, pH 8.2, the sections were transferred to a 1.5-dilution of gold-conjugated goat anti-rabbit IgG (Auroprobe, EM GAR G10; Amersham Pharmacia Biotech) in TBS, pH 8.2, containing 0.1% bovine serum albumin at ambient temperature for 60 min. The sections were further washed in TBS, pH 8.2, fixed with 2.5% glutaraldehyde for 10 min, washed extensively with double distilled water, and counterstained with 4% aqueous uranyl acetate for 10 min. Immunogold labeling was assessed with a Philips 30 transmission electron microscope operated at 60 kV.

RNA Extraction and Northern Blot Analysis—Total RNA was isolated and analyzed by Northern blots using specific cDNA probes or oligonucleotides for MMP-1, c-fos, c-ergos, and 18 S ribosomal RNA for sequential hybridization (36, 48, 49). Briefly, after extraction of total RNA, equal amounts of total RNA (5–20 µg/lane) were fractionated by size on a 0.9% 2.2 M formaldehyde gel and blotted to nitrocellulose filters (Schleicher & Schuell). Hybridizations were performed using denatured [32P]-labeled cDNA probes. For 3′-end labeling of the 24-mer 18 S ribosomal RNA probe, 5′I terminal deoxyxynucleotidyl transferase buffer (0.5 M potassium cacodylate, pH 7.2, 10 µM CoCl2, 1 M dithiothreitol), 10 pmol of 3′-ends (80 ng of DNA of the 24-mer), 1.5 µCi of α-32PdUTP, 150 mM Tris/20 mM MgCl2, and 50 U of terminal deoxynucleotidyl transferase (Life Technologies) were incubated at 37 °C for 1 h. Densitometric analysis was performed using the ScanPackII system (Biometa, Göttingen, Germany).

Transfection with CAT Reporter Gene Constructs and CAT Enzyme-linked Immunosorbent Assay—The collagenase CAT constructs were kindly provided by Peter Angel (DKFZ, Heidelberg, Germany) and have been described previously (50, 51). The −517/+63 CAT construct contains the 5′ control region of the collagenase gene running from base −517 to +63 including an AP-1 binding sequence at positions −73 to −42, which is required for the induction of transcription by the tumor promoter 12-O-tetradecanoylphorbol 13-acetate. The −517/+63 mutTREColCAT construct contains a mutated TPA response element between the wild type, −517/+63 CAT (A+G)G (46–49). The plasmid pCMV-βGal (CLONTECH, Heidelberg, Germany) was used as internal control. Cells were plated at a density of 1.2 × 104 the day prior to transfection. CAT reporter plasmids (25 µg) and the pCMV-βGal plasmid (2.5 µg) were cotransfected by calcium phosphate precipitation (38) using a 2-min glycerol shock at 4 h after treatment of cells with the calcium phosphate–DNA precipitate. Twelve h after transfection, cells were washed twice with PBS and irradiated at a dose of 300 kJ/m2 in PBS. Subsequently, the original “preirradiation medium” was added back to the irradiated cells. For control purposes, cultures were mock-treated. For the quantitation of expressed CAT protein, cells were detached 30 h after transfection with PBS, 10 mM EDTA; collected by centrifugation; resuspended in 200 µl of 250 mM Tris-HCl (pH 7.6), 5 mM EDTA, and lysed by four freezing/thawing cycles. Fifty mg of total cellular protein was assayed by a CAT enzyme-linked immunosorbent assay (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Luciferase activity was determined in a type 2010 luminometer (ALL, San Diego), using a commercial assay system (Promega, Heidelberg, Germany). All transfections were performed in duplicate and assayed at least three times.

RESULTS

Generation and Characterization of the Fibroblast Cell Line 1306 Stably Overexpressing Human Mn-SOD—The human fibroblast cell line 1306 was transfected with the eukaryotic expression vector pcDNA3 containing the cDNA of human Mn-SOD and a neomycin resistance cassette. Several clones with G418-resistant cell clones were isolated. The Mn-SOD activity of these clones was 2.2–4.6-fold increased compared with that of the parental wild type 1306 cells and 1306 cells transfected with the neomycin resistance expression vector pcDNA3 alone. The Mn-SOD clones, Mn-SOD3 with a 4.6-fold increase, Mn-SOD12 with a 3.2-fold increase, and Mn-SOD14 with a 2.5-fold increase in Mn-SOD activity were further studied as indicated. Increased expression of clone Mn-SOD3 did not significantly alter the activities of other antioxidant enzymes including copper, zinc superoxide dismutase (Cu,Zn-SOD), catalase, glutathione peroxidase, and the phospholipid hydroperoxide glutathione peroxidase (Table 1). To ascertain that the overexpressed Mn-SOD was properly transported into the mitochondria, immunoelectron microscopy was performed using an antibody against Mn-SOD (Fig. 1). Control cells transfected with the neomycin resistance expression vector alone revealed only low labeling (Fig. 1A). By contrast, the Mn-SOD-transfected cell clone Mn-SOD3 revealed a strong labeling in mitochondria, indicating that Mn-SOD is increased at their physiological site of production (Fig. 1B). There was very low, slightly above background labeling in the extramitochondrial cytosol. This low amount of extramitochondrial labeling may be due to antibody detection of the Mn-SOD precursor form on its way from the nucleus to the mitochondria. Substitution of the primary antibody by normal rabbit serum did not show any immunolabeling in both control and Mn-SOD-expressing cells (data not shown). Overall, these data show that in this system the overexpression of the recombinant Mn-SOD is correctly routed and processed within the cells.

Mn-SOD Overexpression Confers Cell Protection against Oxidant Injury Induced by Paraquat but Not by UVA Irradiation—UVA irradiation and paraquat are known to intracellularly induce oxidative stress. Upon UVA irradiation particularly, singlet oxygen, hydrogen peroxide, and to a minor extent superoxide anions are generated, while paraquat cytotoxicity is mainly due to the increased intracellular production of superoxide anions. To determine whether an increase in Mn-SOD activity protects against these different forms of oxidant injury, two control cell clones, including 1306 cells transfected with the neomycin resistance vector alone, and three Mn-SOD-overexpressing cell clones were exposed to UVA irradiation at doses ranging from 100 to 1500 kJ/m2, and in a different set of experiments they were exposed to paraquat at concentrations ranging from 200 to 750 µM. The tested Mn-SOD clones showed an increase in Mn-SOD activity ranging from 2.5-fold (Mn-SOD14) to 3.2-fold (Mn-SOD12) and 4.6-fold (Mn-SOD3). Only modest alteration in viability of Mn-SOD-overexpressing cells, as assessed by the MIT assay, was detected upon UVA irradiation compared with the control cells (Fig. 2A). Apparently, the clones expressing 4.6- and 3.2-fold more Mn-SOD are slightly less viable in response to high UVA doses of 1200 kJ/m2 (Fig. 2A). It is possible that the detoxification of UVA-generated superoxide anions to hydrogen peroxide adds to the overall hydrogen peroxide load directly generated by UVA irradiation, finally resulting in cytotoxic concentrations. High levels of hydrogen peroxide, in fact, may drive the Fenton reaction resulting in the production of highly toxic hydroxyl radicals. By contrast, treatment of Mn-SOD overexpressing cells with para-

| Table I | Activities of different antioxidant enzymes do not differ in Mn-SOD-overexpressing cells compared with mock-transfected (V3) and parental controls (1306 (wt)) |
|----------|---------------------------------------------------------------|
| Mn-SOD       | Cu,Zn-SOD     | GSHPase<sup>a</sup> | PHGPx<sup>b</sup> | Catalase |
| I306 (wt)   | 3.0 ± 0.2   | 9.0 ± 0.6 | 53.2 ± 1.3 | 81.1 ± 0.3 | 3.8 ± 0.3 |
| V3         | 2.9 ± 0.2   | 8.5 ± 0.4 | 49.4 ± 1.7 | 88.7 ± 0.9 | 4.2 ± 0.4 |
| MnSOD3      | 13.8 ± 1.0  | 9.3 ± 0.5 | 48.7 ± 2.1 | 85.4 ± 0.4 | 4.2 ± 0.3 |

<sup>a</sup> Glutathione peroxidase.

<sup>b</sup> Phospholipid hydroperoxide glutathione peroxidase.

*p = 0.0029 compared with mock-transfected control cells V3 (alternate (Welch) t test).
Mn-SOD Modulates the Induction of MMP-1

Immunogold labeling of cultured 1306 fibroblasts was performed with the polyclonal rabbit serum against human Mn-SOD and subsequent incubation with gold-conjugated goat anti-rabbit IgG as described in detail under “Experimental Procedures.” In vector-transfected control cells (V) only minor labeling was detected in mitochondria (arrow), representing physiological expression of Mn-SOD (A). In Mn-SOD-overexpressing 1306 cells (Mn-SOD3), a strong labeling occurred in the mitochondrial matrix (arrow), indicating that the overexpressed recombinant Mn-SOD is correctly routed to the mitochondria (B). Original magnification is × 17,000 (A) and × 18,000 (B) M, mitochondrion.

UVA Irradiation and Paraquat Treatment Increase Specific MMP-1 mRNA Levels in Mn-SOD-overexpressing Cells—To study the effect of Mn-SOD overexpression under different oxidative stress on gene expression of MMP-1, the Mn-SOD-overexpressing cell clone Mn-SOD3 (Mn) was either exposed to UVA irradiation at a dose of 300 kJ/m² or exposed to 150 μM paraquat, conditions that have previously been shown to be nontoxic (Fig. 3). Total RNA was isolated at different time points (6, 12, 24, and 48 h) post-treatment and subjected to Northern blot analysis. UVA irradiation led to a marginal induction in vector-transfected control cells V3 (V) at 6 h with a maximal induction at 24 h post-treatment and a subsequent decrease to basal levels. By contrast, exposure of Mn-SOD-overexpressing cells (Mn) to UVA irradiation resulted in a 9.5-fold induction of specific MMP-1 mRNA levels at 24 h compared with UVA irradiated vector transfected control cells (V) (Fig. 3A). While vector-transfected control cells (V) did not show any induction of MMP-1 mRNA levels even at a high paraquat concentration of 400 μM, treatment of Mn-SOD-overexpressing cells (Mn) with a paraquat concentration of 150 μM resulted already in a time-dependent induction of MMP-1 mRNA levels with a maximum at 24 h post-treatment. This induction was further enhanced with a maximal induction at 48 h after exposure of cells to paraquat at a concentration of 400 μM (Fig. 3B). These results suggest that the increase in MMP-1 mRNA levels upon exposure of Mn-SOD-overexpressing cells to increasing superoxide anion (O₂⁻) concentrations may be caused by unbalanced production of hydrogen peroxide.

Enhanced Spontaneous and Paraquat-induced Release of Hydrogen Peroxide from Mn-SOD-overexpressing Cells—Since all tested Mn-SOD-overexpressing cell clones did not show any alteration in those antioxidant enzymes responsible for further detoxification of hydrogen peroxide (Table I), we hypothesized that hydrogen peroxide may accumulate in Mn-SOD-overexpressing cells. In order to test this hypothesis, cells were analyzed for their release of hydrogen peroxide into culture supernatants by the scopoletin/horseradish peroxidase method. Both the spontaneous release of hydrogen peroxide and the hydrogen peroxide release of cells exposed to the redox cycling superoxide anion-generating agent paraquat were studied. As a consequence of its higher superoxide anion dismutating capacity, the clone Mn-SOD3 (Mn) showed an increase in the spontaneous cellular hydrogen peroxide release of about 60% compared with the parental cells 1306 and the control cells V3 (V) (Fig. 4A). Increasing the intracellular O₂⁻ concentration upon exposure of cells to 1 mM paraquat for 2 h resulted in a further increase in hydrogen peroxide production (Fig. 4B).

Further Evidence for the Central Role of Increased Hydrogen...
Peroxide Concentrations in the Induction of MMP-1 mRNA Levels—

We have used a combined genetic and biochemical approach with stably transfected Mn-SOD-overexpressing cells and inhibitors of H$_2$O$_2$ detoxifying pathways to intracellularly increase the concentration of H$_2$O$_2$. For this purpose, we have incubated the clone Mn-SOD3 with ATZ, an inhibitor of catalase, and BSO, an indirect inhibitor of glutathione peroxidase. Both agents led to a further increase in intracellular hydrogen peroxide. Furthermore, Mn-SOD-overexpressing and vector-transfected cells were incubated with the iron chelator DFO to block the H$_2$O$_2$-consuming Fenton reaction. Total RNA was isolated and subjected to Northern blot analysis. Three independent experiments were performed showing the same results as the presented blot.

UVA Induction of c-fos and c-jun Protooncogene Expression Is Enhanced by Increased Intracellular H$_2$O$_2$ Concentrations—

The transcriptional induction of MMP-1 depends on the transactivation of the AP-1 site, a DNA recognition sequence within the MMP-1 promoter. Transactivation of the AP-1 site occurs by binding of newly synthesized and heterodimerized c-Jun and c-Fos, which constitutes the AP-1 transcription factor. To examine whether increased H$_2$O$_2$ concentrations may enhance the steady state mRNA levels of c-fos and c-jun, the Mn-SOD-overexpressing clone Mn-SOD3 (Mn) and the vector-transfected control clone V3 (V) were UVA-irradiated or exposed to paraquat (Fig. 6). Subsequently, specific c-jun and c-fos mRNA levels were determined at different time points using Northern blot analysis. Upon UVA irradiation, c-jun and c-fos mRNA levels were increased 2- and 4-fold, respectively, in Mn-SOD-overexpressing cells compared with vector-transfected cells.

**Fig. 3.** UVA irradiation and paraquat resulted in a stronger induction of MMP-1 mRNA levels in Mn-SOD-overexpressing cells. Mn-SOD-overexpressing cells (Mn-SOD3) and vector control cells (V3) were irradiated with a UVA source at a dose of 300 kJ/m$^2$ (A) or treated with paraquat at the indicated concentrations (B). Total RNA was isolated at 6, 12, 24, and 48 h post-treatment and subjected to Northern blot analysis with sequential hybridization of specific probes for MMP-1 and the 18S ribosomal RNA (rRNA). The Northern blot reveals representative data reproduced in three independent experiments.

**Fig. 4.** Spontaneous and paraquat-induced H$_2$O$_2$ release in supernatants is enhanced in Mn-SOD-overexpressing cells. The rate of spontaneous (A) and paraquat (PQ)-induced (B) H$_2$O$_2$ release was determined in confluent fibroblast cultures of the parental wild type 1306 cells (wt), vector-transfected control cells (V), and Mn-SOD-overexpressing cells (Mn) using the scopoletin/horseradish peroxidase assay as described under "Experimental Procedures." Hydrogen peroxide (H$_2$O$_2$) release is expressed in pmol/1$\times 10^6$ cells. The values represent the mean of three independent experiments with S.D. A and B, *: $p < 0.0001$ compared with vector-transfected 1306 fibroblasts (V) (Student's t-test).

Peroxide Concentrations in the Induction of MMP-1 mRNA Levels—

We have used a combined genetic and biochemical approach with stably transfected Mn-SOD-overexpressing cells and inhibitors of H$_2$O$_2$ detoxifying pathways to intracellularly increase the concentration of H$_2$O$_2$. For this purpose, we have incubated the clone Mn-SOD3 with ATZ, which inhibits the catalase; BSO, which indirectly inhibits the glutathione peroxidase; and DFO, an iron chelator, which blocks the H$_2$O$_2$-consuming Fenton reaction (52). After incubation of confluent monolayer cultures of the Mn-SOD-overexpressing cell clone Mn-SOD3 (Mn) and the vector-transfected control clone V3 (V) for 12 and 24 h with the above mentioned chemical compounds, total RNA was isolated and subjected to Northern blot analysis. In control cells of clone V3, no induction of MMP-1 mRNA levels could be detected upon treatment with different chemical compounds (Fig. 5), suggesting that a threshold concentration of hydrogen peroxide responsible for MMP-1 induction has apparently not been reached. A low constitutive expression of MMP-1 mRNA was detectable in untreated cells of the Mn-SOD-overexpressing cell clone Mn-SOD3. Treatment of these cells with ATZ, BSO, or DFO alone or in any combination resulted in a 15-fold increase in the steady state levels of MMP-1 mRNA after 12 and 24 h. These data provide further evidence that intracellular increase in hydrogen peroxide may play a major role in the induction of MMP-1 mRNA levels.

**Fig. 5.** Modulation of steady-state mRNA levels of MMP-1 in Mn-SOD-overexpressing and control cells by different pro- and antioxidant compounds. Mn-SOD-overexpressing cells (Mn) and vector-transfected control cells (V) were incubated with ATZ, an inhibitor of catalase, and BSO, an indirect inhibitor of glutathione peroxidase. Both agents led to a further increase in intracellular hydrogen peroxide. Furthermore, Mn-SOD-overexpressing and vector-transfected cells were incubated with the iron chelator DFO to block the H$_2$O$_2$-consuming Fenton reaction. Total RNA was isolated and subjected to Northern blot analysis. Three independent experiments were performed showing the same results as the presented blot.
with similar induction kinetics, suggesting that H$_2$O$_2$, in fact, enhances UVA induction of c-fos and c-jun mRNA levels (Fig. 6). Similar results were obtained following exposure of Mn-SOD-overexpressing cells to 150 μM paraquat (data not shown). Increase...
Mn-SOD Modulates the Induction of MMP-1

...dependent antioxidant enzymes. This may be of particular relevance for the understanding of tumor susceptibility and tumor progression. There are several examples for the detrimental effects of imbalances in interdependent antioxidant enzymes occurring in genetic disorders, transgenic organisms, and cell biology (27, 28, 69). The most prominent example is Down’s syndrome (trisomy 21) with an aberration in chromosome 21. The copper, zinc superoxide dismutase (Cu,Zn-SOD) is localized on chromosome 21, and due to increased gene dosage its activity is increased with no compensatory increase in the activities of the interdependent catalase or glutathione peroxidase. These individuals with trisomy 21 suffer from premature aging, and their fibroblasts reveal typical morphological and functional features of postmitotic senescent cells (70). Furthermore, the complete absence of Mn-SOD results in perinatal lethality (27). Hence, effective cellular protection requires a balance between interdependent antioxidant enzymes with an appropriate relationship to each other. Further in vivo support comes from studies on double transgenic Drosophila with overexpression of interdependent antioxidant enzymes revealing substantial protection against oxidant-dependent injury and an overall increase in organismic lifespan (71).

Here, we have used different strategies to increase the intracellular hydrogen peroxide concentration to study its effect on key steps in the regulation of steady state mRNA levels of matrix-degrading enzymes. Accordingly, we have generated Mn-SOD-overexpressing cell clones with increased spontaneous release of hydrogen peroxide, which was further enhanced when these cells were challenged by different oxidants like paraquat or UVA irradiation. Moreover, we have used different inhibitors and chemical compounds to block all hydrogen peroxide-detoxifying pathways including inhibition of catalase, glutathione peroxidase, and the hydrogen peroxide-consuming Fenton reaction, thus further increasing hydrogen peroxide in Mn-SOD-overexpressing cells. In all of these experimental settings, we found MMP-1 mRNA levels to be up to 9.5-fold increased compared with vector-transfected control cells, indicating that hydrogen peroxide as suggested previously (66) plays a major role in the induction of MMP-1. These results are of considerable interest because they imply potential caveats in the uncritical use of therapeutic antioxidant strategies. It remains to be seen whether co-expression of catalase could correct the unbalanced hydrogen peroxide levels and the subsequent MMP-1 induction in Mn-SOD-overexpressing cells. To further dissect regulatory effects of Mn-SOD overexpression on the induction of MMP-1, specific mRNA levels of c-fos and c-jun, as well as their transactivating effect on the AP-1 site within the MMP-1 promoter were studied. We focused on these regulatory key steps because it was shown that induction of AP-1, a heterodimer of Jun and Fos proteins, primarily relies on de novo synthesis of these two DNA binding and transactivating subunits. We found that UVA irradiation of Mn-SOD-overexpressing cells with subsequently increased hydrogen peroxide levels resulted in a marked increase in the inducibility of c-jun and, to a lesser extent, of c-fos mRNA levels and furthermore resulted in a significant increase in transactivation of the AP-1 site-containing MMP-1 promoter CAT construct. A variety of reports have outlined the regulatory effects of reactive oxygen species in the expression of several genes including c-fos and c-jun (72–74). However, in these studies a potential role for imbalances in the enzymatic antioxidant defense has not been addressed.

Although we did not provide direct evidence, the observed increase in the steady state c-fos and c-jun mRNA levels in Mn-SOD-overexpressing cells are most likely due to phosphorylation of preexisting transcription factors, like the ternary complex factor or the c-Jun/ATF2 (activating transcription factor) by defined kinase families (75, 76). In fact, there is some evidence that reactive oxygen species are involved in the activation of extracellular stimulus-responsive kinases and Jun N-terminal kinases preceding the phosphorylation of the ternary complex factor, which together with the serum response factor activates c-fos transcription, and of the c-Jun-ATF2 complex, which in its phosphorylated form initiates c-jun transcription (52, 77).

The observed discrepancy between the 9.5-fold induction of MMP-1 mRNA levels and the weaker 4-fold induction of AP-1-dependent CAT activity in Mn-SOD-overexpressing cells upon UVA irradiation points to the possibility that besides transcriptional regulation of AP-1 other mechanisms and/or transcription factors may be involved in the enhanced inducibility of MMP-1 mRNA levels. In fact, the relatively weak activation of AP-1 under oxidative conditions is not an unprecedented observation (21, 73, 79–81). In this context, it is most interesting that DNA binding and transactivation of NF-κB and PEA3 have earlier been shown to cooperate with AP-1 sites in MMP transcription (82, 83). In fact, Stein et al. (84) have demonstrated that Fos and Jun proteins are capable of physiologically interacting with NF-κB p65 through a Rel homology domain with subsequent enhanced DNA binding and transactivation via the p65 and the AP-1 response elements. The exact biophysical and molecular mechanisms underlying signal transduction induced by reactive oxygen species are as yet unknown. Possible mechanisms include oxidant-macromolecule interaction, alteration in the overall and local cellular redox status, and calcium signaling (85). Also, the upstream signaling steps preceding the induction of activity of Jun N-terminal kinase and extracellular stimulus-responsive kinase, c-jun, c-fos, and MMP-1 mRNA levels after oxidative challenge of Mn-SOD-overexpressing cells and the potential involvement of cytokine networks in these processes (65, 78, 86, 87) have not been elucidated. However, there are some indications that activation of AP-1 is mediated by membrane-associated Src-tyrosine kinases and Ha-Ras GTP-binding proteins after UVC irradiation (60).

Our data are consistent with a model whereby ROS preferentially initiate the transcription of c-fos and c-jun (75, 76). Here, we corroborate and extend these data and, in addition, provide the first evidence that unbalanced enzymatic antioxidant defense with subsequent intracellular accumulation of hydrogen peroxide drives the accumulation of c-jun mRNA and, in their heterodimerized form, the enhanced transactivation of AP-1. However, our experiments do not allow us to distinguish whether enhanced steady state MMP-1 mRNA levels are due to enhanced transcription, enhanced mRNA stability, or a combination thereof. Overall, our results perfectly fit with an earlier published model for the role of increased load of reactive oxygen species in carcinogenesis (69) and connective tissue disorders in that imbalances in the interrelated and interdependent antioxidant enzymes drive the accumulation of intracellular ROS, as in our case hydrogen peroxide, which subsequently activates signal transduction pathways and modulates the activity of genes that regulate effector genes related to tissue degradation.

Further understanding may provide therapeutic approaches to substitute and balance antioxidant deficiencies in pathological states.

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