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Genetic Modulation of the Cellular Antioxidant Defense Capacity

by Paul Amstad* and Peter Cerutti*

Oxidants are ubiquitous in our aerobic environment. While they are always toxic, they can also exert pathophysiological effects at low concentrations and play an etiological role in human disease. For example, oxidants can stimulate cell growth and act as tumor promoters. The cellular antioxidant defense system attenuates the effect of oxidants and consists of low molecular weight components and several enzymes. Most important are catalase (CAT), superoxide dismutases (SOD), and glutathione peroxidase. We are attempting to elucidate the role of CAT and Cu,Zn-SOD in oxidant tumor promotion of mouse epidermal cells JB6. We have found that the promotable clone 41 possesses 2- to 3-fold higher levels of activity, protein, and stationary mRNA of CAT and Cu,Zn-SOD than does the nonpromotable clone 30. We propose that the growth-stimulatory effect of oxidants is more pronounced in promotable clone 41 because it is better protected from oxidant toxicity. In order to corroborate this model, we have constructed JB6 cells with higher levels of Cu,Zn-SOD and CAT by transfection with expression vectors containing cDNA for these genes. On the other hand, cells with decreased amounts of Cu,Zn-SOD have been obtained by their stable transfection with a vector containing SOD-cDNA in the antisense orientation. These cell clones with modified antioxidant enzyme complements are being characterized. In particular, their promotability by oxidants and their sensitivity to killing and oxidative macromolecular damage are being measured. Certain tumor promoters that lack oxidizing properties may generate a cellular prooxidant state by a variety of mechanisms. For example, it had been reported that the phorbol ester TPA decreases the activities of CAT and SOD in mouse skin. We found for JB6 cells that this loss of enzyme activity was due to a decrease in the steady-state concentrations of CAT- and SOD-mRNA. The observed decreases in CAT and SOD can be considered as part of the complex reprogramming of gene expression that is set in motion by TPA.

Introduction

Oxidants are toxic to most cells. They inflict damage on several targets, most importantly to lipids, proteins, and DNA. Cells are protected by multiple levels of antioxidant defenses. The notion that a gradual decrease in these defenses might play a role in the aging process has been considered for many years but remains controversial. Comparative studies of the antioxidant capacity of tissues of different animal species have suggested a positive correlation with the maximal lifespan potential (*1*). Interestingly, the increase in lifespan of rats by dietary restriction was accompanied by an increase in the activities, mRNA contents, and rates of transcription of the antioxidant enzymes catalase (CAT) and Cu, Zn-superoxide dismutase (SOD) (A. Richardson, personal communication). Besides playing a role in aging, oxidants possess carcinogenic potential. There is convincing evidence that low doses of exogenous oxidants can exert a growth-promoting effect and act as natural promoters in tumorigenesis (*2–4*). The following principal alternatives can be distinguished for the action of oxidant promoters. Oxidants and cellular prooxidant states represent signals for the selective induction of differentiation or growth in a particular subpopulation of epidermal cells. Indeed, a burst of extracellular active oxygen (AO) induced the competence-related protooncogenes *c-fos* and *c-myc* in mouse epidermal cells JB6 (*5,6*). Specific subpopulations may differ in their susceptibilities to the cytostatic and cytotoxic effects of oxidants (*7,8*). In support of the second alternative, we found that the cytostatic effect of AO, DNA strand breakage, and poly-ADP-ribosylation were more pronounced in the nonpromotable clone 30 than in the promotable clone 41 (*9*).

Because of the role of oxidants in degenerative disease, aging, and carcinogenesis, much research is being concentrated on the elucidation of the mode of action of oxidants and on the characterization of the cellular antioxidant defense systems. All cells contain low molecular weight antioxidants such as glutathione, cysteine, ascorbate, α-tocopherol, urate, and carotinoids. In addition, they contain several antioxidant enzymes, most importantly glutathione peroxidase (GPx), glutathione-*S*-transferase, several SODs, and CAT. The individual

*Department of Carcinogenesis, Swiss Institute for Experimental Cancer Research, 1066 Epalinges/Lausanne, Switzerland.
Address reprint requests to P. Amstad, Department of Carcinogenesis, Swiss Institute for Experimental Cancer Research, 1066 Epalinges/Lausanne, Switzerland.

contributions of these multiple, interacting components in intact cells is only poorly understood. In particular, the relative contributions of the antioxidant enzymes under *in vivo* conditions remain unknown despite the fact that their enzymology has been investigated in detail (*10*).

Work with expression vectors containing Cu,Zn-SOD indicates that an oversupply of this enzyme is not necessarily beneficial to a mammalian cell and can even cause increased lipid peroxidation (*11*). It has been suggested that the presence of three genes for Cu,Zn-SOD in the aging-related disease Down's Syndrome may play a role in its etiology (*10,12*). Interestingly, overexpression of Cu,Zn-SOD in human cells can result in a compensatory increase in GPx activity (*13*). While work with expression vectors can yield useful information, it has the drawback that cells are flooded with a particular protein and that there is no guarantee for proper intracellular compartmentalization.

In our work we have investigated four aspects of the cellular antioxidant defense in mouse epidermal cells JB6: *a*) We have compared the constitutive levels of the major antioxidant enzymes in promotable clone 41 and nonpromotable clone 30. *b*) Since the promotional action of the phorbol-ester promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on JB6 clone 41 cells is suppressed by the addition of Cu,Zn-SOD (*4*), we have studied the effect of TPA on the expression of the antioxidant enzymes. *c*) We have increased the level of Cu,Zn-SOD and CAT by the transfection of JB6 cells with expression vectors containing cDNA for these enzymes. *d*) Alternatively, we are attempting to selectively reduce the level of Cu,Zn-SOD by the antisense RNA/hybrid arrest approach in JB6 clone 41 cells. We feel that the use of genetic manipulation to construct cells that specifically lack a single function represents a physiological avenue to an understanding of the role of the individual antioxidant enzymes *in situ*. Although we have obtained insights into the role of CAT in human fibroblasts in studies of cells from patients with the autosomal recessive disease acatalasia (*14*), the poor growth properties of these strains prohibited their complete biochemical characterization. We hope that the availability of stable transfectants of JB6 cells that differ only in their content of a particular antioxidant enzyme will allow insights into their role in cellular aging and carcinogenesis under physiological conditions.

Promotable Mouse Epidermal Cells JB6 Clone 41 Have an Elevated Antioxidant Defense Capacity

When we measured the specific activities of SOD, CAT, and GPx in monolayer cultures of JB6 cells, we discovered that the promotable clone 41 contained approximately twice the activity of SOD and CAT relative to the nonpromotable clone 30, whereas the activities of GPx were comparable (*15*). We took advantage of the cross-activity of a rabbit antibody against human CAT to assess CAT protein concentrations by Western-blot analysis. Constitutive amounts of CAT were 2- to 3-fold higher in clone 41 than in clone 30, in agreement with the enzyme activity (*15*).

For the determination of steady-state mRNA concentrations by Northern blots, we constructed SP6 plasmids with c-DNA inserts of human CAT (*16*) and Cu,Zn-SOD (*17*) and for bovine GPx for the preparation of RNA probes. Figure 1 shows that the mRNA concentrations for CAT and SOD were considerably higher in clone 41 than in clone 30, whereas the GPx mRNA levels were comparable. We conclude that the antioxidant defense of JB6 clone 41 is superior to that of clone 30. The difference between the two clones is particularly remarkable because the two antioxidant enzymes SOD and CAT are increased coordinately in clone 41. Since the product of the action of SOD is H_2O_2, an increase in its activity is only beneficial to the cell if it is counterbalanced by a sufficient capacity for the destruction of H_2O_2 (*11*). This is apparently accomplished in clone 41 by an increase in CAT.

It should be mentioned that SOD and CAT may mutually protect each other from inactivation by active oxygen (*18*). A relatively high SOD activity in clone 41 has also been measured by Colburn et al. (*19*). In contrast, the third major antioxidant enzyme GPx was present at comparable levels in the two clones. The observed difference appears to be at the level of expression, as both clones contained the same complement of SOD and CAT genes according to Southern-blot analysis. Clone 41 also possessed a slightly higher level of glutathione than did clone 30 (*15*). The intracellular capacity for antioxidant defense cannot be assessed with present technologies. Therefore, it is not possible to

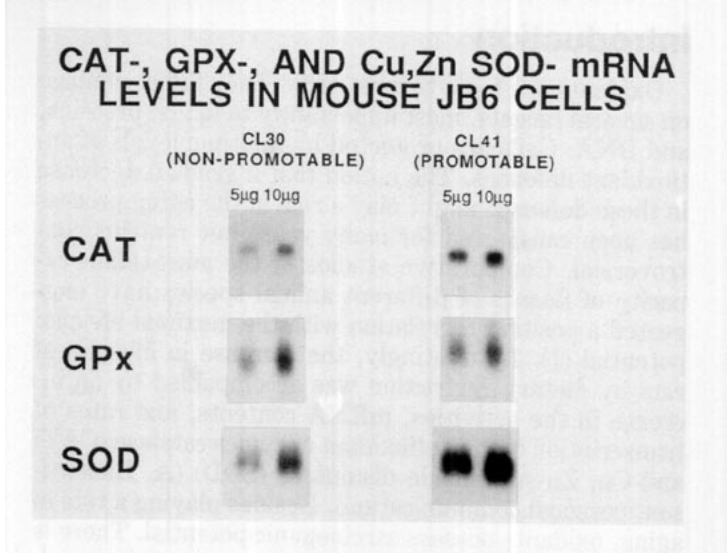

FIGURE 1. Catalase, glutathione peroxidase, and Cu,Zn-superoxide dismutase mRNA levels in nonpromotable JB6 clone 30 and in promotable clone 41. Total RNA was prepared, and 5 or 10 µg were electrophoresed in 1.4% agarose/formaldehyde denaturing gels and then transferred to gene screen. The filters were hybridized with ^{32}P-labeled transcripts from SP6 recombinants that contained cDNA fragments of human CAT, bovine GPx, or human Cu,Zn-SOD, respectively.

judge whether the coordinate 2- to 3-fold higher level of SOD and CAT represents a large or small difference in total antioxidant defense (*15*).

The difference in constitutive antioxidant defense of clone 30 and clone 41 may play a role in their promotability by oxidant promoters. Gindhart and Colburn and their collaborators had found that only clone 41 was promotable to anchorage-independent growth by benzoyl peroxide (*20*), IO_4 (*21*), and AO produced by xanthine/xanthine oxidase (*22*). The latter observation was confirmed in our own laboratory (*9*). In accordance with its higher capacity for antioxidant defense, clone 41 was better protected from AO-induced DNA strand breakage, poly-ADP-ribosylation and cytotoxicity than was clone 30 (*9*). AO strongly induced the growth-competence-related protooncogenes *c-fos* and *c-myc* from very low basal levels in the less protected JB6 clone 30. Only weak induction of these protooncogenes was observed in clone 41, but it occurred on the background of high, persistent *c-myc* expression in untreated controls (*5,6*). We speculate that the superior constitutive antioxidant defense of clone 41 protects it from excessive AO toxicity and allows growth-related genes to exert their functions. In contrast, in the nonpromotable clone 30, cytotoxicity predominates, and the strong transient induction of *c-fos* and *c-myc* by AO does not suffice for growth stimulation.

Downregulation of SOD and CAT by Phorbol Ester in Mouse Epidermal Cells JB6

The question whether other promoters that lack oxidizing properties may owe at least part of their effects to the induction of a cellular prooxidant state has not been resolved. There are several indications that TPA can induce a prooxidant state in epidermal cells. TPA induces chemiluminescence (*23*) and decreases the ratio of reduced to oxidized glutathione (*24*). Several antioxidants including exogenously added SOD antagonize TPA promotion (*2,4,20,25*). In order to exert its antipromotional effect, SOD had to be added during the first 2 hr of TPA treatment of JB6 clone 41 or clone 22, and it was speculated that a protein kinase-C-mediated process induced a cellular prooxidant state (*22*). The report by Solanki et al. (*26*) that TPA caused a decrease in the activities of SOD and CAT in mouse skin suggested that it might induce a prooxidant state by weakening the antioxidant defense. The possibility had been considered that this decrease reflects the elimination of a subpopulation of cells with high SOD activity (*27*). Evidence had also been presented suggesting that the apparent decrease in the specific activity of CAT in mouse epidermis 15 hr after TPA treatment could be due to an increase in total extractable protein (*28*).

In our own experiments with mouse epidermal cells JB6, we found that TPA induced a coordinate loss in SOD and CAT activities that was at least in part a consequence of a slow decrease in stationary mRNA concentrations. It occurred to a comparable degree in promotable and nonpromotable cells. The observed decreases preceded and were more pronounced than the consequent changes in the enzyme activities. Within the time frame of our experiments, TPA had no effect on GPx activity nor GPx mRNA levels (*15*). Similar results for TPA-induced changes in the activities of CAT and SOD in JB6 clone 41 were reported by Nakamura et al. (*22*). While the extent of the TPA-induced decreases in CAT and SOD activities were comparable to the present work, the drop in activities occurred earlier, i.e., after 2 hr rather than 4 to 8 hr. For CAT, the slower time course has been corroborated by Western blot analysis in our work. However, it is noteworthy that a drop in steady-state CAT and SOD mRNA concentrations was already discernible 2 hr after TPA treatment in clone 41 and clone 30. Although the decrease in mRNA concentrations indicates that the loss in SOD and CAT activities is at least in part due to genetic downregulation, it does not exclude enzyme inactivation as a contributing factor. Nuclear run-off experiments indicate that TPA did not affect the rate of transcription of CAT and SOD in JB6 cells, and the observed downregulation of these genes may involve a decrease in messenger stability (*15*).

Our results support the notion that TPA can induce a cellular prooxidant state in mouse epidermal cells. In contrast to oxidants, low concentrations of TPA are nontoxic to JB6 cells. Therefore, it is unlikely that differential resistance to oxidative stress plays a crucial role for the promotability of JB6 cells by TPA. Despite considerable indirect evidence for a role of AO, the importance of the cellular antioxidant defense in the promotion of epidermal cells by TPA remains unclear.

Transfection of Mouse Epidermal Cells with Cu,Zn-SOD and CAT Expression Vectors

The model derived from results described so far proposes that a fine balance between the level of the antioxidant defense, cellular toxicity, and the induction of growth-related genes determines promotability by oxidants (*7,8,10*). While a weak antioxidant defense resulting in high toxicity is incompatible with growth, a very potent defense may scavenge the signal that is required for genomic induction. We are testing this model by preparing mouse epidermal cells JB6 clone 41 with moderately increased levels of Cu,Zn-SOD or CAT. For this purpose we have transfected cells with antibiotic-selectable expression vectors containing human cDNA for Cu,Zn-SOD or a hybrid containing 5′-cDNA sequences of rat CAT spliced to human CAT cDNA. [cDNA and/or genes for the major human antioxidant enzymes have been recently cloned in several laboratories: Cu,Zn-SOD (*17*), Mn-SOD (*29*), EC-SOD (*30*), CAT (*16*), GPx (*31*).] The expression vector for Cu,Zn-SOD is pD5-derived, containing adenovirus type 5 and 2 promoters as well as the SV40-neomycin re-

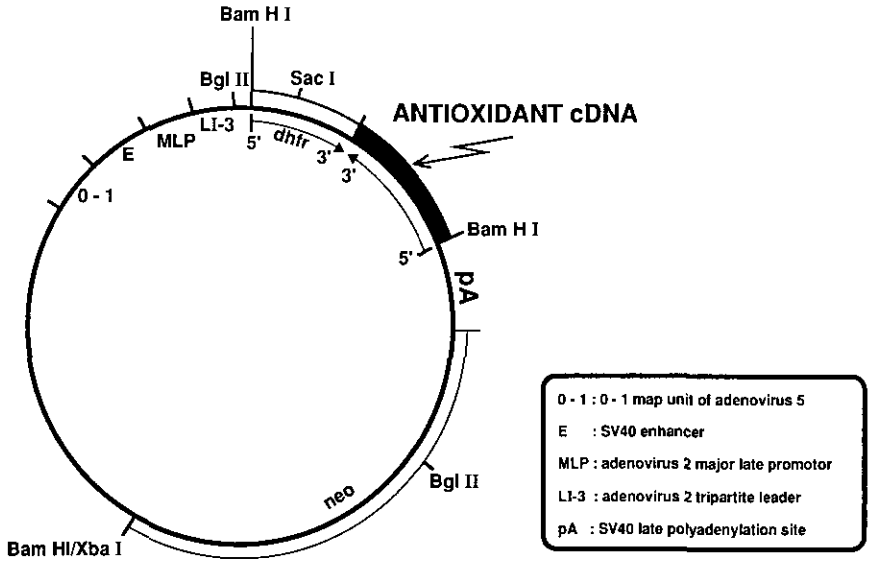

FIGURE 2. pD5-neo-dhfr-based construct containing antioxidant (Cu,Zn-SOD) gene sequences inserted in the antisense direction. Transfected cells are selected with geneticin, and amplification of the antioxidant gene sequences is attempted by growth in increasing concentrations of methotrexate.

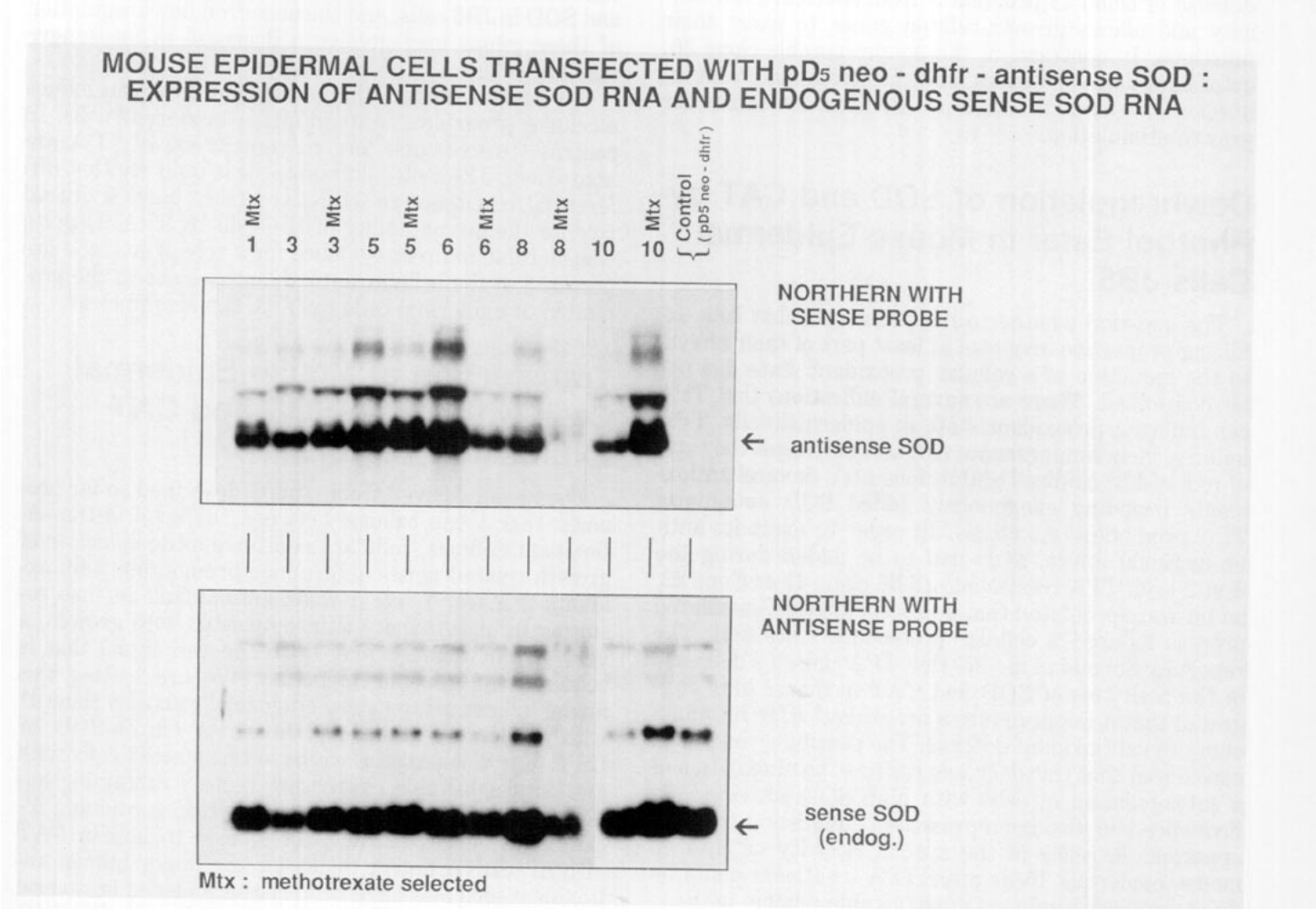

FIGURE 3. Northern blot of geneticin- and methotrexate-selected JB6 clones that had been transfected with pD5-neo-dhfr-antisense SOD plasmid. The upper half of the figure shows a blot with a sense-SOD probe reflecting expression of antisense SOD RNA. Although all transfected clones show expression, there are large variations in intensity. The filter was then stripped and hybridized with a corresponding anti-sense probe for the determination of sense SOD from the endogenous gene (lower half of figure).

sistance gene cassette; the vector for CAT contains the HCMV early promoter and an SV40-hygromycin resistance gene cassette.

Of the many antibiotic resistant clones which have been analyzed so far, only two in each series (i.e., two SOD and two CAT clones) possess moderate 2- to 3-fold increased enzyme activities over prolonged periods in culture (note: the selected clones are cultured routinely in the presence of low concentrations of antibiotic in order to prevent the loss of the transfected gene). Northern-blotting of total RNA from these clones revealed increased expression of Cu,Zn-SOD or CAT, respectively, and Southern blots indicated the presence of the transfected DNA. Western blots with antibodies against the human proteins showed the presence of bands corresponding to the human enzymes in addition to the cross-reacting endogenous mouse protein. The biological and biochemical characterization of these cell clones is under way. So far, survival experiments following treatment with active oxygen generated by extracellular xanthine/xanthine oxidase (X/XO) have not revealed dramatic differences in sensitivity relative to the nontransfected parent.

Transfection of Mouse Epidermal Cells with Vectors Containing Cu,Zn-SOD-cDNA in the Antisense Orientation

We have constructed a pD5-based expression vector containing the complete cDNA of human Cu,Zn-SOD inserted in the antisense orientation downstream of the mouse dihydrofolate reductase gene. Figure 2 shows the principal features of the plasmid. This expression vector allows the selection of successfully transfected cells with geneticin and co-amplification of Cu,Zn-SOD cDNA sequences by cell growth in increasing concentrations of methotrexate. At present we can only give a progress report. As documented in Figure 3, several antibiotic-resistant cell clones that show good expression of antisense Cu,Zn-SOD RNA have been selected. In several instances (but not always), higher amounts of message are detectable following methotrexate selection. The lower half of Figure 3 shows the same stripped blot reprobed with a corresponding antisense SOD probe, indicating that all clones express sense SOD RNA from the endogenous gene. All antibiotic-selected clones were also characterized by Southern-blotting of Bgl II-restricted DNA with a 600 bp Hind III/EcoRI fragment of human Cu,Zn-SOD cDNA. A characteristic 2.7 kb fragment was detectable in all clones, but there was no clear evidence for amplification of the antisense SOD sequence upon methotrexate selection. Preliminary measurements of SOD activity revealed values below controls in some but not all antisense RNA expressing clones. Activities returned to normal after long-term culturing (in low concentrations of geneticin) in several clones.

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