Genomic Instability and Catalase Gene Amplification Induced by Chronic Exposure to Oxidative Stress


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ABSTRACT

Chronic exposure (>200 days) of HAI fibroblasts to increasing concentrations of H2O2 or O2 results in the development of a stable oxidative stress-resistant phenotype characterized by increased cellular antioxidant levels, particularly catalase (D. R. Spitz et al., Arch. Biochem. Biophys., 279: 249-260, 1990; D. R. Spitz et al., Arch. Biochem. Biophys., 292: 221-227, 1992; S. J. Sullivan et al., Am. J. Physiol. (Lung Cell. Mol. Physiol.), 262: L748-L756, 1992). Acutely stressed cells failed to develop a stably resistant phenotype or increased catalase activity, suggesting that chronic exposure is required for the development of this phenotype. This study investigates the mechanisms underlying increased catalase activity in the H2O2- and O2-resistant cell lines.

In H2O2- and O2-resistant cells, catalase activity was found to be 20-30-fold higher than that in the parental HAI cells and correlated with increased immunoreactive catalase protein and steady-state catalase mRNA levels. Resistant cell lines also demonstrated a 4-6-fold increase in catalase gene copy number by Southern blot analysis, which is indicative of gene amplification. Chromosome banding and in situ hybridization studies identified a single amplified catalase gene site located on a rearranged chromosome with banding similarities to Z-4 in the hamster fibroblast karyotype. Simultaneous in situ hybridization with a Z-4-specific adenine phosphoribosyltransferase (APRT) gene revealed that the amplified catalase genes were located proximate to APRT on the same chromosome in all resistant cells. In contrast, HAI cells contained only single copies of the catalase gene that were not located on APRT-containing chromosomes, indicating that amplification is associated with a chromosomal rearrangement possibly involving Z-4. The fact that chronic exposure of HAI cells to either H2O2 or 95% O2 resulted in gene amplification suggests that gene amplification represents a generalized response to oxidative stress, contributing to the development of resistant phenotypes. These results support the hypothesis that chronic exposure to endogenous metabolic or exogenous environmental oxidative stress represents an important factor contributing to gene amplification and genomic instability.

INTRODUCTION

In all O2-metabolizing cells, a delicate steady-state balance exists between the production of prooxidant species (i.e., hydrogen peroxide, superoxide, and organic hydroperoxides) believed to arise from respiration and cellular antioxidants that act in concert to detoxify these species (i.e., catalases, glutathione peroxidases, glutathione reductases, superoxide dismutases, glutathione, and NADPH). When this balance is disrupted by increases in prooxidant production and/or decreases in antioxidant capacity, a condition referred to as oxidative stress exists (1). If oxidative stress persists, oxidative damage to critical biomolecules (i.e., DNA, RNA, proteins, and lipids) accumulates and eventually disrupts normal metabolism, resulting in a wide variety of biological effects ranging from alterations in signal transduction and gene expression (2-5) to mitogenesis (6), transformation (7-9), mutagenesis (10, 11), and cell death (12-14). To survive oxidative stress, the cell must reestablish the steady-state balance between prooxidants and antioxidants, because steady-state metabolism requires a highly reducing environment. This is accomplished by decreasing prooxidant production, increasing antioxidant capacity, and/or increasing the repair of oxidative damage. The basic mechanisms whereby cells reestablish this balance in response to oxidative stress are fundamental to understanding the biological effects resulting from exogenous exposure to prooxidants as well as endogenous increases in prooxidants caused by changes in respiration.

To investigate the basic mechanisms underlying resistance to oxidative stress in mammalian cells, stable oxidative stress-resistant cell lines have been isolated from the HAI Chinese hamster fibroblast cell line after chronic (>200 days) exposure to progressively increasing concentrations of H2O2 (50-800 μM) or O2 (80-95%); Refs. 12-15). In this regard, H2O2 represents chronic exposure to a well-defined exogenously applied oxidant, and 95% O2 represents chronic exposure to an endogenous source of increased prooxidant production believed to be derived from mitochondrial respiration (12-14). Both the H2O2 (designated OCS and OC14) and 95% O2-resistant (designated O2R95) cell lines demonstrated stable (>75 days after oxidant exposure) increases in several cellular antioxidants including glutathione, glutathione peroxidase activity, superoxide dismutase activity, and catalase activity (12-14). The greatest magnitude of increase (20-30-fold) was noted in catalase activity; therefore, the molecular mechanism(s) responsible for this dramatic change is of considerable interest.

To define the mechanisms responsible for this increase in catalase activity, we examined the relationship between increased catalase activity, immunoreactive protein, steady-state mRNA levels, and the catalase gene copy number. When the catalase gene was found to be amplified, the mechanisms by which this stable amplification event could have occurred were investigated using chromosomal in situ hybridization and chromosome banding studies. The results indicate that chronic exposure of HAI cells to oxidative stress causes amplification of the catalase gene by a process involving recombination between chromosomes containing the catalase and APRT7 genes. This work supports the hypothesis that gene amplification may represent a fundamental cellular response underlying the development of resistant phenotypes after the chronic exposure of immortalized cells to oxidative stress.
MATERIALS AND METHODS

Cell and Culture Conditions. HA1, OC14, OC5, and Q2R95 cell lines were maintained in Eagle's MEM supplemented with 10% FCS (Hyclone), penicillin/streptomycin, and phenol red as described previously (12-14). Monolayers of cells were grown exponentially to 50-80% confluence and then used for the preparation of whole cell homogenates and chromosome spreads or extracted for RNA or DNA. Cells for survival experiments and the isolation of acutely treated clones were grown in a similar fashion and treated in complete media with H2O2 or 95% O2 as described previously (12-14). After treatment, the cultures were trypsinized and plated into cloning dishes and incubated for 7-9 days, and the clones were ring-isolated or stained for clonogenic survival analysis as described previously (15).

Sample Preparation for Enzymology, Immunoblotting, RNA, and DNA Analysis. For the enzymology and determination of immunoreactive protein, cell monolayers were harvested by scraping at 4°C in PBS, pelleted at 400 x g, frozen at —20°C, thawed at room temperature, and homogenized on ice in 50 mM potassium phosphate buffer (pH 7.8) containing 1.34 mM diethyleneriaminopentaacetic acid (13). Whole homogenates were used to measure catalase activity by the method of Beers and Sizer (16) as detailed previously (12). Activity units were normalized to protein content as determined by the method of Lowry et al. (18), and equal aliquots of homogenates were processed and loaded onto polyacrylamide gels for immunoblotting based on this determination. Total RNA was isolated from monolayers directly from the culture plate using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Extracted samples were stored in 100% ethanol at —80°C until analysis. Genomic DNA was isolated by lysing cells in 0.5 M EDTA and 100 µg/ml proteinase K and then digesting them to overnight at 50°C. The mixture was extracted twice with phenol-chloroform and the DNA precipitated with ethanol. The DNA pellets were recovered by centrifugation, resuspended in TE buffer (10 mM Tris and 1 mM EDTA (pH 8.0)), and treated with 10 µg/ml DNase-free RNase for 1 h before extraction with phenol-chloroform. The genomic DNA was then reprecipitated and stored in absolute ethanol at —80°C until analysis. RNA and DNA concentrations were determined spectrophotometrically at 260 nm.

Determination of Immunoreactive Catalase Protein. A rabbit polyclonal antibody made against bovine catalase (Sigma; C-40) was kindly provided and characterized by Spitz et al. (12). Polyacrylamide gels (10%) were loaded with samples of protein extracts, electrophoresed, and stained for protein. The proteins were transferred to immobilized nitrocellulose membranes and blocked with powdered milk, and incubated with a 1:1000 dilution of antibody. The antibody made against bovine catalase (Sigma: C-40) was kindly provided and used for the preparation of whole cell homogenates and chromosome spreads or extracted for RNA or DNA. Cells for survival experiments and the isolation of acutely treated clones were grown in a similar fashion and treated in complete media with H2O2 or 95% O2 as described previously (12-14). After treatment, the cultures were trypsinized and plated into cloning dishes and incubated for 7-9 days, and the clones were ring-isolated or stained for clonogenic survival analysis as described previously (15).

Oxidative Stress and Gene Amplification

Northern blot analysis was carried out by fractionating approximately 10 µg of total cellular RNA from each sample by electrophoresis on a formaldehyde-agarose gel, followed by transfer to a GeneScreen Plus membrane (DuPont). The membrane was then hybridized with 32P-labeled DNA probes prepared by random priming (Amersham) in 1% SDS, 1 M NaCl, and 10% dextran sulfate overnight at 65°C. The membrane was washed twice in 1% SDS and 2 x SSC at 65°C and once in 1% SDS and 0.4 x SSC at 65°C before being exposed to film for autoradiography. The membranes were probed initially with the CAT-specific probe (either pCat10 or SaeI), followed by a β-actin probe as an internal loading control. Southern blot analysis was carried out by electrophoresing 10-µg samples of β-globin digested genomic DNA on a 1% agarose gel in TEA buffer and then transferring the DNA onto GeneScreen Plus membrane by blotting with 0.4 M NaOH. Hybridization and wash conditions were the same as those for the Northern blot analysis. The membranes were hybridized with a radiolabeled catalase probe (1.0-kb SacI fragment), followed by the 3.9-kb BamHI APRT gene probe as a loading control. Quantiﬁcation was carried out by densitometric scanning of the resulting autoradiographs.

Chromosome Banding and in Situ Hybridization. In the initial in situ hybridization and banding experiments, the metaphases were collected by treating the cells in 200 µg/ml bromodeoxyuridine for 2.5 h, followed by a 4 h in 5 µg/ml thymidine and then 1.5 h in 0.1 µg/ml Colcemid, and then the metaphases were harvested by shaking the flask and collecting the media. After centrifugation, the media were discarded, and the cells were resuspended in 0.4% KCl (10 ml) and then incubated at 37°C for 30 min. Cells were then fixed in 3:1 (v/v) methanol/acetic acid (22), and air-dried chromosome spreads on slides were prepared. For in situ hybridization, the slides were pretreated with RNase and proteinase K before denaturation in 70% formamide. The 1.5-kb insert from pCat10 was excised with EcoRI and HindIII and labeled with biotin using the Oncor nick translation kit. The probe was dissolved in hybridization mix (containing 50% formamide, RNA, and herring sperm DNA) to a final concentration of 2 ng/µl and denatured at 70°C. Slides were incubated with the probe overnight, and unincorporated probe was removed in 50% formamide. The signal was amplified with biotin-avidin, and a total of three layers of avidin-FITC were applied. Slides were mounted in antifade containing propidium iodide counterstain. FITC signals were visualized with epifluorescence, and color slides were taken with Kodak EKTACHROME P800/1600 transparency film. The metaphases were photographed twice to view DAPI-banded chromosomes and FITC signals. This method (using the cdNA pCat10 probe) was adequate to determine that the amplified genes resided on a chromosome that had some banding characteristics consistent with hamster chromosome Z-4, as determined by comparison with a previously published hamster fibroblast karyotype (23). However, experiments using this probe were unable to detect single copies of the catalase gene in the parental HA1 cell line and were therefore unable to yield information as to the mechanism of how the amplification event may have occurred.

The second set of experiments designed to determine the chromosomal localization of single copies of the catalase gene in relation to the APRT gene known to reside on hamster chromosome Z-4 (23) was carried out by in situ hybridization of the homologous gene sequences present in pCat3 and simultaneous 32P hybridization with a λ-phage clone containing the hamster APRT gene. These experiments were done with pCat3 (a 12-kb genomic catalase gene probe) labeled with digoxigenin (green) and S78 (a recombinant λ-phage clone containing the genomic APRT gene probe) labeled with biotin (red), according to a previously published method (24, 25). Briefly, in situ hybridization and washing procedures were performed essentially as described previously (24, 25), with the following modification. Two-color hybridizations were done with 100 ng each of digoxigenin-labeled pCat3 and biotin-labeled S78 DNA plus 50 µg of hamster Corl DNA (Life Technologies, Inc.), 5 µg of sheared salmon sperm DNA, and 5 µg of Escherichia coli rRNA in 10 µl of 10% dextran sulfate, 50% formamide, and 1x SSC and hybridized overnight at 37°C. After washing, the bromodeoxyuridine-substituted metaphase chromosome preparations were treated to obtain replication banding by staining with Hoechst.
Fig. 1. Catalase activity in HA1, OC14, OC5, O2R95, H2O2, and 95% O2 acutely selected clones from HA1. Cell lines were maintained and harvested as described in "Materials and Methods," and catalase activity was determined on whole homogenates and normalized per milligram of protein. The N values represent the number of separate samples that were analyzed and, in the case of the acutely selected cells, the number of separately collected clones. Errors represent ±1 SD, and asterisks indicate those groups that are significantly different from HA1 (P < 0.05).

33258 (0.5 mg/ml) and irradiating for 10 min at a 20-cm distance from two 15-W UV bulbs. Biotin-labeled probe detection was performed with 10 μg/ml Cy3-conjugated avidin (Sigma), and digoxigenin-labeled probe detection was performed with a 1:200 dilution of FITC-conjugated antidigoxigenin (Boehringer Mannheim) in 4× SSC and 0.2% Tween 20 for 30 min. The digoxigenin signal was amplified with FITC-conjugated F(ab')2 fragment rabbit antiserum IgG (H + L; Jackson ImmunoResearch Laboratories, Inc.). Finally, the slides were stained in DAPI and mounted.

Statistics. Statistical comparisons were made using ANOVA with Duncan’s new multiple range test. The mean ±1 SD is reported. Differences were declared significant if P < 0.05.

RESULTS

The mean catalase activity in the parental HA1 cell line and in the oxidative stress-resistant OC14, OC5, and O2R95 cell lines was measured throughout the course of the experiments (approximately 100 population doublings) shown in Figs. 2–5. Both the H2O2-resistant (OC14 and OC5) and 95% O2-resistant (O2R95) cell lines demonstrated significant 20–30-fold increases in catalase activity (Fig. 1). The increase in catalase activity was the result of corresponding increases in the immunoreactive catalase protein (Fig. 2) as measured by Western blot analysis. Experiments in which resistant cell samples were diluted until the band intensity was approximately equal to that of HA1 indicated that catalase protein levels increased approximately 25-fold in the resistant cell lines (data not shown).

Within the limits of resolution, the protein appeared to be of identical size in all cell lines. Northern blot analysis of total cellular RNA isolated from the four cell lines also indicated that the steady-state levels of catalase mRNA (Fig. 3) had similarly increased in the resistant cell lines. These results show that the increased catalase activity observed in the oxidative stress-resistant cell lines as compared with that of the parental cell line is due to increased protein levels that are the consequence of increased catalase mRNA levels.

To determine whether this stable increase in catalase expression resulted from a selection of preexisting cells from the HA1 population that overexpressed catalase before oxidant exposure or from a process that required chronic exposure to develop, the following experiment was carried out. Populations of the parental HA1 cells were selected with a single acute exposure to 1 mm H2O2 for 1 h or 95% O2 for 92 h (surviving fraction < 2 × 10−3), and nine surviving clones from each treatment group were ring-isolated and expanded into clonally selected cell lines. Each H2O2 or 95% O2 clonally selected cell line was
This question was addressed by in situ hybridization and banding at multiple sites or a single amplification event involving one site. Due in part to amplification of the catalase gene.

Levels of the APRT gene varied little from the heterogeneous HAI parental cell line acutely exposed to oxidative stress and not by the selection of a preexisting phenotype. Selection with either H2O2 or 95% O2 resulted in cell lines that demonstrated catalase activity that was not significantly greater than that of the nonselected parental HAI cell populations. These results support the hypothesis that catalase overexpression in the OC5, OC14, and O2R95 cell lines is a result of chronic exposure to oxidative stress and not by the selection of a preexisting phenotype from the heterogeneous HAI parental cell line acutely exposed to oxidative stress.

The remarkable stability (>200 days; approximately 200 population doublings) observed for increased catalase activity in cell lines chronically exposed to oxidative stress indicated that stable genetic changes had occurred. In contrast, OC5 (Fig. 5B), OC14 (Fig. 5C), and O2R95 (Fig. 5D) all contained a single amplified catalase gene site that was now located on a chromosome with an APRT gene. Neither the Southern blot analysis (Fig. 4) nor in situ hybridization results (Fig. 5) indicates amplification of the APRT gene. These results suggest that a translocation event involving a chromosome carrying a copy of the APRT gene (possibly Z-4) and a separate chromosome carrying a single copy of the catalase gene occurred during chronic exposure to oxidative stress. Amplification of the catalase gene was only observed in cells that had undergone translocation, and gene amplification was always restricted to a single APRT-associated chromosomal site. Because both the H2O2- and 95% O2-selected cell lines exhibited similar rearrangements and gene amplification, the translocation/amplification event does not seem to be specific to the conditions of oxidant exposure but rather seems to occur in response to chronic oxidative stress in general.

**DISCUSSION**

The most significant finding in the current study is that chronic exposure of a mammalian cell line to an exogenous (H2O2) or endogenous (95% O2) metabolic source of oxidative stress results in the amplification of an antioxidant gene (catalase) critical to the development of the stable oxidative stress-resistant phenotype. Overexpression of catalase could not be detected in HAI cells that survived a single acute exposure to either H2O2 or 95% O2. It is unlikely, therefore, that chronic oxidant exposure selected a preexisting subpopulation already stably expressing catalase at elevated levels from the parental cell line. These data support the hypothesis that prolonged...
exposure to oxidative stress is the critical step in the genetic changes that lead to catalase overexpression.

Currently, there are two nonexclusive models for gene amplification. The first mechanism proceeds through the reintegration of extrachromosomal copies of the selected gene in the form of episomal or double minute chromosomes. It is unlikely that this mechanism contributes to catalase gene amplification, because no double minute chromosomes containing catalase genes were detected by in situ hybridization in either the resistant or parental cell lines. The second process by which gene amplification is believed to occur is the chromatid breakage-fusion-bridge mechanism commonly seen in drug-selected hamster cell lines (27, 28). In this model, a chromatid break is induced on the chromosome between the resistance gene and the telomere. After replication, the broken end is repaired by fusion with the sister chromatid, generating a dicentric chromosome with duplicated copies of the target gene. Repetition of the cycle generates multiple copies of the gene located distal to the original locus (see Ref. 27 for more details). Evidence from recent studies of drug-induced gene amplification has found that these breakage sites are associated with fragile sites (27, 28). In addition, the literature suggests that chromosomal instability and recombinational events may also be related to fragile site sequences (27–29). Chronic exposure of
Chinese hamster ovary fibroblasts to 95% O₂ has been shown to give rise to what appear to be fragile sites as well as chromosomal instability (aberrations and breaks; Ref. 30), and it is reasonable to postulate similar effects from other oxidants such as H₂O₂. Even more relevant is the localization of 95% O₂-induced fragile sites to chromosomes Z-3 and Z-4 (30), the latter being a tentatively identified catalase gene amplification site in the current study as well as a site prone to deletions in a previous study (26). These data lead us to speculate that perhaps fragile site sequences near APRT on Z-4 and near the catalase gene on another chromosome are activated by agents causing oxidative stress, thereby initiating DNA strand breakage, the critical first step leading toward gene amplification of the protective protein, catalase. This hypothesis is consistent with the observation that many of the drugs and agents (including H₂O₂) that give rise to gene amplification cause DNA breaks (27, 31).

A second unresolved mechanistic question is whether the gene amplification/translocation event alone can account for the increased levels of catalase mRNA in the oxidative stress-resistant phenotype. There is a very good correlation between the 20–30-fold increases in catalase activity, the increases in immunoreactive protein, and the steady-state levels of mRNA. The observed 4–6-fold increase in catalase gene copy number, although a major component contributing to increased catalase levels, does not seem to be able to completely account for the increase in steady-state catalase mRNA levels. Chromosomal translocations can disrupt normal gene structure and alter gene expression by bringing new regulatory elements such as enhancers or sequences that govern mRNA stability into the gene structure (32). Translocations involving the catalase gene could involve such a phenomenon. In this model, oxidative stress would activate fragile sites, leading to chromosome translocations resulting in both catalase gene amplification and the juxtaposition of new sequences that enhance transcriptional activity or increase the mRNA stability of the amplified genes. Alternatives to this hypothesis could also include specific secondary mutations in a transcription factor(s) that binds to the catalase gene and/or the loss of negative regulatory elements in the gene itself. Regardless of the specific mechanism, it does seem that mechanisms other than gene amplification are contributing to the increase in catalase mRNA.

The overall significance of oxidative stress-induced gene amplification in immortalized cell lines potentially relates to a wide variety of biologically significant situations due to the ubiquitous nature of oxidative stress. Many cytotoxic therapies used for the treatment of cancer result in increased oxidative stress. Anthracycline antibiotics (i.e., Adriamycin) are known to cause oxidative stress, and gene amplification seems to be one mechanism by which cells can become resistant to these chemotherapeutic agents (33). Many other anticancer agents such as mitomycin C, nitrogen mustards, and ionizing radiation have been suggested to result in oxidative stress, and repeated treatment with these agents is associated with the acquisition of cellular resistance to drugs as well as gene amplification (34–39). Previously, we have shown that the oxidative stress-resistant phenotype in OC5 and OC14 cells is accompanied by an increased resistance to cisplatin (40), and we have data showing that OC5 cells become resistant to Adriamycin-induced cell killing. The data in this report as well as data from other studies showing that hamster as well as human tumor cells are capable of undergoing antioxidant gene amplification in response to chronic oxidative stress (41, 42) suggest that gene amplification may be a generalized response to oxidative stress with potential relevance to a wide variety of situations in which tumor cells become resistant to cytotoxic therapy.

Perhaps one of the most speculative but intriguing applications of the concept that gene amplification and genomic instability represent a generalized response to oxidative stress may involve situations in which gene amplification is known to occur, but the origin of the selective pressure responsible for inducing the amplification event is unknown. Several reports document the amplification of presumptive oncogenes in human tumors, but the origin of the selective pressure responsible for inducing the amplification event is as yet unknown (reviewed in Ref. 43). In addition, human tumor cells seem to have increased intracellular production of prooxidants such as peroxides as well as decreases in cellular antioxidants such as catalase and manganese superoxide dismutase (Ref. 44; reviewed in Ref. 45) that would be expected to result in a prooxidant state. It is also well accepted that oxidative stress can initiate as well as promote carcinogenesis, and antioxidants are known to be anticarcinogens (7, 8). It is therefore intriguing to speculate that the amplification of oncogenes could be induced as a generalized response to an endogenous oxidative stress associated with the carcinogenic process and the altered metabolism of cancer cells that allows the tumor cell to continue to proliferate in the face of an increasingly prooxidant state. In support of this speculation, it has been recently reported that cell lines transfected with myc and ras (both presumptive oncogenes) become resistant to oxidative stress induced by exposure to ionizing radiation (46) and demonstrate significant alterations in oxidative metabolism (47). However, rigorous testing of this hypothesis awaits further experimentation.

In summary, our results demonstrate that chronic exposure of the HA1 cell line to exogenous bolus doses of H₂O₂ or endogenous metabolic increases in prooxidant production caused by 95% O₂ (presumably O₂⁻, H₂O₂, and organic hydroperoxides) results in the amplification of catalase genes in a process that seems to involve genomic instability. The extra copies of the gene are stably integrated into the chromosomal structure and seem to be the result of a recombinational event. This work suggests that gene amplification could represent a generalized response to oxidative stress in immortalized cell lines that contributes to the development of resistant phenotypes after chronic exposure. This work also supports the hypothesis that chronic exposure to environmental or metabolic oxidative stress could represent an important factor contributing to gene amplification and genomic instability.

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REFERENCES


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