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Peroxidase deficiency of nickel-transformed hamster cells correlates with their increased resistance to cytotoxicity of peroxides

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Using a procedure aimed at isolation of genes that are inactivated during nickel-induced carcinogenesis in Chinese hamster cells, a homolog of genes encoding human and mouse heme containing peroxidases has been cloned. Northern blot analysis of normal cultured fibroblasts and two nickel-transformed cell lines confirmed that this gene was expressed in normal but not in transformed cells. Nickel-transformed cells also tested negative for peroxidase activity using a sensitive fluorescence assay. Cultured embryo cells or fibroblasts that express peroxidase activity and their nickel-transformed peroxidase-deficient counterparts were employed to investigate the role of peroxidase-catalyzed processes in cytotoxicity induced by *tert*-butyl hydroperoxide or cumene hydroperoxide. It has been found that peroxidase-deficient cells were significantly more resistant to cytotoxic effect of these compounds suggesting that cytotoxic effect of hydroperoxides may be mediated in part by free radicals generated in the course of peroxidase-catalyzed reactions.

Keywords: nickel-transformed hamster cells, molecular cloning, peroxidase gene, Northern blot, fluorometric assay, organic hydroperoxides, cytotoxicity, free radicals

Introduction

Peroxidases, catalases and superoxide dismutases are considered to be the first line of defense against oxidative stress. Out of these three, peroxidases are remarkable for their ability to catalyze the formation of a variety of highly reactive intermediates including diverse categories of free radicals (Mason 1982, Yamasaki 1987, O'Brien 1988, Rao *et al.* 1988, Ramos *et al.* 1992, Chamulitrat *et al.* 1991). Although the primary role of peroxidases is at the level of cell defense, their ability to catalyze the formation of free radicals may lead to cell injury and death. The cytocidal activity promoted by the various peroxidases has received a considerable amount of attention since it may contribute to the cell killing exerted by many anticancer drugs (Everse 1991). For example, the most widely used quinone-containing drugs, such as adriamycin, daunorubicin or mitomycin C, are activated by peroxidase oxidation which results in the formation of semiquinone radicals as well as superoxide and possibly hydroxyl radicals (Bachur *et al.* 1978, Lown 1983).

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To study the role of peroxidases in cytotoxicity induced by externally added organic hydroperoxides, a commonly used model for effects of lipid peroxidation inside cells (Trotta *et al.* 1982, Starke & Faber 1985, Trible *et al.* 1988), we utilized cell lines that express or do not express peroxidase, so the effect of intracellular levels of the enzyme could be tested. In doing this, we also identified peroxidase as a gene that was inactivated during nickel-induced cell transformation. We found that cells that do not express this gene are much more resistant to cytotoxicity of organic hydroperoxides suggesting the peroxidase-catalyzed metabolism contributes to the cell injury induced by these compounds.

Materials and methods

Chemicals

Tert-butyl hydroperoxide (*t*-BHP), cumene hydroperoxide (CHP), horseradish peroxidase and 30% solution of hydrogen peroxide were purchased from Sigma (St Louis, MO), and dichlorofluorescein diacetate (DCF-dAc) from Eastman Kodak (Rochester, NY).

Cell lines

Primary Chinese hamster embryo (CHE) cell cultures were initiated from individual male embryos which were aseptically removed on the 17th day of gestation. Chinese hamster fibroblast cell line (CH-1) was initiated from the fragment of tissue obtained through ear punch biopsy of male adult donors. All cells were routinely maintained in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 units ml^{-1} pencillin, 100 $\mu g\,ml^{-1}$ of streptomycin (Gibco, Gaithersburg, MD) in an atmosphere of 5% CO_2 and 95% air. At passage 2, embryo cells were treated with either $NiCl_2$ (1 mM for 24 h) to obtain Ni-2 or crystalline NiS (10 $\mu g\,ml^{-1}$ for 24 h) to obtain Ni-6 transformed cell lines (Convary & Costa 1988).

Isolation of white blood cells

Cells sedimented from 50 ml of freshly collected human blood were treated for 10 min with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 1 mM EDTA) to lyse erythrocytes. The remaining cells were washed with PBS and analyzed for peroxidase activity.

Molecular cloning, sequencing and searching for homologous sequences

cDNA from CHE cells was subtracted with an excess of mRNA from Ni-2 cells in order to obtain a probe for isolation of genes inactivated during nickel carcinogenesis. This subtracted cDNA was used for screening a CHE cell cDNA library (Salnikow *et al.* 1994). The library has been constructed in lambda ZAP vector (Stratagene, La Jolla, CA), which contains a pBluescript plasmid that can be excised from the phage vector. pBluescript clones were subjected to sequencing using the chain termination method with Sequenase 2.0 (US Biochemical, Cleveland, OH) according to the manufacturer's recommendations. Each clone has been sequenced from the regions flanking both ends of the insert using T3 and T7 primers. In an attempt to identify newly acquired sequences, a computer search through gene bank databases has been performed. This search was run by FASTA Search program of the University of Wisconsin Genetic Computer Group sequence analysis package (Devereux *et al.* 1989), and included all sequences deposited in the GenBank, EMBL and NBRF databases.

Northern blot analysis

Total cytoplasmic RNA was isolated using RNAzol (Biotecx, Houston, TX) according to the manufacturer's protocol. Poly $(A)^+$ was selected by the system which uses streptovidin coupled to paramagnetic particles and a biotinylated oligo(dT) primer (Promega, Madison, WI). Typically, 200 μg of total RNA yielded approximately 5 μg of poly $(A)^+$ RNA. Then, 1 μg of each mRNA was analyzed by electrophoresis in 1.2% agarose formaldehyde gel and transferred onto nitrocellulose BA-S85 membrane (Schleicher & Schuell, Keene, NH). Membranes were processed for hybridization to ^{32}P-labeled riboprobes synthesized by T7 and T3 DNA-dependent RNA polymerases (Stratagene) in the presence of [^{32}P]rCTP (Amersham, Arlington Heights, IL). RNA blots were hybridized overnight at 65°C in a hybridization solution (5 × SSC, 5 × Denhardts, 5% dextran sulfite, 0.1 M NaH_2PO_4, pH 7.0) and washed in 0.1 × SSC at 65°C. Blots were rehybridized to a ^{32}P-labled β-actin probe to determine the quantity of RNA loaded in each lane of the gel.

Assessment of peroxidase activity

Peroxidase activity was measured with a fluorometric assay. The assay was first devised by Keston & Brandt (1965) for measuring microquantities of H_2O_2 and uses DCF as a fluorescent probe. In our previous study (Huang *et al.* 1993), it has been shown that the oxidation of non-fluorescent 2,7-difluoroescein (DCFH) to fluorescent DCF in the presence of H_2O_2 is catalyzed by peroxidase in a dose-dependent manner. DCFH was freshly prepared from DCF-dAc as follows: 0.05 ml of 10 mM DCF-dAc in ethanol was mixed with 2 ml of 0.01 N NaOH at room temperature for 30 min and the mixture was then neutralized with 17.95 ml of 25 mM phosphate-buffered saline (PBS), pH 7.4. This solution was kept on ice in the dark until use. Cell extracts were prepared from 2–3 million cells by lysis in 0.5% Triton X-100 for 30 min on ice. The cell lysates were then centrifuged for 10 min at 560 *g* to separate nuclei and unbroken cells. The supernatants were collected and dialyzed overnight at 4°C against PBS to remove halides or thiocyanates that might inhibit peroxidase activity by binding to the heme group of the enzyme. After dialysis, the supernatants were divided into two parts, one for the peroxidase assay and the other for determination of protein content by the Lowry method using a protein kit from BioRad (Hercules, CA).

Samples (0.4 ml) of cell extracts were mixed with 0.4 ml of 25 μM solution of DCFH, 0.4 ml of 1 M Tris–HCl buffer (pH 7.4) and 2.8 ml of distilled water (37°C) in a fluorescence-free cuvette. All reactions were carried at 37°C with constant stirring. The fluorescence was measured in the fluorometer (Spex Fluorolog, Edison, NJ) with excitation wavelength of 502 nm (bandwidth 1.8 nm) and emission wavelength of 522 nm (bandwidth 4.5 nm). The changes in fluorescence were recorded after addition of 40 μl of 100 mM *t*-BHP or H_2O_2. Peroxidase activity measured by changes in fluorescence units per second was corrected for protein concentration.

Measurement of cytotoxicity

To measure the cytotoxic effect of hydroperoxides, 300 cells of each cell line were plated in triplicate in 60-mm dishes. After overnight growth, the attached cells were treated for 2 h at 37°C with *t*-BHP, CHP or H_2O_2, in complete α-MEM medium containing 10% fetal bovine serum (Sigma).

Following treatment, cells were washed twice with PBS and incubated in the same medium at 37°C in the atmosphere of 5% CO_2 and 95% air. After 7 days of incubation, colonies were stained with Giemsa:methanol (50% v:v) and counted. For all untreated cells, plating efficiency was in the range of 20–30%.

Results

Several clones were obtained following screening of CHE cDNA library with probes obtained by subtraction of CHE cDNA with an excess of Ni-2 mRNA. Clone 3 carried an 1.6 kb insert that displayed a high degree of sequence homology to the family of mammalian genes encoding heme-containing peroxidases, particularly to human eosinophil peroxidase (EPO). The 150 nucleotides fragment adjacent to the T7 promoter of the vector was 72% identical to the peroxidase, whereas homology of a 460 base long sequence from the opposite end of the insert was even higher parentheses around (84%). The region of highest homology encompassed exons 7 and 8 of human EPO. This fragment also displayed 74, 62 and 60% homology of mRNA of human and mouse myeloperoxidase (MPO), bovine lactoperoxidase (LPO) and human thyroid peroxidase (TPO), respectively. Thus, clone 3 must represent the hamster homolog of either of the above mentioned peroxidase genes. Since the cDNA fragment has been cloned in a procedure aimed at the selection of genes that are expressed in normal but not in nickel-transformed cells (Salnikow *et al.* 1994), the expression of the corresponding gene should be restricted to normal cells represented here by fibroblasts. This was tested in the experiment in which mRNA isolated from cultured fibroblasts (CH-1) and two (Ni-2 and Ni-6) nickel-transformed cell lines was hybridized to riboprobes synthesized from clone 3. The result of this experiment is presented in Figure 1. Clone 3 riboprobe detects a single mRNA species which was present in normal but not in nickel-transformed cells. The size of this transcript has been estimated to be 2.6 kb, which closely corresponds to the size of the coding strand for human EPO (Ten *et al.* 1989).

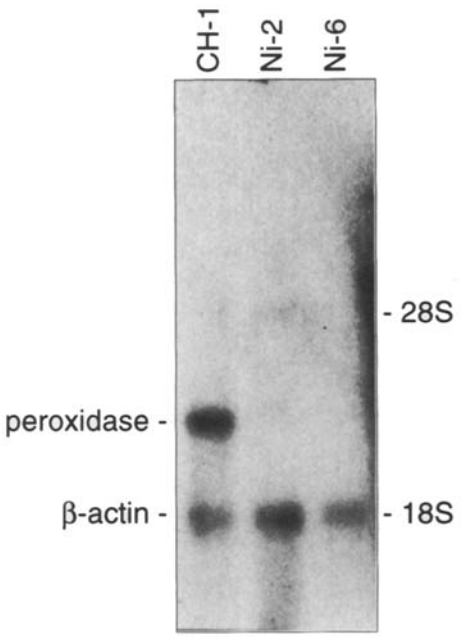

Figure 1. Expression of peroxidase gene in Chinese hamster fibroblast (CH-1) and two nickel-transformed cell lines (Ni-2 and Ni-6). Poly(A)$^+$ selected RNA of each cell line (1 μg) was electrophoresed in 1.2% agarose formaldehyde denaturing gel and transferred onto nitrocellulose membranes. The membrane was hybridized to ^{32}P-labeled riboprobe generated from the plasmid carrying a 1.6 kb cDNA fragment of the hamster peroxidase gene. To assess the RNA load in each lane, the blot was reprobed with radioactive β-actin.

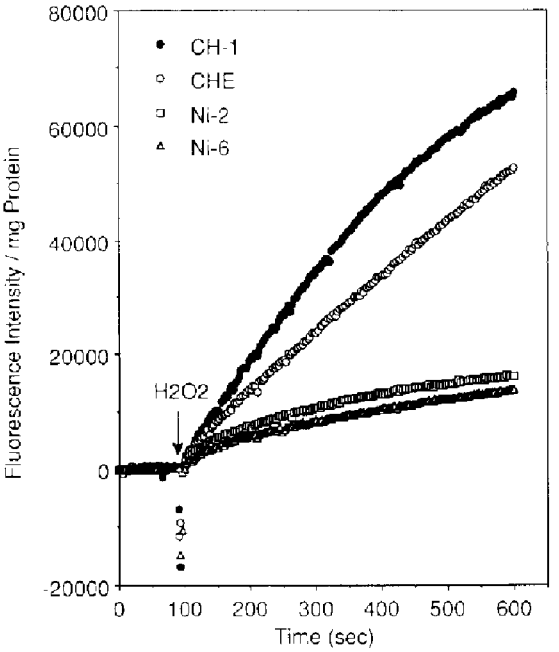

Figure 2. Cytotoxic effect of H_2O_2 (A), *t*-BHP (B) and CHP (C). CH-1 or Ni-2 cells (300) were plated in 60-mm dishes. After overnight incubation, cells were treated with indicated doses of H_2O_2, TBHP and CHP for 2 h in complete α-MEM. Following treatment, cells were washed and incubated for an additional 7 days. Colonies were stained with Giemsa:methanol and counted. The results represent a mean value of three independent experiments and are expressed as percent of the number of colonies formed by untreated cells.

Figure 2 shows peroxidase activity recorded in cell lysates of normal (CH-1, CHE) and nickel-transformed (Ni-2, Ni-6) cell lines. The activity is expressed as changes in DCF fluorescence per second per mg of protein. It can be seen that cell extracts alone did not enhance the fluorescence intensity of DCF, but when H_2O_2 was added to the reaction mixture 90 s later, an increase in fluorescent reading was immediately recorded in the extracts of normal cells. A very small but noticeable increase of DCF fluorescence with nickel-transformed cell extracts was also observed.

Linear regression analysis of the peroxidase activity data yielded two parameters; the initial fluorescence change, dFI/dt, and ΔFI, which represents the difference between the initial fluorescence intensity (FI) and the FI measured after 30 s. The data collected from all measurements and presented in Table 1 show that both ΔFI and initial rate dFI/dt in CHE and CH-1 cell extracts are much higher than those in cell extracts of nickel-transformed cell lines and this was true for either H_2O_2 or *t*-BHP substrates. These results are in good agreement with findings from Northern blot analysis.

Most assays for peroxidase were developed for the measurement of activity in inflammatory cells. Using purified horseradish peroxidase or polymorphonuclear leukocytes (PMNs), we compared the DCF method with the homovanillic acid (HVA) oxidation assay (Menegazzi *et al.* 1991). A good correlation was found between these two assays (data not shown). However, the HVA assay was not sensitive enough to detect peroxidase activity in cell extracts utilized in the study. As shown in Table 1, with DCF, the activity in PMNs was approximately 50 times higher than those in CH-1 and CHE cells. A negative control (BSA) showed a small initial rate which was probably due to the oxidation of DCF by H_2O_2 alone. Results presented in Table 1 also show that H_2O_2 seems to be better substrate than *t*-BHP, since the values of both parameters recorded for normal cells in the presence of H_2O_2 were significantly higher. Having cell lines with such differences in peroxidase activity afforded a system to study the role of peroxidase-catalyzed processes in the cytotoxic effects of peroxides.

Table 1 Peroxidase activity in different cells lines[a]

	t-BHP as substrate		H_2O_2 as substrate	
	initial rate[a]	ΔFI_{max} at 30 s[c]	initial rate[b]	ΔFI_{max} at 30 s[c]
CH-1	2555.3	9356.5	3816.6	16283.8
CHE	2362.2	7498.6	5799.9	26592.3
Ni-2	879.3	3438.6	691.9	3392.7
Ni-6	1227.8	4429.0	524.2	2357.5
PMNs	12839.8	131550	—[d]	
BSA	—	—	256.1	831.3

[a]Peroxidase activity is expressed as changes of DCF fluorescence per second per mg of protein. Experimental conditions were performed as described in Materials and methods. The data represent a mean value of two separate experiments.
[b]The initial rate was calculated from the addition of substrates to 30 s.
[c]ΔFI_{max} was the maximum value of DCF at 30 s after addition of *t*-BHP or H_2O_2 as substrates.
[d]Not tested.

The cytotoxicity of H_2O_2, *t*-BHP and CHP was examined in CH-1 and Ni-2 cells by the colony formation assay (Figure 3A–C). A 2 h treatment with increasing doses of either compound resulted in a marked decrease in the number of colonies scored 7 days later. When *t*-BHP and CHP were used (Figure 3B and C), nickel-transformed cells appeared more resistant to cytotoxicity induced by these compounds. LD_{50} values calculated for Ni-2 cells treated with *t*-BHP (36 μM) and CHP (10 μM) were significantly higher than corresponding values (14 and 5.8 μM, respectively) for CH-1 cells. H_2O_2 (Figure 3A) induced equal cytotoxicity in CH-1 and Ni-2 cells and considerably lower ($LD_{50} = 38$ μM) than *t*-BHP or CHP. This could be attributed to the protective effect of catalase present in both cell lines which decomposes H_2O_2 but not organic peroxides.

Discussion

We cloned a cDNA fragment, expressed in normal cells but not in their nickel-transformed counterparts, which shows very high homology to the family of mammalian genes encoding heme-containing peroxidases. The highest homology was found to mRNA for human EPO. Not only was sequence homology high, but the size of the transcript

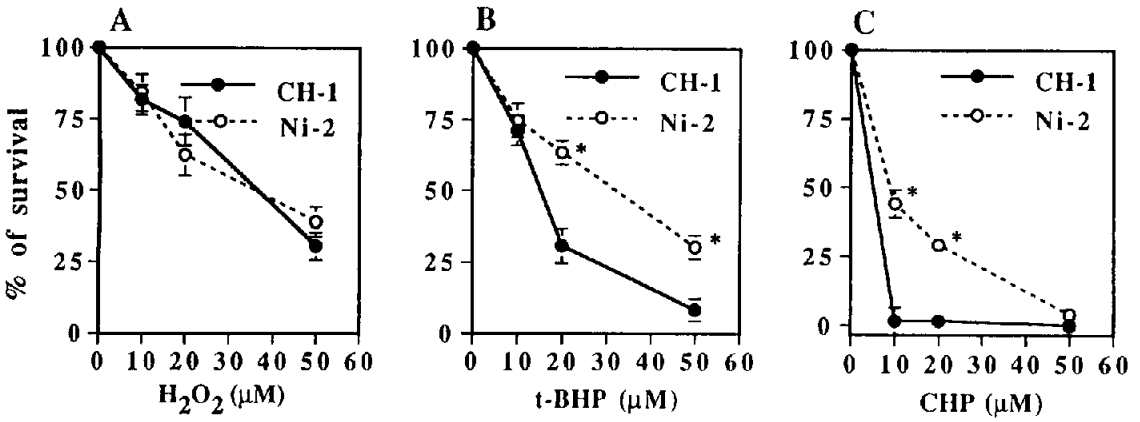

Figure 3. Time-dependent changes in the fluorescence intensity (FI) per mg of protein as a measure of peroxidase activity in normal (CH-1, CHE) and nickel-transformed (Ni-2, Ni-6) cell lines. The assay was performed as described in Materials and methods. To initiate the reaction, 40 μl of 100 mM H_2O_2 was added 90 s after starting reading. The fluorescence background before addition of H_2O_2 was subtracted at each data point.

closely corresponded to that of the human gene (Ten *et al.* 1989). In view of the fact that EPO is limited to eosinophils, the observation of the gene being expressed in fibroblasts seems to argue against its identification as the hamster homolog of human EPO. The sequenced segment of the cDNA clone might represent part of the gene vital for the function of the enzyme that is conserved across all species. This could explain why despite gross structural differences between MPO and EPO, the sequence homology of this fragment to both enzymes was high. There is also the possibility that an EPO-like enzyme is present in more cell types and tissues but the level of its expression is below the sensitivity of detection by most biochemical assays compared with highly sensitive molecular probing of mRNA. The transcript of the gene was found in normal cells, but not in two nickel-transformed cell lines.

Identification of the cloned cDNA as a peroxidase gene was further confirmed by the measurement of enzyme activity. The assay employed has been used for detection of H_2O_2 as a measure of nickel-induced oxidative stress in CHO cells (Huang *et al.* 1993, 1994). In our previous study (17), it has been found that the conversion of non-fluorescent CDFH reducing substrate into measurable fluorescent oxidized CDF exhibited a dose-dependent requirement for peroxidase, H_2O_2 or organic hydroperoxides. Thus, the observed very low level of fluorescence in cell extracts of nickel-transformed cells can be interpreted as a consequence of peroxidase deficiency in those cells.

Evidence from molecular cloning together with biochemicaal data establishes the peroxidase deficiency in nickel-transformed cells but not in cultured normal embryonic cells or fibroblasts. Cells having a low, but apparently normal intracellular level of peroxidase, and their enzyme-deficient counterparts, can be used for studying the role that peroxidase might play in cellular damage caused by oxidative stress. One of the major consequences of oxidative stress is lipid peroxidation which leads to the formation of strong oxidants such as lipid hydroperoxides. These may enter a chain of reactions catalyzed by peroxidase generating highly reactive free radical intermediates which can further amplify cell damage. If peroxidase-driven metabolism contributes to the cytotoxicity resulting from oxidative stress, then peroxidase-deficient cells should be more resistant to toxicity induced by organic hydroperoxides. This was the case with nickel-transformed cells that showed a 2-fold higher value of LD_{50} compared with peroxidase expressing fibroblasts. Although the level of free radicals has not been determined, their formation in peroxidase containing cells seems to be the most plausible explanation for the observed difference. Decomposition of organic hydroperoxides to free radicals by MPO of human PMNs has been reported (Chamulitrat *et al.* 1991). Peroxidase-mediated lysis of erythrocytes involving reactive oxygen species (Lin *et al.* 1988) and the cytostatic effect of horseradish and thyroid peroxidase derived free radicals to cultured CHO cells (Moore & Mehlhorn 1993) has been also observed. Many studies have shown that free radicals are products of the peroxidase-catalyzed oxidation of a large and diverse group of small molecules, including both xenobiotics and natural metabolites (Mason 1982, Lown 1983, Yamasaki 1987, O'Brien 1988, Rao *et al.* 1988) supporting a free radical mechanism of cytotoxicity. Peroxidase-derived free radicals are also involved in the cytotoxicity of some anti-tumor drugs (Bachur *et al.* 1978, Lown 1983, O'Brien 1988, Everse 1991), but it must be pointed out that in none of these studies have cells expressing normal intracellular levels of enzymes been used. Chinese hamster fibroblasts containing low but detectable peroxidase activity and nickel-transformed peroxidase-deficient cells could supplement the existing systems for studying mechanisms of cell killing by those drugs. To evaluate the role of the cloned peroxidase in cytocidal activity by a more direct way, attempts are being made to transfect peroxidase-deficient transformed cells with the peroxidase cDNA.

Nickel has been shown to cause inactivation of certain genes (Klein *et al.* 1991, Salnikow *et al.* 1994), as well as induction of oxidative stress (Huang *et al.* 1993, 1994). Switching off expression of peroxidase would render cells more resistant to nickel-induced oxidative stress injury, resulting in positive selection of those cells. There is a large body of evidence that transformed cells are far more resistant to lipid peroxidation than their normal counterparts (Geleotti *et al.* 1991, Trush & Kensler 1991) supporting the proposed mechanism of selection. Studies are under way to determine whether hemoperoxidase deficiency coincides with the acquisition of the transformed phenotype.

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