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Necrotic cell death by hydrogen peroxide in immortal DF-1 chicken embryo fibroblast cells expressing deregulated MnSOD and catalase

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Abstract

The reactive oxygen species are known as endogenous toxic oxidant damaging factors in a variety of cell types, and in response, the antioxidant genes have been implicated in cell proliferation, senescence, immortalization, and tumorigenesis. The expression of manganese superoxide dismutase mRNA was shown to increase in most of the immortal chicken embryo fibroblast (CEF) cells tested, while expression of catalase mRNA appeared to be dramatically decreased in all immortal CEF cells compared to their primary counterparts. The expression of copper–zinc superoxide dismutase mRNA was shown to increase slightly in some immortal CEF cells. The glutathione peroxidase expressed relatively similar levels in both primary and immortal CEF cells. As primary and immortal DF-1 CEF cells were treated with 10–100 μM of hydrogen peroxide (concentrations known to be sublethal in human diploid fibroblasts), immortal DF-1 CEF cells were shown to be more sensitive to hydrogen peroxide, and total cell numbers were dramatically reduced when compared with primary cell counterparts. This increased sensitivity to hydrogen peroxide in immortal DF-1 cells occurred without evident changes in either antioxidant gene expression, mitochondrial membrane potential, cell cycle distribution or chromatin condensation. However, the total number of dead cells without chromatin condensation was dramatically elevated in immortal DF-1 CEFs treated with hydrogen peroxide, indicating that the inhibition of immortal DF-1 cell growth by low concentrations of hydrogen peroxide is due to increased necrotic cell death, but not apoptosis. Taken together, our observation suggests that the balanced antioxidant function might be important for cell proliferation in response to toxic oxidative damage by hydrogen peroxide.

Keywords: Manganese superoxide dismutase; Catalase; Hydrogen peroxide; Chicken embryo fibroblast; DF-1

1. Introduction

We have established a number of non-virally and non-chemically immortalized chicken embryo fibroblast (CEF) cells that include the spontaneously immortalized cell line, DF-1, and several immortal CEF cell lines derived from various embryonic tissues [1,2]. In previous studies, we demonstrated that immortal DF-1 cells divided more rapidly than primary

Abbreviations: CAT, catalase; CEF, chicken embryo fibroblast; CuZnSOD, copper–zinc superoxide dismutase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; MMP, mitochondrial membrane potential; MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SA-β-gal, senescence-associated β-galactosidase

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and other immortal CEF cells. The accelerated proliferation of DF-1 cells was found to be correlated with increased mitochondrial transcription and ATP generation [3].

Due to normal cellular oxygen metabolism, intracellular reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide are generated via the electron transport chain in mitochondria. Furthermore, approx. 2% of the oxygen taken up in cells is known to be converted to ROS which results in the cumulative oxidative damage to proteins, lipids, and nucleic acids [4]. In the present study, we determined the expression of various antioxidant genes and cellular response to hydrogen peroxide in primary and immortal DF-1 CEF cells.

Cellular ROS toxicity can be eliminated by serial activation of a number of antioxidant enzymes such as manganese superoxide dismutase (MnSOD), copper–zinc superoxide dismutase (CuZnSOD), catalase (CAT), and hydrogen glutathione peroxidase (GPX). MnSOD and CuZnSOD, located in the mitochondria and cytoplasm, respectively, are the initial antioxidant enzymes that convert superoxide anions to hydrogen peroxide, while CAT and GPX continue the process to detoxify hydrogen peroxide to water. Although the expression patterns of each of the antioxidant genes have been shown to be quite different depending on specific cell types, tissue origin and species [5–15], the overall coordinated expression among the antioxidant genes has been demonstrated to be more important to detoxify notorious oxidants [16].

Within cellular environments, levels of intracellular toxic oxidants are elevated during inflammatory reactions [17] and through a variety of cellular signaling pathways that occur as a result of growth factors such as platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, transforming growth factor-β and interleukin-1 [18–22]. Numerous studies have demonstrated that increased oxidants, especially hydrogen peroxide, are directly involved in the perturbation of cellular homeostasis resulting in either cell proliferation/cell growth arrest, or cell death by either apoptosis or necrosis [23–26].

In several mouse cell lines, oxidants such as superoxide and hydrogen peroxide have been shown to function as mitogens and activate cellular proliferation [27,28]. By contrast, in human diploid fibroblasts, sublethal concentrations of hydrogen peroxide (200 μM) caused replicative cessation and a senescence-like phenotype with the loss of mitogenic response, enlarged size, and reduced saturation density [29], due to the activation of p53, $p21^{WAF1/CIF1}$ and Rb [24]. In human lymphocytes, physiologically relevant levels of hydrogen peroxide (25–100 μM) were detected in the inflammatory process and shown to induce rapid cell death [30–32]. When concentrations of hydrogen peroxide were low (25–50 μM), these cells were prone to die by apoptosis, whereas higher levels (>100 μM) caused cell death by necrosis [30]. The cellular responses to hydrogen peroxide are likely to be quite different depending on cell type, tissue origin, and even species.

2. Materials and methods

2.1. Cell culture

All cell culture reagents were purchased from Gibco BRL. All immortal CEF cells (DF-1, BCEFi, HCEFi, SkCEFi, BoCEFi, VCEFi [1,2]) and their primary counterparts were grown in DMEM high glucose medium (without sodium pyruvate) enriched with 10% fetal bovine serum (FBS), 1% antibiotic–antimycotic solution, and 1% 200 mM L-glutamine. Primary (passage 8) CEF cells were made quiescent by growing in DMEM medium enriched with 0.2% FBS for 72 h; cell growth arrest was determined by [^{3}H]thymidine labeling. After 72 h, a second set of quiescent cultures was serum-stimulated for 5 h with 20% FBS-DMEM medium. Primary passage 17 and 19 senescent CEF cells were allowed to grow for 2 weeks and then assayed for senescence-associated β-galactosidase activity, a histochemical marker of replicative senescence [33]. Primary and DF-1 CEF cells were seeded at 5×10^5 cells/10 cm dish and treated with different concentrations of hydrogen peroxide (0, 10, 25, 30, 50, 75 and 100 μM; Sigma) for 2 days. The trypan blue exclusion method was used to assess viability after cells were allowed to grow in the presence of different concentrations of hydrogen peroxide as described above.

2.2. RNA isolation and Northern analysis

Total RNA was isolated from all primary and immortal CEF cells using TRIzol (Gibco BRL) following the manufacturer's instructions. Twenty micrograms of total RNA from different passages of primary and immortal CEF cells were separated by electrophoresis on a 1% agarose gel containing formaldehyde and blotted onto nylon membranes. Four different [α-^{32}P]dATP labeled chicken-specific antioxidant cDNA probes (MnSOD, CuZnSOD, CAT, and GPX) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; for RNA loading control) were prepared by reverse transcription polymerase chain reaction (RT-PCR) and hybridized to membranes at 42°C for 16 h in the presence of 50% formamide, 5×SSPE, 1% SDS, 10×Denhardt's solution, and 200 μg/ml sonicated salmon sperm DNA.

2.3. RT-PCR analysis

For semiquantitative RT-PCR, 3 μg of DNase I-treated RNA was converted to cDNA with Superscript II reverse transcriptase (Gibco BRL) following the manufacturer's instructions. A portion (1 μl) of the RT reaction was used to amplify MnSOD, CuZnSOD, CAT, GPX, and GAPDH fragments using chicken-specific primers. The GenBank accession number, the forward (F) and reverse (R) primers and predicted size (in base pairs) of each product are given for the following: MnSOD (AF299388, F5′-GCAGCTGCATCATTGAAAACACCACG-3′, R5′-CCAGCCGGTGCCCTGAACACCGACTG-3′; 300 bp); CuZnSOD (U28407, F5′-GAAGGCCGTGTGCGTGATGA-3′, R5′-CACGGAAGAGCAAGTACAGC-3′; 532 bp); CAT (AW198495, F5′-GCATGTCCGTTTCAGGAGAT-3′, R5′-CGCCATAGTCAGGATGAACA-3′; 207 bp); GPX (AI981938, F5′-GGTGAATTACACTCAGCTCGTC-3′, R5′-CCCTTCACGGTTGATAAGAAAC-3′; 292 bp); GAPDH (K01458, F5′-TGCAGGTGCTGAGTATGTTGTGGA-3′, R5′-CCACAACACGGTTGCTGTATCCAA-3′; 709 bp). After denaturing at 95°C for 1 min, all cDNA fragments were amplified for 18 cycles (verified to be in the linear range) using *TaKaRa Ex Taq* (TaKaRa Biomedicals) at 95°C for 30 s, 62°C for 1 min, and 72°C for 30 s for each amplification cycle. Chicken GAPDH mRNA was amplified to normalize the RT-RCR reaction. All RT-PCR products were verified by Southern hybridization to their respective cDNA probes and by sequence analysis (Advanced Genetic Analysis Center, University of Minnesota).

2.4. Determination of mitochondrial membrane potential

To study mitochondrial membrane potential, primary and immortal DF-1 CEF cells were treated with different concentrations (0, 10 and 30 μM) of hydrogen peroxide for 2 days, and then stained with 0.5 μg/ml rhodamine 123 (Sigma) which is easily sequestered by the mitochondrial membrane. Once the mitochondrial membrane potential is lost, rhodamine 123 is subsequently washed out of the cells. The mitochondrial membrane potential was determined using FACScan and analyzed by a Cell Quest software program.

2.5. Cell cycle analysis

The DNA content of trypsinized primary and DF-1 CEF cells grown in the presence (10 and 30 μM) or absence of hydrogen peroxide was determined by staining cells with propidium iodide using a Cellular DNA Flow Cytometric Analysis (Roche). Cell cycle analysis was carried out using a FACScan (Becton Dickinson), and the percentages of each cell stage were analyzed using a Cell Quest software program (Becton Dickinson).

2.6. Cell viability and death assays

Primary and immortal DF-1 CEF cells were allowed to grow in the presence (30 μM) or absence of hydrogen peroxide for 2 days. Cell death was determined by staining cells with propidium iodide (0.5 μg/ml) for 30 min. Chromatin condensation was specifically detected in apoptotic cells under fluorescent light. At least 100 cells were counted randomly using inverted fluorescence microscopy. Propidium iodide-stained cells or both propidium iodide-stained and chromatin-condensed cells were counted from three independent experiments.

3. Results and discussion

3.1. The altered expression of MnSOD and catalase mRNA in primary and immortal CEF cells

Identification of genes differentially expressed in immortal cells could be an important step in understanding the mechanisms underlying cellular immortalization. In previous studies, we determined that both the expression of mitochondrial-encoded genes and the generation of cellular ATP production are higher in immortal DF-1 CEF cells compared to primary CEF cells [3]. In general, the generation of more energy seems to be positively correlated with the production of ROS in the mitochondria, since at least 2% of the oxygen utilized by cells is known to be converted to ROS [4]. The accumulation of ROS results in oxidative damage that is believed to be one of several possible factors leading cellular senescence and crisis [34–36], suggesting that immortal cell lines that continuously undergo cell division (such as the DF-1 CEF cell line) should have a cellular detoxification system to efficiently eliminate toxic ROS. In the present studies, we have compared the expression patterns of antioxidant genes in primary and immortal CEF cells.

To characterize the expression of chicken antioxidant genes, we first obtained partial cDNA sequences for MnSOD, CuZnSOD, CAT, and GPX by RT-PCR. Chicken MnSOD was amplified using degenerate primers that were designed by determining the regions of highest homology existing between hu-

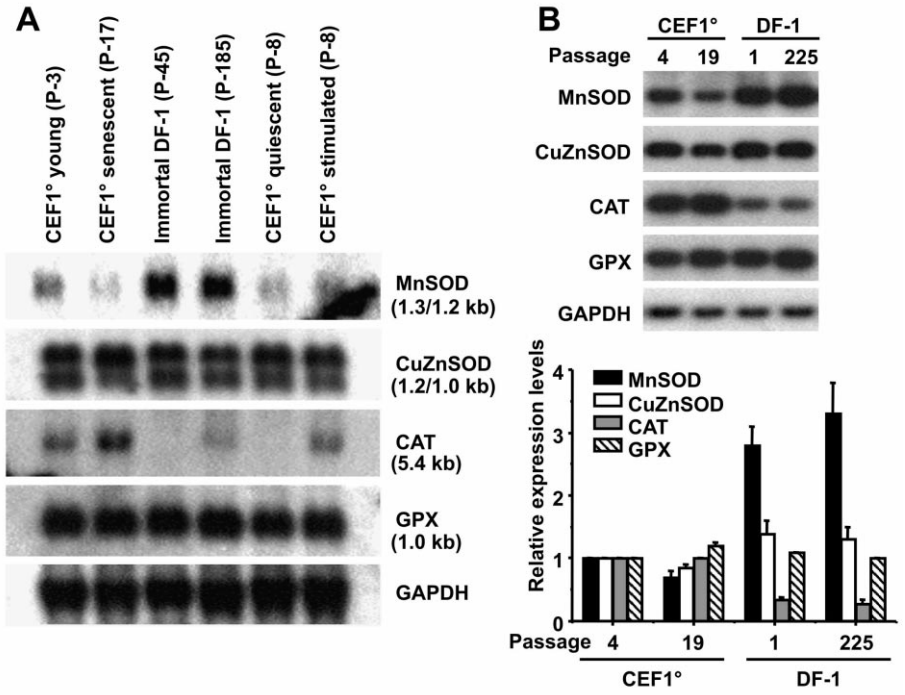

Fig. 1. Antioxidant gene expression in primary and immortal CEF cells from different cell states. (A) Steady-state levels of chicken MnSOD, CuZnSOD, CAT, and GPX mRNA in primary (CEF1°) and immortal DF-1 CEF cells prepared from different cell passages and culture conditions were determined by Northern blot hybridization using ^{32}P-labeled chicken-specific MnSOD, CuZnSOD, CAT and GPX cDNA probes. Equal RNA loading was determined by reprobing the membrane with a chicken GAPDH cDNA. The P-numbers indicate cell passages when total RNA was isolated. (B, top) Expression levels of chicken MnSOD, CuZnSOD, CAT, and GPX mRNA were determined by semiquantitative RT-PCR using RNA prepared from different cell passages of primary (CEF1°) and immortal DF-1 cells (primary presenescent passage 4, primary senescent passage 19, earliest immortal passage 1, and immortal passage 225). RT-PCR products were visualized by hybridizing to their corresponding ^{32}P-labeled MnSOD, CuZnSOD, CAT and GPX cDNA probes. (B, bottom) The chicken MnSOD, CuZnSOD, CAT and GPX mRNA levels from three independent experiments were quantitated using the NIH image soft program, and values normalized to GAPDH mRNA levels were plotted as relative expression levels.

man, mouse, and rat MnSOD mRNA, while CuZnSOD, CAT, and GPX were amplified with chicken-specific primers (see Section 2).

We determined the steady-state mRNA expression of these antioxidant genes in primary and spontaneously immortalized DF-1 CEF cells (Fig. 1A). Expression of chicken MnSOD mRNA (both 1.3 and 1.2 kb transcripts) markedly increased in immortal DF-1 cells (either passage 45 or 185) compared to primary early passage 3 CEF cells (Fig. 1A). The expression of MnSOD slightly decreased in primary senescent passage 17 CEF cells compared to primary early CEF cells, but was not elevated in serum-stimulated quiescent cells (Fig. 1A). In contrast to MnSOD mRNA expression in immortal DF-1 cells, the steady-state levels of the 5.4 kb CAT transcript decreased in immortal DF-1 cells compared to primary CEF cells. Expression of CAT mRNA increased in senescent CEF cells relative to primary early CEF cells. CAT mRNA was barely detectable in primary quiescent CEF cells, but was increased in primary serum-stimulated CEF cells (Fig. 1A). However, expression of CuZnSOD (both 1.2 and 1.0 kb transcripts) and the 1.0 kb GPX transcript were relatively similar in both primary and immortal CEF cells as well as in serum-stimulated and senescent cells compared to quiescent and early CEF cells, respectively (Fig. 1A). Together, these results indicate that there is a major difference between primary and immortal CEF cells regarding the altered expression of the antioxidant genes, MnSOD and CAT. In addition, the steady-state levels of MnSOD and CAT mRNA, but not CuZnSOD and GPX mRNA seem to be differentially regulated by cell growth conditions as well as cell senescence.

Since most of our immortal CEF cells were initially established from populations of primary senescent cells, it was of interest to determine if the altered expression of MnSOD and CAT mRNA occurred at different cell passages. We analyzed the steady-state expression levels of four antioxidant mRNAs in different passages of primary and immortal CEF cells: primary presenescent passage 4, primary senescent passage 19, earliest immortal passage 1 and immortal passage 225 cells. We found that MnSOD mRNA increased approx. 3-fold in the earliest passage of immortal CEF cells that could be tested, whereas CAT mRNA decreased approx. 4-fold in the same immortal CEF cell line (Fig. 1B).

We analyzed the expression patterns of four of the major antioxidant genes in all of the immortal CEF cells recently established in our laboratory to determine if their altered gene expressions were a common event. The steady-state expression of MnSOD mRNA increased in all immortal CEF cells tested compared to their primary cell counterparts (Fig. 2). In contrast, the steady-state levels of CAT mRNA markedly decreased in all of the immortal CEF cells analyzed (Fig. 2). Interestingly, the expression of CuZnSOD was found to be relatively higher in some of the immortal CEF cells (BoCEFi, SkCEFi, BCEFi and HCEFi) compared their primary cell counterparts (Fig. 2). However, the expression of GPX mRNA was not changed in any of the primary

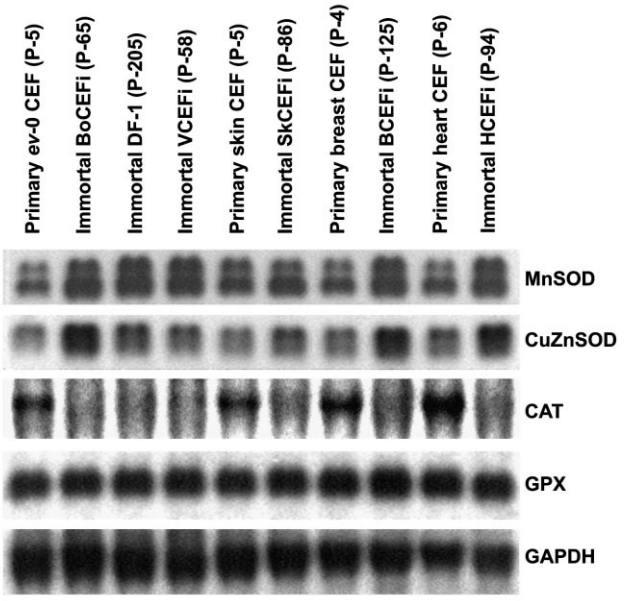

Fig. 2. Antioxidant gene expression in primary and immortal CEF cells. Two independent Northern blots were prepared using RNA from various immortal CEF cells established in our laboratory and their primary counterparts which included *ev*-0 (endogenous virus-free), BoCEFi (Bone-derived CEF immortal), DF-1 (spontaneously immortalized CEF), VCEFi (Vector-derived CEF immortal), SkCEFi (Skin-derived CEF immortal), BCEFi (Breast-derived CEF immortal), HCEFi (Heart-derived CEF immortal) cells. The blots were hybridized with ^{32}P-labeled chicken-specific MnSOD, CuZnSOD, CAT and GPX cDNA probes, then reprobed with a chicken GAPDH cDNA to monitor equal RNA loading. The P-numbers indicate cell passages when total RNA was isolated.

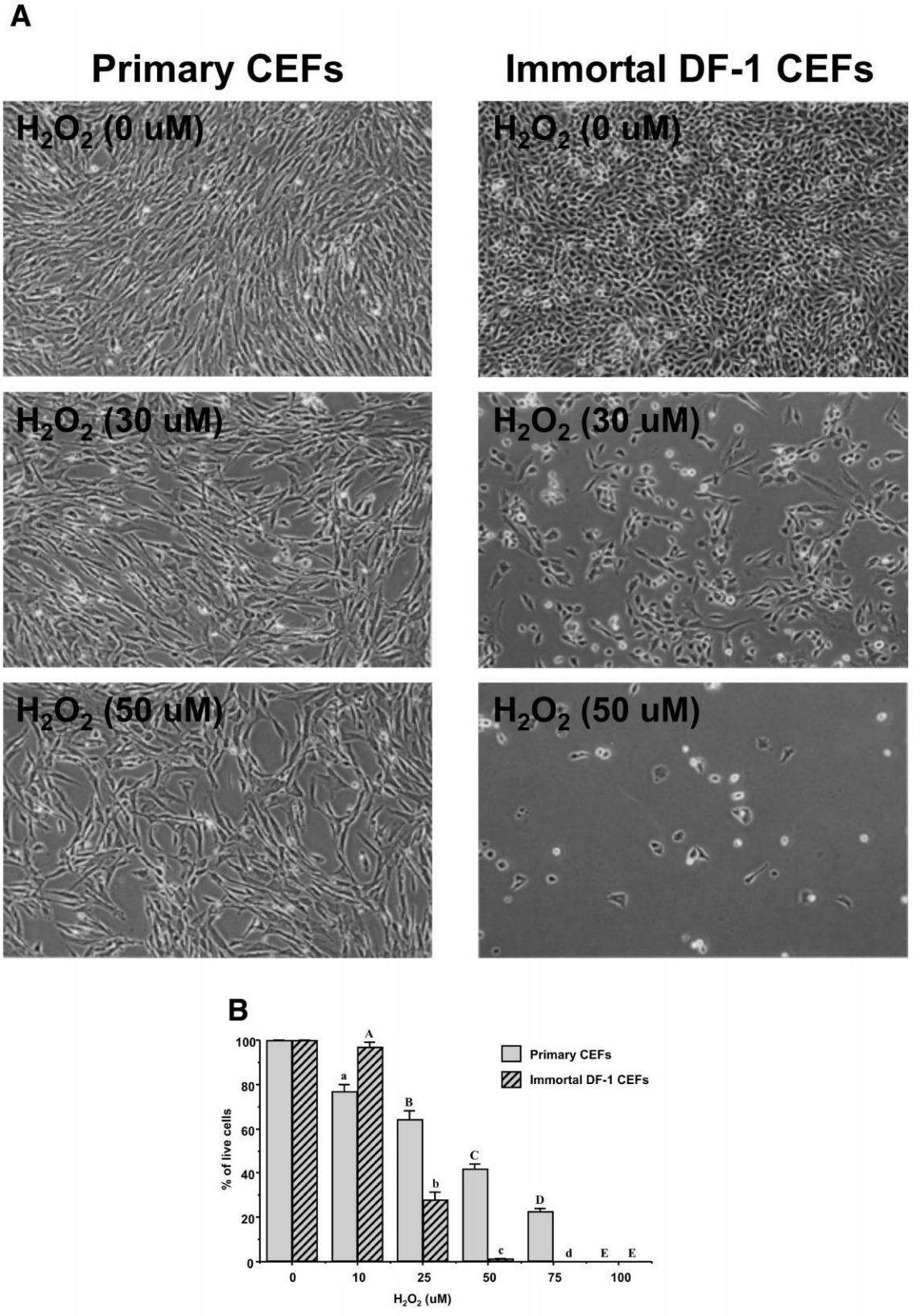
A
Primary CEFs
Immortal DF-1 CEFs
H2O2 (0 uM)
H2O2 (0 uM)
H2O2 (30 uM)
H2O2 (30 uM)
H2O2 (50 uM)
H2O2 (50 uM)
B
% of live cells
H2O2 (uM)
Primary CEFs
Immortal DF-1 CEFs
0
10
25
50
75
100
A
a
B
b
C
c
D
d
E
E

Fig. 3. The proliferation of primary and immortal DF-1 CEF cells in response to extracellular hydrogen peroxide. (A) Photomicrographs (76× magnification) were obtained from primary and immortal DF-1 CEFs grown in the presence (10 and 30 μM) or absence of hydrogen peroxide (H_2O_2). (B) Primary and immortal DF-1 CEFs seeded at 5×10^5 cells/10 cm dish were treated with different concentrations of hydrogen peroxide (0, 10, 25, 50, 75 and 100 μM) for 2 days. Trypan blue-negative cells were counted and are represented as the relative percentage of live cells. The significant differences observed in the relative percentage of live primary and immortal DF-1 CEF cells in each hydrogen peroxide treatment group were determined by Student's two-tailed *t*-test. The small letters (a–d) represent significant decreases ($P<0.01$) in the relative percentage of live cells compared to the corresponding capital letters (A–D). There was no significant difference ($P>0.05$) found in the relative percentage of live primary (E) and immortal DF-1 CEF (E) cells treated with 100 μM hydrogen peroxide.
←

and immortal CEF cells tested (Fig. 2). Together, these results suggest that the altered expression of MnSOD and CAT mRNA could be an early and common event in the process of cellular immortalization (at least for all the immortal CEF cells tested here). However, these genetic alterations in antioxidant gene expression do not seem to be directly associated with cellular immortalization, since the enforced overexpression of antioxidant genes such as MnSOD has been shown to only extend cellular life span in normal primary CEF cells (data not shown). Furthermore, numerous studies have documented that: (1) MnSOD expression was upregulated in a number of tumor cell lines such as melanoma cells [11] and cervical carcinoma cells [12]; and (2) CAT expression was shown to be downregulated in immortalized mouse liver cells [13], hepatoma cells [14], and keratinocyte cells [15]. However, none of these studies have shown the direct relationship between altered regulation of antioxidant genes and cellular immortalization or tumorigenesis.

3.2. The proliferation of CEF cells is profoundly inhibited by hydrogen peroxide

Hydrogen peroxide is a major component of the ROS. This small diffusible ubiquitous molecule that is rapidly destroyed in cells has been shown to be involved in a variety of phenotypic changes such as senescence-like cell growth arrest or cell death depending on the cell type and its intracellular concentration [24,29–32]. We determined the effects of hydrogen peroxide in two different types of CEF cells, normal primary and spontaneously immortalized. When primary and immortal DF-1 CEF cells were treated with sublethal concentrations of hydrogen peroxide (30 and 50 μM) for 2 days, the proliferation rate of both cell types was dramatically inhibited although none of the senescence-like phenotypic changes were displayed [29,33] such as larger and flattened cellular morphology or cell senescence-associated β-galactosidase (SA-β-gal; data not shown) activity (Fig. 3A). We examined the effects of various concentrations of hydrogen peroxide (0, 10, 25, 50, 75, and 100 μM) on the cell proliferation of primary and immortal DF-1 CEF cells. In primary CEF cells, the number of live cells (determined by trypan blue exclusion) gradually decreased in the presence of hydrogen peroxide in a dose-dependent manner (Fig. 3B). However, in immortal DF-1 CEF cells, only approx. 26% of the cells were living while in the presence of 25 μM hydrogen peroxide approx. 63% of the primary CEF cells were shown to be alive (Fig. 3B). Therefore, the current studies demonstrate that immortal DF-1 CEF cells were found to be sig-

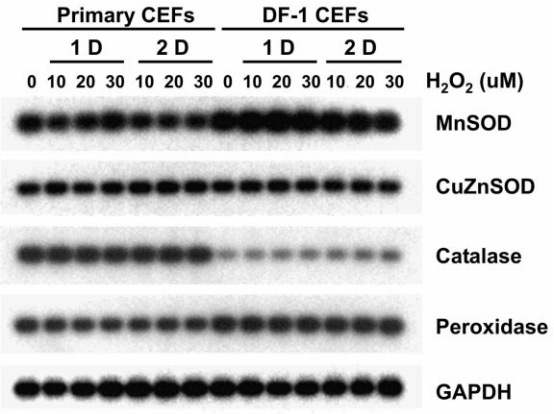

Fig. 4. The expression of antioxidant genes in response to hydrogen peroxide in primary and immortal DF-1 CEFs. Both primary and immortal DF-1 CEFs were allowed to grow in the different concentrations of hydrogen peroxide (0, 10 and 30 μM) for 1 or 2 days. The expression pattern of antioxidant genes was determined in three independent experiments by RT-PCR using gene-specific primer pairs and visualized by hybridizing the PCR products to their corresponding ^{32}P-labeled cDNA probes.

nificantly more sensitive to hydrogen peroxide-mediated toxicity than their primary cell counterparts. Furthermore, most of the CEF cells were dead within 30 min following treatment with higher concentrations of hydrogen peroxide (100 μM in primary CEF cells and 75–100 μM in immortal DF-1 CEF cells; Fig. 3B). These results were quite different from human diploid fibroblasts such as WI38 cells that were shown to be resistant to cell death and prone to be in a senescence-like growth arrest at higher levels (100–1000 μM) of hydrogen peroxide [24,29]. Taken together, these observations indicate that the sublethal concentrations of hydrogen peroxide used in this study (which were based on human diploid fibroblasts studies [24,29]) had a severe cytotoxic effect on CEFs (although immortal DF-1 CEFs were far more sensitive to hydrogen peroxide compared to primary CEFs).

3.3. Expression patterns of antioxidant genes in primary and immortal DF-1 CEF cells treated with different levels of hydrogen peroxide

Since increased ROS was found to elevate endogenous antioxidant gene expression in mouse skeletal muscle cells [37], we examined the expression patterns of antioxidant genes in primary and immortal DF-1 CEF cells grown at different times and concen-

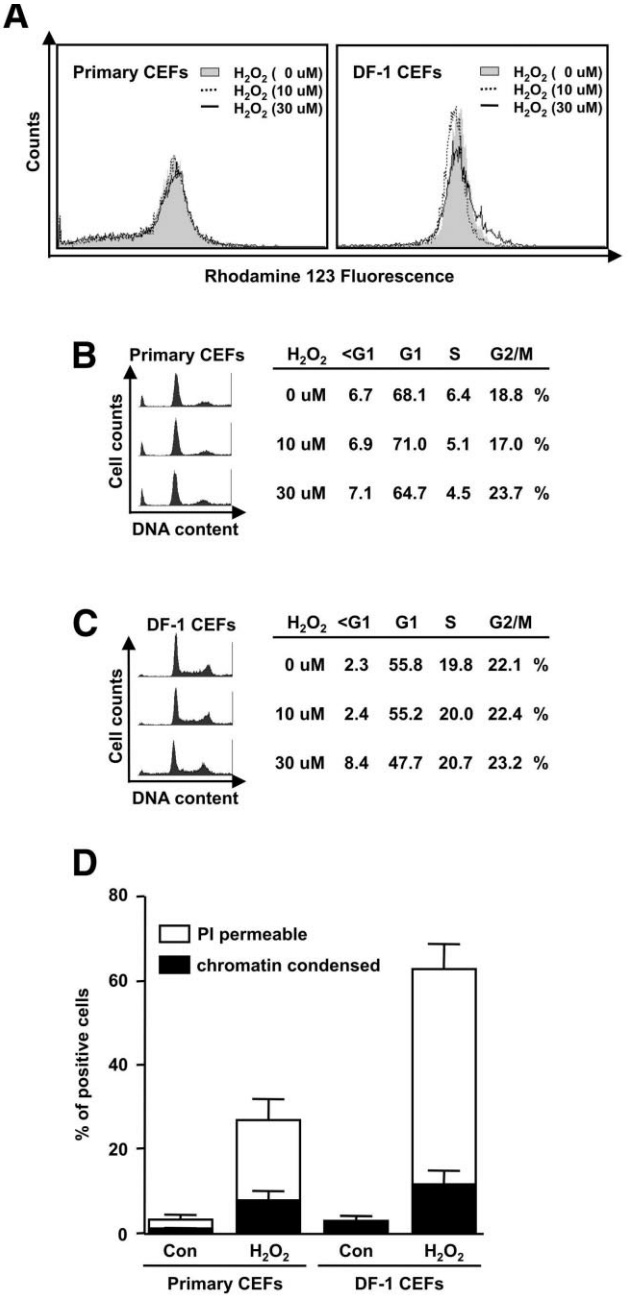

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Fig. 5. Hydrogen peroxide-mediated cell death in CEFs occurs by necrosis, not apoptosis. Primary and immortal DF-1 CEFs were grown in the presence (10 and 30 μM) or absence of hydrogen peroxide for 2 days. Cells were stained with 0.5 μg/ml of rhodamine 123 to determine mitochondrial membrane potential using FACScan analysis and the Cell Quest software program. (B) Primary and (C) immortal DF-1 CEFs were treated with different concentrations of hydrogen peroxide (0, 10 and 30 μM). After 2 days, the DNA content of trypsinized cells was determined by staining with propidium iodide. Cell cycle analysis was performed using FACScan and the Cell Quest software program. The percentage of each cell cycle stage from three independent experiments is shown on the right side. (D) The cell death rate of primary and immortal DF-1 CEFs grown in the absence (Con) or presence (H_2O_2) of hydrogen peroxide (30 μM) was determined by staining with propidium iodide (PI). 'PI permeable' represents cell death by necrosis, while 'chromatin condensed' indicates cell death by apoptosis. Significant differences between the percentage of both PI permeable and chromatin condensed primary and immortal DF-1 CEF cells treated with hydrogen peroxide were determined by Student's two-tailed *t*-test. There was no significant difference ($P > 0.05$) in the percentage of chromatin condensed primary and immortal DF-1 CEF cells treated with hydrogen peroxide. In the presence of hydrogen peroxide, the percentage of PI permeable immortal DF-1 CEF cells was significantly higher ($P < 0.01$) compared to the percentage of PI permeable primary CEF cells.

trations of hydrogen peroxide. To avoid severe cytotoxic effects of hydrogen peroxide, both cell types were treated with low concentrations (10, 20, and 30 μM) of hydrogen peroxide. We demonstrated that the expression levels of antioxidant genes such as MnSOD, CuZnSOD, catalase and GPX were not changed, regardless of the concentrations of hydrogen peroxide used (Fig. 4). It is possible that the obvious differences in antioxidant gene expression patterns between CEF cells and mouse skeletal muscle cells treated with hydrogen peroxide may be due to differences in species and cell types.

3.4. Cell death by hydrogen peroxide without the typical hallmarks of apoptosis

Hydrogen peroxide has been shown to cause cell death by either apoptosis or necrosis [30–32]. Several different biochemical changes such as increased ROS generation, cytochrome *c* release into the cytoplasm, activation of caspases and the loss of mitochondrial membrane potential (MMP) are commonly detected in the apoptotic processes. The loss of MMP should be an essential apoptotic event that can cause the release of ROS and cytochrome *c* from the mitochondria to the cytoplasm, which, in turn, results in the oxidative damage to most macromolecules in the cell and the activation of caspases, respectively [38,39]. We determined MMP in primary and DF-1 CEF cells grown in the presence (10 and 30 μM) or absence of hydrogen peroxide. Interestingly, MMP was not altered in either type of CEF cells when treated with hydrogen peroxide (Fig. 5A). These results suggest that hydrogen peroxide did not disrupt mitochondrial integrity in immortal DF-1 CEF cells, and that any CEF cell death due to hydrogen peroxide was not likely to be a result of apoptosis.

We next determined the accumulated sub-G_1 cell population, (one of the biochemical hallmarks of apoptosis), in primary and immortal DF-1 CEF cells using FACS analysis. In primary CEF cells, the sub-G_1 cell population was 6.7–7.1% in the presence or absence of hydrogen peroxide, suggesting that hydrogen peroxide did not induce genomic DNA fragmentation in primary CEF cells (Fig. 5B). Immortal DF-1 CEF cells treated with 30 μM hydrogen peroxide slightly increased the sub-G_1 population to 8.4% compared to 2.3 and 2.4% of the cells in sub-G_1 when treated with either 0 or 10 μM hydrogen peroxide, respectively (Fig. 5C). However, it is difficult to determine if the sub-G_1 cell population increase is directly associated with immortal DF-1 cell death via apoptosis. Furthermore, we observed that the majority of genomic DNA in both primary and immortal DF-1 CEFs was not fragmented by hydrogen peroxide treatment as determined by gel electrophoresis (the standard method to study genomic DNA fragmentation; data not shown). Taken together, our observations indicate that increased cell death and decreased cell numbers in the presence of hydrogen peroxide are due to neither apoptosis nor senescence-like cell growth arrest.

3.5. Immortal DF-1 CEF cell death by hydrogen peroxide is a result of necrosis

Necrosis is known to occur due to the disruption of cellular and nuclear membranes leading to the breakdown of cellular homeostasis by an influx of water and extracellular ions when cells are exposed to extreme physiological stimuli. Rupture of the cellular membrane is one of the crucial criteria used to distinguish necrosis from apoptosis. We examined the integrity of cellular membranes in primary and immortal DF-1 CEF cells treated with hydrogen peroxide by staining them with propidium iodide (which cannot enter the membranes of living cells but can enter into cells that have disrupted membranes). The presence of propidium iodide-stained cells without chromatin condensation [40] would be indicative of necrotic cells. When CEF cells were treated with hydrogen peroxide and stained with propidium iodide, approx. 24 and 61% of primary and immortal DF-1 CEF cells, respectively, were propidium iodide-permeated, whereas less than 10% of primary and immortal DF-1 CEF cells were shown to be both propidium iodide-permeated and chromatin-condensed (Fig. 5D). These results indicate that the significant increase in cell death due to hydrogen peroxide treatment in immortal DF-1 CEF cells (compared to their primary cell counterparts) is caused by necrosis, and not apoptosis. Furthermore, we have observed that immortal DF-1 CEF cells have higher levels of endogenous hydrogen peroxide than their primary cell counterparts, and that immortal DF-1 CEF cell death caused by oxidative stress through treatment

with antimycin A (which induces endogenous hydrogen peroxide generation) was inhibited by treatment with hydrogen peroxide scavengers such as sodium pyruvate and *N*-acetylcysteine (Kim et al., manuscript submitted). Together, the hypersensitive necrotic death shown by immortal DF-1 CEF cells in response to hydrogen peroxide might be due to different physiological conditions such as deregulated intracellular oxidant/antioxidant homeostasis.

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