A Newly Established Neuronal ρ-0 Cell Line Highly Susceptible to Oxidative Stress Accumulates Iron and Other Metals

RELEVANCE TO THE ORIGIN OF METAL ION DEPOSITS IN BRAINS WITH NEURODEGENERATIVE DISORDERS*

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From human neuroblastoma-derived SILA cells we have established a ρ-0 cell line that is deficient in both respiration and mitochondrial DNA. Lactate dehydrogenase activity, lactate production, and growth in the medium without glucose indicate that these cells shift from aerobic to anaerobic metabolism. Electron microscopic observations revealed abnormal mitochondria with unique cristae structures. Staining with Mito-Tracker dye showed that the mitochondrial transmembrane potential was reduced by 30–40% from the parent cell levels. These cells were markedly susceptible to H$_2$O$_2$ and died apparently by a necrotic mechanism, a process blocked by deferoxamine in the parent cells but not ρ-0 cells. Analysis by inductively coupled plasma-mass spectrometry revealed an approximately 3-fold accumulation of iron in the ρ-0 cells at confluence (n = 4–6, three clones, *p < 0.05). Iron and four other metals were all elevated in the cells of one of the ρ-0 clones and were similar to control levels in the control hybrid cells, which were replenished with normal mitochondrial DNA. Their sensitivity to H$_2$O$_2$ was also similar to that of the parent cells. These results indicate that a newly established neuronal related ρ-0 cell line is highly susceptible to active oxygen species and that these toxicity effects appear to be related to an accumulation of transition metals, which probably occurs through the respiratory impairment.

Iron and other transition metals exacerbate and in some cases initiate the degeneration of neurons (e.g. 1–3) through the Fenton reaction (4). In the brain of patients with Alzheimer’s disease (AD), an increase in the content of iron (5–9) and aluminum (4, 7) has been reported, and treatment of AD patients with iron chelators has been discussed (10). In the brain of patients with Parkinson’s disease and Huntington’s disease, iron and other metals also appear to accumulate (8, 11). It is important to note that all of these diseases show mitochondrial abnormalities to some extent (12–19), suggesting a coupling of metal accumulation with mitochondrial deficiency. More direct evidence of mitochondrial and iron association in neurodegenerative disorders comes from an increase in mitochondrial iron in the fibroblasts of patients with Friedreich’s ataxia, whose responsible gene is the mitochondrial frataxin (3, 20, 21). It would also be intriguing to uncover an association of mitochondrial respiratory deficiency and cell death with an accumulation of metals because a new, pivotal, regulatory role for mitochondria in cell survival and death has emerged from a growing body of evidence (for a review, see Ref. 22).

We considered mtDNA-depleted ρ (rho)-0 cells (23) to be a useful cellular model in an analysis of the consequences of chronic mitochondrial impairment and decreased respiration. These cells have served as a recipient for diseased mtDNAs (13, 24, 25) and are a valuable cellular tool for analyzing the coupling between cellular phenotypes and chronic respiratory deficiency (26–28) and for searching for genes coupled to the respiration-deficient status (29, 30). Generation of such ρ-0 cell types that have a neuronal background appears difficult because neuronal and glial cells are susceptible to respiratory crisis, and only one human neuroblastoma-derived ρ-0 line is available (SH-SY5Y origin (31)).

In this report, first we established a novel ρ-0 line from a human neuroblastoma line, characterized it, and discovered that its phenotype might be relevant to mitochondrial and neurodegenerative disorders. We then examined whether these cells are vulnerable to oxidative stress through a metal-mediated mechanism. The present results strongly suggest that chronic deficiency in the mitochondrial respiration of cells produces an accumulation of iron and other metals, rendering them highly susceptible to oxidative stress.

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¶ The abbreviations used are: AD, Alzheimer’s disease; ANOVA, analysis of variance; DFX, deferoxamine; EtBr, ethidium bromide; ICP-MS, inductively coupled plasma-mass spectrometry; LDH, lactate dehydrogenase; m, mitochondrial transmembrane potential; mtDNA, mitochondrial DNA;
Susceptibility of a Novel p-0 Line to Oxidative Stress

Table I
Biochemical characterization

| O2 | Lactate | ATP | GSH |
|----|---------|-----|-----|
|    | fmol/min/cell | unit/mg | mg/mg | pmol/mg |
| SILA | 0.56 ± 0.18 | 3.63 ± 0.29 | 88.6 ± 12.3 | 12.3 ± 5.4 |
| Sp-0/1–6 | ND | 4.92 ± 0.64 | 224 ± 9.5*** | 68.3 ± 5.2 |
| Sp-0/4–2 | ND | 4.51 ± 0.34 | 204 ± 8.1*** | 34.2 ± 4.4 |
| Sp-0/6H10 | ND | 12.2 ± 3.46*** | 272 ± 67.3*** | 111 ± 39.1*** |

EXPERIMENTAL PROCEDURES

Cell Type, Ethidium Bromide (EtBr) Treatment, and Establishment of p-0 Lines—The SILA cell line was initially isolated from a child’s neuroblastoma by Matsumura et al. (32) at our university. These cells produced neuronal and epithelial subtypes in vitro, but we employed a neuronal type 2B4 subline in this study (provided by Dr. Matsumura). SILA cells were grown in Dulbecco’s modified Eagle’s medium (high glucose, 4.5 g/liter, Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal calf serum. For establishing p-0 cells, pyruvate and uridine were also added to the medium at the reported concentrations (31). Initially, cells were treated with EtBr (Bio-Rad) at different concentrations ranging from 0.25 to 10 μg/ml. Those concentrations, at which cells showed no morphological response or immediate death, were excluded from the subsequent experiment. When most of the SILA cells remaining in the culture started to grow continuously after a few months of EtBr treatment, we subcloned them either by limiting dilution or cloning rings. Cloned cells were maintained in the culture medium supplemented with pyruvate, uridine, and 1 μg/ml EtBr for the next month. We monitored respiration of cells periodically as described below. After ~10 passages, EtBr-resistant and respiration-deficient cells were used for subsequent experiments.

O2 Consumption—Cells at confluence in a 10-cm diameter dish were detached and suspended in 10 ml of Tyrode’s solution (+), which consisted of 0.134 mM NaCl, 3 mM KCl, 3 mM Na2HPO4, 2 mM MgCl2, 5 mM Hepes, 5 mM glucose, 12 mM NaHCO3, 1 mM EGTA, and 3.5 mg/ml bovine serum albumin, pH 6.5. Then, the cells were collected, and a certain number of them, ~4 × 10^6 cells, were resuspended in 1.2 ml of Tyrode’s solution (+), pH 7.4, which omitted EGTA from Tyrode’s solution (+). Cells were then transferred to the chamber of the device (Oxigraph type 9, Central Science, Co., Ltd., Tokyo, Japan), which was equipped with the Clarke-type electrode to measure the rate of O2 consumption polarographically, and O2 consumption was calculated using the following formula: rate of O2 consumption (in fmol/min/cell) = (the rate of O2 consumption of cells (in mg/liter/min) – the rate of determination of the concentration of Tyrode’s solution (+) (in mg/liter/min) × 1.2 ml) × 10^{-15} (pmol/62/g cell), where the volume of the chamber is 1.2 ml and the molecular weight of O2 is 32.

PCR in Determining Mitochondrial DNA—A portion of the D-loop region of mtDNA was amplified using serially diluted DNA samples isolated from the parent and three representative p-0 clones. The intensities of these amplified bands in gel visualized with EtBr were as above, which was determined using bicinchoninic acid reagent (BCA Protein Assay Reagent Kit, Pierce Chemical Co.).

Dependence of p-0 Cells on Pyruvate, Uridine, and Glucose—To confirm the dependence of their growth on the supplementation of pyruvate and uridine, cell growth was monitored by the trypan blue exclusion method over 5 days in culture with or without these substrates. The dependence of energy metabolism on glucose was evaluated by counting cells in 3-cm diameter dishes for 24 h in galactose-substituted (for glucose) medium and by comparing cellular ATP and lactate production (as described above).

ΔΨm-sensitive MitoTracker Staining—Cells cultured in wells of a 24-well plate in which glass coverslips were placed were labeled with MitoTracker dye (CMTMRos, Molecular Probes, Eugene, OR) at 100 nM for 15 min, postfixed with 4% paraformaldehyde solution (0.01 M phosphate buffer, pH 7.4), and observed using a fluorescent microscope (ECLIPS E1000, Nikon, Kyoto) with a red filter for interference (± 590 nm). Fluorescent intensities in the unit area of the cytoplasm and nucleus of SILA and Sp-0 cells were measured densitometrically, and from these values, the intensity of the glass slide was subtracted as a background. The fluorescent intensity of the nucleus was used to normalize intercellular and interexperimental differences in MitoTracker staining. Products obtained by dividing the intensity of cytoplasmic fluorescence with that of nucleic fluorescence were calculated (n = 10–12 cells) and statistically compared.

Electron Microscopy—We observed three clones of the Sp-0 line, clone 1-6, 4-2, and 6H10, under electron microscopy. Cells at confluence in a T75 flask were fixed with 2% glutaraldehyde for 30 min and collected from the flask with a scraper. They were postfixed with 1% OsO4 and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead and observed with a H7000 electron microscope (Hitachi Co. Ltd., Tokyo).

H2O2 Stress and Effect of an Iron/Copper Chelator—For this experiment, we used the clone 4-2 from three Sp-0 lines because its ATP level was similar to that of the parent cells. Cells grown in 3-cm diameter dishes were incubated with H2O2 (analytical grade for atomic absorption, Wako Pure Chemical Industries, Kyto) at various concentrations with or without deferoxamine mesylate (DFX, Wako) for 24 h. 10 μl of culture medium from each dish was then incubated with substrate solution for determining LDH activity released from cells with the above described kit (LDH-D). Meanwhile, cells in the dish were lysed with lysis buffer (5 mM Tris/HCl, pH 7.4) containing 0.5% Triton X-100 and 20 mM EDTA, and the total DNA was extracted with phenol. DNA precipitated with ethanol and dissolved in Tris/EDTA buffer was incubated with RNase A (Sigma) at 100 μg/ml for 1 h, extracted again with phenol, precipitated with ethanol, and electrophoresed in 2.2% agarose gel.

Determination of Metals—To measure the total cellular metals, control and p-0 cells grown in and confluent at 10-cm diameter dishes were detached with trypsin, washed twice with phosphate buffer that included mannosil at 0.25 M, and then were completely reduced to ash by treatment with nitric acid for (poisonous metal determination, Wako), hydrogen peroxide (for atomic absorption spectrochemical analysis, Wako), and perchloric acid for (poisonous metal determination, Wako) under heat (8, 35). Cellular ashes were dissolved with 10 ml of 6% nitric acid and then analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) using a Shimadzu ICPS-8500 (Shimadzu, Kyoto, Japan). We first measured the iron content (m/z 57) in three representative Sp-0 clones to confirm that an accumulation of iron in this cell type was general. Then, we established a control cybrid line (see below) and measured the levels of other metals, aluminum (m/z 27), manganese (m/z 55), copper (m/z 63), and zinc (m/z 66) together with iron, in the parent SILA, Sp-0, and the control cybrid cells. We selected these metals because they are the major transition metals within cells. Contamination from tubes and other sources was avoided for these metals. The concentration of cellular metals was calculated according to a linearly regressed curve prepared for each metal using a standard
solution (Multielement Standard Solution BM, Wako). Values of a 
seriously diluted multielement standard solution showed linear regression 
with a line in the range from 5 to 1,000 ng/ml for copper, iron, and 
and from 0.5 to 100 ng/ml for copper and manganese, respectively. 
The measurement was performed at least twice (n = 3–4) to verify the results.

Production of Normal Cybrid Cells and Sensitivity to Oxidative Stress—To clarify whether an elevation of metal contents in Sp-0 cells is mtDNA-dependent, we produced a cybrid cell line replenished with 
mormal mtDNA by fusing Sp-0 cells with platelets from a normal volunteer (23-year-old Japanese female) by the standard protocol (24, 25). The mtDNA level and the metabolic shift were evaluated by the PCR 
procedure and by lactate production as described above, respectively. 
We succeeded in producing the control cybrids from Sp-0 clones 1-6 and 
and 6H10 and used Sp-0/1-6 and its corresponding control cybrid cells for determination of the five metals. To assess the sensitivity of parent, 
Sp-0/1-6, and the control cybrid cells to oxidative stress, the cells were incubated individually with H2O2 at 0.5 mM for 24 h because the results 
obtained in the earlier experiment (see above) indicated that this concentration of H2O2 was critical for evaluating its effect. The LDH 
activity released to culture media from cells thus treated was measured as 
discussed previously.

Statistical Analysis—Using an appropriate computer program (Win 
Stat, version 1.2, Abacus Concepts, Berkeley, CA), we compared two 
groups and multigroups with the unpaired t test and ANOVA with 
Bonferroni’s multiple comparison, respectively. Significance was set at 
p < 0.05.

RESULTS

Establishment of Sp-0 Lines—In initial experiments, we de-
termined the range of appropriate concentrations of EtBr in the 
medium to be from 1 to 10 μg/ml, as reported previously (31). 
SILA cells were markedly resistant to EtBr toxicity and were 
able to grow even at the highest concentration. Subsequently, 
more than 10 clones were isolated, termed Sp-0, and we used 
three Sp-0 clones for subsequent analysis. The rate of O2 con-
sumption of the parent line was 0.56 ± 0.18 fmol/min/cell (n = 
3), whereas that of the Sp-0 cells was immeasurable (Table I). 
Targeting 3 ng and 30 pg (corresponding to 1 and 10−6 in Fig. 
1) of the total DNA isolated from SILA cells, portions of both 
the D-loop region of mtDNA and β-actin gene were amplified by 
PDR. When the lowest quantity of the genomic DNA, 0.3 pg 
(10−4), was used, the fragment of the D-loop region still was 
produced, but the β-actin fragment was not (Fig. 1, SILA). The 
sensitivity of this determination was therefore calculated as a 
couple copies of a gene, given that the DNA quantity of a single 
cell comprises 6 pg (2 (diploid) × 3 × 109 (bp/haploid of a 
human genome) × 600 (molecular weight/bp) × 10−23). While 
using DNA samples isolated from Sp-0 clones, regardless of the 
quantities, no D-loop region-derived bands were detectable 
(Fig. 1, e.g. Sp-0 clones 1-6 and 4-2). We examined all other 
clones in the same way, and we periodically performed PCR 
using DNA samples as 10 ng (see also production of control 
cybrids).

Biochemical Characterization of Sp-0 Cells—The LDH activ-

Fig. 1. Determination of the mtDNA level. Determination of the 
level of mtDNA in SILA and two representative clones of the Sp-0 line 
is shown. Numbers at the top (1, 10−3, 10−4) indicate dilution factors of 
the DNA samples used in PCR amplification with the starting concen-
tration of the total DNA as 3 ng. D-loop and actin indicate the D-loop 
region of human mtDNA and the nuclear DNA-encoded β-actin. PCR 
products were electrophoresed in 1.5% agarose gel, which was subse-
quently stained with EtBr bromide and photographed. m indicates the 
100-bp ladders with an intensified band at 500 bp.

Fig. 2. Effect of substitution of glucose with galactose in the culture medium. Sp-0 clone 4-2 cells were cultured in the 3-cm diameter dishes, and at con-
fluence the medium was replaced with fresh medium supplemented with either 
glucose or galactose. After 24 h, the cell number was counted by the trypan blue 
exclusion method, and the lactate production and ATP level were measured with 
its as described under “Experimental Procedures.” Error bars indicate the 
means ± S.E.; * and ** indicate p < 0.05 and 0.001, respectively. To evaluate the 
growth pattern in the medium with 
(closed circles) or without (open circles) 
pyruvate and uridine, ~1 × 106 cells were 
plated in 3-cm diameter dishes on day 0 
and allowed to grow for the next 5 days 
(n = 3). Growth curves indicate that they 
were unable to grow without these 
substrates.

Dependency of Sp-0 cells to pyruvate, uridine and glucose

| Glucose | Galactose |
|---------|-----------|
| ATP level (µmol/mg) | ** | * |
| Lactate production (µg/mg) | * |  |

| Glucose | Galactose |
|---------|-----------|
| Cell growth (x10^6) | * |  |

| Glucose | Galactose |
|---------|-----------|
| Lactate production (µg/mg) | * |  |
The level of ATP of Sp-0 cells (Table I; Sp-0/1-6 and Sp-0/4-2) were increased slightly, whereas that of the other clone (Sp-0/6H10) was increased significantly \((n = 3-4, ***, p < 0.0001)\) compared with parent SILA cells. Lactate release of all three clones of Sp-0 were increased 60% \((***, p < 0.0001)\). Without pyruvate and uridine in the culture medium, Sp-0 cells did not grow (Fig. 2, cell growth, open circles, \(n = 3\)) and were dead by 1 week (data not shown). When pyruvate and uridine were added, the cells grew constantly over 5 days (Fig. 2, closed circles, \(n = 3\)). All three Sp-0 clones showed similar results.

**Electron Microscopic Observation**—SILA cells contained normal mitochondria with an electron-dense matrix and regular cristae structure (Fig. 3A), whereas all Sp-0 cells contained only swollen mitochondria with a translucent matrix, which were not observed in parent cells (Fig. 3B–D). The morphology of cristae was markedly varied, short, extended, and circular. Occasionally, mitochondria with onion-shaped, concentric multilamellae cristae (Fig. 3D, arrows) were observed regardless of Sp-0 clones.

**MitoTracker Staining for \(\Delta \Psi_m\)**—The mitochondrial structure in SILA cells was clearly stained with CMTMRos (Fig. 4A), whereas in Sp-0 cells the amorphous structure was only stained weakly (Fig. 4B). Determination of the red fluorescent intensity of CMTMRos incorporated into mitochondria of SILA and two clones of Sp-0 cells demonstrated a 30–40% reduction in \(\rho\)-0 cells compared with controls (Fig. 4C, \(n = 10–12\) cells, ***, \(p < 0.0001\)).

**Oxidative Stress and Effect of an Iron/Copper Chelator**—Parent cells were affected when treated with \(\text{H}_2\text{O}_2\) in a dose-dependent manner as evidenced by an elevation of LDH activity in the medium (Fig. 5A, SILA, \(n = 3\), ***, \(p < 0.0001\), versus without \(\text{H}_2\text{O}_2\) treatment, open bar). The electrophoresed DNA of SILA cells showed a ladder pattern in a corresponding way (Fig. 5B, left half). The LDH release from Sp-0/4-2 cells into the culture medium occurred by the addition of \(\text{H}_2\text{O}_2\) even at 0.25 mM and had already reached the maximum at 0.5 mM (Fig. 5A, Sp-0, \(n = 3\)). DNA isolated from the Sp-0/4-2 cells treated with \(\text{H}_2\text{O}_2\) showed a smear in gel in a dose-dependent manner (Fig. 5B, right half). When SILA cells were treated with DFX, an iron/copper chelator (43), together with \(\text{H}_2\text{O}_2\), both the LDH release (Fig. 5C, SILA, \(n = 3\), ***, \(p < 0.0001\) and **, \(p < 0.001\), versus without \(\text{H}_2\text{O}_2\) treatment, open bar) and DNA fragmentation (Fig. 5D, left half) were completely abolished in a dose-dependent manner. On the other hand, the DFX treatment up to 500 \(\mu\)M had no inhibitory effect on either the LDH release from Sp-0/4-2 cells (Fig. 5C, Sp-0, \(n = 3\)) or the smear DNA pattern (Fig. 5D, right half).

**Determination of Iron**—ICP-MS revealed the concentration...
of iron in SILA and three Sp-0 clones, 1-6, 4-2, and 6H10, to be 124 ± 24, 395 ± 126, 359 ± 94, and 356 ± 23 ng/mg, respectively (Fig. 6, n = 4–5, *, p < 0.05).

Production of Normal Cybrid—We could establish a normal and control cybrid line using Sp-0/1-6 cells as a recipient by fusing them with platelets from a healthy volunteer. Replenishment of the mtDNA in the ρ0 cells was evidenced by PCR (Fig. 7A, cybrid clones 1 and 2). Correspondingly, the lactate production by control cybrid clones 1 and 2 was similar to that of the parent cells, which was significantly lower than that of Sp-0 cells (Fig. 7B, n = 3, ***, p < 0.0001).

Measurement of Other Metals and Sensitivity to H2O2 in Parent, Sp-0, and Control Cybrids—The levels of five metals, aluminum, iron, zinc, manganese, and copper were all found to be elevated significantly in Sp-0 clone 1-6 cells compared with the parent cells (Fig. 8, A and B). With the exception of the zinc levels, the other four metals in control cybrid cells were similar to those in parent cells. The levels of zinc in the cybrids also showed a decreasing trend. LDH release from parent, ρ0, and control cybrid cells (Fig. 8C, LDH, n = 3, ***, p < 0.001) and their morphologies (Fig. 8, D-F) after treatment with H2O2 at 0.5 mM for 24 h indicated that the sensitivity of the control cybrids to this treatment was similar to that of the parent cells, with Sp-0/1-6 cells being more sensitive than the other two cell types.

Iron levels in SILA and three Sp-0 clones

![Graph showing iron levels in SILA and three Sp-0 clones](image)

**Fig. 6. Iron contents.** Cells confluent in 10-cm diameter dishes were washed twice with phosphate buffer containing 0.25 mM mannitol and reduced completely to ash, which was dissolved in 10 ml of 6% nitric acid. Cellular ashes were subjected to the ICP-MS. A standard curve for iron was obtained using a serially diluted metal standard, and the regression coefficient with a line was greater than r = 0.999 in all experiments. Total iron levels were standardized with cellular protein and are shown as ng/mg. Three Sp-0 clones (1-6, 4-2, 6H10, closed bars) contained more iron than SILA (open bar) cells (n = 4–6, mean ± S.E., *, p < 0.05).

**DISCUSSION**

Given the considerable phenotypic differences among lines and because only one human neuroblastoma-derived ρ0 cell line (SH-SY5Y origin) is currently available (31), it becomes
necessary to isolate additional ρ-0 lines from nervous tissue for comparison. We established ρ-0 lines from human neuroblastoma-derived SILA cells to determine whether a deficiency in mitochondrial respiration renders cells susceptible to metal-mediated, oxidative stress.

It is expected, a priori, that ρ-0 cells shift their energy metabolism from aerobic to anaerobic. That cybrid cells replenished with normal mtDNA produced lactate at the same level as the parent cells indicates that a glycolytic shift of ρ-0 cells had taken place and that it is respiration state-dependent and reversible. These results are consistent with those reported by Vaillant et al. (36). It is not surprising that the ATP level of these respiration-lacking cells was at least not reduced compared with that of the parent cells when glycolysis was highly up-regulated to overcome the reduction of the cellular ATP. Cells of a glial nature, such as astrocytes and Schwann cells, are known to up-regulate glycolysis under ischemic conditions (37, 38). It is necessary to establish more ρ-0 lines with neuronal or glial origin and compare them with each other to elucidate mechanisms by which cells adapt to the respiration-deficient state.

Electron microscopic observation of ρ-0 cells revealed swollen mitochondria with translucent matrix and quite irregular cristae structure, which were seen regardless of the clones. Unique onion-shaped concentric multilamellae-containing mitochondria are specific not only to the present ρ-0 cells but were also observed in other ρ-0 cells (39, 40). Progressive external ophthalmoplegia and Kearns-Sayre syndrome, which are caused by deletion of mtDNA (41, 42), are known to develop similar morphological changes in the mitochondria in muscle tissues (43, 44). Heart muscles and other tissues affected with anoxia and ischemia (45, 46) and neurons whose mitochondrial respiration is inhibited with methyl mercury (47) also develop concentric lamellae and other types of mitochondrial morphologies as seen in the present and other ρ-0 cells. Concentric lamellae-containing mitochondria could thus represent a morphological hallmark of impaired respiration of muscle and neuronal cells. Importantly, this structure was observed in brains of AD patients (48) as well as in ρ-0 cells to which mtDNAs from AD patients were transferred (25). These findings suggest a strong association of mitochondrial impairment with the pathogenesis of AD. The number of abnormal mitochondria in ρ-0 cells seemed to be similar to that in the parent cells. However, it is necessary to determine precisely whether the number of mitochondria was altered in ρ-0 cells.

CMTMROS, a MitoTracker dye, which is a formaldehyde-resistant fluorochrome, is sensitive to ∆Ψm unlike other MitoTrackers (49). The ∆Ψm of the present ρ-0 cells was reduced approximately 30–40% compared with the parent cell level. This result concurs with the report that the ∆Ψm of 143B-ρ0 cells is 80–90% less than that of their parent cell level but is different from the result of ρ-0 cells derived from HeLa S3, which showed no reduction (50). It is conceivable that even the pathological mitochondria found in the present ρ-0 cells still hold a reduced ∆Ψm because the F1-ATPase (50, 51) and the adenine nucleotide translocator (50) of mitochondria are still functional, at least in those reported ρ-0 lines.

Mitochondrial abnormalities may be a final, common pathway leading to neuronal death in neurodegenerative disorders (12). To identify the consequences of chronic respiratory deficiency, we examined whether ρ-0 cells are susceptible to oxidative stress, another major factor in neurodegeneration (4, 9, 12, 14). LDH release and the DNA electrophoretic pattern of cells after incubation with the different concentration of H2O2 clearly demonstrated their hypersensitivity to this stress. We are unable to address the precise cell death mechanism only with these results, but as evidenced by nucleosomal DNA fragmentation and the smear DNA electrophoretic pattern, the parent SILA cells apparently underwent apoptosis, and ρ-0 cell death probably occurred through necrosis.

Cells are permeable to H2O2, which is converted into a highly toxic hydroxyl radical through a mechanism known as the Fenton and Haber-Weiss reaction when transition metals such as iron, copper, and zinc are present (4). Therefore, we hypothesized that these metals, particularly iron, are accumulated in ρ-0 cells. DFX is distinctly hydrophilic as the partition coefficients indicate (0.01 with iron and 0.03 without iron) (52). However, many researchers successfully utilized DFX to remove cellular iron/copper, relying on the fact that it influxes/effluxes across cell membranes in time- and dose-dependent
Reversal in metal accumulation and susceptibility to \( \text{H}_2\text{O}_2 \) of Sp-0 cells by replenishment with normal mtDNA

**Fig. 8. Comparison of three cell types in contents of five metals and sensitivity to \( \text{H}_2\text{O}_2 \).** A and B, samples prepared for ICP-MS were applied to the ICP-8500 apparatus as described before. For this experiment we programmed it to measure five different metals in SILA (open bars), Sp-0/1-6 (closed bars), and the control cybrids (gray bars) sequentially \((n = 4-6, \text{mean} \pm \text{S.E.}, \ast, p < 0.05, \ast\ast, p < 0.001, \ast\ast\ast, p < 0.0001, \text{ANOVA with Bonferroni's multiple comparison})\). The metal contents were measured and standardized as described above and are shown as ng/mg. These metals were grouped into categories A and B because the basal levels of manganese and copper are about 1 order of magnitude different from those of aluminum, iron, and zinc. C, SILA (open bars), Sp-0/1-6 (closed bar) and the control cybrid (gray bar) cells were incubated with \( \text{H}_2\text{O}_2 \) at 0.5 mM for 24 h, and the LDH activities released into culture media were measured as described under “Experimental Procedures.” The total LDH activities of cells without the treatment were also measured using the cell lysate. In this graph, LDH activities in the media were expressed as a percentage of the total cellular LDH activities of each line. *** indicates \( p < 0.0001 (n = 3, \text{ANOVA with Bonferroni's multiple comparison}) \). D–F, morphologies of SILA (D), Sp-0/1-6 (E), and control cybrid (F) cells after treatment with \( \text{H}_2\text{O}_2 \) at 0.5 mM for 24 h. The scale bar in F indicates 33 \( \mu \text{m} \). Sp-0 cells were detached from the dish and shrunk in morphology, whereas the parent and cybrid cells showed only a slight morphological change.

and saturable manner (52). Coincubated together with \( \text{H}_2\text{O}_2 \), DFX completely abolished the parent cell death, whereas Sp-0 cell death was not suppressed, indicating an accumulation of iron and copper in these cells. An inhibitory effect on the SILA cell death was observed at DFX concentrations greater than 50 \( \mu \text{M} \) and a complete suppression at more than 200 \( \mu \text{M} \). In Sp-0 cells, although we were unable to examine the effect of DFX at greater than 500 \( \mu \text{M} \) because of its toxicity, an accumulation of iron and other metals is therefore expected to be at least severalfold higher than that in the parent cells because DFX coordinates iron at the molar ratio 1:1 (52). Other explanations such as a decrease in the level of the antioxidant systems may also be likely. To seek out the underlying mechanisms, we first examined the total reduced GSH level in parent and Sp-0 cells and found that the levels in both these cell types were not significantly different from each other. We then evaluated the total cellular iron level in SILA and three Sp-0 clones using ICP-MS. This technique is highly sensitive and quantitative and is also applicable to any elements included in cells and tissues (8, 35). As expected, in all three Sp-0 clones, the iron contents were approximately 3-fold higher compared with the parent SILA cells. This is the first study to reveal an accumulation of iron in mtDNA-depleted cells. To clarify whether the \( \text{H}_2\text{O}_2 \)-induced Sp-0 cell death is metal-dependent and whether accumulation of metals and cell-death is dependent on mtDNA, the levels of other transition metals in parent, \( \rho \)-0, and the control cybrid cells and the sensitivity of these three cell types to \( \text{H}_2\text{O}_2 \) were evaluated. The contents of aluminum, zinc, manganese, and copper as well as iron were all elevated in Sp-0 cells compared with those of parent cells. These levels in the control cybrids were similar to the parent cell levels except for zinc, which nevertheless showed a decreasing trend. Moreover, unlike Sp-0 cells, the susceptibility of the control cybrid cells to \( \text{H}_2\text{O}_2 \) was as similar to that of the parent cells. These findings strongly suggest that both SILA and Sp-0 cell death include a metal-dependent mechanism and that accumulated transition metals in Sp-0 cells exacerbated the toxic effect of \( \text{H}_2\text{O}_2 \). Evaluation and comparison of contents of metals in other Sp-0 clones and \( \rho \)-0 cells from other cell lines will be necessary to conclude whether the accumulation of metals shown above is reproducible. It is not surprising that an increase in the cellular iron is observed in an association with an increase of other metals because of the coupling of metabolisms of iron, copper, and other metals (2, 4, 9, 11, 53).

The evidence that the contents of metals in the control cybrid cells were similar to those in the parent cells suggests that iron...
accretion is the result of impairment in mitochondrial respiration. Deficiency in mitochondrial Fe-S enzymes occurs in mitochondria in muscle tissues of mice, whose frataxin gene was genetically disrupted (21). Importantly, an impairment of the mitochondrial enzymes preceded an accumulation of iron in the tissue by several weeks. The substantia nigra and globus pallidus of the brain of patients with Parkinson’s disease are known to accumulate iron (4, 9, 11), which is concomitant with the dysfunction of complex I (12). Moreover, in vulnerable regions of brains affected with AD, accumulation of iron and aluminum (5–9) and mitochondrial abnormalities (12, 14, 17–19) were also reported. It appears therefore plausible that the accumulation of iron and other metals repeatedly observed in brains with these neurodegenerative disorders could be caused by or at least coupled with mitochondrial impairment. Hence, it is important to elucidate the mechanisms of accumulation of metals in p0 cells.

In conclusion, we have prepared a novel p0 line from a human neuroblastoma SILA line with which complementary experiments are available. These mutant cells exhibit unique features including metabolic adaptation, altered mitochondrial morphologies, and reduced ∆Ψm. They were highly susceptible to oxidative stress likely because of an accumulation of iron and other metals. Some of these unique phenotypes are mtDNA dependent because replenishment of p0 cells with normal mtDNA reversed the altered phenotypes. These phenotypes of newly established p0 cells resemble, in part, the phenotypes of some forms of mitochondrial and neurological diseases caused with or without alterations in mtDNA.

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