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Expression of the Apoptosis-Mediator Fas Is Enhanced by Dysfunctional Mitochondria

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We applied an antibody against an apoptosis mediator, Fas/APO-1/CD95, to HeLa-derived cells that completely lack mitochondrial DNA (mtDNA) or have mutant mtDNAs. The anti-Fas antibody killed the cells completely lacking mtDNA (EB8), at concentrations as low as 1 ng/ml, but not control cells harboring wild-type mtDNA (Ft2-11). TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) and analysis of fragmented DNA indicated that the cell death of EB8 was due to apoptosis. The antibody was cytotoxic to other two cell lines harboring mutant mtDNA with a point mutation or a large-scale deletion. RT-PCR (reverse transcriptase-polymerase chain reaction) showed that the mRNA content of the Fas gene was 2 to 19-fold higher in the cells with deficient mtDNA than in the control cells. In addition, the expressed Fas protein was detected by immunohistochemical staining in the cells without mtDNA but not in the control cells. Incubating the cells containing wild-type mtDNA with the respiratory inhibitors rotenone and antimycin A enhanced the content of mRNA of the Fas gene 2 to 4-fold and sensitized cells to the antibody. Thus, defects in mitochondria caused apoptotic cell death by anti-Fas antibody and enhanced Fas gene expression.

Key words: apoptosis, Fas antigen, gene expression, mitochondria disease, respiratory inhibitor.

In mitochondrial encephalomyopathies, muscle and brain mitochondria are functionally and morphologically abnormal. They have been classified into three distinct clinical subgroups, CPEO (chronic progressive external ophthalmoplegia), MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes), and MERRF (myoclonus epilepsy associated with ragged red fibers) (*1*). Each syndrome is caused by a mutation on mitochondrial genome. A point mutation at nucleotide number (nt) 3243 in the mitochondrial $tRNA^{Leu(UUR)}$ gene (*2, 3*), a point mutation at nt 8344 in the $tRNA^{Lys}$ gene and large-scale deletions of mitochondrial DNA (mtDNA) including several tRNA genes are associated with MELAS, MERRF, and CPEO, respectively (*4*). Another point mutation at nt 4269 in the $tRNA^{Ile}$ gene has also been found in a patient with fatal cardiomyopathy (CM) (*5, 6*). In order to exclude the possibility of involvement of defects in the nuclear genome, enucleated cells (cytoplasm) of the patients with mitochondrial encephalomyopathy were fused with human cells lacking mtDNA (cybrids), because the biogenesis of mitochondria is directed by both nuclear and mitochondrial genomes. Studies with cybrid clones obtained by intercellular mtDNA transfer showed that these mutations are responsible for pathogenesis of the associated diseases (*6-10*). It, however, remains unknown how the cells with mutant mitochondrial DNAs die. The mutant mtDNAs usually coexist with wild-type mtDNA and are often accumulated in some affected cells. To cure the mitochondrial diseases, it might be useful to remove the mutant mtDNAs by killing the cells in which they are accumulated.

Apoptosis is distinct from accidental cell death (*11*) and its pathogenic importance has recently been recognized, as well as its physiological meanings during the maturation of the immune system, embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover (*12-14*). Morphological and biochemical features typical of apoptosis are reduction of the cell volume, nuclear shrinkage, condensation and margination of chromatin, the formation of apoptotic bodies, and cleavage of genomic DNA into high-molecular-weight segments measured in kilobases and nucleosomal length fragments in multiples of about 180 bp (*13-17*).

Fas/APO-1/CD95 induces apoptosis when cells are stimulated by an agonistic monoclonal antibody (*18, 19*) or the natural Fas ligand (*20*). Fas-antigen is a 48-kDa transmembrane glycoprotein that belongs to the nerve growth factor (NGF) receptor/tumor necrosis factor (TNF) receptor superfamily (*19, 21*) and is expressed in many types of tissues, including thymus, spleen, ovary, and heart (*22*) and in a wide variety of normal and malignant cells (*23*).

In order to cure mitochondrial diseases, conventional gene therapy involving introduction of a functional gene into cells cannot be applied, since patients' cells usually

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.

have both wild-type and mutant mtDNAs (*3, 4, 24*). Apoptosis is a physiological process that eliminates useless cells from the body without inflammatory response. We speculated that the cells with deficient mitochondria could be killed by anti-Fas antibody. As the first step in developing the therapy for mitochondrial diseases, we show that apoptotic cell death was induced by anti-Fas antibody in the cells harboring deficient mitochondria and that expression of the Fas gene was enhanced by deficient mitochondria.

MATERIALS AND METHODS

Cells and Culture—The cells used in this study were all derived from human HeLaCot cells (*7*). EB8 and EB3 cells, which are totally devoid of mitochondrial DNA, were isolated independently (*7, 24*). CP8, CM1-1-4-5, and Ft2-11 are cybrid clones which were constructed by fusion between EB8 cells and enucleated fibroblasts from patients with mitochondrial encephalomyopathies or from a normal fetus. CP8 cells have mutant mtDNA (Δ-mtDNA) with a deletion 5,196 bp long (nt 8563 to 13758) transferred from a CPEO patient (*7*). This cybrid clone contains 40% of wild-type mtDNA and 60% of the deleted one, resulting in 90% less of respiratory enzymatic activity (*7*). CM1-1-4-5 cells harbor mutant mtDNA with a point mutation in the $tRNA^{Ile}$ gene (nt 4269), which was obtained from a patient with fatal cardiomyopathy (*6*). This cybrid clone contains 100% of the mutant mtDNA, but maintains 10% of the respiratory enzymatic activity (*6*). Ft2-11 cells have wild-type mtDNA from cells of a normal fetus (*25*). Cells were maintained in F-12 nutrient mixture (GIBCO BRL) containing 10% fetal bovine serum (Irvine Scientific, California) at 37°C in 5% CO_2/95% air.

Cytotoxicity of the Anti-Fas Antibody to Cells—The IgM isotype of the monoclonal antibody (clone CH-11) against human Fas-antigen (anti-Fas mAb) was purchased from MBL (Nagoya). The respiratory chain inhibitors rotenone and antimycin A were obtained from Sigma. Cells (5×10^4) were plated in 35-mm dishes and given fresh medium 2 h before experiments. For experiments with respiratory inhibitors, cells were incubated with them at indicated concentrations in the presence of uridine (50 μg/ml, GIBCO BRL) for 6 h. The cells were then incubated with anti-Fas mAb at various concentrations for the indicated periods. Surviving cells were harvested with trypsin and counted by Trypan blue exclusion. The number of cells without anti-Fas mAb or inhibitors was taken as 100%.

Nuclear Staining Method—Condensed chromatin was detected by the TUNEL [terminal deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling] method with an Apop Tag kit (Oncor, Gaithersburg, MD). Cells were cultured for 5 h on sterile glass cover slips in the presence of anti-Fas IgM antibody (clone CH-11) (20 ng/ml). The cells fixed with acetone on the cover slip were subjected to TUNEL, followed by counterstaining with hematoxylin.

Immunohistochemical Staining Procedure—A streptavidin-biotin (SAB) procedure (Histofine SAB-PO(M) kit; Nichirei, Tokyo) was employed for detecting Fas-antigen by using an IgG isotype of anti-human Fas monoclonal antibody (clone UB2, dilution of 1:50) (MBL, Nagoya). Final peroxidase-conjugated products were stained with diaminobenzidine as a chromogen. Each cell was counterstained with hematoxylin.

DNA Fragmentation—One million cells in a 100-mm dish were incubated with anti-Fas mAb (clone CH-11: 20 ng/ml) for various periods. Harvested cells were incubated in 10 mM Tris, pH 7.4, containing 10 mM EDTA, and 0.5% Triton X-100 on ice for 10 min. Intact nuclei were sedimented by centrifugation at $10,000\times g$ for 20 min. After intensive digestion with RNase A and proteinase K, fragmented DNAs were precipitated with 2-propanol, and stained with ethidium bromide after 1.2% agarose gel electrophoresis.

RT-PCR Analysis—Poly(A)$^+$ RNA was prepared using a QuickPrep Micro mRNA Purification kit (Pharmacia). Each sample (0.2 μg) was incubated with SuperScript reverse transcriptaseII (GIBCO BRL) with 100 pmol of oligo(dT) to synthesize the first strand of cDNA in a 20-μl reaction mixture (cDNA pool). Several microliters of 100-diluted cDNA pool were used for PCR. Endogenous mRNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard to normalize the relative levels of Fas mRNA. To effectively amplify Fas and GAPDH cDNAs, pairs of primer sequences were searched by means of computer analysis using the software GENETYX (Software Development, Tokyo). To amplify Fas cDNA, 5′-dGCCTAATGCACCCCCAAACA-3′ for the sense strand and 5′-dAACGTAGGATAGTAGTAAGGAG-3′ for the antisense strand were selected. These primers gave a PCR product of 346 bp, corresponding to nt 1985 to 2330 of Fas cDNA (*19*). To amplify GAPDH cDNA, 5′-dCCCCTGGCCAAGGTCATCCATG-3′ for the sense strand and 5′-dCAGTGAGCTTCCCGTTCAGCTC-3′ for the antisense strand were selected. These primers gave a PCR product of 214 bp, corresponding to nt 508 to 721 of human GAPDH cDNA (Accession No. M17851 in GenBank). For quantifying a small amount of mRNAs, PCR was performed in the presence of 0.2 mM dGTP, dATP, and dTTP, 0.1 mM dCTP, 30 μCi/ml [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham), and 0.5 μM each primer. The thermal cycle profile was as follows: denaturation at 94°C for 45 s, annealing at

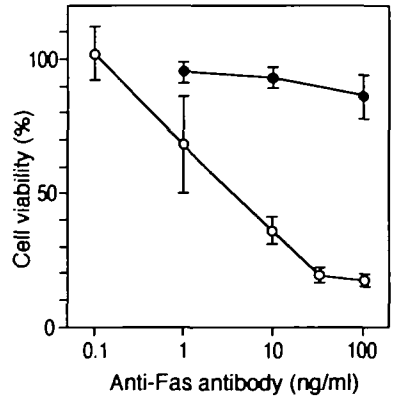

Fig. 1. **Dose-dependent cell death of EB8 and Ft2-11 by anti-Fas antibody.** Cells were incubated with or without anti-Fas antibody at various concentrations for 24 h, then surviving cells were counted by the Trypan blue exclusion method. Cell viability is shown as the average value of triplicate experiments. The number of cells incubated without the anti-Fas antibody was taken as 100%. The vertical bars represent standard deviations. Open and filled circles represent EB8 and Ft2-11 cells, respectively.

55°C for 45 s, and extension at 72°C for 2 min. PCR initially proceeded with the primers for Fas cDNA for the optimal number of cycles, then the GAPDH primers were added for the following 15 cycles. The PCR products are amplified exponentially under these conditions. PCR products were separated by 10% polyacrylamide gel electrophoresis and radioactivity levels were measured using a bioimaging analyzer, Fujix BAS 2000 (Fuji Photo Film, Tokyo). The radioactivity level of Fas-specific PCR products was normalized against that of GAPDH-specific PCR products to calculate the enhancement of the Fas gene transcription.

RESULTS

EB8 Cells Died in the Presence of the Anti-Fas Antibody—The EB8 cell line was isolated from human HeLaCot cells and it completely lacks mtDNA. During characterization of this cell line, we found that anti-Fas monoclonal antibody (anti-Fas mAb) was extensively cytotoxic to it. We, therefore, examined the viability of EB8 cells incubated with various concentrations of anti-Fas mAb for 24 h. Compared with EB8 cells incubated in the absence of anti-Fas mAb, 29% of cells died in the presence of anti-Fas mAb at a concentration as low as 1 ng/ml, as shown in Fig. 1. Only 18% of the cells survived in the presence at 33 ng/ml of anti-Fas mAb and they were killed by anti-Fas mAb in a dose-dependent manner. On the other hand, Ft2-11 cells, which have wild-type mtDNA and the same nuclear background as EB8 cells, were resistant to anti-Fas mAb. The viability of Ft2-11 declined gradually from 97% at 1 ng/ml to 84% at 100 ng/ml. A 14-h incubation of EB8 cells with anti-Fas mAb gave the same result, although Ft2-11 cells were completely resistant to anti-Fas mAb (not shown).

Anti-Fas mAb Caused Pyknotic Chromatin and DNA Fragmentation in EB8 Nuclei—In apoptotic cells, the

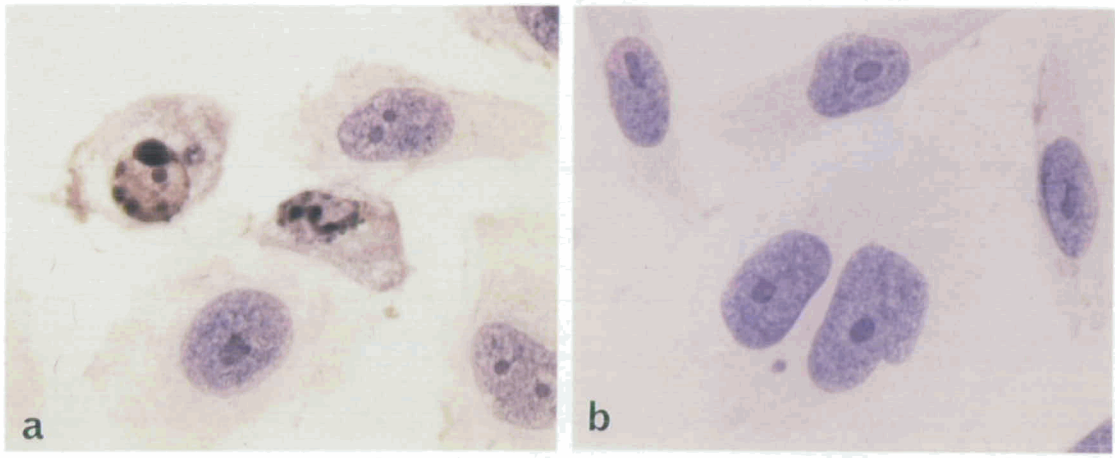

Fig. 2. **Pyknotic chromatin of EB8 cells incubated with anti-Fas antibody.** EB8 (a) and Ft2-11(b) cells were seeded on glass cover slips one day before experiments. The cells were incubated with the anti-Fas IgM antibody (20 ng/ml) for 5 h, then fixed on the cover slips with acetone and stained brown by the TUNEL method. The cells were also counterstained with hematoxylin (purple). Original magnification is ×200.

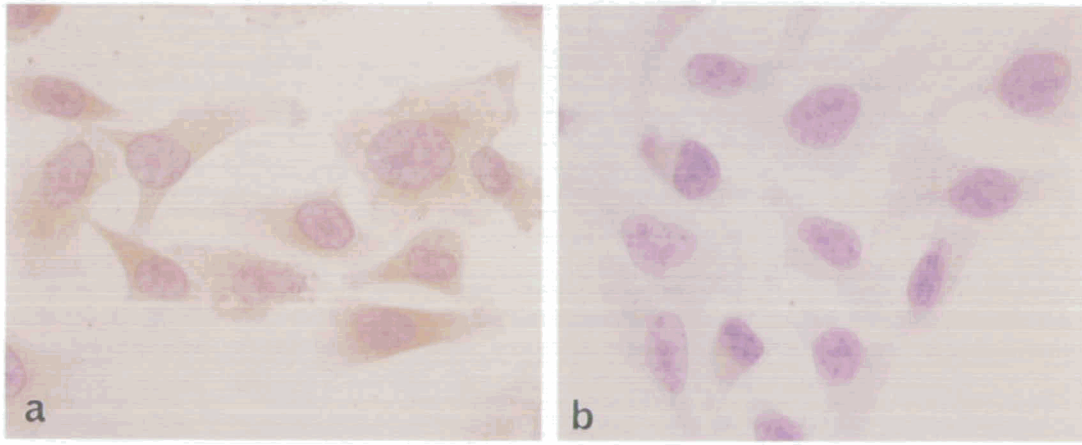

Fig. 6. **Immunohistochemical staining of Fas-antigen of EB8 and control cells.** EB8 (a) and Ft2-11 (b) cells were seeded on glass cover slips one day before experiments. The cells were fixed on the cover slips with methanol containing 0.3% H_2O_2 and incubated with anti-Fas IgG antibody (50-fold dilution) followed by an SAB procedure. Diaminobenzidine was used for brown color development of the anti-Fas antibody. The cells were also counterstained with hematoxylin (purple).

chromatin morphologically becomes pyknotic and the DNA is biochemically broken down into small segments. EB8 cells exposed to anti-Fas mAb were examined by the TUNEL method. About half of the EB8 cells incubated with anti-Fas mAb for 5 h showed typical condensed chromatin that stained brown, indicating fragmentation of chromosomal DNA (Fig. 2a). These cells were also shriveled and developed vacuoles. However, Ft2-11 cells incubated with anti-Fas mAb had no morphological changes in their nuclei (Fig. 2b). Fragmented DNAs of EB8 cells incubated with anti-Fas mAb for 2 to 12 h, were extracted (Fig. 3). Fragmented DNAs were detected in cells after 4 h and the amount dramatically increased after 6 h of incubation. Small DNA fragments that were multiples of about 180 bp (base pairs) gave definite signals indicating a DNA ladder. We concluded that EB8 cells underwent cell death induced by anti-Fas mAb in an apoptotic process.

HeLa Cells with Mutant mtDNA Were Also Killed by Anti-Fas mAb—The HeLa-derived EB8 cell line lacking mtDNA underwent apoptotic cell death in the presence of anti-Fas mAb. To clarify whether this process was specific to EB8 cells, we examined other cell lines. EB3 cell line, which was independently isolated from HeLa cells by the same selection as EB8, also completely lacks mtDNA. Cybrid clones CM1-1-4-5 and CP8-4, which are both derivatives of EB8 cells, harbor mtDNA with a point mutation in the $tRNA^{Ile}$ gene and Δ-mtDNA with a 5-kb deletion, respectively, and both clones maintain about 10% of respiratory enzymatic activity. All cells examined died in the presence of anti-Fas mAb (20 ng/ml) with a viability of 22 to 31% as shown in Fig. 4. These results indicate that anti-Fas mAb is cytotoxic to cells with genetically deficient mtDNA as well as to cells lacking whole mtDNA.

The Level of mRNA of the Fas Gene Was Elevated in Cells Lacking mtDNA or Harboring Mutant mtDNA—The cytotoxicity of anti-Fas mAb is mediated by Fas antigen (*18, 19*). Relative levels of Fas mRNA in Ft2-11, EB8, CP8-4, and CM1-1-4-5 cells were quantified by the RT-PCR method under conditions in which the PCR products were amplified exponentially (*26, 27*), using GAPDH (glyceraldehyde 3-phosphate dehydrogenase; EC 1.2.1.12) mRNA as an endogenous internal standard (*28, 29*). We synthesized cDNAs from poly(A)$^+$RNA by means of reverse transcriptase using oligo(dT) as a primer, and amplified them with Fas cDNA-specific primers by PCR in the presence of [α-^{32}P]dCTP (Fig. 5). For the last 15 cycles, GAPDH-specific primers were added. The cDNAs of Ft2-11 cells generated similarly low levels of Fas- and GAPDH-specific PCR products after 27 cycles. Using cDNAs of CM1-1-4-5 and CP8-4 cells, the levels of amplified Fas- and GAPDH-specific PCR products after 26 cycles were similar. The cDNAs of EB8 cells amplified more Fas- than GAPDH-specific products after 26 cycles. A Fas-specific PCR product was purified from a gel and sequenced to confirm that it was derived from the cDNA of the Fas gene (data not shown). To exclude the possibility of contaminating genomic DNAs in the poly(A)$^+$RNA preparations, cDNA amplified by PCR was confirmed to be absent when reverse transcriptase was omitted in mixtures for synthesizing the first strand of cDNA (lanes marked as −RT in Fig. 5). The radioactivity of each band was measured by the bioimaging analyzer BAS2000, and the radioactivity of the Fas-specific band was normalized to that of the GAPDH-specific band. In EB8, CP8-4, and CM1-1-4-5 cells, mRNA of the Fas gene was enhanced by

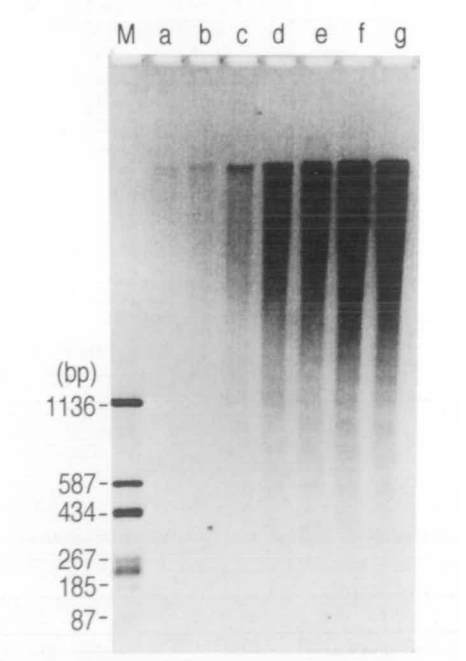

Fig. 3. **DNA fragmentation of EB8 cells incubated with anti-Fas antibody.** EB8 cells (1×10^6) were incubated with the anti-Fas antibody (20 ng/ml) for 2 (b), 4 (c), 6 (d), 8 (e), 10 (f), and 12 h (g). Cells were lysed with Triton X-100 to obtain fragmented DNAs, which were resolved by agarose gel electrophoresis (1.2%) as described in "MATERIALS AND METHODS." Fragmented DNA was also prepared from EB8 cells without the anti-Fas mAb (a). DNA fragments of *Hae*III-digested pUC18-derived CAT vector were used as molecular weight standards (M).

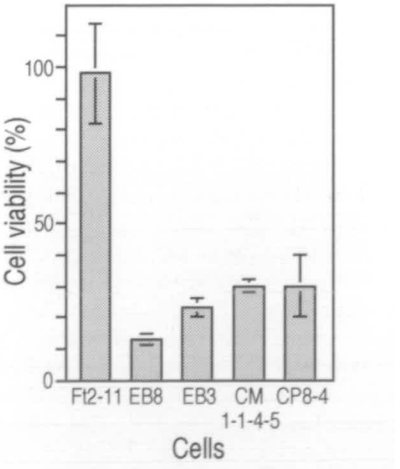

Fig. 4. **Viability of Ft2-11, EB8, EB3, CM1-1-4-5, and CP8-4 cells.** EB3 cells isolated independently of EB8 cells also completely lacked mtDNA. CM1-1-4-5 and CP8-4 have mutant mtDNA with a point mutation in the $tRNA^{Ile}$ gene and a large-scale deletion, respectively. Cells were incubated with or without anti-Fas mAb (20 ng/ml) for 24 h. Surviving cells were counted by Trypan blue exclusion. Cell viability is shown as the average of values obtained from triplicate experiments. The number of cells incubated without anti-Fas mAb was taken as 100%. The vertical bars represent standard deviations.

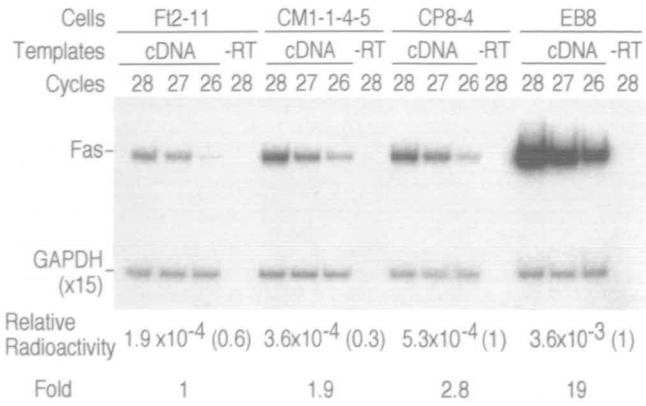

Fig. 5. **Quantitation of Fas mRNA by RT-PCR.** Poly(A)-tailed RNA prepared from Ft2-11, CM1-1-4-5, CP8-4, and EB8 cells was used to generate a cDNA pool by means of reverse transcriptase using oligo(dT) as a primer. Diluted cDNA pool was amplified by PCR in the presence of [α-^{32}P]dCTP with a pair of Fas-specific primers as indicated on the top of each lane. For the last 15 cycles, a pair of GAPDH-specific primers was added to the reaction mixture. Fas- and GAPDH-specific primers generated 346- and 214-bp PCR products, respectively. To estimate the effects of genomic DNA in poly(A)$^+$ RNA preparations, cDNA reactions were also performed without reverse transcriptase (−RT). The relative radioactivity level of Fas PCR product to GAPDH PCR product was calculated from 8 to 11 PCR experiments using 5, 2, 1, and 3 poly(A)$^+$RNA preparations of Ft2-11, CM1-1-4-5, CP8-4, and EB8 cells, respectively. SD is shown in parenthesis.

19, 2.8, and 1.9-fold, respectively, compared with that in Ft2-11 cells.

Fas-Antigen Protein Is Detected at Higher Level in EB8 than Ft2-11 Cells—We examined whether the level of Fas-protein in EB8 cells is increased, as was expected from the results of RT-PCR. EB8 and Ft2-11 cells were immunohistochemically stained by the SAB method using anti-Fas IgG. As shown in Fig. 6, EB8 cells were stained uniformly, but the control Ft2-11 cells were not. This result suggests the Fas-antigen is expressed on the surface of the cell membrane, as expected.

Respiratory Chain Inhibitors Induced the Fas Gene Transcription—As shown above, the cells with mutant mtDNA increased the level of Fas mRNA. The mutant mtDNAs used here had mutations at different regions. It is unlikely that a specific DNA sequence of mtDNA is involved in the induction of the Fas gene. We investigated whether inhibitors of mitochondrial respiration induce the Fas gene expression. Ft2-11 cells, which have normal mitochondria, were incubated with two respiratory chain inhibitors: rotenone, which inhibits NADH-CoQ reductase (complex I); and antimycin A, which inhibits CoQ-cytochrome *c* reductase (complex III). The mRNA levels of the Fas gene were determined by RT-PCR (Fig. 7A) as described above. Rotenone at 1 μM enhanced the gene expression 2-fold in 2 h. Antimycin A at 50 μM also induced the gene expression 4-fold in 5 h. Adding anti-Fas mAb after a 6-h incubation of the cells with rotenone (0.3 and 1 μM) or antimycin A (50 μM) caused 30 to 50% of them to die, although 1 μM rotenone alone was highly cytotoxic (Fig. 7B). Therefore, respiration inhibitors altered the sensitivity of the cells to anti-Fas mAb and elevated the level of Fas mRNA.

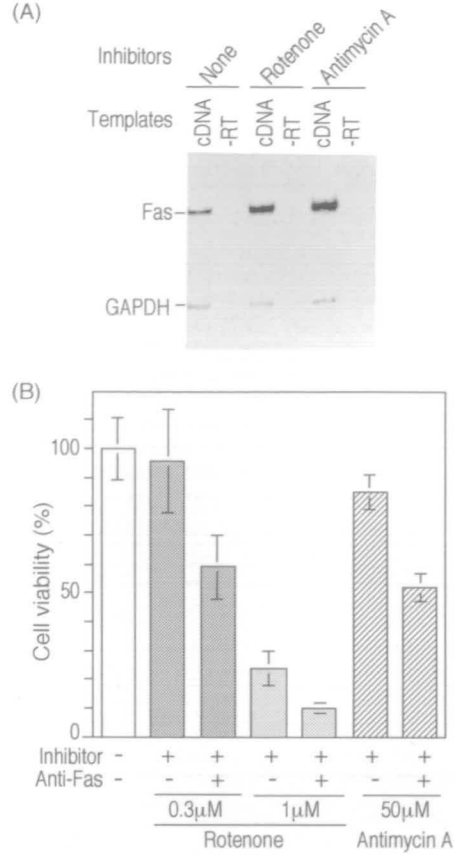

Fig. 7. **Effects of the respiratory chain inhibitors, rotenone and antimycin A, on Fas gene expression and sensitivity to the anti-Fas antibody of cells having wild-type mtDNA.** (A) Quantitation of Fas mRNA levels by RT-PCR. Ft2-11 cells having wild-type mtDNA were incubated with rotenone (1 μM) for 2 h or antimycin A (50 μM) for 5 h in the presence of uridine (50 μg/ml). Cells were lysed to prepare poly(A)$^+$RNA. After synthesizing cDNA, diluted cDNA pool was amplified with a pair of Fas-specific primers alone for the first 15 cycles in the presence of [α-^{32}P]dCTP. For the following 15 cycles, PCR proceeded with a pair of GAPDH-specific primers. The cDNA synthesis reactions also proceeded without reverse transcriptase (−RT). (B) The sensitivity of cells to the anti-Fas antibody. Ft2-11 cells were incubated with rotenone or antimycin A at the indicated concentrations for 6 h in the presence of uridine (50 μg/ml), then the antibody (20 ng/ml) was added. After 18 h, cells were counted by Trypan blue exclusion. The number of cells incubated without inhibitors or the antibody was taken as 100%. Data were obtained from triplicate experiments.

DISCUSSION

Mitochondrial encephalomyopathies are genetic diseases in which mutations are found on the mitochondrial genome. The mitochondrial genome is fundamentally different in its genetic characteristics from the nuclear one. First, mtDNA is contained in multiple copies in a cell, ranging from hundreds to several thousands depending on the tissue-type. Since mutant mtDNAs usually coexist with the wild-type, a mutant phenotype is observed only when the responsible mutant mtDNA is accumulated in excess of the wild-type. In addition, the mutant mtDNAs have a propagation advantage by unknown mechanisms (*7, 30, 31*). Therefore, even if wild-type mtDNA were introduced by a novel method, no improvement of the phenotype would be expected. As a possible method for therapy, it might be

useful to eliminate the affected cells, because the mutant mtDNA usually accumulates in specific affected cells. In addition, as cells rich in wild-type mtDNA are expected to be advantageous for growth, the healthy cells might complement the affected cells. If an apoptotic process takes place, the affected cells could be eliminated without inflammatory response. In this study, the EB8 derivatives with mutant mtDNA were shown to be sensitive to anti-Fas mAb, as was EB8 itself.

To investigate the relation of deficient mitochondria with apoptosis, the effects of nuclear genes should be taken into consideration, because all the components involved in the mitochondrial genetic system are encoded by nuclear genes. Since the cybrid clones used in this study, including the control, were constructed from EB8 by cytoplasmically transferring mitochondria, all the cell lines share the same nuclear background. Recently, Gamen *et al.* have reported that mtDNA-depleted promonocytic leukemia cells became less sensitive to TNF and Fas-mediated cytotoxicity than the control cells and that this change in TNF-sensitivity was due to the loss of the surface of the TNF receptor possibly during long exposure to ethidium bromide to eliminate mtDNA (*32*). On the other hand, although the Fas expression was not changed, the sensitivity was still maintained but declined. These findings disagreed with ours. This discrepancy may be due to a difference in cell types: they used 500 ng/ml of anti-Fas mAb, whereas we used 20 ng/ml. Another possibility is that their mtDNA-less cells may have undergone a change in sensitivity to anti-Fas mAb during long exposure to ethidium bromide. In our study, we used as cotrol a cybrid cell line which had been constructed by fusing the parental EB8 cells with cytoplasm of normal fetus to exclude the possibility of any alternations during isolation of mtDNA-depleted cell lines.

The transcription level of the Fas gene was investigated to understand the cause(s) of the high sensitivity of cells harboring deficient mitochondria against anti-Fas mAb. As estimated by PCR-amplified cDNA (Fig. 5), the mRNA level of the Fas gene is about 1×10^{-4} that of GAPDH in Ft2-11 cells. Thus, it was difficult to quantify the level of mRNA by Northern blotting. We therefore applied RT-PCR with an endogenous internal standard to quantify the relative mRNA level. If a linear evaluation over a wide range is achieved by plotting the amplification curves, this approach is one of the most sensitive and reliable means of quantitative analysis (*26, 27, 33*). To effectively amplify the cDNAs, we searched for the optimal pair of primers using a computer. The GAPDH mRNA used here as endogenous internal standard in RT-PCR is widely used as an endogenous internal standard for gene expression (*28, 29*).

EB8 enhanced the Fas gene expression 19 times over that of Ft2-11 cells. Other cells harboring mutant mtDNAs also increased the Fas mRNA level somewhat (2 to 3-fold). Since the mutant cybrid clones still maintain 10% of respiratory enzymatic activity (*6, 7*), such increase appears reasonable. As far as examined, gene expressions of nuclear-encoded mitochondrial proteins including a subunit of cytochrome *c* oxidase and mitochondrial transcription factor 1 were not changed by eliminating mtDNA (*34, 35*). The 20-fold expression of the Fas gene in EB8 cells should be emphasized as a marked effect of the mtDNA deficiency.

The human Fas gene is a single copy gene of over 25 kb and consists of nine exons (*36, 37*). It contains multiple transcription initiation sites and no canonical "TATA" boxes. Northern blotting has revealed two transcripts of around 2 and 2.7 kb in human T cell lymphoma KT-3 cells (*19*) and in HeLa cells exposed to influenza virus or poly(I)-poly(C) (*38*). RT-PCR has revealed three variant forms of the Fas transcript, which were alternative splicing products in the coding region, in phytohemagglutinin-activated peripheral blood mononuclear cells (*39*). Many factors stimulate Fas expression, including cerebral ischemia (*40*), viral antigens of cytomegalovirus and varicella-zoster virus (*41*), infection with influenza virus (*42*), murine cytomegalovirus (*43*) and human immunodeficiency virus (*44*), a synthetic double-stranded RNA poly-(I)-poly(C) treatment (*38*), cytokines TNFα, interferons α and γ (*22, 45-48*) and interleukin-2 (*49*), NF-IL6 (nuclear factor for interleukin-6 expression) (*50*), and the tumor suppressor protein p53 (*51*). However, none of them enhanced the Fas expression as much as the loss of mitochondrial DNA presented in this study. There are many putative binding sites for transcription factors in the 5′-upstream region of the Fas gene, including AP-1, NF-$\varkappa$B, Sp1 (*36, 37*). The factor(s) involved in the enhancement of Fas gene expression in cells harboring deficient mitochondria remains to be determined. Although the enhancement of the Fas expression is associated with the sensitivity to apoptosis, it is unknown whether the enhanced expression of Fas is directly responsible for the cell death. Since the mitochondrial-deficient cells have no function, the cells could signal the onset of apoptosis.

Susceptibility to apoptosis may be induced by decline of the respiratory level of mitochondria or complementary enhancement of glycolysis. Mitochondrial production of oxygen radicals is proposed to be involved in the mechanism of TNF cytotoxicity (*52*). In addition, some respiratory chain inhibitors are able to induce apoptosis to some extent in the absence of cycloheximide and actinomycin D (*53*). We found that incubating cells harboring wild-type mitochondria with rotenone or antimycin A rendered the cells susceptible to anti-Fas mAb (Fig. 7). The cells exposed to these respiratory inhibitors probably produce oxygen radicals in the mitochondria. It is notable that these inhibitors caused enhancement of the Fas gene expression. However, the cells without mtDNA also showed enhanced Fas gene expression at the highest level. These cells are unlikely to produce oxygen radicals, because of their lack of respiratory activity.

Finally, this study demonstrated that it is possible to kill specifically cells with accumulated mutant mtDNA. This finding may be useful for therapeutic application to mitochondrial diseases because of the expected complemental growth of normal cells.

REFERENCES

1. DiMauro, S., Bonilla, E., Zeviani, M., Nakagawa, M., and DeVivo, D.C. (1985) Mitochondrial myopathies. *Ann. Neurol.* **17**, 521-538
2. Kobayashi, Y., Momoi, M.Y., Tominaga, K., Momoi, T., Nihei, K., Yanagisawa, M., Kagawa, Y., and Ohta, S. (1990) A point mutation in the mitochondrial tRNA(Leu)(UUR) gene in MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes). *Biochem. Biophys. Res. Commun.* **173**, 816-822

3. Kobayashi, Y., Momoi, M.Y., Tominaga, K., Shimoizumi, H., Nihei, K., Yanagisawa, M., Kagawa, Y., and Ohta, S. (1991) Respiration-deficient cells are caused by a single point mutation in the mitochondrial tRNA-Leu(UUR) gene in mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes (MELAS). *Am. J. Hum. Genet.* **49**, 590-599
4. Wallace, D.C. (1992) Diseases of the mitochondrial DNA. *Annu. Rev. Biochem.* **61**, 1175-1212
5. Taniike, M., Fukushima, H., Yanagihara, I., Tsukamoto, H., Tanaka, J., Fujimura, H., Nagai, T., Sano, T., Yamaoka, K., Inui, K., and Okada, S. (1992) Mitochondrial tRNA(Ile) mutation in fatal cardiomyopathy. *Biochem. Biophys. Res. Commun.* **186**, 47-53
6. Hayashi, J.-I., Ohta, S., Kagawa, Y., Takai, D., Miyabayashi, S., Tada, K., Fukushima, H., Inui, K., Okada, S., Goto, Y.-i., and Nonaka, I. (1994) Functional and morphological abnormalities of mitochondria in human cells containing mitochondrial DNA with pathogenic point mutations in tRNA genes. *J. Biol. Chem.* **269**, 19060-19066
7. Hayashi, J.-I., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y.-i., and Nonaka, I. (1991) Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. *Proc. Natl. Acad. Sci. USA* **88**, 10614-10618
8. Chomyn, A., Meola, G., Bresolin, N., Lai, S.T., Scarlato, G., and Attardi, G. (1991) In vitro genetic transfer of protein synthesis and respiration defects to mitochondrial DNA-less cells with myopathy-patient mitochondria. *Mol. Cell. Biol.* **11**, 2236-2244
9. King, M.P., Koga, Y., Davidson, M., and Schon, E.A. (1992) Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the tRNA(Leu(UUR)) mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes. *Mol. Cell. Biol.* **12**, 480-490
10. Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., Lai, S.T., Nonaka, I., Angelini, C., and Attardi, G. (1992) MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. *Proc. Natl. Acad. Sci. USA* **89**, 4221-4225
11. Majno, G. and Joris, I. (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* **146**, 3-15
12. Wyllie, A.H., Kerr, J.F., and Currie, A.R. (1980) Cell death: The significance of apoptosis. *Int. Rev. Cytol.* **68**, 251-306
13. Wyllie, A.H. and Morris, R.G. (1982) Hormone-induced cell death. Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. *Am. J. Pathol.* **109**, 78-87
14. Arends, M.J., Morris, R.G., and Wyllie, A.H. (1990) Apoptosis. The role of the endonuclease. *Am. J. Pathol.* **136**, 593-608
15. Roy, C., Brown, D.L., Little, J.E., Valentine, B.K., Walker, P.R., Sikorska, M., Leblanc, J., and Chaly, N. (1992) The topoisomerase II inhibitor teniposide (VM-26) induces apoptosis in unstimulated mature murine lymphocytes. *Exp. Cell. Res.* **200**, 416-424
16. Oberhammer, F., Wilson, J.W., Dive, C., Morris, I.D., Hickman, J.A., Wakeling, A.E., Walker, P.R., and Sikorska, M. (1993) Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.* **12**, 3679-3684
17. Brown, D.G., Sun, X.-M., and Cohen, G.M. (1993) Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. *J. Biol. Chem.* **268**, 3037-3039
18. Yonehara, S., Ishii, A., and Yonehara, M. (1989) A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* **169**, 1747-1756
19. Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S.-I., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**, 233-243
20. Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75**, 1169-1178
21. Oehm, A., Behrmann, I., Falk, W., Pawlita, M., Maier, G., Klas, C., Li-Weber, M., Richards, S., Dhein, J., Trauth, B.C., Ponsting, H., and Krammer, P.H. (1992) Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. *J. Biol. Chem.* **267**, 10709-10715
22. Watanabe-Fukunaga, R., Brannan, C.I., Itoh, N., Yonehara, S., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992) The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J. Immunol.* **148**, 1274-1279
23. Krammer, P.H., Dhein, J., Walczak, H., Behrmann, I., Mariani, S., Matiba, B., Fath, M., Daniel, P.T., Knipping, E., Westendorp, M.O., Stricker, K., Baumler, C., Hellbardt, S., Germer, M., Peter, M.E., and Debatin, K.-M. (1994) The role of APO-1-mediated apoptosis in the immune system. *Immunol. Rev.* **142**, 175-191
24. Hayashi, J.-I., Yonekawa, H., Watanabe, S., Nonaka, I., Momoi, M.Y., Kagawa, Y., and Ohta, S. (1991) Somatic cell genetical approaches to mitochondrial diseases in *Progress in Neuropathology* (Sato, T. and DiMauro, S., eds.) Vol. 7, pp. 93-102, Raven Press, New York
25. Hayashi, J.-I., Ohta, S., Kagawa, Y., Kondo, H., Kaneda, H., Yonekawa, H., Takai, D., and Miyabayashi, S. (1994) Nuclear but not mitochondrial genome involvement in human age-related mitochondrial dysfunction. Functional integrity of mitochondrial DNA from aged subjects. *J. Biol. Chem.* **269**, 6878-6883
26. Noonan, K.E., Beck, C., Holzmayer, T.A., Chin, J.E., Wunder, J.S., Andrulis, I.L., Gazdar, A.F., Willman, C.L., Griffith, B., Von Hoff, D.D., and Roninson, I.B. (1990) Quantitative analysis of *MDR1* (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **87**, 7160-7164
27. Kinoshita, T., Imamura, J., Nagai, H., and Shimotohno, K. (1992) Quantification of gene expression over a wide range by the polymerase chain reaction. *Anal. Biochem.* **206**, 231-235
28. Bosma, P.J. and Kooistra, T. (1991) Different induction of two plasminogen activator inhibitor 1 mRNA species by phorbol ester in human hepatoma cells. *J. Biol. Chem.* **266**, 17845-17849
29. Zentella, A., Weis, F.M.B., Ralph, D.A., Laiho, M., and Massagué, J. (1991) Early gene responses to transforming growth factor-β in cells lacking growth-suppressive RB function. *Mol. Cell. Biol.* **11**, 4952-4958
30. Yoneda, M., Chomyn, A., Martinuzzi, A., Hurko, O., and Attardi, G. (1992) Marked replicative advantage of human mtDNA carrying a point mutation that causes the MELAS encephalomyopathy. *Proc. Natl. Acad. Sci. USA* **89**, 11164-11168
31. Kobayashi, Y., Ichihashi, K., Ohta, S., Nihei, K., Kagawa, Y., Yanagisawa, M., and Momoi, M.Y. (1992) The mutant mitochondrial genes in mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) were selectively amplified through generations. *J. Inherit. Metab. Dis.* **15**, 803-808
32. Gamen, S., Anel, A., Montoya, J., Marzo, I., Pineiro, A., and Naval, J. (1995) mtDNA-depleted U937 cells are sensitive to TNF and Fas-mediated cytotoxicity. *FEBS Lett.* **376**, 15-18
33. Uenaka, R., Kuwajima, M., Ono, A., Matsuzawa, Y., Hayakawa, J.-I., Inohara, N., Kagawa, Y., and Ohta, S. (1996) Increased expression of carnitine palmitoyltransferase I gene is repressed by administering L-carnitine-deficient juvenile visceral steatosis mice. *J. Biochem.* **119**, 533-540
34. Hayashi, J.-I., Tanaka, M., Sato, W., Ozawa, T., Yonekawa, H., Kagawa, Y., and Ohta, S. (1990) Effects of ethidium bromide treatment of mouse cells on expression and assembly of nuclear-coded subunits of complexes involved in the oxidative phosphorylation. *Biochem. Biophys. Res. Commun.* **167**, 216-221
35. Tominaga, K., Hayashi, J.-I., Kagawa, Y., and Ohta, S. (1993) Smaller isoform of human mitochondrial transcription factor 1: Its wide distribution and production by alternative splicing. *Biochem. Biophys. Res. Commun.* **194**, 544-551
36. Behrmann, I., Walczak, H., and Krammer, P.H. (1994) Struc-

ture of the human APO-1 gene. *Eur. J. Immunol.* **24**, 3057-3062
37. Cheng, J., Liu, C., Koopman, W.J., and Mountz, J.D. (1995) Characterization of human Fas gene. Exon/intron organization and promoter region. *J. Immunol.* **154**, 1239-1245
38. Takizawa, T., Fukuda, R., Miyawaki, T., Ohashi, K., and Nakanishi, Y. (1995) Activation of the apoptotic Fas antigen-encoding gene upon influenza virus infection involving spontaneously produced beta-interferon. *Virology* **209**, 288-296
39. Cascino, I., Fiucci, G., Papoff, G., and Ruberti, G. (1995) Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. *J. Immunol.* **154**, 2706-2713
40. Matsuyama, T., Hata, R., Tagaya, M., Yamamoto, Y., Nakajima, T., Furuyama, J.-i., Wanaka, A., and Sugita, M. (1994) Fas antigen mRNA induction in postischemic murine brain. *Brain Res.* **657**, 342-346
41. Ito, M., Watanabe, M., Ihara, T., Kamiya, H., and Sakurai, M. (1995) Fas antigen and bcl-2 expression on lymphocytes cultured with cytomegalovirus and varicella-zoster virus antigen. *Cell. Immunol.* **160**, 173-177
42. Takizawa, T., Matsukawa, S., Higuchi, Y., Nakamura, S., Nakanishi, Y., and Fukuda, R. (1993) Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells. *J. Gen. Virol.* **74**, 2347-2355
43. Yoshida, H., Sumichika, H., Hamano, S., He, X., Minamishima, Y., Kimura, G., and Nomoto, K. (1995) Induction of apoptosis of T cells by infecting mice with murine cytomegalovirus. *J. Virol.* **69**, 4769-4775
44. Katsikis, P.D., Wunderlich, E.S., Smith, C.A., Herzenberg, L.A., and Herzenberg, L.A. (1995) Fas antigen stimulation induces marked apoptosis of T lymphocytes in human immunodeficiency virus-infected individuals. *J. Exp. Med.* **181**, 2029-2036
45. Maciejewski, J., Selleri, C., Anderson, S., and Young, N.S. (1995) Fas antigen expression on CD34+ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression in vitro. *Blood* **85**, 3183-3190
46. Panayiotidis, P., Ganeshaguru, K., Foroni, L., and Hoffbrand, A.V. (1995) Expression and function of the FAS antigen in B chronic lymphocytic leukemia and hairy cell leukemia. *Leukemia* **9**, 1227-1232
47. Quirk, S.M., Cowan, R.G., Joshi, S.G., and Henrikson, K.P. (1995) Fas antigen-mediated apoptosis in human granulosa/luteal cells. *Biol. Reprod.* **52**, 279-287
48. Nagafuji, K., Shibuya, T., Harada, M., Mizuno, S.-i., Takenaka, K., Miyamoto, T., Okamura, T., Gondo, H., and Niho, Y. (1995) Functional expression of Fas antigen (CD95) on hematopoietic progenitor cells. *Blood* **86**, 883-889
49. Tartaglia, L.A., Ayres, T.M., Wong, G.H.W., and Goeddel, D.V. (1993) A novel domain within the 55 kd TNF receptor signals cell death. *Cell* **74**, 845-853
50. Wada, N., Matsumura, M., Ohba, Y., Kobayashi, N., Takizawa, T., and Nakanishi, Y. (1995) Transcription stimulation of the Fas-encoding gene by nuclear factor for interleukin-6 expression upon influenza virus infection. *J. Biol. Chem.* **270**, 18007-18012
51. Owen-Schaub, L.B., Zhang, W., Cusack, J.C., Angelo, L.S., Santee, S.M., Fujiwara, T., Roth, J.A., Deisseroth, A.B., Zhang, W.-W., Kruzel, E., and Radinsky, R. (1995) Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol. Cell. Biol.* **15**, 3032-3040
52. Schulze-Osthoff, K., Bakker, A.C., Vanhaesebroeck, B., Beyaert, R., Jacob, W.A., and Fiers, W. (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. *J. Biol. Chem.* **267**, 5317-5323
53. Wolvetang, E.J., Johnson, K.L., Krauer, K., Ralph, S.J., and Linnane, A.W. (1994) Mitochondrial respiratory chain inhibitors induce apoptosis. *FEBS Lett.* **339**, 40-44