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Inhibition of glucocorticoid-induced apoptosis by the expression of antisense gene of mitochondrial ATPase subunit 6[1]

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Abstract To isolate the apoptosis-linked genes involved in the cell death of thymocytes induced by glucocorticoids, we developed a functional cloning assay. Murine $CD4^+CD8^+$ thymic cell line 2-257-20 cells were transfected with cDNA expression libraries obtained from a dexamethasone-resistant cell line. The transfected cells were selected in the presence of dexamethasone, and the plasmids which episomally expanded were then extracted from the surviving cells. One of the rescued cDNAs was found to be an antisense cDNA fragment identical to the mouse mitochondrial ATPase 6 gene. In the stable transfectants with the ATPase 6 antisense gene, the induction of apoptosis by dexamethasone was significantly delayed. Furthermore, the ATP synthesis in these transfectants was also reduced to some extent. ATPase 6 is a subunit of F_oF_1 ATPase and our results support that ATP synthesis from the mitochondria is necessary for the induction of apoptosis induced by glucocorticoids.

Key words: Dexamethasone-induced apoptosis; ATP synthesis; Antisense cDNA; F_oF_1 ATPase; Functional cloning assay

1. Introduction

Apoptosis, or programmed cell death, is a naturally occurring process of cell suicide that plays a critical role in the development and maintenance of metazoans by eliminating either superfluous or unwanted cells [1,2]. More than 50 years ago, the thymus gland was first observed to undergo hypertrophy in association with adrenocortical insufficiency and atrophy in association with adrenocortical excess [3]. We now know that this phenomenon is due to the gain or loss of apoptosis in the thymocytes induced by glucocorticoids (GCs) [4]. Subsequently, it was clarified that GC-induced apoptosis is mediated through GC receptor and requires the translocation of the receptor from the cytoplasm into the nucleus. In the nucleus, the GC receptor functions as a transcription factor, either enhancing or repressing the expression of a selected repertoire of genes [5]. Furthermore, GC can modify the cellular functions by cross-talking with other transcription factors, notably AP-1 [6] and NF-κB [7]. Although a previous paper has reported GC-induced apoptosis to be primarily mediated by interference with other transcription factors essential for cell survival [8], Reichardt et al. presented compelling evidence that GC-induced apoptosis in murine thymocytes requires DNA binding of the GC receptor by introducing a point mutation into the GC receptor gene [9].

After the transcription of several genes, the signals from GC receptor are expected to be conveyed to the mitochondria. Thereafter, cytochrome *c* will be released and interact directly with Apaf-1. This complex activates caspase 9, which in turn activates caspase 3 or 7, followed by the cleavage of various death substrates [10]. The final steps of apoptosis induced by GCs have thus been rather well defined, but all the steps, especially those between the binding of GC receptor to DNA and the mitochondria, still remain to be elucidated.

In addition to the regulation of the cell number of thymocytes in the thymus, GC mainly exerts its lethal effect on the immature $CD4^+CD8^+$ thymocytes located in the cortex, and $CD4^+CD8^-$ or $CD4^-CD8^+$ cells in the medulla are relatively resistant [11]. This differential effect is similar to that observed in apoptosis induced via T cell receptor. Interestingly, although both stimuli are known to be lethal by themselves, GC- and T cell receptor-mediated signals prevent each other's induction of apoptosis in $CD4^+CD8^+$ thymocytes [12]. It is speculated that the balance of these two stimuli may in part thus regulate the process of thymocyte selection [13]. A clarification of all the steps of apoptosis induced by GCs will hopefully shed further light on this speculation.

We have designed a method to select genes involved in apoptosis in thymocytes by using a murine $CD3^+CD4^+CD8^+$ thymocyte cell line (2-257-20). This selection system is based on the assumption that a transfected cDNA library, constructed in the mammalian expression vector pPyori-SRα-BX, should protect some recipient cells from death [14–16]. Such an inhibition may depend on either the inactivation of apoptotic genes by antisense RNA or dominant negative mutants, or on the expression of proteins with anti-apoptotic activity. Using this system, we isolated several genes, designated dexamethasone-induced apoptosis related genes (DIARGs), and one of them was found to act as antisense of the ATPase 6 gene. The significance of ATP synthesis in the development of apoptosis by GCs was discussed.

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[1] The cDNA sequence data reported in the manuscript have been submitted to the GenBank database under accession number AF093677.

Abbreviations: BSD, blasticidin S hydrochloride drug-resistant; Dex, dexamethasone; DIARG, dexamethasone-induced apoptosis-related gene; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; GC, glucocorticoid; PI, propidium iodide; PS, phosphatidylserine; Py, polyoma virus; $\Delta\Psi_m$, mitochondrial membrane potential

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2. Materials and methods

2.1. Plasmids

pPyori-SRα-BX was constructed as follows. The *Bgl*II site of pPyOICAT [17], which was kindly supplied by Dr. Y. Ito (Kyoto University, Japan), was changed to the *Hin*dIII site. Then the *Hin*dIII–*Eco*RI fragment containing the polyoma virus (Py) replication origin core sequence was isolated. This fragment was inserted between the *Hin*dIII and *Eco*RI sites of pBluescript II SK (Stratagene, La Jolla, CA, USA). Next, the *Hin*dIII–*Sal*I fragment of pSRα-BX3 (Dr. Takebe, Japanese National Institute of Health), containing the SRα promoter sequence constructed from a pSRα296 expression vector, was inserted between the *Hin*dIII and *Eco*RI sites of the vector. The cDNA library was inserted into pPyori-SRα-BX. pPyLT-BSD was constructed as follows. The *Sma*I–*Eco*RI fragment of pMAM2-BSD (Funakoshi, Tokyo, Japan) containing the blasticidin S hydrochloride drug-resistant (BSD) sequence was isolated. The fragment was blunt ended using T4 polymerase (Toyobo, Osaka, Japan), ligated and then digested with *Bgl*II (pMAM2-BSDΔ*Eco*RI-Δ*Sma*I). The *Bgl*II–*Bam*HI fragment of pRSV-LT containing polyoma virus large T antigen was isolated and inserted into the *Bgl*II site of pMAM2-BSDΔ*Eco*RI-Δ*Sma*I.

2.2. Sequencing

The samples were prepared by performing fluorescence-based dideoxy sequencing reactions using the BigDye™ DNA Sequencing kit (ABI, Columbia, MD, USA). The samples were analyzed by the Applied Bio-systems model 310 DNA Sequencing System (PE Applied Biosystems, Foster City, CA, USA).

2.3. Cell lines

$CD3^+CD4^+CD8^+$ thymic cell line 2-257-20 was established from a cell suspension culture of $CD4^+CD8^+$ thymic lymphoma RVC [18] which was induced with a radiation leukemia virus in new born BALB/c mice. The cells were sensitive to the apoptosis induced by dexamethasone (Dex), cyclic AMP and anti-CD3 antibody. Dex-resistant cell line 7-15 was selected from ethyl methanesulfonate-treated 2-257-20 cells. Tag2 cells were established by transfecting 2-257-20 with pPyLT-BSD plasmid and express polyoma virus large T antigen. All of the murine thymocyte cell lines were maintained in a culture medium RPMI 1640 supplemented with 2 mM glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES and 10% fetal calf serum (FCS; Hyclone: lot AGD 6389, Logan, UT, USA).

2.4. Obtaining stable transfectants with the antisense ATPase 6 gene

Logarithmically growing 2-257-20 cells were harvested, washed and resuspended in phosphate-buffered saline (PBS). Next, 25 μg of expression plasmid pMAM2-BSD containing an antisense cDNA fragment was added to the cell suspension, and thereafter, electroporation was carried out at 300 mV and 960 μF with a Gene Pulsar (Bio-Rad, Hercules, CA, USA). The cells were allowed to recover in 10 ml of liquid culture medium for 24 h, and then the cells were distributed into each well of a 24-well plate at 2×10^5 cells/ml/well in the presence of blasticidin S hydrochloride (Funakoshi, Tokyo, Japan) at a final active concentration of 10 μg/ml. Drug-resistant colonies were visible 10–14 days later, removed from the plate, and grown in liquid culture medium. Mock transfectant M10 and stable transfectants with ATPase 6 antisense MMK4, MMK6, MMK8, MMK10, MMK12 and MMK27 were also used in this study.

2.5. Cell viability and cytofluorometric determination of apoptosis-associated alterations

The cells were suspended at 2×10^5/ml and 1 ml of cell suspension was seeded into each well of a 24-well plate. Cell death was induced by treatment with 1 μM Dex (Merck, Rahway, NJ, USA) and the viability was determined with trypan blue. The frequency of subdiploid cells was determined by staining Nonidet P40-permeabilized cells with propidium iodide (PI) (Sigma, St. Louis, MO, USA), and then the phosphatidylserine (PS) exposure on the outer membrane was assessed after treatment with FITC–annexin V (Boehringer Mannheim, Germany) (15 min at room temperature) and PI. To evaluate mitochondrial membrane potential ($\Delta\Psi_m$) [19], cells were incubated with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Molecular Probes, Eugene, OR, USA) at 20 nM for 15 min at 37°C in 5% CO_2. Cytofluorometry was performed on a FACSCalibur analyzer (Becton Dickinson, Mountain View, CA, USA).

2.6. Ribonuclease protection assay

To determine the expression of ATPase 6 antisense mRNA in transfected cell lines (MMK6, MMK12), the ribonuclease protection assay was performed using a RPA III™ kit (Ambion, Austin, TX, USA). Transfected cell lines were either untreated or stimulated with 1 μM Dex for 4 h. The cDNA fragment of ATPase 6 was cloned into pBluescript II SK (Stratagene, La Jolla, CA, USA) to synthesize an antisense cRNA probe labeled with [α-^{32}P]CTP (Amersham, UK) using T7 RNA polymerase (Gibco BRL, Rockville, MD, USA). In brief, 7.0×10^5 cpm of probe was hybridized to 20 μg of total RNA at 42°C for 12 h. Unhybridized RNA molecules were then digested with 112.5 units of RNase A and 4500 units of RNase T1. The protected molecules were resolved by electrophoresis and then were visualized by autoradiography.

2.7. ATP assays

The intracellular ATP levels of thymocyte cell lines were determined by the luciferin-luciferase method [20] after the cells were incubated in RPMI 1640 (10% FCS) medium for 24 h. Next, 3×10^6 cells were rapidly collected and washed with PBS, then the cells were resuspended in 500 μl of ice-cold 3% perchloric acid. The samples were centrifuged at 13 000 rpm for 10 min. A 400-μl aliquot of the supernatant was neutralized with KOH and HCl. The volume was adjusted to 500 μl with H_2O, and the samples were centrifuged. 10 μl of sample was injected into 200 μl of an ATP Determination kit standard reaction solution containing luciferin and luciferase (Molecular Probes, Eugene, OR, USA). The resulting light emission was then measured in a luminometer (Lumat LB 9501, Berthold, Germany). The inhibition of ATP synthesis by oligomycin was evaluated by incubating M10 and MMK12 cells in the presence of 10 μM oligomycin in a glucose-free RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and 10% dialyzed FCS.

3. Results

3.1. Cloning DIARG-1, an antisense cDNA fragment identical to the mouse mitochondrial ATPase 6 gene

A thymus cell line, Tag2, expressing polyoma LT antigen (pPyLT-BSD) was transfected with the cDNA library constructed from the Dex-resistant mutant subclone, 7-15, in pPyori-SRα-BX vector, and selected with both blasticidin S (10 μg/ml) and Dex (1 μM). Transfection was carried out with the DEAE dextran method modified by Takai et al. [21], and the transfection efficiency was determined to be around 1% using the lacZ expression vector (pSRα-lacZ). After 21 days, the cells that survived in the presence of Dex were collected, and then the episomal DNA was extracted. Several rescued pPyori-SRα plasmids were pooled (50 clones), re-transfected further and selected with both blasticidin S and Dex. After a

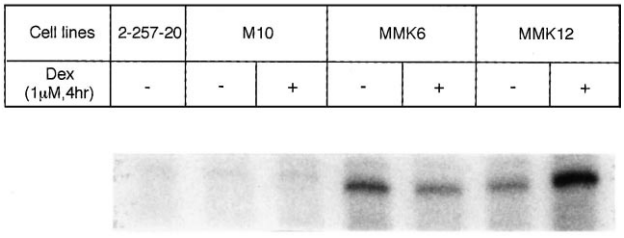

Fig. 1. Expression of ATPase 6 antisense mRNA in transfected cell lines. Transfectants of antisense ATPase 6 cDNA (MMK6 and MMK12) and mock transfectant (M10) were stimulated with or without 1 μM Dex for 4 h. Total RNA was extracted and the expression of antisense mRNA was detected with the ribonuclease protection assay as described in Section 2.

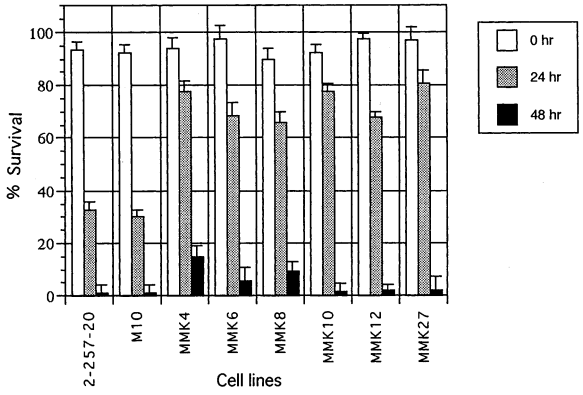

Fig. 2. Inhibition of cell death in transfectants with antisense ATPase 6 gene. The cells were resuspended in RPMI 1640 (10% FCS) medium and subjected to the following treatments at 37°C: 1 μM Dex for 0, 24 and 48 h. The percentage of surviving cell was calculated from the percentage of the trypan blue-negative populations.

third screening, we isolated several cDNA clones, designated DIARGs. The size of the cDNA inserts ranged between 300 and 4000 bp. Next, the cDNA inserts were sequenced. One of them, DIARG-1, was found to be an antisense cDNA fragment identical to the mouse mitochondrial ATPase 6 gene. This cDNA was 840 bp long, and the open reading frame started from the initiation codon at nucleotide positions 139–142 and ended at the termination codon TAA at positions 818–820. The homology among several species (mouse, rat, hamster, bovine and human) ranged between 71 and 85% (data not shown).

3.2. *Expression of ATPase 6 antisense mRNA in transfected cell lines*

To detect the expression of antisense mRNA of the ATPase 6 gene, MMK6 and MMK12 cells were incubated either with or without 1 μM Dex for 4 h. Total RNA was extracted and an RNA protection assay was performed. As shown in Fig. 1, the expression of mRNA was clearly detected in MMK6 and MMK12 cells without the stimulation of Dex, and in MMK12 cells the level of expression increased to some extent when stimulated with Dex. In contrast, no such expression was detected in either 2-257-20 cells or mock transfectant M10 cells.

3.3. *Inhibition of Dex-induced apoptosis in transfectants with antisense ATPase 6 cDNA*

Cell death was induced by treatment with 1 μM Dex in various transfectants. At 0, 24, and 48 h the cells were harvested and the cell viability was determined (Fig. 2). All of the six stable transfectants with antisense ATPase 6 showed a reduced susceptibility to the killing effect of Dex, especially when the viability was assessed at 24 h.

The induction of apoptosis causes various alterations in the cells, and it was investigated cytofluorometrically whether or not the expression of antisense ATPase 6 gene could also interfere with these alterations. One of the typical events in apoptosis is the fragmentation of nuclear DNA and this phenomenon can be detected as the appearance of subdiploid cells when the cell cycle was analyzed with PI. As shown in Fig. 3a, the increase in the frequency of subdiploid cells at 24 h was greatly reduced in MMK12 cells, although no large difference was observed between the M10 and MMK12 cells at 48 h.

Furthermore, in the advanced stage of apoptosis, PS is exposed to the outer plasma membrane, and this event is detectable as the binding of annexin V. The results in Fig. 3b again demonstrated that the alteration of the cell membrane at 24 h

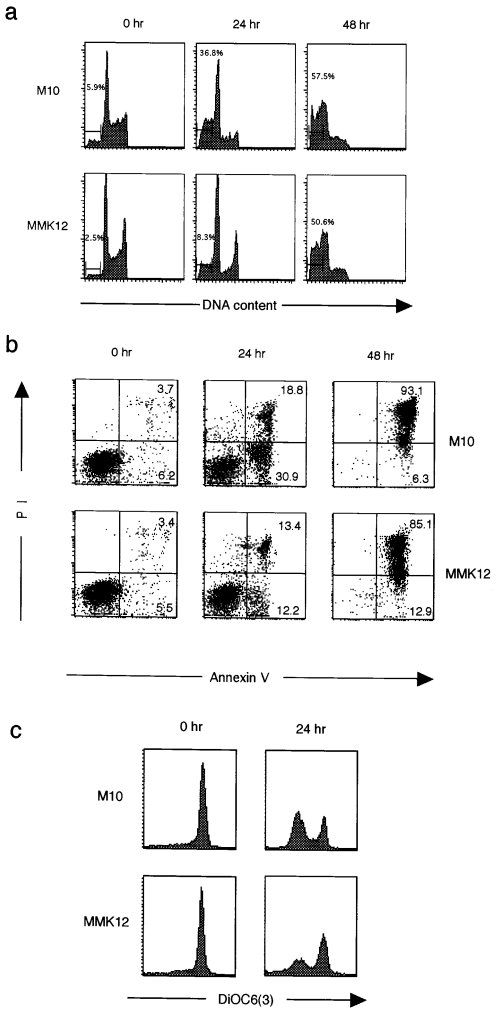

Fig. 3. Thymocyte cell lines expressing antisense transcripts to ATPase 6 are protected from apoptosis-associated alterations. a: A cell cycle analysis of thymocyte cell lines expressing antisense transcripts to ATPase 6. Exponentially growing M10 or MMK12 cells were washed twice and resuspended at a concentration of 2×10^5 cells/ml. After 0, 24 or 48 h, cells were stimulated with 1 μM Dex. At the indicated times, cells were collected, and the cell cycle distribution was assessed by PI staining and analyzed by FACSCalibur. The figures indicate the frequency of subdiploid cells. b: The kinetics of Dex-induced PS externalization on thymocyte cell lines expressing antisense transcripts to ATPase 6. Single cell suspensions of M10 and MMK12 cells were washed, resuspended at 5×10^5/ml in complete medium and plated in a final volume of 1 ml in 24-well flat-bottom tissue culture plates. The following cell death stimuli were administered: 1 μM Dex for 24 or 48 h. Treated and untreated cell lines were cultured at 37°C in 5% CO_2, followed by harvesting, staining with FITC–annexin V and PI for 15 min at room temperature. After washing twice, the cells were analyzed using a FACSCalibur using Cell Quest software. From each sample, 1×10^5 events were collected. c: The disruption of mitochondrial transmembrane potential was evaluated by staining M10 and MMK12 cells with Di-$OC_6(3)$ at 20 nM for 15 min, 37°C in 5% CO_2 24 h after stimulation with 1 μM Dex. Cytofluorometric analysis was performed on a FACSCalibur analyzer.

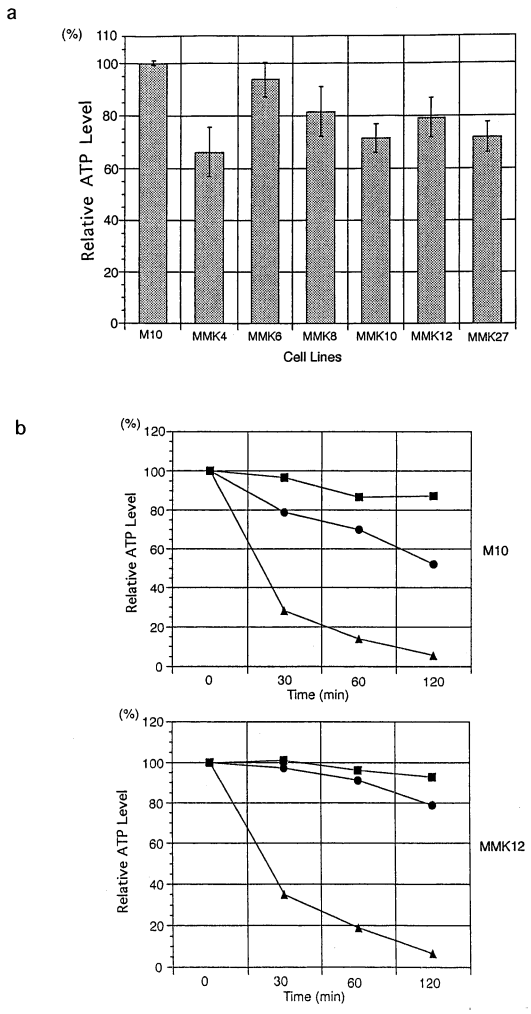

Fig. 4. Intracellular ATP level of thymocyte cell lines expressing antisense transcripts to ATPase 6. a: Determination of intracellular ATP. Intracellular ATP levels of thymocyte cell lines expressing antisense transcripts to ATPase 6 were determined by the luciferin-luciferase method after the cells were incubated without oligomycin in 0.35% glucose-containing RPMI. The ATP level of control cells (Mock; M10) cultured under normal conditions was regarded as 100%. b: Depletion of intracellular ATP level of M10 and MMK12 cells was determined by the luciferin-luciferase method after the cells were incubated for the indicated periods with 10 μM oligomycin in glucose-free RPMI (▲), without oligomycin in glucose-free RPMI (●) and without oligomycin in 0.35% glucose-containing RPMI (■). The ATP level of the cells cultured under normal conditions was regarded as 100%.

was also inhibited in MMK12 cells, although the difference at 48 h was marginal between M10 and MMK12 cells.

During the various steps before cell death, a disruption of $\Delta\Psi_m$ was also observed, and it is interesting to ask whether this disruption precedes or follows the inhibitory effects due to the expression of the antisense gene of mitochondrial ATPase 6. The potential-sensitive dye $DiOC_6(3)$ was used to evaluate the change in the mitochondrial membrane potential. As shown in Fig. 3c, the loss of $\Delta\Psi_m$ was less in MMK12 cells than that observed in M10 cells. This result suggests that the suppression of ATP production by the expression of antisense ATPase 6 thus appears to exert its effect upstream of the disruption of $\Delta\Psi_m$.

3.4. Suppression of ATP production in transfectants with the antisense ATPase 6 gene

To analyze the production of the ATP levels in the thymocyte cell lines transfected with control or antisense ATPase 6 gene vectors, the luciferin-luciferase method was used. Under regular culture conditions, maintained with 0.35% glucose-containing RPMI 1640, the intracellular ATP levels were more or less reduced in all the tested cell lines (Fig. 4a). However, by incubating the cells in a glucose-free medium with 10 μM oligomycin (an inhibitor of mitochondrial F_oF_1 ATPase), the kinetics in the reduction of intracellular ATP levels were not substantially changed between M10 and MMK12 cells (Fig. 4b). Furthermore, the addition of glucose or the omission of oligomycin also helped to maintain the level of ATP.

4. Discussion

In this report, we developed a functional cloning method to isolate the genes involved in the apoptosis of thymocytes induced by GCs. The cDNA library was prepared from the Dex-resistant thymic cell line, 7-15, and we expected the isolated genes to work as either antisense RNA, a dominant negative mutant or to show an overexpression of anti-apoptotic activity. We here described one of the genes isolated which was found to be identical to the antisense DNA of mitochondrial ATPase 6 gene which encodes a subunit of the F_oF_1 ATPase. Similar cloning methods have been introduced to identify apoptosis-linked genes. Kimchi et al. isolated a series of genes called DAP responsible for interferon-γ-induced cell death by transfection with antisense cDNA expression libraries [14]. Furthermore, by means of a functional selection strategy, the 'Requiem' gene was identified to be essential for apoptosis in the myeloid cell–interleukin 3 deprivation system [15], and ALG-2 and ALG-3 for apoptosis in the T cell hybridoma–TCR stimulus system [16]. It is interesting to note that all of the genes so far isolated with these methods are considered to work as antisense RNA.

As the isolated gene was a mitochondrial gene, the argument remains as to whether or not antisense RNA could cross the mitochondrial membrane. However, tRNA in the cytoplasm has been reported to be able to cross the mitochondrial membrane using specific receptors which can recognize specific nucleotide sequences and integrate tRNA into the mitochondria [22]. Furthermore, Shirafuji et al. demonstrated that antisense RNA for cytochrome *c* oxidase could cross the mitochondrial membrane to exert its inhibitory effect [23]. We also demonstrated that ATP production was suppressed to some extent in transfectants with the antisense ATPase 6 gene.

The altered function of mitochondria has been recognized for many years as an important contributor to ischemic and necrotic death. Recently, however, evidence has accumulated suggesting these organelles to play a critical role in apoptosis [24]. One of the functions of the mitochondria is assumed to be the production of ATP via the F_oF_1 ATPase proton pump [25]. By employing oligomycin, a specific inhibitor of the proton pump by binding to the F_o portion, it was demonstrated that a depletion of the intracellular ATP completely blocked apoptosis [26]. Furthermore, Matsuyama et al. screened for yeast mutants that do not exhibit pGal-Bax-induced death on a galactose-containing medium [27]. The authors isolated one

recessive Bax-resistant yeast mutant, and the isolated gene ATP 4 was found to encode a subunit of the F_o portion in the yeast F_oF_1 ATPase. Interestingly, ATPase 6 is also a subunit of the F_o portion in the mammalian F_oF_1 ATPase proton pump. In our experimental series, it is noteworthy that the ATPase 6 antisense gene was repeatedly isolated (unpublished observation), but the antisense genes against other subunits of F_oF_1 ATPase have not been identified so far. The F_oF_1 ATPase consists of F_o and F_1 portions and the crystal structure of the water-soluble F_1 portion has been solved. However, a transmembrane F_o portion is more complicated and little structural information exists. Therefore, most of the information regarding the F_o portion has been obtained based on an analysis of the F_oF_1 ATPase derived from *Escherichia coli*. The ATPase 6 is assumed to be equivalent to subunit *a* of the bacterial F_o portion, and F_o consists of three subunits $a_1b_2c_{12}$. According to Rastogi and Girvin [28], subunit *a* plays a critical role to lead rotation of the c_{12} oligomer. This rotation in turn causes a rotation of the ε and γ units of the F_1 portion. Since ATPase 6 works as a single molecule as compared to other subunits, it might thus be more easily affected by antisense gene expression.

Regarding apoptosis in thymocytes, there are at least two principal signaling pathways. Fas antigen (CD95)-mediated stimulation activates caspase 8 by rapid formation of the death-induced signalling complex, followed by the activation of caspase 3. The activation of this pathway is not blocked by Bcl-2 [10]. In contrast, apoptosis by various stimuli including GCs, γ-irradiation and anti-CD3 antibody is initiated at the mitochondrion. Cytochrome *c* is released from the mitochondria into the cytosol, and together with ATP it binds to Apaf-1 [29,30]. This event unmasks the caspase recruitment domain motif in Apaf-1 and allows the binding of procaspase 9. This pathway is inhibited by Bcl-2 [31]. Following the activation of the initiator caspase 8 or caspase 9, the two pathways converge on the activation of caspase 3, which finally brings cell death. However, in caspase 3 knockout mice, thymocytes remain sensitive to Dex, and the death pathway by Dex may use caspase 6, 7 or some other molecules instead of caspase 3 [10]. In these pathways, it is reasonable to assume that an overexpression of antisense transcription of ATPase 6 reduces ATP production, which in turn prevents the complex formation of Apaf-1 and cytochrome *c*. This prevention delays the activation of caspase 9, and consequently various changes characteristic of apoptosis are thus blocked as demonstrated herein. However, ATP is also assumed to be involved in the step(s) downstream of caspase 3 [32]. Furthermore, Chvatchko et al. reported that $P_{2\times1}$ molecules, a non-selective cation channel activated by ATP, are upregulated in thymocytes during Dex-induced apoptosis and that antagonists to these receptors protect thymocytes from cell death [33]. In addition, Stefanelli et al. [26] demonstrated that ATP depletion (in the presence of oligomycin) inhibits GC-induced thymocyte apoptosis, and they presented evidence that the inhibition of apoptosis may thus be due to a reduction in the binding of GC receptor to the thymocyte nuclei. It is, therefore, possible that these functions of ATP may also contribute to the effects observed in this experiment. We hope that other genes obtainable from this series of experiments will clarify more precisely the mechanism of apoptosis induced by GCs and the role of GCs in the maturation and differentiation of thymocytes.

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