Regulation of human male germ cell death by modulators of ATP production

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The understanding of testicular physiology, pathology, and male fertility issues requires knowledge of male germ cell death and energy production. Here, we induced human male germ cell apoptosis (detected by Southern blot analysis of DNA fragmentation, TUNEL, activation of caspases-3 and -9, and electron microscopy) by incubating seminiferous tubule segments under hormone- and serum-free conditions. Inhibitors of complexes I to IV of mitochondrial respiration, exposure to anoxia, and inhibition of F0F1-ATPase (with oligomycin) decreased the ATP levels (analyzed by HPLC) and suppressed apoptosis at 4 h. Uncoupler 2,4-dinitrophenol (DNP) and oligomycin combination also suppressed death at 4 h, as did the DNP alone. Inhibition of glycolysis by 2-deoxyglucose neither suppressed nor further induced apoptosis nor altered the antia apoptotic effects of the mitochondrial inhibitors. Furthermore, Fas system activation did not modify the effects of mitochondrial modulators. After 24 h, delayed male germ cell apoptosis was observed despite the presence of the mitochondrial inhibitors. We conclude that the mitochondrial ATP production machinery plays an important role in regulating in vitro-induced primary pathways of human male germ apoptosis. The ATP synthesized by the F0F1-ATPase seems to be the crucial death regulator, rather than any of the complexes (I-IV) alone, the functional electron transport chain, or the membrane potential. We also conclude that there seem to be secondary pathways of human testicular cell apoptosis that do not require mitochondrial ATP production. The present study emphasizes the role of the main catabolic pathways in the complex network of regulating events of male germ cell life and death.

testis; spermatogenesis; apoptosis; mitochondria; oxidative phosphorylation

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germ cell death (22) strongly suggests that the ATP machinery is not only the target of the death events but also a regulator of male germ apoptosis. However, whether it is the ATP (or its concentration) as such or whether it is a certain site of the machinery that controls death is not known. For nontesticular cells, there are multiple proposals for the crucial death-regulating site, including the ATP itself or certain OXPHOS component(s), such as individual complexes, the ΔΨm, or the ATP synthase (7, 10, 11, 17, 25, 32, 39, 40, 52, 57).

The purpose of the present study was to evaluate the relationship(s) of the following two fundamental entities of testicular functions: the male germ cell death and the ATP-producing machinery. The goal was to find out whether the ATP-producing machinery or a particular part of OXPHOS would regulate human testicular cell death. More specifically, we aimed at investigating the roles of individual mitochondrial complexes, the FoF1-ATP synthase, by using specific inhibitors and of uncoupling of OXPHOS in human male germ cell apoptosis. In addition, the study was designed to determine whether blocking of glycolysis or activating the Fas system would modulate male germ cell death, which was induced by incubating segments of human seminiferous tubules under hormone- and serum-free conditions. Characterizing and understanding the basic mechanisms involved in male germ cell apoptosis are essential steps for the development of novel therapeutic regiments to control accelerated apoptosis during abnormal spermatogenesis as well as for more targeted approaches to male contraception.

MATERIALS AND METHODS

**Patients.** Testicular tissue was obtained from 41 adult men aged 52–89 yr undergoing orchidectomy as a treatment for prostate cancer. The patients had received neither hormonal nor chemotherapeutic medication nor had they received radiotherapy before the operation, and none of them had suffered from cryptorchidism. The operations were performed between November 1998 and July 2005 at the Department of Urology, Helsinki University Central Hospital (Helsinki, Finland). The Ethics Committees of the Departments of Pediatrics and of Urology, University of Helsinki, approved the study protocol (number 14/95).

**Tissue preparation and induction of apoptosis.** To maintain the physiological cell-to-cell interactions of testicular cells, segments of seminiferous tubules were cultured instead of isolated cells. The tissue culture, including the apoptosis-inducing conditions and the time points, was based on our previously described in vitro model (18–20). Briefly, immediately after the testis tissues from the operations were obtained, segments of seminiferous tubules (1–5 mm in length) were microdissected in petri dishes containing tissue culture medium (Nutrient mixture Ham’s F-10 containing 6.1 mmol/l of D-glucose and 1.0 mmol/l of sodium pyruvate; Gibco Europe, Paisley, UK) supplemented with 0.1% of human serum albumin (Sigma Chemical, St. Louis, MO) and 10 μg/ml of gentamicin (Gibco). For induction of apoptosis, segments of seminiferous tubules were incubated to culture plates containing the hormone- and serum-free culture medium described above and incubated for 4 or 24 h at 34°C in humidified room air with CO2 adjusted to 5%.

**Inhibition of the complexes of mitochondrial respiratory chain.** To evaluate the effects of the different complexes of the mitochondrial respiratory chain on human testicular cell apoptosis, specific inhibitors of the complexes were added to the culture medium. NADH-ubiquinone oxidoreductase (complex I) was inhibited with rotenone; succinate-ubiquinone oxidoreductase (complex II) with thenoyltrifluoroacetone (TTFA); ubiquinol-cyt c oxidoreductase (the bc1-complex or complex III) with antimycin A; and cyt c oxidase (cytochrome aa3 or complex IV) with KCN (all derived from Sigma Chemical). The final concentrations of rotenone were 10 and 100 μM, of TTFA 100 and 200 μM, of antimycin A 10 and 20 μM, and of KCN 5 and 10 mM. In all experiments, the pH was neutralized before culture.

**Exposure to anoxia.** Exposure to anoxia was performed by culturing the samples in humidified tight glass chambers under continuous gas flow (minimum 1 l/h) from gas bottles containing 5% of CO2 and 95% of nitrogen (maximum <10 parts/million of O2; Aga, Espoo, Finland).

**Inhibition of the FoF1-ATP synthase and uncoupling of OXPHOS.** The mitochondrial FoF1-ATP synthase was inhibited by adding oligomycin ABC (Sigma) to the cultures at final concentrations of 200 and 400 μM. Uncoupling of OXPHOS was achieved with 2,4-dinitrophenol (DNP; Sigma) at final concentrations of 200 and 400 μM. To create a situation in which the respiratory chain is active, but where ATP synthase is inhibited, a combination of uncoupler (DNP) and the FoF1-ATP synthase inhibitor oligomycin was used. In all experiments, the pH was neutralized before culture.

**Blockade of glycolysis.** Cytosolic ATP production was inhibited by preventing glycolysis with 2-deoxyglucose (2-DG, Sigma) at concentrations of 5 or 10 mmol/l. To inhibit both cytosolic and mitochondrial ATP production, combinations of 2-DG and inhibitors of mitochondrial respiration (rotenone, TTFA, antimycin, KCN, or oligomycin) were used in the cultures.

**Activation of the Fas receptors.** To evaluate whether activation of the Fas system would modulate the effects of mitochondrial inhibitors, or vice versa, agonistic anti-Fas antibody (Roche Molecular Biochemicals, Mannheim, Germany) was added in the cultures. The final concentrations were 2 and 5 μg/ml, and it was used in the absence or the presence of KCN, DNP, or oligomycin. In the pilot experiments, recombinant human Fas ligand (Fas-L; Calbiochem-Oncogene Research Products, San Diego, CA) was also tested, instead of anti-Fas antibody being activated. The final concentrations of Fas-L were 0.5 and 1.0 μg/ml.

**Detection of cell death by Southern blot analysis of DNA fragmentation.** Segments of seminiferous tubules were snap-frozen in liquid nitrogen and stored at −80°C until isolation of DNA. DNA was extracted using the Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals), as previously described (50). After isolation, the DNA was quantified by spectrophotometry (absorbance at 260 nm). DNA samples (1 μg) were 3’-end-labeled with digoxigenin-dideoxy-UTP (Roche) using the terminal transferase (Roche) reaction, subjected to electrophoresis on 2% agarose gels, and blotted on nylon membranes. DNA was cross-linked to the membranes by ultraviolet irradiation. The membranes were washed and blocked with 1% Blocking reagent (Roche). After 3’-end-labeling of DNA, the membranes were localized with alkaline phosphatase-conjugated anti-digoxigenin antibody (Anti-Digoxigenin-AP; Roche) as described previously (19). After being washed with washing buffer (0.1% Tween 20 in maleic acid buffer), the membrane was equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl2, pH 9.5), and it was used in the absence or the presence of KCN, DNP, or oligomycin. In the pilot experiments, recombinant human Fas ligand (Fas-L; Calbiochem-Oncogene Research Products, San Diego, CA) was also tested, instead of anti-Fas antibody being activated. The final concentrations of Fas-L were 0.5 and 1.0 μg/ml.

**In situ end-labeling (TUNEL) of DNA and propidium iodide staining.** In situ end-labeling of DNA was performed using the TUNEL kit (Roche), as previously described. Briefly, the microscopic slides were fixed in liquid nitrogen, the cover
slips were removed, and the frozen slides were dipped in ice-cold ethanol and then fixed for 10 min in formalin and washed in PBS. The slides were then kept in ethanol-acetic acid (2:1) for 5 min at −20°C, then washed in PBS, dehydrated, and stored at −20°C until they were stained. DNA in situ 3′-end-labelling was performed as described earlier (50) with modifications. Briefly, after rehydration and permeabilization in a microwave oven for 5 min in 10 mmol/l citric acid (pH 6.0), the samples were preincubated with terminal transferase reaction buffer (1 mol/l sodium cacodylate, 125 mmol/l Tris·HCl, and 1.25 mg/ml BSA, pH 6.6). The DNA in the samples was 3′-end-labeled with Dig-dd-UTP (Roche) by the terminal transferase (Roche) reaction for 1 h at 37°C. For the negative controls, the terminal transferase enzyme was replaced with the same volume of distilled water. The samples were then treated with blocking solution [2% Blocking reagent (Roche) in 150 mmol/l NaCl and 100 mmol/l Tris·HCl, pH 7.5]. Antidigoxigenin antibody conjugated with horseradish peroxidase (Anti-Digoxigenin-POD; Roche) was used to detect the Dig-dd-UTP-labeled DNA. The bound antibody was then localized, using diaminobenzidine (Sigma), after which the slides were weakly counterstained with hematoxylin and dehydrated. Alternatively to TUNEL, the samples were stained with propidium iodide according to the manufacturer’s protocol (Vector Laboratories, Burlingame, CA) to test the viability of the cells.

**Evaluation of caspase activation.** In addition to our previously reported detection of the activation of caspase-3 during the cultures (61, 62), we evaluated the activation of caspase-9 by Western blot analysis, which was performed by the NuPage Bis-Tris gel system (Invitrogen, Frederick, MD) according to the manufacturer’s instructions. In brief, total tissue proteins (50 µg) were separated on 4–12% NuPage Bis-Tris gradient gels, and electrophoresis was performed at 180 volts. The proteins from the gels were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA), and the transfer was checked by staining with 0.2% Ponceau S in 3% TCA. After being blocked, caspase-9 was detected with caspase-9 rabbit polyclonal antibody (H-170; Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes both the precursor and the cleaved forms of this caspase. After washes, the membranes were incubated with peroxidase-conjugated anti-rabbit IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA). The bound secondary antibodies were located with an electron chemiluminescence (ECL plus) detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The loading was controlled by reprobing with α-tubulin antibody (Sigma).

**Electron microscopy.** For electron microscopy (EM), segments of seminiferous tubules were fixed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.2, postfixed with 1% osmium tetroxide in 0.1 mol/l phosphate buffer, dehydrated, and embedded in epoxy resin. Tissue blocks were then sectioned at 50 nm with a Reichert E ultramicrotome (Reichert Jung, Vienna, Austria). The samples were stained with uranyl acetate and lead citrate with a Leica EMstain apparatus (Leica, Vienna, Austria). Observations were made with a JEOL JEM 1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

**Determination of adenine nucleotides by HPLC.** Samples of testicular tissue were snap-frozen in liquid nitrogen. To extract the adenine (ANs; ATP, ADP, and AMP), the tissues were homogenized in 0.42 N ice-cold perchloric acid. The homogenates were then neutralized with 4.42 N KOH and centrifuged. During these procedures, the samples were kept on ice. The AN concentrations in the supernatants were determined by HPLC using a Shimadzu LC 10AD vp liquid chromatograph with a reversed-phase column (Ultra Techsphere 5 ODS; Labtronic Oy, Vantaa, Finland) and an ultraviolet detector set at 254 nm. The published method (12) was modified as follows: buffer A (0.1 M KH2PO4 and 8.0 mM tetrabutylammonium hydrogen sulfate, pH 6.0) was run at 1.5 ml/min for 2.5 min, followed by a linear increase during 10 min to 100% buffer B (buffer A with 30% methanol), which was continued for 2.5 min and followed by a linear increase during 1 min to 100% buffer A, which was run for 4 min. The compounds were identified and quantified by comparison with the retention times and peak areas of known standards, calibrated by spectrophotometry. The AN concentrations were expressed in relation to testis tissue wet weight (µmol/g of testis).

**Statistical analysis.** The experiments were repeated on at least three independent occasions. For statistical comparisons, data obtained from the replicate experiments (means ± SE) were analyzed by one-way ANOVA followed by the post hoc test with Bonferroni correction. A P value <0.05 was considered significant.

**RESULTS**

**Inhibitors of mitochondrial respiratory complexes prevent human testicular apoptosis and suppress the ATP levels at 4 h.** In line with our previous results (18–20), incubation of human seminiferous tubule segments under serum- and hormone-free culture conditions induced apoptosis in male germ cells (Figs. 1 and 2). Apoptosis was detected by Southern blot analysis of DNA fragmentation (Fig. 1, A and B), by activation of the caspase-3 (as previously described and Refs. 61 and 62) and caspase-9 (Fig. 1F), and by in situ end labeling of the apoptotic DNA (TUNEL; Fig. 2, A–D). The apoptotic nature of cell death and the identity of dying cells were further confirmed by EM. Consistent with our previous studies (17–19), the dying cells were identified as mainly spermatocytes and spermatids.

The testicular cell death was effectively prevented by all the specific inhibitors of individual mitochondrial complexes (I–IV) at 4 h (Figs. 1 and 2). Low-molecular-weight DNA fragmentation was suppressed by 82% (P < 0.001), 82% (P < 0.001), 70% (P < 0.001), and 78% (P < 0.001) after 4 h of exposure to the inhibitor of OXPHOS complex I (100 µM rotenone), complex II (200 µM THTFA), complex III (20 µM antimycin A), and complex IV (10 mM KCN), respectively, relative to the 4-h control (Fig. 1, A and B). The lower concentrations of the inhibitors (10 µM rotenone, 100 µM THTFA, 10 µM antimycin, and 5 mM KCN) also had antia apoptotic effects that did not significantly differ from those obtained with the higher concentrations of the inhibitors (data not shown). In addition to the apoptotic laddering in the Southern blot analysis of DNA fragmentation, some unspecific smearing of DNA, indicating necrotic death, was seen in occasional experiments (data not shown). Consistent with the Southern blot analysis and TUNEL, most of the testicular cells showed normal morphology under the EM (Fig. 2, E and H, top) after treatment with the mitochondrial inhibitors (rotenone, THTFA, antimycin A, KCN, or oligomycin) for 4 h. Occasional spermatocytes and spermatids showed signs of typical apoptosis (Fig. 2F). Germ cells showing atypical ultrastructural morphology or necrosis were rarely seen (Fig. 2, G and H). This was further confirmed by propidium iodide staining for cell viability (data not shown). In addition, the suppression of the caspase activation by the OXPHOS inhibitors (Fig. 1F) demonstrates that the effects of these compounds on the apoptotic pathways occur both at the level of caspase activation and DNA fragmentation.

Each inhibitor significantly suppressed the ATP level compared with the control samples but did not totally deplete ATP (Fig. 1C). The AMP levels were also diminished significantly compared with the 4-h control samples (Fig. 1D), whereas the ADP levels showed more variability, and the decline did not reach statistical significance (Fig. 1E).
Exposure to gas anoxia suppresses germ cell death after 4 h of culture. To further assess the role of mitochondrial respiration, the samples were exposed to anoxic conditions. Culture of the testicular samples for 4 h in these conditions significantly suppressed germ cell death and resulted in a 69% (P < 0.01) decrease of DNA laddering relative to the 4-h control sample (Fig. 1, A and B). Exposure to anoxia resulted in a decline of the ATP levels (Fig. 1C) and of ADP and AMP (data not shown), but these effects were not significant when compared with control samples. There are several possible explanations for this relatively slight AN decline. For example, although the glass chamber in which the samples were cultured was very tight and the anoxic gas flow was continuous, the possibility of some minor oxygen leakage cannot be excluded totally. More-
over, the samples were not totally anoxic during the microdissection before the incubation period, which differs from the case with mitochondrial inhibitors, which were present already during microdissection. Nevertheless, anoxic conditions during the 4-h incubation effectively suppressed germ cell apoptosis.

**Inhibition of the F_0F_1-ATP synthase and/or uncoupling of mitochondrial respiration suppress apoptotic DNA fragmentation at 4 h.** Similar to the effect of respiratory chain inhibitors and anoxia, oligomycin, an inhibitor of the ATP-forming mitochondrial ATP synthase (F_0F_1-ATPase), suppressed apoptotic DNA fragmentation at 4 h (Fig. 3A and B). A combination of oligomycin and DNP, which allows activity of the respiratory chain while ATP synthase is blocked, also prevented male germ cell death at 4 h, as did DNP by itself (Fig. 3A and B). Oligomycin and DNP separately, and their combination, again significantly suppressed the ATP (and ADP and AMP) levels, as measured by HPLC and compared with the 4-h control samples (Fig. 3C). These effects of oligomycin and DNP were similar with the lower concentration of inhibitor (see MATERIALS AND METHODS).

**Inhibition of glycolysis decreases ATP levels but does not suppress germ cell apoptosis.** To study the role of glycolysis in testicular cell death, 2-DG, an antimetabolite of glucose, was added to the culture. Exposure to 2-DG (5 and 10 mM) decreased the ATP levels measured by HPLC compared with the samples cultured without 2-DG (4 h control vs. 4 h 2-DG; Fig. 4C). However, the decline in ATP concentration was statistically significantly less than that observed with the mitochondrial inhibitors (Fig. 4C). Also, 2-DG did not notably affect apoptotic DNA laddering (Fig. 4, A and B). Apparently, a blockade of glycolysis did neither inhibit nor further stimulate apoptosis in the present model.

A combination of 2-DG with an inhibitor of mitochondrial respiration (rotenone, TTFA, antimycin, KCN, or oligomycin) was then used to create a situation where no ATP production would take place via glycolysis or OXPHOS. Such combinations depleted the ATP levels (Fig. 4C) and suppressed the apoptotic DNA laddering at 4 h (Fig. 4, A and B), much like the cases with the inhibitors of mitochondrial OXPHOS alone. In the Southern blot analysis, some mild unspecific smearing of DNA, indicating necrotic death, was seen sporadically (data not shown).

**Exposure to the inhibitors of mitochondrial ATP production (+ 2-DG) results in delayed testicular apoptosis at 24 h.** After 24 h of incubation, the inhibitors of mitochondrial ATP production (rotenone, TTFA, antimycin A, and oligomycin), with or without 2-DG, still suppressed germ cell death (Fig. 4D), although this effect was no longer significant, and clear DNA laddering indicating delayed testicular apoptosis was observed (Fig. 4, D and E). The levels of ATP (Fig. 4F), ADP, and AMP (data not shown) were further depleted compared with the 4-h samples. In Southern blot analysis, in addition to laddering, some unspecific smearing of DNA, indicating necrotic death, was seen in occasional experiments (data not shown). EM results were consistent with the Southern blot analysis and showed typical apoptotic death in numerous germ cells (data not shown) at 24 h. In addition, sporadic cells appeared necrotic or intoxicated, with dark and dense nuclei with irregular clumping of chromatin, swollen unrecognizable cytoplasmic organelles, and nondetectable plasma membranes (data not shown), which suggests that some individual testicular cells may be more sensitive to the toxic/necrotic effects than others (data not shown).

**Activation of the Fas system does not induce apoptosis in the presence of mitochondrial inhibitors.** The antiapoptotic effects of the mitochondrial inhibitors KCN, DNP (Fig. 5), or oligomycin were not modified by activation of the Fas system at 4 h. Even though the activating anti-Fas antibody maintains its activity in nontesticular systems, the use of antibodies may have tissue-specific (and other, such as target specificity) concerns. Of note, in terms of concerns, we do not, in this particular tissue model, mean inability of the antibodies to pass through the blood-testis barrier. This is because, first, we are using segments of tubules in vitro, and the antibodies are introduced to testicular cells not only from outside the tubules but also from the inside, i.e., from lumen, and second because we have previously used other antibodies, such as antagonist antibodies to the Fas system (48), that do affect germ cell apoptosis in the present culture model. Nevertheless, in the
present study, we used a recombinant human Fas-L protein, instead of the antibody, in the preliminary experiments. The results obtained with the use of either agonistic anti-Fas antibody (Fig. 5) or Fas-L (data not shown) (± KCN) did not differ from each other, thus supporting the use of the activating anti-Fas antibody in further studies.

**DISCUSSION**

In the present investigation, we studied the roles of modulators of ATP production in human male germ cell death. Because cell-to-cell interactions play an important role in the regulation of male germ cell energy metabolism, catabolism, and death, as well as in the overall physiology and pathology of the testis (6, 14, 27, 51, 60), incubation of isolated cells was considered inappropriate. Therefore, we used a tissue culture model in which segments of human seminiferous tubules are cultured. The tissue culture model, like all in vitro models, has limitations. However, it has the advantage of maintaining the physiological contacts between the Sertoli cells and the germ cells, which allows studies on apoptotic mechanisms involving different cell types. Furthermore, physiological concentrations of testicular compounds, testosterone, estradiol, and lactate, prevent germ cell apoptosis in this model (18, 19, 50). To our knowledge, there are currently no other methods that would be more sophisticated for manipulating human male germ cell death in an environment maintaining the physiological interactions. Therefore, we think that, at present, the in vitro tissue culture model gives the best available way to investigate apoptotic mechanisms within the human seminiferous tubules.

In the present study, each of the inhibitors of mitochondrial complexes I to IV at 4 h (i.e., rotenone, TTFA, antimycin A, and KCN, respectively; Fig. 1) effectively prevented the apoptotic death of spermatocytes and spermatids induced by hormone- and serum-free culture conditions. Hence, it is very unlikely that any one of the complexes alone is crucial for the primary apoptotic pathways triggered by the culture conditions. In other words, if one of the inhibitors alone, such as the previously reported KCN (22), had shown the antiapoptotic effect while others did not, it would have indicated the importance of the particlar complex (e.g., IV), but this was not the case. Thus our findings strongly suggest that the antiapoptotic effect of the respiratory chain inhibitors must be explained by the effect of each of them in blocking collective mitochondrial ATP machinery activity. This conclusion is further supported by the similar effect of anoxia, which also blocks the flux of reducing equivalents in the respiratory chain.

ATP generation by the F0F1-ATP synthase is crucially dependent on an active respiratory chain. Specific inhibition of ATP synthase resulted in an antiapoptotic effect of similar magnitude as that resulting from respiratory chain inhibition by 10.220.33.6 on April 12, 2017 http://ajpendo.physiology.org/ Downloaded from Fig. 3. Inhibition of the F0F1-ATP synthase and/or uncoupling of the mitochondrial respiration suppress testicular apoptosis at 4 h. Segments of human seminiferous tubules were incubated under hormone- and serum-free culture conditions. Mitochondrial F0F1-ATP synthase was inhibited with oligomycin (200 μM), and uncoupling was chemically performed with 2,4-dinitrophenol (DNP, 200 μM). A: Southern blot analysis of DNA fragmentation. After 4 h of culture, oligomycin effectively suppressed testicular apoptosis. A combination of oligomycin and DNP, which allows activity of the respiratory chain while ATP synthase is blocked, also inhibited male germ cell death at 4 h, as did the DNP by itself. B: quantification of low-molecular-weight (mw) DNA (<1.3 kb) fragmentation. C: HPLC analysis. Compared with the 4-h controls, testicular ATP levels were further decreased by oligomycin and/or DNP. Each value represents a mean of replicate experiments ± SE. **p < 0.01 and ***p < 0.001. Nos. in parentheses indicate the no. of replicate experiments in each treatment.
Fig. 4. Inhibition of glycolysis neither suppresses germ cell apoptosis nor alters the testicular apoptosis, which is delayed by inhibitors of mitochondrial OXPHOS. Segments of human seminiferous tubules were cultured for 4 or 24 h. Glycolysis was prevented by adding 2-DG (10 mM), an antimetabolite of glucose, to the cultures. Complex I of the respiratory chain was inhibited with rotenone (100 nM) and F1F0-ATP synthase with oligomycin (oligo, 200 nM).

A: Southern blot analysis of DNA fragmentation. After 4 h of culture, blocking of glycolysis by 2-DG did not suppress (or further induce) testicular apoptosis nor alter the antiapoptotic effects of the mitochondrial modulators.

B: quantification of low-molecular-weight DNA (<1.3 kb) fragmentation.

C: HPLC analysis. Compared with the 4 h controls, testicular ATP levels were further decreased by 2-DG and/or by the mitochondrial inhibitors. D: after 24 h of incubation, the inhibitors of mitochondrial ATP production (rote and oligo), with or without 2-DG, still suppressed germ cell death, but this antiapoptotic effect was no longer significant, and clear DNA laddering indicating delayed testicular apoptosis was observed. E: quantification of low-molecular-weight DNA (<1.3 kb) fragmentation. F: at 24 h, the ATP levels measured by HPLC were declined in the control cultures (24 h control) and further depleted by 2-DG, rotenone, and oligomycin. Each value represents a mean of replicate experiments ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001. Nos. in parentheses indicate the no. of replicate experiments in each treatment.
A mean of 3 replicate experiments of low-molecular-weight DNA (1.3 kb) fragmentation. Each value represents coupler presensitize the testicular cells to Fas-induced death. mitochondrial ATP production was impaired, nor did the mitochondrial un-

Fas receptor by agonistic anti-Fas antibody did not induce apoptosis when (5 mg/ml).

different primary action as, e.g., antimycin, oligomycin, and
dissipated by blocking the respiratory chain, or by uncoupling, IM, because, although the electrochemical proton gradient is

makes a distinction between the cell types. Therefore, the possibility that the observed ATP concentration decrease (mediated by 2-DG) reflects the decrease taking place in other cells than those undergoing apoptosis. Supportive of this notion is the demonstration that these other nondying cell types, such as Sertoli cells and spermatogonia, are known to use glycolysis for their energy production (whereas the dying cell types prefer OXPHOS; see Refs. 3, 28, 45, 53, 64). Of note, when using OXPHOS inhibitors, we cannot exclude the possibility that the ATP decrease would occur in different cells than the death process. However, this seems unlikely, and we rather think that, with these mitochondrial inhibitors, the de-

tective ATP (because the ATP levels were not totally de-

2-DG) were no longer significant after 24 h of incubation, and clear DNA laddering indicating delayed testicular cell apoptosis was observed (Fig. 4, D and E). EM further con-
firmed that the type of death was mainly apoptotic. That the germ cells were able to die despite the inhibitors of the ATP production machinery indicates activation of secondary apoptotic pathways within these cells. These secondary pathways appear not to be regulated by the OXPHOS-associated factors. Furthermore, that the germ cells were able to die by apoptosis when ATP was depleted indicates that these secondary pathways may not require ATP either. This is supported by findings with nontesticular cells showing, on one hand, the existence of pathways that appear not to require mitochondrial functions or ATP (2, 10, 17, 23, 36, 44, 66) and, on the other, activation of

Fig. 5. Antiapoptotic effect of the mitochondrial inhibitor was not modified by activating the Fas system. Segments of seminiferous tubules were cultured under serum- and hormone-free culture conditions in the absence or presence of mitochondrial uncoupler DNP (200 μM) and/or agonistic anti-Fas antibody (5 mg/ml). A: Southern blot analysis of DNA fragmentation. Activation of the Fas receptor by agonistic anti-Fas antibody did not induce apoptosis when mitochondrial ATP production was impaired, nor did the mitochondrial uncoupler presensitize the testicular cells to Fas-induced death. B: quantification of low-molecular-weight DNA (<1.3 kb) fragmentation. Each value represents a mean of 3 replicate experiments ± SE.

It seems unreasonable to assume that compounds with such different primary action as, e.g., antimycin, oligomycin, and 2,4-DNP would all block apoptosis, unless their action would lead to a common effect. Indeed, the obvious common feature is blockade of the F₁F₀-ATP synthase activity. This occurs specifically with oligomycin and indirectly by either blocking the respiratory chain (at any respiratory chain complex and including anoxia) or by uncoupling the OXPHOS. Furthermore, the results specifically exclude other potentially important mitochondrial parameters, such as the membrane potential, as being involved in germ cell apoptosis. Although the ATP synthase itself has been suggested to be important for the death control (39, 40, 57), the most obvious explanation for the observed effects is the lowered concentration of mitochondrial ATP. In some nontesticular cells, ATP is, indeed, proposed to be necessary for the apoptotic program, which is an active process that consequently may require energy in the form of ATP (9, 33, 52). Of note, apoptotic pathways independent of ATP production have also been described, and the role of the ATP production machinery in controlling cell death appears to depend on the cell type and on the inducer of apoptosis, as seems to be the case with most of the regulators of apoptosis (2, 10, 17, 23, 36, 44, 59, 66).

In the present study, 2-DG, an antimetabolite of glucose, decreased the ATP levels significantly less than inhibition of the OXPHOS, and apoptosis was unaffected (Fig. 4). Hence the first possibility is that ATP concentration may have to be decreased under a critical threshold to achieve the antiapoptotic effect. Here it must be emphasized that the concentrations of ATP were determined from total samples of segments of seminiferous tubules. Thus the determination neither distinguishes between mitochondrial and cytoplasmic ATP nor makes a distinction between the cell types. Therefore, the second possibility is that the observed ATP concentration decrease (mediated by 2-DG) reflects the decrease taking place in other cells than those undergoing apoptosis. Supportive of this notion is the demonstration that these other nondying cell types, such as Sertoli cells and spermatogonia, are known to use glycolysis for their energy production (whereas the dying cell types prefer OXPHOS; see Refs. 3, 28, 45, 53, 64). Of note, when using OXPHOS inhibitors, we cannot exclude the possibility that the ATP decrease would occur in different cells than the death process. However, this seems unlikely, and we rather think that, with these mitochondrial inhibitors, the de-

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firmed that the type of death was mainly apoptotic. That the germ cells were able to die despite the inhibitors of the ATP production machinery indicates activation of secondary apoptotic pathways within these cells. These secondary pathways appear not to be regulated by the OXPHOS-associated factors. Furthermore, that the germ cells were able to die by apoptosis when ATP was depleted indicates that these secondary pathways may not require ATP either. This is supported by findings with nontesticular cells showing, on one hand, the existence of pathways that appear not to require mitochondrial functions or ATP (2, 10, 17, 23, 36, 44, 66) and, on the other, activation of
different types of apoptotic cascades within certain cell types (39, 67).

Because cell-to-cell interactions play an important role in germ cell survival, it is possible that the antiapoptotic compounds act on the Sertoli cells rather than the germ cells by modulating the supply of pro- or antiapoptotic paracrine factors. The present in vitro model, having the advantage of maintaining physiological contacts between the different cell types, allows investigation of paracrine systems. One such system, which has been suggested to regulate germ cell death in the testis and in our in vitro model, is the Fas-Fas-L system (12, 13, 24, 34, 48, 49). The proapoptotic Fas-L expressed by the Sertoli cells, and perhaps also by the germ cells, has been suggested to activate the Fas receptors and consequently the apoptotic cascade in the germ cells (12, 13, 24, 34, 48). Here we aimed at investigating whether additional Fas activation would be able to modulate the effects of the mitochondrial inhibitors. Another aspect in terms of adding Fas activators to the culture was based on the literature, in which uncouplers of OXPHOS have been shown to presensitize certain nontesticular cells to the Fas death signal (35). Moreover, it has been suggested that cross talk between the Fas receptor and mitochondrial signaling can occur when Fas is activated by increased amounts of the agonistic Fas antibody (35, 63). In our study, the antiapoptotic role of the mitochondrial inhibitors was not modified by the activating anti-Fas antibody or human recombinant Fas-L (Fig. 5). That the activation of the Fas receptors did not induce apoptosis in the presence of the mitochondrial inhibitors may have several explanations. It may suggest that 1) these compounds inhibit the particular apoptotic pathway that is triggered by Fas or a pathway that has steps common in with it and 2) that the inhibitory actions of the compounds take place downstream of the Fas receptors in the germ cells (i.e., not e.g., via the action of Sertoli cells). However, 3) that these compounds would act primarily on the Sertoli cells or 4) that the Fas receptors would not activate germ cell death pathways are not totally excluded. The first of these possibilities (i.e., suggestion 3) could be explained by induction of Sertoli cell production of antiapoptotic factor(s), which could act on germ cells downstream of the Fas receptors. Whichever the explanation, the results show that activators of the Fas system failed to induce apoptosis when the mitochondrial ATP production was impaired and that the mitochondrial inhibitors or uncouplers did not presensitize the testicular cells to Fas-induced death.

From the present study, we conclude that the mitochondrial ATP production machinery plays an important role in regulating primary pathways of human male germ cell apoptosis, triggered through the hormone- and serum-free culture conditions. The results indicate that it is unlikely that any of the complexes (I-IV) of the mitochondrial respiration alone, the functional electron transport chain, or the membrane potential is/are important. Rather, a straightforward conclusion from our experiments is that ATP synthesized by the F0F1-ATPase is crucial for the primary pathways of testicular cell apoptosis. We also conclude that there seem to be secondary pathways of human testicular apoptosis that do not require mitochondrial ATP production. The present study increases the understanding of the role of the mitochondrial catabolic pathways in the complex network of regulatory events of male germ cell life and death.

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