Fas regulates germ cell apoptosis in the human testis in vitro

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Pentikäinen, Virve, Krista Erkkilä, and Leo Dunkel. Fas regulates germ cell apoptosis in the human testis in vitro. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E310–E316, 1999.—The Fas-Fas ligand (FasL) system has been implicated in maintaining the immune privileged nature of the testis; it seems to be involved in the regulation of germ cell apoptosis. This system is recognized as a key physiological regulator of apoptosis in many cell types (16, 17). Fas (APO-1/CD95) is a type I transmembrane protein belonging to the tumor necrosis factor receptor superfamily (19, 30). FasL, in turn, is a member of the tumor necrosis factor family of proteins (27, 35). It is produced as a type II membrane protein and may be cleaved by a metalloproteinase to produce a soluble ligand (37–39). Interaction of cell surface Fas molecules with FasL or with agonistic anti-Fas antibody activates the apoptotic death machinery in Fas-bearing cells (26, 27). Recently, the link between Fas and the effectors of the cell death machinery has been elucidated. Fas contains a cytoplasmic death domain that, upon activation, recruits another death domain-containing protein FADD/MORT1 (7, 9). FADD/MORT1, in turn, recruits caspase 8 (FLICE/MACH; see Refs. 6, 25), a member of the family of cysteine proteases that are recognized to mediate the specific proteolytic cleavage events in the dying cells. Self-activation of caspase 8 initiates activation of a cascade of these proteases, caspases, which results in rapid death of the cell (10, 29).

In the testis, the Fas-FasL system has been implicated in maintaining the immune-privileged nature of this organ (3). Sertoli cells expressing FasL eliminate Fas-positive activated T cells, thus protecting the germ cells against the immune system and preventing rejection reactions in the testicular environment. Recent studies in the rat and mouse testes have suggested another important function for the Fas-FasL system in the testis; it seems to be involved in the regulation of testicular germ cell apoptosis (24). However, all of these results concerning the role of the Fas-FasL system in the testis have been obtained from rat and mouse studies and so do not necessarily apply to humans. FasL has been previously localized to the Sertoli cells (26, 42) and Fas to the germ cells (36) of the human testis, but the function of these proteins in the human testis has remained questionable. Therefore, the aim of the present study was to elucidate the functional role of the Fas-FasL system in the human testis. Germ cell apoptosis was induced in vitro, and the ability of an antagonistic antibody to FasL, or a potent inhibitor of caspases, to block this apoptosis was tested. Furthermore, the expression and localization of Fas protein in the human testis was studied and compared with the localization of apoptotic DNA.

METHODS

Patients. Testis tissue was obtained from men, aged 68–83 yr, undergoing orchidectomy as a treatment for prostate...
cancer. They had not received hormonal or chemotherapeutic medication or radiotherapeutic treatment for the cancer before the operation. They had no endocrinological disease, and none of them had suffered from cryptoorchidism. The operations were performed between September 1997 and February 1998 at the Helsinki City Health Department, Surgical Unit (Helsinki, Finland). The Ethics Committees of the Department of Pediatrics and the Department of Urology, University of Helsinki, approved the study protocol.

Tissue culture. Segments of seminiferous tubules were cultured, instead of isolated germ cells, to maintain the environment for the germ cells as physiological as possible. Testis tissue was microdissected under a transillumination stereomicroscope in a petri dish containing PBS. Segments of seminiferous tubules (~1 mm in length) were isolated and transferred to 96-well culture plates. Each well contained 100 μl of tissue culture medium (Ham's F-10; Gibco Europe, Paisley, UK) supplemented with 0.1% human albumin (Sigma Chemical, St. Louis, MO) and 10 μg/ml gentamicin (Gibco). The samples were incubated for 4–24 h under serum- and hormone-free conditions (i.e., without survival factors) at 34°C in a humidified atmosphere containing 5% CO₂.

Inhibition of germ cell apoptosis in the tissue cultures. To test the effect of anti-FasL antibody, rabbit polyclonal antibody to FasL (sc-957/C-20; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the above-described tissue cultures at final concentrations of 1 and 20 ng/ml. Non-specific rabbit IgG (Sigma Chemical) was used as a control at the same concentrations. The role of caspases in germ cell apoptosis was studied by using a universal caspase inhibitor, benzylxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK; Enzyme Systems, Dublin, CA), to the tissue cultures at 2 and 20 μM.

Southern blot analysis of apoptotic DNA fragmentation. Tissue samples were snap-frozen in liquid nitrogen and were stored at −70°C until DNA isolation. Genomic DNA was extracted as described (4, 13, 15). After quantification, the DNA samples were 3′ end labeled with digoxigenin-dideoxy-UTP (Dig-dd-UTP; Boehringer Mannheim) using the terminal transferase (Boehringer Mannheim) reaction, subjected to electrophoresis on 2% agarose gels, and blotted on nylon membranes overnight. The membranes were cross-linked to the membranes by ultraviolet irradiation. The membranes were then washed and blocked for 30 min at room temperature. Apoptotic, 3′ end-labeled DNA was detected by the antibody reaction (anti-digoxigenin antibody, AFOS-conjugated; Boehringer Mannheim) as described recently (13, 14). For the luminescence reaction, the membranes were incubated in CSPD solution for 5 min at room temperature (CSPD; Boehringer Mannheim). The membranes were then enclosed in hybridization bags, incubated for 15 min at 37°C, and exposed to X-ray films. The films were scanned with a tabloptop scanner (Microtek), and the digital image was analyzed with National Institutes of Health-Image (L.61) analysis software. The digitized quantification of low-molecular-mass DNA fragments (185-bp multiples) of the sample incubated for 4 h under serum-free culture conditions without any treatments was set as 1.0 (100% apoptosis), and the amounts of low-molecular-mass DNA fragments in the other samples were presented as relative to it.

In situ labeling of apoptotic DNA. Small segments of seminiferous tubules (~1 mm in length) were squashed under coverslips and fixed as described previously (13, 14, 16, 31). The squash preparations were rehydrated and washed two times for 5 min in distilled water. They were then incubated for 10 min with terminal transferase reaction buffer (1 mol/l potassium cacodylate, 125 mol/l Tris-HCl, and 1.25 mg/ml BSA, pH 6.6). DNA 3′ end labeling with Dig-dd-UTP (Boehringer Mannheim) by the terminal transferase reaction, detection of Dig-dd-UTP with the anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1,000, Boehringer Mannheim), and exposure to the substrates for alkaline phosphatase were performed as described previously (13, 16). For the negative controls, the terminal transferase (Tdt) enzyme was replaced with the same volume of distilled water. Light counterstaining was performed with hematoxylin.

Electron microscopy. Segments of seminiferous tubules were microdissected under a transillumination microscope and cultured as described in Tissue culture. They were then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, dehydrated, and embedded in epoxy resin. The samples were then sectioned at 50 nm with a Reichert E Ultramicrotome (Reichert Jung, Vienna, Austria) and were stained with uranyl acetate and lead citrate. Observations were made with a Jeol EM 1200 EX transmission electron microscope (Jeol, Tokyo, Japan). Germ cells were identified according to their characteristic morphology (21). Apoptosis was recognized by typical ultrastructural changes, including condensation of nuclear chromatin and degeneration of cytoplasmic organelles.

Immunohistochemical staining of Fas protein. Squash preparations of human seminiferous tubules were rehydrated and incubated in 1% H₂O₂ for 30 min at room temperature to quench endogenous peroxidases. They were then washed twice for 5 min in PBS and were blocked with 5% normal goat serum in PBS containing 0.1% Tween 20 for at least 1 h at room temperature. Rabbit polyclonal antibody to human Fas (sc-715/C-20; Santa Cruz Biotechnology) was added to the preparations at 2 μg/ml in 0.1% Tween 20 in PBS, and incubation was performed overnight at 4°C. After incubation, the slides were washed three times for 5 min in PBS. The primary antibody was detected using a biotin-conjugated goat anti-rabbit IgG secondary antibody from the ABC-Elite Kit (Vector Laboratories, Burlingame, CA). For location of the antibody, 0.05% diaminobenzidine substrate (Sigma Chemical) was added. Light counterstaining was performed with hematoxylin. Inhibitor peptide (20 μg/ml; Santa Cruz Biotechnology) was used to verify the specific staining of the Fas protein on the DNA slides.

Western blot analysis. Small tissue sections were homogenized in PBS containing proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). To separate Fas from the cell membranes, SDS was added at 1%, and the samples were incubated at 95°C for 5 min. After centrifugation at 17,000 g for 30 min, the supernatants were collected, and their protein concentrations were determined by Bio-Rad DC protein assay (Bio-Rad Laboratories). Proteins (10–15 μg) were loaded in 12% SDS-polyacrylamide gels, and electrophoresis was performed at 150 V in the presence of a low-range marker standard (Bio-Rad). The proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) by electrophoresis overnight at 4°C in transfer buffer (26 mM Tris, 192 mM glycine, and 20% methanol) at 30 V (80 mA). The transfer was checked by staining with 0.2% Ponceau S in 3% trichloroacetic acid. Western blotting was performed, using the same rabbit polyclonal antibody to human Fas (sc-715/C-20; Santa Cruz Biotechnology) at 0.1 μg/ml, followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The antibody was located with the enhanced chemiluminescence detection kit (Amersham). The specificity of the anti-Fas antibody was confirmed, using inhibition peptide (Santa Cruz Biotechnology).
RESULTS

In vitro induction of human testicular apoptosis and identification of apoptotic cells by electron microscopy. In the present study, human germ cells were cultured in their physiological environment, i.e., in the seminiferous tubules. Germ cell apoptosis was induced in this in vitro model by incubating segments of seminiferous tubules under serum- and hormone-free conditions (i.e., without survival factors). The apoptotic cells were identified by electron microscopy, using morphological criteria of apoptosis. Characteristics for the apoptotic cells were, e.g., condensation of nuclear chromatin and degeneration of cytoplasmic organelles. During the late stages of apoptosis, the structure of the nuclear envelope dispersed and finally disappeared. The apoptotic cells were most often identified as spermatocytes (Fig. 1, A–C) and occasionally as spermatids (Fig. 1, D–E). Some of the apoptotic spermatids showed a ring-like condensation of chromatin around the nuclear periphery, characteristic for apoptosis of this type of germ cell. Late apoptotic cells were impossible to identify.

Localization of the Fas protein in the human seminiferous epithelium. Localization of the Fas protein was studied immunohistochemically using the rabbit polyclonal antibody to human Fas. Representative samples of cells from human seminiferous epithelium were obtained by squashing segments of seminiferous tubules under coverslips. In this technique, cells from seminiferous epithelium migrate under the coverslip to produce a monolayer. The cells maintain their morphological characteristics, allowing identification of individual cell types. In these squash preparations, strong positive staining for the Fas protein was observed in the same germ cell types that were identified as apoptotic in the electron microscopy, namely spermatocytes and spermatids (Fig. 2A). There was no staining when the antibody was replaced with PBS (negative control, Fig. 2B). The specificity of the antibody was confirmed with inhibition peptide. Colocalization of Fas in the same types of germ cells that were undergoing apoptosis suggests that the Fas-FasL system is involved in germ cell apoptosis.

Inhibition of germ cell apoptosis by antibody to FasL or by caspase inhibitor Z-VAD.FMK. To study the functional role of the Fas-FasL system in human germ cell apoptosis, we used the rabbit polyclonal antibody to human FasL (C-20) to inhibit apoptotic cell death in the testis tissue culture. This antibody binds to the carboxy-terminal part of the FasL, thus preventing interaction between Fas and FasL with activation of the Fas-FasL system.
system. The clear inhibitory effect of this antibody on germ cell apoptosis was detected by in situ 3’ end labeling (ISEL) of apoptotic DNA with Dig-dd-UTP of squash preparations from human seminiferous tubules. With this technique, no or only few positively staining cells were observed in the samples taken immediately after the operation (Fig. 3A). In contrast, strong staining was seen in the samples cultured for 4 h under serum-free conditions without survival factors (Fig. 3B). The amount of positively staining cells was effectively decreased by the anti-FasL antibody at the concentration of 20 µg/ml (Fig. 3C). No staining was seen when the terminal transferase enzyme was replaced with the same volume of distilled water (negative control).

Because the downstream effectors of Fas include various caspases, we next tested the ability of a universal inhibitor of caspases, Z-VAD.FMK, to block germ cell apoptosis. As shown in Fig. 3D, the amount of ISEL-positive, apoptotic germ cells was dramatically decreased in squash preparations of seminiferous tubules treated with 20 µM Z-VAD.FMK compared with tubules cultured for 4 h without survival factors.

Quantification of the inhibitory effect of the anti-FasL antibody or Z-VAD.FMK on testicular apoptosis. To confirm the results of ISEL analysis and to obtain quantitative information on the inhibitory effect of the anti-FasL antibody or Z-VAD.FMK on germ cell apoptosis, we further performed Southern blot analysis of low-molecular-mass DNA fragments (185-bp mul-

Fig. 3. In vitro inhibition of germ cell apoptosis by an antagonistic anti-Fas ligand (FasL) antibody C-20 or a caspase inhibitor benzylxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD.FMK). Segments of human seminiferous tubules were incubated under serum- and hormone-free conditions in the presence or absence of C-20 or Z-VAD.FMK. Samples were squashed and fixed, and apoptotic cells were detected by in situ 3’ end labeling of apoptotic DNA as described in METHODS. A: no apoptotic cells were observed in a sample taken immediately after the operation; B: apoptotic cells in a squash preparation of a sample incubated for 4 h without survival factors. Germ cell apoptosis was effectively inhibited when the anti-FasL antibody C-20 at 20 µg/ml (C) or the caspase inhibitor Z-VAD.FMK at 20 µM (D) was added to the culture medium.

Fig. 4. Effect of an antagonistic anti-FasL antibody (C-20) on in vitro-induced human testicular apoptosis. Segments of seminiferous tubules were incubated under serum- and hormone-free conditions in the presence or absence of C-20. DNA was extracted, 3’ end labeled with digoxigenin-dideoxy-UTP, and subjected to electrophoresis on 2% agarose gels (1 µg/lane). To detect the labeled DNA, staining with CSPD was performed as described in METHODS. A: no apoptotic cells were observed in a sample taken immediately after the operation; B: apoptotic cells in a squash preparation of a sample incubated for 4 h without survival factors. Germ cell apoptosis was effectively inhibited when the anti-FasL antibody C-20 at 20 µg/ml (C) or the caspase inhibitor Z-VAD.FMK at 20 µM (D) was added to the culture medium.

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The anti-FasL antibody reduced the total amount of low-molecular-mass DNA fragmentation by 39% at the concentration of 20 µg/ml (Fig. 4), indicating that the Fas-FasL system does function in the human testis. Lower concentrations of the antibody were not effective. Furthermore, nonspecific rabbit IgG, used as a control, had no effect on the amount of apoptosis (data not shown). Z-VAD.FMK, in turn, inhibited germ cell apoptosis in a dose-dependent manner (Fig. 5). DNA fragmentation was suppressed by 45% at the Z-VAD.FMK concentration of 20 µM and by 35% at 2 µM (Fig. 5B). These findings suggest that germ cell apoptosis is mediated largely via the caspase pathway. Thus the apoptotic process initiated by cross-linking of Fas by means of FasL seems to involve activation of caspases.

Western blot analysis of Fas expression during the apoptotic process of germ cells. The time course of the Fas expression during the apoptotic process of germ cells was studied by Western blot analysis, in which a strong band at the expected molecular mass (50 kDa) was observed (Fig. 6). In addition, three faint bands were seen. One of them (100 kDa) disappeared when the primary antibody was incubated with the inhibition peptide before use, suggesting that it represented the 97-kDa band reported to be formed after activation of Fas (20). The other two bands did not disappear after using the inhibition peptide, suggesting that they represent impurities of the primary antibody. Expression of Fas remained constant at 0, 4, and 24 h. Thus, in the human testis, the Fas protein does not seem to be upregulated during the apoptotic process of germ cells.

**DISCUSSION**

In the present study, we show that germ cell apoptosis in the human testis is mediated by the Fas-FasL system. Germ cell apoptosis was induced in vitro by incubating segments of seminiferous tubules under serum- and hormone-free conditions (i.e., without survival factors), and this apoptosis was inhibited by blocking the Fas-FasL system with an antibody to FasL. This antibody has previously been successfully used for inhibition of Fas-mediated apoptosis in human monocyte cell culture (22). Furthermore, a potent caspase inhibitor, Z-VAD.FMK, effectively inhibited germ cell apoptosis, implying that germ cell apoptosis is mediated via the caspase pathway. The Fas protein was localized immunohistochemically to spermatocytes and spermatids, which were also the cells that were identified as apoptotic in electron microscopy. Finally, Western blot analysis indicated strong expression of the Fas protein in the testis tissue. All these data suggest an important role of the Fas-FasL system in human testicular apoptosis.

The Fas-FasL system is an important mediator of apoptotic cell death in many cell types. In the testis, it has been shown to maintain the immune-privileged nature of this organ (3); the FasL, constitutively expressed by the somatic Sertoli cells, eliminates Fas-positive activated T cells, which might otherwise cause a rejection reaction in the immune-privileged environment. In the present report, we suggest another important function for the Fas-FasL system in the human testis. From our observations, that germ cell death can be inhibited by blocking the interaction between Fas and FasL and that Fas is present in germ cells undergo-
ing apoptosis, we infer that the Fas-FasL system mediates germ cell apoptosis in the human testis. The FasL constitutively expressed by the Sertoli cells is suggested to bind to the Fas of the germ cells and so cause death of these Fas-bearing germ cells. A selection process of this kind is proposed to be necessary for normal spermatogenesis, since the supportive capacity of the Sertoli cells is limited.

In the rat testis, upregulation of Fas was observed in germ cells undergoing apoptosis after in vivo administration of Sertoli cell toxicants (24). In the human testis, however, the expression of Fas did not seem to be upregulated during the increased apoptotic process after withdrawal of survival factors. Therefore, there would appear to be additional pathways leading to increased apoptosis in the human testis, or; alternatively, Fas activation may be more effective in unfavorable conditions, thus enhancing the ability of the Fas-FasL system to mediate apoptotic germ cell death. Furthermore, there may be regulation of the Fas-FasL system at the level of the production and modification of the FasL. Finally, we cannot exclude that the lack of upregulation of Fas might be explained by the background of nonexpressing cell types present in seminiferous tubules or by sample timing.

The downstream effectors of Fas include various caspases, the most upstream of them being caspase 8 (10, 29). Activation of the caspase 8 is followed by activation of caspase 3, which then degrades substrates involved in genome function, such as poly(ADP-ribose) polymerase, the 70-kDa protein component of the DNA-dependent protein kinase (8, 11, 23, 28, 33, 41). To our knowledge, no reports have been published concerning the role of caspases in male germ cell apoptosis. Therefore, we tested the ability of a universal caspase inhibitor, Z- VAD.FMK, to inhibit testicular apoptosis in vitro. As shown in the present study, Z- VAD.FMK effectively inhibited germ cell apoptosis, suggesting that Fas-associated germ cell apoptosis was mediated by caspases. Additional pathways for activation of caspases may also exist in the testis. This seems likely, because the apoptotic processes known today have all been shown to involve activation of caspases, which ultimately mediate the highly specific cleavage of key structural and functional proteins in the dying cells (10, 29).

The importance of understanding the mechanisms of germ cell death has become evident during recent years, when sperm counts and semen quality have been reported to be declining in Western countries (34). Our recently developed in vitro model enables us to study the mechanisms of human germ cell apoptosis and to evaluate the possible risk factors of male reproductive functions. An in vitro culture may naturally have some limitations as to approximation of germ cell physiology in vivo. However, we believe that, in the present in vitro model, conditions are sufficiently close to the situation in vivo, first because in the present model the germ cells are allowed to stay in their natural environment (i.e., in the seminiferous tubules) and second because germ cell death can be blocked by a physiological germ cell survival factor, testosterone (13). The present study, in which this model was used, provides evidence for the function of one of the key apoptosis-related systems in the turnover of human testicular germ cells. In conclusion, the Fas-FasL system seems to play an important role in the regulation of human male germ cell apoptosis.

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