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TNFα Down-Regulates the Fas Ligand and Inhibits Germ Cell Apoptosis in the Human Testis

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The cytokine TNFα is known to be secreted by testicular germ cells. However, its effect on maturing germ cells is unknown, and its role in the regulation of spermatogenesis is unclear. Here we aimed at characterizing the effects of TNFα on germ cell survival in the human testis. We found that TNFα effectively and dose-dependently inhibited germ cell apoptosis, which was induced *in vitro* by incubating segments of human seminiferous tubules under serum-free culture conditions. EMSAs indicated increased activity of nuclear factor κB in seminiferous tubules cultured under apoptosis-inducing conditions. However, we did not observe any significant effect of TNFα on the activation of this transcription factor, which is often considered to be a mediator of TNFα-induced survival signals. As the expression of the TNF receptor protein in the human seminiferous epithelium was predominantly found in the Sertoli cells, the antiapoptotic effect of TNFα is probably mediated via these somatic cells. Interestingly, expression of the Fas ligand, a known inductor of testicular apoptosis, was down-regulated by TNFα. Thus, in the seminiferous tubules, germ cell-derived TNFα may regulate the level of the Fas ligand and thereby control physiological germ cell apoptosis. (*J Clin Endocrinol Metab* 86: 4480–4488, 2001)

TNFα IS A pleiotropic cytokine that exerts a wide range of cellular effects, including inflammatory and immunoregulatory responses and modulation of apoptotic cell death (1–3). In the testis, TNFα is produced by male germ cells and is held to be one of the testicular paracrine factors that, together with the hormones of the hypothalamic-pituitary-testicular axis, regulate spermatogenesis. Within mouse seminiferous tubules, pachytene spermatocytes and round spermatids were found to express TNFα mRNA, and the bioactive cytokine was mainly produced by the round spermatids (4). TNFα has been shown to be secreted not only by germ cells, but also by the activated interstitial macrophages of the mouse and rat testis (5, 6). The type I (55-kDa) TNFα receptor (TNFR) has been identified in porcine Sertoli and Leydig cells (7, 8) and mouse Sertoli cells (4, 9). Several effects of TNFα on these somatic cells have been documented. In the Sertoli cells, TNFα acts as a proinflammatory cytokine that induces IL-6 production and intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression (9–11). It has also been suggested to play a role in the local control of spermatogenesis, because in the Sertoli cells it regulates the production of lactate (12, 13), transferrin (14), cAMP response element-binding protein (15), and IGF-binding protein (16). In addition, cultured Leydig cells have been shown to respond to TNFα by decreasing their biosynthesis of T (8, 17, 18).

Although the effects of TNFα on Sertoli cells and interstitial Leydig cells have been studied previously, the effects of this cytokine on maturing germ cells remain unknown. During spermatogenesis, a number of germ cells undergo physiological apoptotic death before reaching maturity (19). In this respect, it is of interest that TNFα is a potent modulator of apoptotic cell death (3, 20). It has been shown to induce apoptosis in a variety of transformed cell lines, whereas in nontransformed cells and normal tissues, proliferative responses rather than cell death may dominate (21, 22). The cellular responses of TNFα are mediated by the TNFRs TNFR-I (55 kDa) and TNFR-II (75 kDa) , which belong to the large TNFR family. TNFR-I is the main receptor responsible for transduction of the TNFα-induced death signal, which is mediated by its cytoplasmic death domain and is usually associated with activation of the caspase pathway (3). On the other hand, the survival signals induced by TNFα are often mediated by activation of the transcription factor nuclear factor κB (NF-κB) after stimulation of one or another of the two TNFRs (3, 22, 23). Identification of the TNFR-I in the Sertoli and Leydig cells, but not in the germ cells, in the porcine and mouse testes suggests that the effects of TNFα on germ cells are paracrine rather than direct. However, receptor expression may be species specific, and no reports have shown localization of the TNFRs in the human testis.

Studies of mouse, rat, and human testicular apoptosis have shown that Fas, another death domain-containing member of the TNFR family, is a powerful mediator of male germ cell death (24, 25). Interestingly, a recent study showed that in cultured mouse Sertoli cells, TNFα regulates the expression and function of the Fas system, suggesting a role for TNFα in testicular apoptosis (26). As there were no reports of the effects of TNFα on human male germ cell apoptosis, we aimed at characterizing the effects of TNFα on germ cell survival in the human testis, using our recently described *in vitro* model (27). In addition, we used immunohistochemistry and Western blotting to study the expression of TNFR-I and

Abbreviations: Dig-dd-UTP, Digoxigenin-dideoxy-UTP; FasL, Fas ligand; ISEL, *in situ* end labeling; NF-κB, nuclear factor κB; TNFR, TNFα receptor.

TNFR-II in the adult human testis. Finally, as the Fas system is a potential target for TNFα action in the seminiferous tubules (26), we tested the ability of TNFα to alter the expression of Fas and Fas ligand (FasL) in human seminiferous tubules.

Subjects and Methods

Patients

Testis tissue was obtained from 18 men, aged 58–87 yr, undergoing orchidectomy as treatment for prostate cancer. They had not received hormonal, chemotherapeutic, or radiotherapeutic treatment for the cancer before the operation. They had no endocrinological disease, and none of them had suffered from cryptorchidism. The operations were performed between July 1998 and December 2000 at the Department of Urology, Helsinki University Central Hospital, and at the Helsinki City Health Department, Surgical Unit (Helsinki, Finland). The ethics committees of the Hospital for Children and Adolescents and the Department of Urology, University of Helsinki, approved the study protocol.

Tissue culture

Apoptosis of the human testicular germ cells was induced *in vitro* by incubating segments of seminiferous tubules under serum-free culture conditions. Segments of seminiferous tubules, rather than isolated germ cells, were cultured to maintain as physiological an environment as possible for the germ cells. The testis tissue was microdissected on a petri dish containing tissue culture medium (nutrient mixture Ham's F-10, Life Technologies, Inc., Paisley, UK) supplemented with 0.1% human albumin (Sigma, St. Louis, MO) and 10 μg/ml gentamicin (Life Technologies, Inc.). Segments of seminiferous tubules (~2 mm in length) were isolated and transferred to culture plates containing the same tissue culture medium. The samples were incubated at 34 C under serum-free conditions in a humidified atmosphere containing 5% CO_2. To study the effects of TNFα on male germ cell apoptosis, recombinant human TNFα (Roche Molecular Biochemicals, Mannheim, Germany; or R&D Systems, Inc., Oxon, UK) was added to the tissue cultures at final concentrations of 1, 10, and 100 ng/ml.

Southern blot analysis of apoptotic DNA fragmentation

Tissue samples were snap-frozen in liquid nitrogen and stored at −80 C until DNA extraction. Genomic DNA was extracted from the testis samples, using an Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals), as previously described (28). DNA was quantified spectrophotometrically (absorbance at 260 nm), and 1 μg of the total DNA from each sample was subjected to 3′-end labeling with digoxigenin-dideoxy-UTP (Dig-dd-UTP, Roche Molecular Biochemicals) by the terminal transferase (Roche Molecular Biochemicals) reaction. The DNA samples were then subjected to electrophoresis on 2% agarose gels, blotted onto nylon membranes, and cross-linked to the membranes by UV irradiation. The membranes were then washed and blocked with 1% blocking reagent (Roche Molecular Biochemicals) in maleic buffer (100 mmol/liter maleic acid and 150 mmol/liter NaCl, pH 7.5) for 30 min at room temperature. The 3′-end-labeled DNA on the membranes was localized with alkaline phosphatase-conjugated antidigoxigenin antibody (Roche Molecular Biochemicals), and the bound antibody was detected by the chemiluminescence reaction (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate, Roche Molecular Biochemicals). The x-ray films exposed to chemiluminescence were scanned with a tabletop scanner (ScanJet 6300C, Hewlett-Packard Co., Palo Alto, CA), and the digital image was analyzed with Scion Image β 4.0.2 (Scion Corp., Frederick, MD) analysis software. The digitized quantification of the low mol wt DNA fragments (<1.3 kb) of the sample cultured for 4 h without survival factors was taken as 1.0 (100% apoptosis), and the amounts of low mol wt DNA fragments in the other samples were expressed in relation to this.

In situ end labeling (ISEL) of apoptotic DNA

Small segments of human seminiferous tubules (~1 mm in length) were squashed under coverslips and fixed as previously described (29). These squash preparations were rehydrated, washed in distilled water, and permeabilized by microwaving at high power for 5 min in citrate buffer (10 mmol/liter citrate, pH 6.0). After incubation for 10 min with terminal transferase reaction buffer (1 mol/liter potassium cacodylate, 125 mmol/liter Tris-HCl, and 1.25 mg/ml BSA, pH 6.6), the apoptotic DNA was 3′-end labeled with Dig-dd-UTP (Roche Molecular Biochemicals) for 1 h at 37 C by the terminal transferase reaction. For the negative controls, the terminal transferase enzyme was replaced with the same volume of distilled water. The preparations were then blocked with blocking solution [2% blocking reagent (Roche Molecular Biochemicals) in 150 mmol/liter NaCl and 100 mmol/liter Tris-HCl, pH 7.5], followed by location of the Dig-dd-UTP with the peroxidase-conjugated antidigoxigenin antibody (Roche Molecular Biochemicals). For detection of the antibody, 0.05% diaminobenzidine substrate (Sigma) was added. Light counterstaining was performed with hematoxylin, whereafter the samples were dehydrated and mounted.

Protein extract preparation

For nuclear protein extracts, freshly isolated seminiferous tubules or seminiferous tubules cultured for 1 or 4 h in the absence or presence of TNFα were gently homogenized with a tight-fitting Potter-Elvehjem homogenizer into ice-cold hypotonic buffer A [50 mmol/liter HEPES (pH 7.4), 10 mmol/liter KCl, 1 mmol/liter EDTA, 1 mmol/liter dithiothreitol, 0.2 mmol/liter phenylmethylsulfonylfluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 0.5% Nonidet P-40], and nuclear protein extracts were prepared as previously described (30). For whole cell protein extracts, sections of testis tissue frozen immediately after orchidectomy or seminiferous tubules cultured in the absence or presence of TNFα were homogenized with an Ultra-Turrax T8 homogenizer into ice-cold homogenization buffer [1% Triton X-100, 150 mmol/liter NaCl, 10 mmol/liter Tris (pH 7.4), 1 mmol/liter EDTA, 1 mmol/liter EGTA, 0.2 mmol/liter phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin]. The homogenates were vortexed vigorously, incubated on ice for 20 min, and centrifuged at 17,000 × *g* for 30 min. The resultant supernatants were stored in aliquots at −80 C until used for EMSAs and Western blotting. Protein concentrations were determined by the Bradford method, using the Bio-Rad Laboratories, Inc. DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA).

EMSA

DNA probes containing a consensus κB enhancer element (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were purchased from Santa Cruz Biotechnology, Inc. (sc-2505, Santa Cruz, CA). The probes were 5′-end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega Corp., Madison, WI). Testicular nuclear protein extracts (10 μg) or whole cell protein extracts (20 μg) were incubated on ice for 10 min with 2 μg poly(dI-dC) (Amersham Pharmacia Biotech) in 20 mmol/liter HEPES (pH 7.9), 10% glycerol (vol/vol), 50 mmol/liter KCl, 0.5 mmol/liter EDTA, 1 mmol/liter dithiothreitol, 1 mmol/liter $MgCl_2$, 0.5 mmol/liter phenylmethylsulfonylfluoride, and 1 μmol/liter leupeptin. A 5′-end-labeled probe (10,000–20,000 cpm) was then added, and incubation was continued at room temperature for 30 min. In the competition experiments, a 100-fold molar excess of unlabeled probe or mutated probe (sc-2511, Santa Cruz Biotechnology, Inc.) was added before the labeled probe. Reaction products were separated on 4% polyacrylamide gels run in 22.5 mmol/liter Tris-borate and 0.5 mmol/liter EDTA at 200 V at room temperature. After electrophoresis, the gels were dried and visualized by autoradiography.

Western blotting

Western blotting was performed on protein extracts from frozen testis tissue. Protein extract from human liver was used as a control for TNFR expression, and human endothelial cell lysate was used as a control for FasL expression. To study possible differences in the glycosylation of the TNFR proteins, in some experiments the protein extracts were subjected to deglycosylation by peptide *N*-glycosidase F (Roche Molecular Biochemicals) according to the manufacturer's instructions or as previously described (31). Proteins (5 -50 μg) were loaded into SDS-polyacrylamide gels, and electrophoresis was performed at 180 V. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA) by electrophoresis for 2 h at 4°C in transfer buffer (26 mmol/liter Tris, 192 mmol/liter glycine, and 10% methanol)

at 100 V. The transfer was checked by staining with 0.2% Ponceau S in 3% trichloroacetic acid. TNFR-I and TNFR-II proteins on the membranes were detected using affinity-purified polyclonal antibodies to human TNFRs. TNFR-I antibodies H-271 (sc-7895, Santa Cruz Biotechnology, Inc.) and CSA-810 (StressGen Biotechnologies Corp., Victoria, Canada) were used at 0.4 and 0.5 μg/ml, respectively. The TNFR-II antibody H-202 (sc-7862, Santa Cruz Biotechnology, Inc.) was used at 0.4 μg/ml. FasL was detected with an antihuman FasL monoclonal antibody (Transduction Laboratories, Lexington, KY), and Fas was detected with an antihuman Fas polyclonal antibody (sc-715, Santa Cruz Biotechnology, Inc.). The primary antibodies were followed with peroxidase-conjugated goat antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or peroxidase-conjugated goat antimouse IgG (DAKO Corp., Glostrup, Denmark). The bound secondary antibody was located with the ECL detection kit (Amersham Pharmacia Biotech, Arlington Heights, IL). The specificity of the bands detected by the TNFR-I antibodies was confirmed using a blocking peptide to the CSA-810 antibody. After detection of the proteins under investigation, the membranes were washed and, as a loading control, probed with an antibody to α-tubulin (Sigma).

To study the alterations in the protein expression of Fas and FasL, the x-ray films exposed to ECL were scanned, and the digital images were analyzed with Scion Image β 4.0.2 (Scion Corp.) analysis software. Standard curves for Fas, FasL, and α-tubulin were generated with a dilution series of a control sample. The amounts of Fas or FasL in the samples were adjusted to the amount of α-tubulin in the corresponding samples.

Immunohistochemical staining of the TNFα receptors

Immunostaining was performed on paraffin-embedded sections from formalin-fixed adult human testes. Testis sections were deparaffinized in xylene, rehydrated, and permeabilized by microwaving at high power for 5 min in citrate buffer (10 mmol/liter citrate, pH 6.0). The sections were then washed and blocked with blocking solution (PBS containing 5% goat normal serum, 3% BSA, and 0.1% Tween 20) for at least 30 min at room temperature. In our preliminary experiments we found that in the negative controls, in which the primary antibody was replaced with PBS, only the erythrocytes in the testicular capillaries stained positively if the endogenous peroxidases were not blocked by methanol containing 1% H_2O_2. Therefore, it appears that in the human testis, endogenous peroxidases are not present in amounts that would affect the immunostaining of the TNFRs. The TNFR-I protein in the sections was detected with two affinity-purified polyclonal antibodies to human TNFR-I (H-271, Santa Cruz Biotechnology, Inc., and CSA-810, StressGen Biotechnologies), which were both used at 0.2 μg/ml. The expression of the TNFR-II was also studied using a polyclonal antibody to human TNFR-II (H-202, Santa Cruz Biotechnology, Inc.) at 0.2–2 μg/ml. The primary antibodies were added to the testis sections in blocking solution, and incubation was performed overnight at 4 C. After incubation, the slides were washed in PBS. The primary antibody was detected using biotin-conjugated goat antirabbit IgG from the corresponding ABC-Elite Kit (Vector Laboratories, Inc., Burlingame, CA), followed by incubation with ABC solution. For location of the antibody, 0.05% diaminobenzidine substrate (Sigma) was added. For the negative controls, the primary antibodies were replaced with nonspecific rabbit IgG (Sigma) or PBS. Double immunostaining of the TNFR-I and the macrophage surface antigen was performed using the H-271 antibody to the human TNFR-I and a monoclonal antibody to the human macrophage surface antigen (HAM56, DAKO Corp., Glostrup, Denmark). The HAM56 antibody was first added to the sections at a dilution of 1:300, and incubation was performed overnight at 4 C. The primary antibody was detected using peroxidase-conjugated goat antimouse IgG (DAKO Corp.), followed by incubation with a solution of diaminobenzidine and nickel chloride from the diaminobenzidene substrate kit for peroxidase (Vector Laboratories, Inc.). After color development, the slides were washed and immunostained with the H-271 antibody as described above. For the negative controls, the primary antibodies were replaced with PBS. After the staining protocols, light counterstaining was performed with hematoxylin, and the sections were dehydrated and mounted.

Statistical analysis

The experiments for Southern blot analysis of DNA fragmentation were repeated on at least three independent occasions. Quantitative data represent low mol wt DNA (integrated OD from scanned x-ray films). The data obtained from samples incubated for 4 h without survival factors was set at 1.0 (100% apoptosis), and the data from samples treated with TNFα were compared with it. Data obtained from 3–13 replicate experiments (mean ± SEM) were analyzed by one-way ANOVA, and when significant differences were found, this was followed by comparison of the groups using unpaired two-tailed *t* test. $P < 0.05$ was considered statistically significant. At least 3 independent experiments were conducted in which the expression of Fas or FasL proteins was studied by Western blotting, and 5 experiments were conducted in which the effect of TNFα on NF-κB activity was studied by EMSA.

Results

In vitro induction of human testicular apoptosis and its inhibition by TNFα

In the present *in vitro* model, human testicular apoptosis was induced by incubating segments of seminiferous tubules under serum-free culture conditions. In our previous studies (32, 33), we have shown with ISEL and electron microscopy that the cells undergoing apoptosis in this model are mainly premeiotic spermatocytes and postmeiotic spermatids. To evaluate the role of TNFα in germ cell apoptosis we added recombinant human TNFα to the culture medium and studied its effects on the amount of apoptosis after 4-h culture in serum-free conditions. Interestingly, TNFα inhibited germ cell death effectively and dose-dependently (Fig. 1). In Southern blot analyses, the total amount of apoptotic low mol wt DNA fragmentation was suppressed by 25% ($P < 0.001$) and 43% ($P < 0.001$) at TNFα concentrations of 10 and 100 ng/ml, respectively. A TNFα concentration of 1 ng/ml was also tested in three experiments, but did not significantly inhibit germ cell death.

To confirm the results of the Southern blot analyses and to obtain information on the morphology of the cells treated with TNFα, we performed ISEL analysis of squash preparations from human seminiferous tubules taken immediately after orchidectomy (0 h) or cultured for 4 h in serum-free conditions in the absence or presence of TNFα. With the squash technique, cells from the seminiferous epithelium move under the coverslip to produce a monolayer and maintain their morphological characteristics, allowing better identification of individual cell types. In agreement with the results of the Southern blot analyses, germ cell apoptosis was clearly inhibited by TNFα (Fig. 2). Because of the presence of different populations of germ cells in the adjacent stages of seminiferous tubules, the varying amounts of ISEL positivity in individual squash preparations could be due to varying amounts of apoptotic activity in distinct types of germ cells present in the preparation. Therefore, the result showing the antiapoptotic effect of TNFα was based on examination of a large number of squash preparations. The majority of the TNFα-treated tubules showed a clearly decreased number of ISEL-positive cells compared with the untreated tubules. Negative controls, in which the terminal transferase enzyme was replaced with distilled water, showed no staining (data not shown).

Activation of NF-κB during testicular apoptosis and the effect of TNFα on this activation

To study the role of NF-κB in TNFα-induced germ cell survival, the ability of TNFα to increase NF-κB activation was tested by EMSAs using extracts from seminiferous tubules cultured for 1 or 4 h under serum-free conditions in the

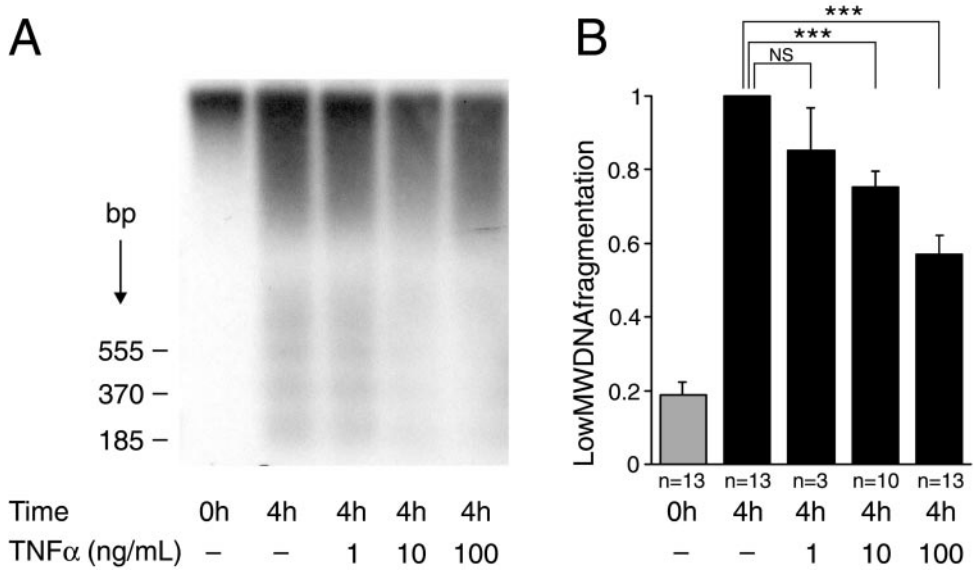

FIG. 1. Inhibition of *in vitro* induced human testicular apoptosis by TNFα. Segments of seminiferous tubules were incubated for 4 h under serum-free culture conditions in the absence or presence of TNFα. DNA from seminiferous tubules was extracted, whereafter 1 μg of the total DNA from each sample was 3′-end labeled with Dig-dd-UTP, and subjected to electrophoresis. The labeled DNA was detected with chemiluminescence as described in *Subjects and Methods*. Immediately after orchidectomy (0 h), the DNA samples showed no apoptotic ladder pattern in Southern blot analysis. In contrast, extensive apoptotic DNA fragmentation was observed in samples from tubules exposed for 4 h to serum withdrawal. This apoptosis was effectively and dose-dependently inhibited by TNFα. A, Radiograph from a representative experiment in which TNFα was added to the culture medium at 1, 10, and 100 ng/ml. B, Quantification of TNFα-mediated inhibition of low mol wt (MW) DNA (<1.3 kb) fragmentation. Each value represents a mean of independent experiments (number of experiments indicated as n) ± SEM. ***, $P < 0.001$.

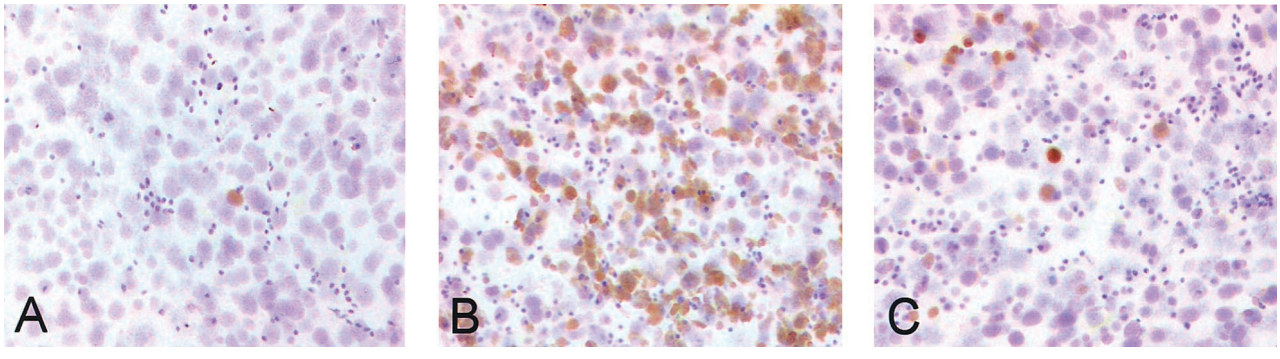

FIG. 2. ISEL analysis of the TNFα-mediated inhibition of *in vitro*-induced apoptosis of human testicular germ cells. Segments of seminiferous tubules were incubated under serum-free culture conditions in the absence or presence of TNFα. After incubation, the tubules were squashed and fixed, and apoptotic cells were detected by 3′-end labeling of apoptotic DNA, as described in *Subjects and Methods*. A, Only a few scattered apoptotic cells were found in the tubules squashed immediately after the orchidectomy (0 h). B, The number of apoptotic cells was greatly increased in the tubules cultured for 4 h in the absence of serum and TNFα. C, Germ cell apoptosis was effectively inhibited by TNFα at 100 ng/ml.

absence or presence of TNFα (Fig. 3). EMSAs indicated the presence of three NF-κB bands, designated A, B, and C. The specificity of these bands was tested by competition experiments in which a 100-fold excess of unlabeled κB oligonucleotide or unlabeled mutated κB oligonucleotide was added in the binding reaction. As shown in Fig. 3, all bands disappeared when the unlabeled κB probe, but not when the mutated κB probe, was included in the reaction.

No change in the intensity of band C was observed in seminiferous tubules in which apoptosis was induced. Band B was also present in 0 h control samples, and its intensity was only slightly increased during incubation of the tubules in serum-free conditions. In contrast, in the tubules in which apoptosis was induced, the intensity of band A was markedly increased, suggesting that it represents an inducible form of NF-κB that most likely plays a role in germ cell apoptosis. Unexpectedly, TNFα did not significantly increase the intensity of any of the NF-κB bands. The result was the same at both 1 and 4 h.

Expression of the TNFα receptors in the human testis

The presence of the TNFα receptors TNFR-I and TNFR-II in the human testis was first studied by Western blot analysis (Fig. 4). Two different antibodies to human TNFR-I (Santa Cruz Biotechnology, Inc., H-271 and StressGen CSA-810)

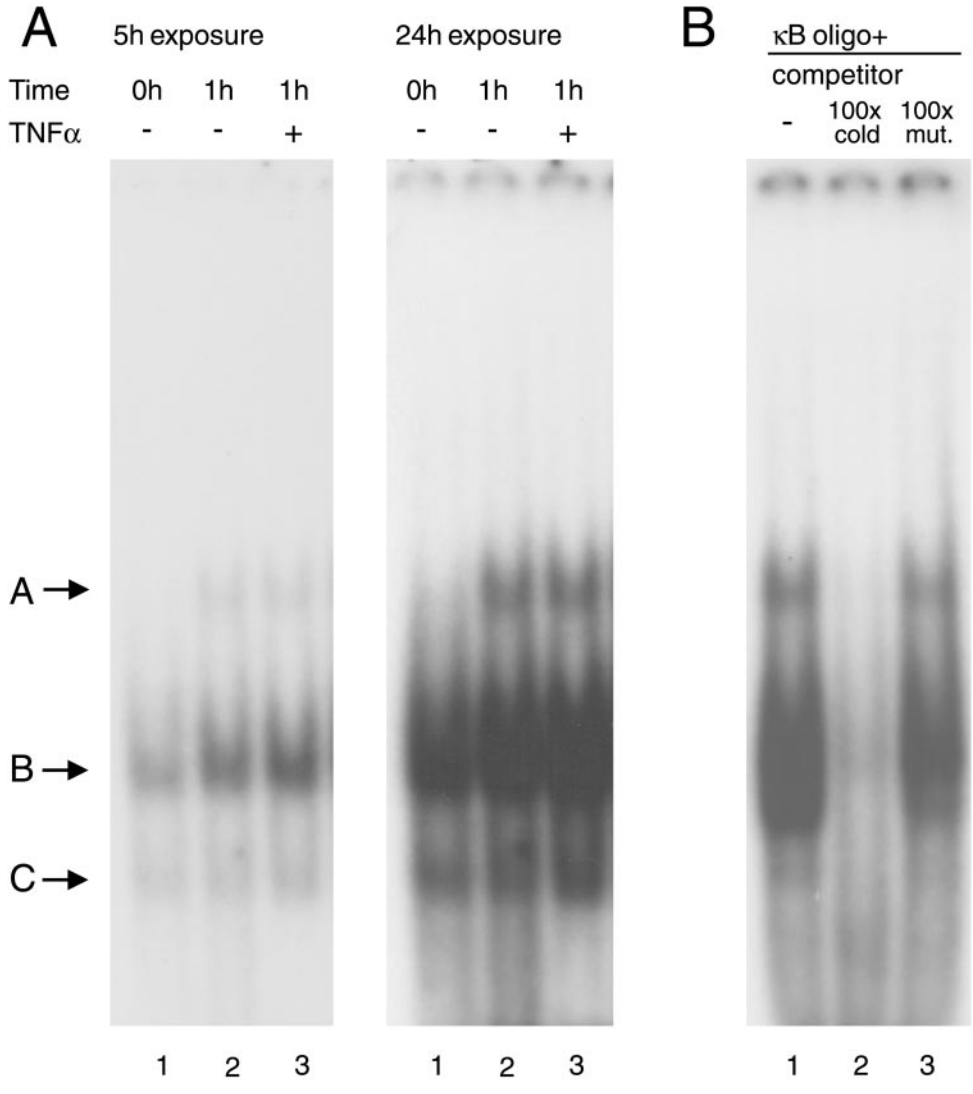

FIG. 3. EMSAs showing activation of NF-κB during human testicular apoptosis. Protein extracts from human seminiferous tubules were incubated with a ^{32}P-labeled κB oligonucleotide. DNA-protein complexes were resolved by nondenaturing PAGE and detected by autoradiography. A, EMSA of nuclear protein extracts from seminiferous tubules that were not exposed to apoptosis-inducing conditions (0 h, lane 1) and were cultured for 1 h under serum-free conditions in the absence (lane 2) or presence (lane 3) of 100 ng/ml TNFα. Three bands, designated A, B, and C, were detected. A slight increase in the intensity of the band B can be observed in cultured samples with short (5-h) exposure time, but not with longer (24-h) exposure time, with which band A is clearly visible. Band A was markedly up-regulated in the tubules that were exposed to apoptosis-inducing conditions. TNFα had no significant effect on the intensity of any of the NF-κB bands. B, Competition experiments demonstrating the specificity of the EMSA bands. Nuclear protein extracts from seminiferous tubules cultured for 1 h under serum-free conditions were incubated with the labeled κB oligonucleotide in the absence of competitors (lane 1) or in the presence of a 100-fold excess of unlabeled κB oligonucleotide (lane 2) or a 100-fold excess of unlabeled mutated κB oligonucleotide (lane 3).

FIG. 4. Western blotting of the TNFRs expressed in the human testis. Protein extracts of adult human testis were subjected to electrophoresis on SDS-PAGE gels, electroblotted on polyvinylidene difluoride membranes, and analyzed with rabbit polyclonal antibodies to human TNFR-I (H-271 or CSA-810) or human TNFR-II as described in *Subjects and Methods*. Both the H-271 and CSA-810 antibodies to the TNFR-I detected an approximately 45-kDa band. In addition, the CSA-810 antibody revealed an approximately 55-kDa band. The specificity of both of these bands was confirmed with a blocking peptide (b.p.). A weak band corresponding to the expected size of the TNFR-II protein was also detected in the testis tissue extracts with the antibody to human TNFR-II.

detected a band of approximately 45 kDa in the testis tissue extracts. A band of similar molecular mass was seen in a human liver extract prepared using the same protocol as that for testis (data not shown). In addition, the CSA-810 antibody revealed a band of approximately 55 kDa in both testis (Fig. 4) and liver (data not shown) extracts. The specificities of both of these bands were confirmed by preabsorption experiments using an inhibitory peptide. The 45-kDa band appears to represent a smaller protein than the usually reported 55-kDa TNFR-I. Deglycosylation experiments indi-

cated that the smaller size does not result from alterations in the degree of glycosylation (data not shown). Accordingly, it is likely that the 45-kDa protein is a fragment of the 55-kDa receptor that has been formed by proteolysis during sample preparation. TNFR-II was also detected in testis tissue from three men. Unfortunately, blocking peptide for the TNFR-II antibody is not available, and we were unable to confirm the data.

Localization of the TNFα receptors in the adult human testis was then studied immunohistochemically in paraffin-embedded sections of formalin-fixed human testis tissue. Although Western blot analysis of the human testis suggested the presence of both TNFα receptors, only TNFR-I was found by immunohistochemistry. TNFR-I was detected by two polyclonal antibodies to human TNFR-I (Santa Cruz Biotechnology, Inc., H-271 and StressGen CSA-810). When the primary antibodies were replaced with nonspecific rabbit IgG, there was no specific staining (Fig. 5A). With the H-271 antibody, strong positive staining was observed in the Sertoli cells and residual bodies of the seminiferous epithelium and in the interstitial Leydig cells (Fig. 5B). Within the Sertoli cells it appeared that the strongest staining was in the nucleus or in very close proximity to the nucleus. Identification of Sertoli cells was based on their typical localization in the seminiferous epithelium, on the morphology of the nucleus, and on the presence of a characteristic nucleolus that can be detected in a phase contrast field (Fig. 5, C and D). In the positive interstitial cells, the staining was found in the cytoplasm. These cells were suggested to be Leydig cells, as they were found to be negative for macrophage surface antigen (Fig. 5E). With the CSA-810 antibody, positively staining interstitial cells were also detected. In the seminiferous epithelium this antibody showed a positive staining pattern that seemed to follow the shape of the Sertoli cell cytoplasm

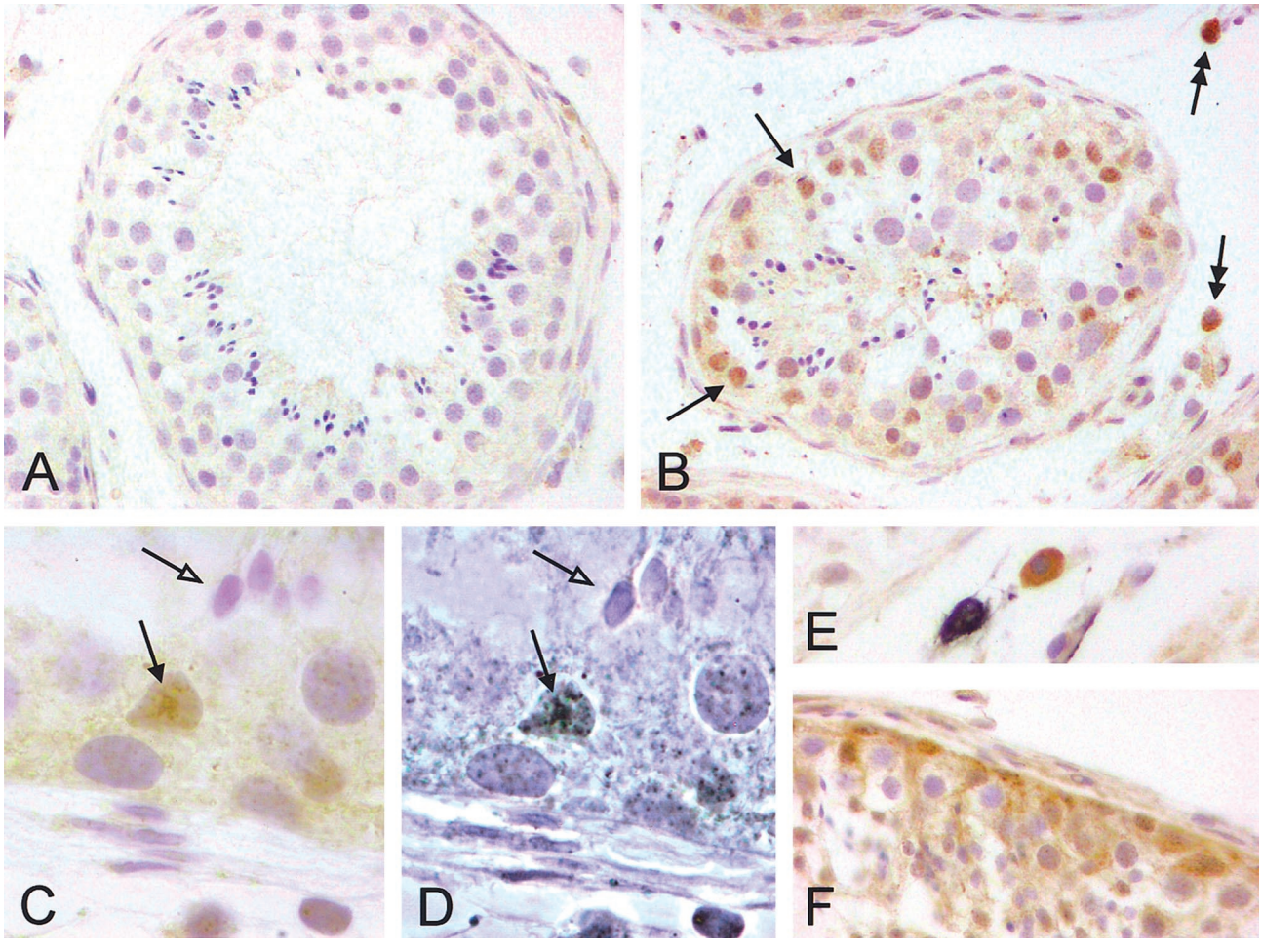

FIG. 5. Immunolocalization of the TNFR-I in the adult human testis. Paraffin-embedded sections of formalin-fixed human testis tissue were immunostained as described in *Subjects and Methods*. A, Nonimmune control in which nonspecific rabbit IgG was used as the primary antibody. Original magnification, ×200. B, Strong positive staining for TNFR-I was observed with the H-271 antibody in Sertoli cells (*arrows*) and in interstitial Leydig cells (*double arrows*). Magnification, ×200. C and D, Higher (×1000) magnification showing the characteristic nucleolus of the Sertoli cell (*arrow*) and the presence of a cluster of spermatids (*open arrows*) in close proximity to the Sertoli cell nucleus, which are typical features of Sertoli cells. A phase contrast field (D) is presented to better illustrate the morphology of these structures. E, The TNFR-I-positive interstitial cells were considered to be Leydig cells, because they were found to be negative for macrophage surface antigen by double immunostaining with the H-271 antibody (*brown*) and an antibody to the macrophage surface antigen (*dark gray*). Original magnification, ×400. F, The CSA-810 antibody to TNFR-I showed a staining pattern that appeared to follow the shape of the Sertoli cell cytoplasm. Magnification, ×400.

(Fig. 5F). In addition, with both the H-271 and the CSA-810 antibodies, some clusters of positively staining spermatogonia and pachytene spermatocytes were found (data not shown), but these cells were far rarer than the positively staining Sertoli cells. No staining was observed in the negative controls, in which the primary antibodies to TNFR-I or macrophage surface antigen were replaced with PBS (data not shown).

Down-regulation of testicular Fas ligand by TNFα

To study whether changes in the function of the Fas system could contribute to the observed antiapoptotic effect of TNFα, the expression of Fas and FasL was studied by Western blot analysis of seminiferous tubules cultured under serum-free conditions in the absence or presence of TNFα. We found that the expression of Fas protein in the seminiferous tubules was not affected by TNFα (data not shown). However, concomitantly with inhibiting testicular apoptosis, TNFα was found to regulate the expression of the FasL (Fig. 6). In five independent experiments, the expression of FasL in tubules cultured under serum-free conditions for 4 h was up-regulated by 48–185% compared with tubules that were not subjected to apoptosis-inducing conditions. TNFα was found to decrease this up-regulated FasL expression by 14–72%. The results were adjusted to the amount of α-tubulin present in the samples.

Discussion

In the testis, TNFα is secreted by germ cells (4) and activated interstitial macrophages (5, 6). The effects of this cytokine on testicular somatic Sertoli and Leydig cells have been previously studied, and it has been suggested to play a role in the regulation of spermatogenesis. However, the effects of TNFα on testicular germ cells have remained unknown. In the present study we found that TNFα effectively inhibited *in vitro*-induced apoptosis of human testicular germ cells. We did not observe any significant effect of TNFα on the activation of NF-κB, which is often considered to be a mediator of TNFα-induced survival signals. The inhibitory effect of TNFα on germ cell apoptosis is likely to be mediated via Sertoli cells, as the type I TNF receptor was predominantly found in the Sertoli cells of the human seminiferous epithelium. Interestingly, our results revealed that the Fas ligand, a known inductor of testicular apoptosis (24, 25), was down-regulated by TNFα. Thus, regulation of the function of the Fas system appears to be a possible mechanism for the TNFα-mediated survival of human testicular germ cells.

TNFα is a pleiotropic cytokine that is known to induce apoptotic pathways in transformed cell lines (20, 22). However, cell death is a rare response to TNFα in nontransformed cells and usually occurs only when gene expression is inhibited by RNA or protein synthesis blockade (21, 22, 34). In cell types resistant to proapoptotic signals of TNFα, the survival signals induced by this cytokine are often associated with activation of the NF-κB pathway (20, 22, 23). The NF-κB transcription factors consist of five known mammalian subunits (p65/RelA, RelB, c-Rel, p50, and p52), which function as homo- or heterodimers. In resting cells, NF-κB dimers remain sequestered in the cytoplasm by inhibitor proteins (IκB). Stimulation by a variety of stimuli, *e.g.* TNFα, leads to degradation of the IκB and translocation of the liberated NF-κB to the nucleus, where it regulates gene expression via interaction with consensus κB enhancer elements (35). In the rat testis, NF-κB p50 and p65 proteins are constitutively expressed and active in the Sertoli cells of all stages of spermatogenesis (36). In addition, nuclear NF-κB expression is elevated in Sertoli cells of stages XIV–VII and is also found transiently in pachytene spermatocytes and spermatids (36). Furthermore, TNFα has been shown to increase nuclear NF-κB binding activity in rat Sertoli cells *in vitro* (36). In the present study TNFα effectively suppressed human testicular apoptosis. As NF-κB is a potential mediator of TNFα-induced survival signals, we tested the ability of TNFα to increase active NF-κB in our *in vitro* model. We found increased NF-κB binding activity in seminiferous tubules cultured under serum-free conditions and showing increased apoptosis. However, TNFα did not increase NF-κB activation. In the present system in which Sertoli cells and germ cells at different stages of maturation are present, we cannot rule out the possibility that in a subpopulation of testicular cells, TNFα would have altered NF-κB activation. Moreover, in cultured rat Sertoli cells, TNFα induces NF-κB activation

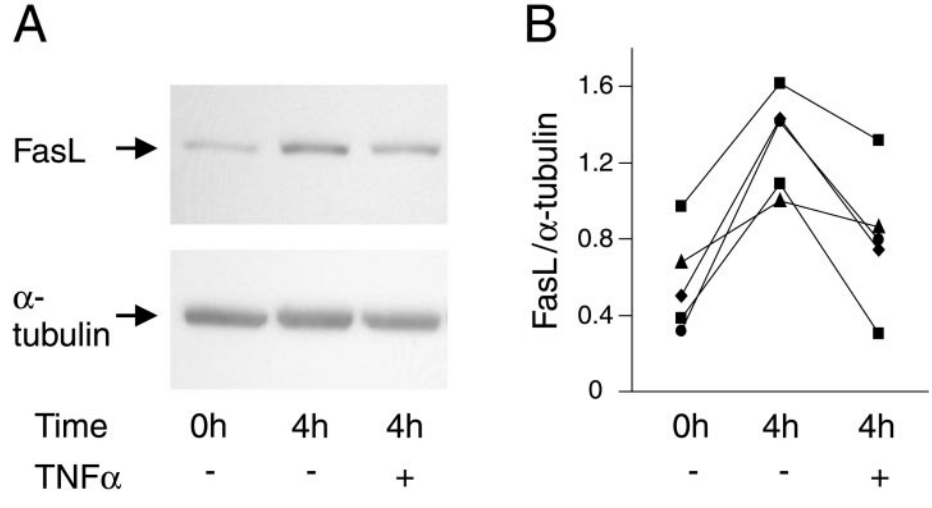

FIG. 6. Down-regulation of testicular FasL by TNFα. Protein extracts from seminiferous tubules cultured for 0 and 4 h in serum-free conditions in the absence or presence of 100 ng/ml TNFα were electrophoresed on 12% SDS-PAGE gels and analyzed by Western blotting as described in *Subjects and Methods*. A, Representative Western blot of FasL and α-tubulin (loading control). B, Quantification of FasL expression (adjusted to the amount of α-tubulin) from Western blots of five independent experiments. In each experiment the expression of the FasL was up-regulated in the tubules that were exposed to apoptosis-inducing conditions. This up-regulated expression of the FasL was decreased by TNFα.

in a biphasic manner, at first with an increase in NF-κB binding within 0.5 h, followed by a gradual decrease and a second rise and fall after 6 h (36). In the present study the effect of TNFα on NF-κB binding activity was first studied 1 h after the orchidectomy. The seminiferous tubules were immediately isolated and placed in TNFα-containing culture medium, but considering the time needed for TNFα to diffuse into several layers of cells in the seminiferous epithelium, the real exposure of the cells to TNFα was less than 1 h. It appears unlikely that an effect of TNFα on NF-κB binding activity would have been observed at an even earlier time point, but this possibility cannot be completely ruled out. It is also possible that in the present culture conditions, the rate and extent of NF-κB activation are already maximal and cannot be enhanced by exogenous TNFα. However, our results suggest that in the human testis, TNFα may induce germ cell survival via a pathway distinct from NF-κB activation.

In the present study we used immunohistochemistry to study the target cells of TNFα in the testis. Although Western blot analysis of the human testis indicated the presence of both TNFα receptors (TNFR-I and TNFR-II), immunohistochemistry detected only TNFR-I. Within the seminiferous epithelium the most abundant expression of TNFR-I was seen in Sertoli cells. This is in agreement with previous reports on porcine and mouse testes, in which both TNFR-I mRNA and protein have been detected (4, 7, 9). In addition, we found that some clusters of spermatogonia and pachytene spermatocytes stained positively for the TNFR-I. However, compared with the number of TNFR-I-expressing Sertoli cells, these cells were rare. When we induced testicular apoptosis in serum-free conditions, the apoptotic cells were identified mainly as spermatocytes and spermatids, which were not usually found to express a receptor for TNFα. Therefore, the TNFα-induced survival of these germ cells is most likely mediated via the adjacent Sertoli cells by a paracrine mechanism. Consistent with this hypothesis, several lines of evidence suggest that TNFα modulates Sertoli cell functions that may affect germ cell survival and spermatogenesis. Firstly, TNFα is probably involved in the metabolic cooperation between Sertoli cells and postmeiotic germ cells. In cultured porcine Sertoli cells, TNFα stimulates the production of lactate, which is used in preference to glucose as an energy substrate by postmeiotic germ cells (12, 13). Thus, it has been suggested that postmeiotic germ cells, which are known to produce TNFα, may control lactate production in Sertoli cells via this cytokine. Secondly, TNFα has been shown to regulate transferrin expression in Sertoli cells (14). Iron transfer from the circulation to the iron-requiring testicular germ cells depends on the synthesis of transferrin, the major iron-transporting protein, by Sertoli cells (37). In cultured rat Sertoli cells, TNFα increases transferrin secretion and the steady state expression of mRNA (14). Thirdly, TNFα may contribute to the cyclical up-regulation of the cAMP response element-binding protein in Sertoli cells via activation of NF-κB (15). cAMP response element-binding protein is an important regulator of a number of cAMP-induced genes in Sertoli cells and consequently is suggested to be a regulator of spermatogenesis. Fourthly, in cultured porcine Sertoli cells, TNFα has been shown to stimulate IGF-binding protein-3 expression (7). An increase in IGF-binding protein-3 is likely to result in a decrease in the bioavailability of IGF-I for its receptors and a decrease in IGF-I action. IGFs stimulate spermatogonial DNA synthesis and may also maintain premeiotic DNA synthesis in rat seminiferous tubules *in vitro* (38). TNFα may therefore alter spermatogenesis by decreasing the bioavailability of the IGFs.

The present study suggests a new paracrine mechanism for the TNFα-induced survival of testicular germ cells. Concomitantly with inhibiting germ cell apoptosis, TNFα was found to down-regulate the expression of FasL, the Sertoli cell-derived cytokine known to induce germ cell death. In a variety of cells, binding of the FasL to its receptor Fas (APO1/CD95) induces apoptosis (39). In the testis, the Fas-FasL system has been shown to contribute to the immune privilege of this organ (40) and to regulate physiological germ cell apoptosis (24, 25). Interestingly, a recent report suggested a role for TNFα in regulation of the expression and function of the Fas system in the seminiferous epithelium (26). Cultured mouse Sertoli cells were shown to express low levels of functionally active membrane-bound Fas protein, which was markedly increased by stimulation with TNFα (26). The researchers suggested that inflammatory cytokines may create a proapoptotic environment by inducing the up-regulation of Fas in Sertoli cells, which leads to Sertoli cell death when contact occurs with FasL-bearing cells, such as activated T lymphocytes. On the other hand, they found that TNFα also induced a soluble antiapoptotic form of Fas at concentrations lower than those needed for the induction of the membrane-bound Fas. Thus, it was suggested that *in vivo* TNFα produced by germ cells may induce soluble Fas, which is a survival factor in the seminiferous tubules. In the present study of the human testis, we did not observe alterations in testicular Fas expression by TNFα. However, the level of FasL in the seminiferous tubules was increased upon induction of apoptosis, and TNFα decreased this up-regulated FasL expression. Down-regulation of the death-promoting FasL may therefore mediate the survival signal induced by TNFα. A similar mechanism of TNFα-mediated inhibition of apoptosis has been observed in the vascular endothelium, where down-regulation of FasL expression in the endothelial cells by TNFα leads to decreased endothelial cytotoxicity toward Fas-bearing leukocytes (41). Thus, secretion of TNFα by activated cells at sites of vascular injury has been suggested to down-regulate FasL expression in the adjacent normal endothelium, promoting more leukocyte extravasation and lesion growth (41).

Taken together, the present results suggest a new mechanism in the paracrine control of spermatogenesis. In cultured human seminiferous tubules, TNFα inhibited germ cell apoptosis by a mechanism that did not appear to be associated with the NF-κB pathway. As the TNF receptor was found to be expressed by Sertoli cells of the human seminiferous epithelium, but not by the majority of the germ cells, the antiapoptotic effect of TNFα on germ cells is most likely mediated via the somatic Sertoli cells. Finally, we found that, concomitantly with inhibiting testicular apoptosis, TNFα down-regulated the expression of the death-promoting FasL. Thus, in the seminiferous tubules, germ cell-derived TNFα may regulate the level of the FasL and thereby control phys-

iological germ cell apoptosis. In light of the present findings from the human testis and the previously reported results from the mouse testis (26), TNFα may modulate testicular apoptosis in both physiological and pathological conditions by regulating the function of the Fas system.

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