EXPERIMENTAL STUDY

Tumor necrosis factor-alpha (TNF- α) promotes cell survival during spermatogenesis, and this effect can be blocked by infliximab, a TNF- α antagonist

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Abstract

Objective: Tumor necrosis factor-alpha (TNF- α) has been shown to inhibit germ cell death in human seminiferous epithelium. In the present study, we wanted to explore the effects of TNF- α in the rat seminiferous epithelium and to study molecular mechanisms of germ cell apoptosis. Furthermore, the effects of infliximab were studied. Infliximab is a TNF- α antagonist used in autoimmune disorders, such as rheumatoid arthritis and Crohn's disease.

Methods: Rat seminiferous tubule segments were cultured in the presence and absence of TNF- α , infliximab and SN50, a NF- κ B inhibitor. TUNEL-staining and cleaved caspase-3 immunohistochemistry combined with squash preparations of rat seminiferous tubule segments were used to evaluate the number of apoptotic cells. Western blot analyses were performed on cultured seminiferous tubule segments for Bcl-2 family proteins (Bax, Bad, Bcl-w, Bcl-xL) and fas ligand.

Results: TNF- α promotes cell survival in the rat seminiferous epithelium, and this prosurvival effect can be blocked by infliximab, a TNF- α antagonist. Bcl-xL was found to be upregulated in mitochondrial membranes by TNF- α , and this upregulation was inhibited by infliximab. Inhibition of NF- κ B translocation to the nucleus prevented the prosurvival effect of TNF- α on seminiferous epithelium.

Conclusions: The present study demonstrates that TNF- α promotes cell survival in the rat seminiferous epithelium, and this effect can be blocked by infliximab. This is the first study to show the effects of infliximab in the testis. The prosurvival effect of TNF- α might be at least partly mediated by modulating the expression and subcellular localization of Bcl-2 family proteins.

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Introduction

Normal spermatogenesis includes apoptotic cell death of some of the developing germ cells. It has been estimated that up to 75% of the hypothetical sperm number is lost due to apoptosis in early spermatogenesis (1, 2). The reason for this cell loss in the testis is explained by maintaining an optimal ratio of Sertoli cells to germ cells. Sertoli cells are essential for germ cell maturation, and the number of maturing germ cells is limited by the number of Sertoli cells (3). Tumor necrosis factor-alpha (TNF- α) belongs to the TNF- α superfamily of death ligands, which at present has 19 members and was first identified 20 years ago as a cytokine with antitumor effects *in vitro* and *in vivo* (4, 5). It causes inflammatory, antiviral and immunoregulatory effects, stimulates proliferation of normal cells, and exerts cytolytic and cytostatic action against tumor cells (6). The effects of TNF- α are mediated through two receptors, TNFR1 and TNFR2. TNFR1 contains the cytoplasmic death domain and belongs to the family of death receptors. Through the death

domain, TNFR1 activates the caspase cascade, leading to cell death. However, TNFR1 also mediates effects that promote cell survival by activation of transcription factors, such as NF- κ B and AP-1, which can induce genes involved in the suppression of apoptosis. In mouse testis, bioactive TNF- α is mainly produced by the round spermatids (7), and TNFR1 is found in porcine and human Sertoli and Leydig cells (8–10). In human testis, TNF- α inhibits germ cell apoptosis and downregulates Fas ligand, another member of the TNF- α superfamily of death ligands (10). Infliximab is a chimeric monoclonal antibody to human TNF- α with potent anti-inflammatory effects, and it is in therapeutic use for rheumatoid arthritis (RA) and Crohn's disease (CD) (11–13). Infliximab antagonizes TNF- α , and it has been shown to cause apoptosis of T lymphocytes in the gut mucosa (14). It is an effective and well-tolerated therapy for the management of RA and CD, although there are concerns that the immunogenicity of the anti-TNF antibody may result in the formation of human antichimeric antibodies as well as lymphoproliferative disorders (12, 13). The Bcl-2 family

of proteins is an important regulator of cell survival. It consists of proapoptotic (such as Bid, Bik, Bim, Bad, Bax, Bak and Bok) and antiapoptotic (such as Bcl-2, Bcl-w, Bcl-xL, A1 and Mcl1) proteins. Proapoptotic family members are divided into two groups, the BH3-only family (such as Bid, Bik, Bim and Bad), which are related by the conserved BH-3 domain (Bcl-2 homology), and the Bax family (Bax, Bak and Bok). Both types of proapoptotic members are needed during the apoptotic process: the BH-3-only proteins act as direct antagonists of prosurvival proteins, whereas Bax family proteins act further downstream, affecting mitochondrial integrity (15). In cellular stress, the Bcl-2 family members congregate at intracellular membranes, of which mitochondria are the best studied in respect of apoptosis machinery. In this study, we wanted to find out whether TNF- α has a pro-survival effect on rat seminiferous epithelium and whether the possible effect could be blocked by infliximab, a TNF- α antagonist. The aim was to evaluate the expression of Bcl-2 family proteins in the rat seminiferous epithelium in the presence of TNF- α and infliximab.

Materials and methods

Experimental animals

Sprague–Dawley rats were housed in a constant temperature (20 °C) and light–dark cycle (lights on, 0600–2000 h) with free access to food and water. Rats in all experiments were killed by CO₂ asphyxiation, and testes were removed for subsequent analysis. All animal experiments were approved by the Turku University Committee on Ethics of Animal Experimentation.

Sequential microdissection of the seminiferous tubules, tissue culture and stimulation

The testes were decapsulated on a Petri dish containing Ham's F12 Dulbecco's MEM (1:1 DMEM/F12; GIBCO BRL, Paisley, UK) supplemented with 0.1% BSA and gentamycin sulfate (50 μ g/ml). By transillumination-assisted microdissection (16, 17), 5-mm-long segments of pooled stages VII–VIII and IX–XII and 1-mm-long segments of stage XII from the rat seminiferous epithelial cycle were dissected for the Western blotting analyses and apoptosis assays. In tissue cultures, for *in situ* 3'-end labeling (ISEL), we used human TNF- α ; for other experiments, we used mouse TNF- α (Roche Diagnostics GmbH, Mannheim, Germany). Infliximab was used at 10 μ g/ml concentration and was purchased from a pharmaceutical supplier (Schering-Plough, Kenilworth, NJ, USA). This concentration has been shown to be effective in inhibition of TNF- α -mediated cell activation (18). The NF- κ B inhibitor SN50 was used at 50 μ g/ml concentration (Biomol,

Plymouth Meeting, PA, USA) (19). For all Western blotting and ISEL experiments, 1:1 DMEM/F12 was supplemented as mentioned above. For the experiment presented in Fig. 2, 1:1 DMEM/F12 was supplemented with 0.001% BSA and gentamycin sulfate (50 μ g/ml). For the experiment presented in Fig. 3, 1:1 DMEM/F12 was supplemented with 0.01% BSA and gentamycin sulfate (50 μ g/ml).

Squash preparations

Segments 1 mm long of rat seminiferous tubule from stage XII were incubated 6–24 h in 100 μ l Ham's F12 Dulbecco's MEM (1:1 DMEM/F12), supplemented, as described above, in a humidified atmosphere (5% CO₂, 34 °C), in the presence or absence of the test chemicals, and then transferred in 15 μ l culture medium onto a microscope slide. The tubules were carefully squashed between microscope slides and cover slips by following the formation of cellular monolayer under phase-contrast microscopy. Next, the squash preparations were snap frozen in liquid nitrogen, and then the cover slips were removed. The slides were dipped briefly in ice-cold 96% ethanol, fixed in 10% formalin for 10 min and transferred to PBS.

In situ 3'-end labeling (ISEL), cleaved caspase-3 immunohistochemistry and quantification of germ cells

After fixation, the slides were washed twice in PBS for 5 min, post-fixed in ethanol:acetic acid (2:1, v/v), again washed in PBS and finally dehydrated in ascending ethanol series, air dried and stored at –70 °C. ISEL was done as previously described (20) with modifications. Each incubation was performed in a humidified box. The squash preparations were rehydrated in ethanol solutions of descending concentrations. Samples were incubated for 10 min in 1 $\times$ TdT buffer (Boehringer Mannheim, Mannheim, Germany) before 3'-end-labeling. A volume of 10 μ l labeling mixture, containing 2 μ l fresh 5 $\times$ TdT (Boehringer Mannheim), 0.16 μ l terminal deoxynucleotidyl transferase (Boehringer Mannheim), 0.05 μ l Dig-11-ddUTP (1 nmol/ μ l, Boehringer Mannheim), 2 μ l 25 mM CoCl₂ (Boehringer Mannheim), 0.09 μ l 5 mM ddATP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and 5.7 μ l water, was applied to each sample, and the incubation was performed at 37 °C for 1 h. After the slides had been washed three times for 10 min each in a Tris buffer (150 mM NaCl and 100 mM Tris–HCl, pH 7.4), about 20 μ l of the same buffer containing 2% (w/v) of blocking reagent (Boehringer) were applied to each slide, and the samples were incubated at room temperature (RT) for 30 min. A volume of 20 μ l 2% (w/v) blocking buffer containing diluted (1/4000) antidigoxigenin alkaline phosphatase conjugate (anti-DIG-AP; Boehringer Mannheim) was

applied to the samples, which were then incubated at RT for 2 h. The slides were washed (three times for 10 min) in the Tris buffer and then left for 5–10 min in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl and 50 mM MgCl₂, pH 9.5). A volume of 20 μ l substrate solution containing nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Boehringer Mannheim) was diluted with 1 ml alkaline phosphatase buffer, and then about 20 μ l of this solution were added to each sample at RT. After the color reaction was estimated to be fully developed, the slides were treated for at least 5 min in EDTA buffer (100 mM Tris-HCl and 1 mM EDTA, pH 8.0) to stop the reaction. Preparations were mounted, and the number of apoptotic cells was calculated under a light microscope. For cleaved caspase-3 immunohistochemistry, the slides were treated with hydrogen peroxide (H₂O₂) for 15 min after fixation and again washed twice in PBS for 5 min. The cell membranes were permeabilized with 0.1% Triton-X in PBS for 15 min and rinsed briefly with PBS. The squash preparations were blocked with buffer containing 5% normal goat serum (NGS), 1% BSA and 0.1% Triton-X for 30 min. Cleaved caspase-3 antibody was added to squash preparation in 1:40 dilution in buffer containing 1% BSA and 0.1% Triton-X in PBS for 2 h. The slides were washed (three times for 10 min) in PBS, and antirabbit horseradish peroxidase (HRP)-linked secondary antibody was added in 1:200 dilution in 1% BSA and 0.1% Triton-X in PBS for 1 h and washed three times for 10 min in PBS. Diaminobenzidine (DAB) staining was done according to the manufacturer's instructions (Zymed Laboratories, South San Francisco, CA, USA). Preparations were mounted, and the number of apoptotic cells was calculated in a blinded fashion under a light microscope.

Protein extraction, subcellular fractionation and SDS-PAGE

Seminiferous tubules for Western blotting analysis were lysed with syringe and needle in buffer A (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate and 20 mM sodium fluoride in PBS, pH 7.4) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) at 4°C for 30 min. Cell lysates were centrifuged for 20 min at 15 000 *g*, and the supernatants were removed to new tubes for concentration measurements. For preparation of cytosolic and mitochondrial fractions, seminiferous tubule segments were homogenized on ice with five strokes of a teflon pestle in a glass homogenizer (Cowie Technology, Middlesbrough, UK) in the presence of buffer B (0.3 M sucrose, 5 mM TES and 200 nM EGTA in PBS, pH 7.4) supplemented with protease inhibitor cocktail (Sigma-Aldrich). The homogenates were centrifuged at 650 *g* for 10 min at 4°C to remove cell debris. The supernatant was centrifuged at 10 000 *g* for 15 min at 4°C to sediment the

fraction containing mainly mitochondria. The mitochondria were washed in buffer B and pelleted. The cytosolic fraction was isolated by centrifuging the 10 000 *g* supernatant fraction at 100 000 *g* for 60 min at 4°C. Protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL, USA). The protein was mixed with sample buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 1.1 M 2-mercaptoethanol and 2% bromophenol blue) and boiled for 5 min. The proteins were separated in a 8–13% SDS-polyacrylamide gel by the Mini Protean II System (Bio-Rad, Hercules, CA, USA). After electrophoresis, the protein was electrophoretically transferred onto a PVDF-membrane (Hybond-P; Amersham), according to the manufacturer's instructions.

Antibodies for immunohistochemistry and Western blotting

A rabbit monoclonal antibody against cleaved caspase-3 (Asp175) was purchased from Cell Signaling Technology (Beverly, MA, USA). A mouse monoclonal antibody against Fas ligand (clone G247-4), a mouse monoclonal antibody against cytochrome c (clone 7H8.2C12) and a rabbit polyclonal antibody against Bax (raised against residues 43–61 of mouse Bax) were purchased from BD Pharmingen (San Diego, CA, USA). A mouse monoclonal antibody against Bcl-xL (clone 2H12) and a rabbit polyclonal antibody against Bcl-w (raised against residues 16–29 of human Bclw) were purchased from Stressgen Biotechnologies (Victoria, Canada). A mouse monoclonal antibody against Ox-Phos complex IV (COX4, clone 20E89) was used to verify the purity of the cytosolic fraction from mitochondria; it was purchased from Molecular Probes (Eugene, OR, USA). A mouse monoclonal anti-actin antibody was purchased from ICN Biomedicals (Costa Mesa, CA, USA).

Western blotting analysis

The membrane was incubated in blocking buffer (0.1% Tween-20 and 5% nonfat milk powder in PBS or TBS for Bax, Bcl-w, Bcl-xL, Bad, actin and cytochrome c, or cleaved caspase-3 and COX4 respectively) at RT for 1 h. The primary antibody incubation was performed at 4°C overnight in blocking buffer for cleaved caspase-3, COX4 and cytochrome c and in PBS-T (PBS containing 0.1% Tween-20) for Bax, Bcl-w, Bcl-xL, Bad and actin. After washes (three times for 10 min) in PBS-T/TBS-T, the membrane was incubated with HRP-linked secondary antibodies (Amersham) for 1 h at RT. After washes (three times for 10 min) in PBS-T/TBS-T, the membrane was subjected to chemiluminescent detection with the ECL Plus Kit (Amersham). The membrane was exposed to Fuji RX-100 film (Eastman Kodak, Rochester, NY, USA) for 15 s–15 min. Stripping of the membrane was done according to the manufacturer's instructions and reprobed with another antibody.

Densitometric analysis of Western blotting results

The X-ray films of Western blotting were first scanned by a UMAX scanner (Super Vista S-20; Binuscan, Mamaroneck, NY, USA) and a Photoperfect software package (Binuscan). The images were saved as TIFF-type files (*.tif, Microsoft, Redmond, WA, USA, and Aldus Co., Seattle, WA, USA) and then quantified by Tina 2.0 densitometric analytic system (Raytest Isotopenmesgerate GmbH, Straubenhardt, Germany), according to the manufacturer's instructions.

Statistical analysis

In Western blotting analyses, the densitometric values of the signals were first normalized against the values obtained from the antiactin control blotting. The values from different experiments were pooled for calculation of means and S.E.M. and for one-way ANOVA, and Dunnett's (in dose-response studies) and Tukey's (multiple comparisons) tests by SPSS 11.0 (SPSS Inc., Chicago, IL, USA). For quantification of ISEL and cleaved caspase-3 results, the experiments were repeated three times, and in each repeat the mean value was obtained from three to four parallel specimens. In the experiments presented in Figs 1 and 3, logarithmic transformation of the values was used to normalize the distribution in treatment groups. *P* values less than 0.05 were considered statistically significant.

Results

TNF- α promotes cell survival in dose-dependent manner, and this effect can be blocked with infliximab, a TNF- α antagonist

Segments 1 mm long from stage XII of the rat seminiferous epithelial cycle were cultured for 24 h in the presence or absence of different concentrations of TNF- α , with or without infliximab. Squash preparations were done from tubule segments to form a monolayer of tubular cells for *in situ* 3'-end labeling (ISEL) to evaluate the number of apoptotic cells in specimens. When compared with the control, the number of apoptotic cells was significantly lower in the presence of 10 ng/ml TNF- α . Infliximab blocked this prosurvival effect of TNF- α (Fig. 1). The prosurvival effect of TNF- α was verified by immunohistochemistry with a cleaved caspase-3 antibody. Segments 1 mm long from stage XII of rat seminiferous epithelium were cultured for 8 h in the presence or absence of different concentrations of TNF- α , with and without infliximab. Squash preparations were done from tubule segments for immunohistochemistry to evaluate the number of apoptotic cells in specimens. The apoptotic cells were identified as germ cells, mostly primary spermatocytes

and spermatids, on the basis of staining pattern and morphology. When compared with the control, the number of apoptotic cells was significantly lower in the presence of 10 and 100 ng/ml TNF- α after 8-h incubation. Infliximab again blocked this prosurvival effect of TNF- α (Fig. 2). Prosurvival ED₅₀ for TNF- α appears to be 1–10 ng/ml in our seminiferous tubule culture.

NF- κ B translocation inhibitor SN50 has proapoptotic effect on rat seminiferous epithelium and abolishes the prosurvival effect of TNF- α

Segments 1 mm long from stage XII of the rat seminiferous epithelial cycle were cultured for 16 h in the presence and absence of 10 ng/ml TNF- α with and without SN50, a NF- κ B translocation inhibitor. Squash preparations were done from tubule segments for immunohistochemistry with cleaved caspase-3 antibody to evaluate the number of apoptotic cells in specimens. The number of apoptotic cells in fresh control was low, as expected. After 16-h incubation, the number of apoptotic cells was significantly lower in the presence of TNF- α compared with control. The number of apoptotic cells in the tubules treated with both TNF- α and SN50 increased sixfold compared with TNF- α -treated tubules. SN50 had a proapoptotic effect on seminiferous epithelium, as the number of apoptotic cells in SN50-treated cells was 2.5-fold higher than in control (Fig. 3).

The expression of Bcl-w, Bcl-xL, Bad or Bax is not affected by TNF- α or infliximab analyzed in total tubule homogenates Segments 5 mm long from pooled stages VII–VIII and IX–XII of the rat seminiferous epithelial cycle were cultured for 21 h in the presence and absence of different concentrations of TNF- α , with and without infliximab. The expression of Bcl-w, Bcl-xL, Bad or Bax was not affected by TNF- α or infliximab analyzed in total tubule homogenates (Fig. 4; data shown from pooled stages IX–XII).

TNF- α stimulation leads to an increased amount of Bcl-xL in mitochondria, and this increase can be blocked by infliximab Segments 5 mm long from pooled stages IX–XII of the rat seminiferous epithelial cycle were cultured for 6 h in the presence and absence of 10 ng/ml TNF- α , with and without infliximab. The protein was extracted from mitochondrial and cytosolic fractions. During 6-h incubation, the amount of Bcl-xL decreased in the mitochondrial fraction compared with the 0-h control (Fig. 5). Stimulation by TNF- α increased the amount of Bcl-xL in the mitochondrial fraction. Infliximab blocked this increase. No changes were observed in

Figure 1 ISEL staining of the rat seminiferous tubule segments cultured in the presence or absence of different concentrations of TNF- α with and without infliximab. (A) Microphotograph of representative specimens showing the ISEL-positive cells at stage XII of the rat seminiferous epithelial cycle after 24 h of treatment with vehicle (control), TNF- α (10 ng/ml), infliximab or TNF- α (10 ng/ml) plus infliximab. Dark nuclei are apoptotic cells, some of which are marked with white arrows as examples. Black arrows indicate the openings of the lumina of the tubules. Bar, 50 μ m. (B) Quantitative analysis of ISEL staining of three independent experiments with three to four parallel samples using different rats. Each bar represents the number of ISEL-positive cells per 1 mm of seminiferous tubule as the mean $\pm$ S.E.M., $n = 3$. * $P < 0.05$ (compared with control).

Figure 2 Immunohistochemistry with cleaved caspase-3 antibody of seminiferous tubule segments cultured in the presence or absence of different concentrations of TNF- α with and without infliximab. (A) Microphotograph of representative specimens showing the cleaved caspase-3-positive cells at stage XII of the rat seminiferous epithelial cycle after 8 h of treatment with vehicle (control), TNF- α (10 ng/ml), infliximab or TNF- α (10 ng/ml) plus infliximab. Dark nuclei are apoptotic cells, some of which are marked with white arrows as examples. Black arrows indicate the openings of the lumina of the tubules. Bar, 50 μ m. (B) Quantitative analysis of cleaved caspase-3 immunohistochemistry of three independent experiments with three to four parallel samples using different rats. Each bar represents the number of cleaved caspase-3-positive cells per 1-mm-long tubule as the mean $\pm$ S.E.M., $n = 3$. * $P < 0.05$ (compared with control).

A

B

Figure 3 Immunohistochemistry with cleaved caspase-3 antibody for seminiferous tubule segments cultured in the presence or absence of TNF- α with and without SN50, a NF- κ B inhibitor. (A) Microphotograph of representative specimens showing the cleaved caspase-3-positive cells at stage XII of the rat seminiferous epithelial cycle after 16 h of treatment with vehicle (control), TNF- α (10 ng/ml), SN50 or TNF- α plus SN50. Dark nuclei are apoptotic cells, some of which are marked with white arrows as examples. Black arrows indicate the openings of the lumina of the tubules. Bar, 50 μ m. (B) Quantitative analysis of cleaved caspase-3 immunohistochemistry of three independent experiments with three to four parallel samples using different rats. Each bar represents the number of cleaved caspase-3-positive cells per 1-mm-long tubule as the mean $\pm$ S.E.M. Bars with different letters are statistically different ($P < 0.05$, $n = 3$).

the cytosolic fraction of Bcl-xL. Bax expression remained unchanged in both cytosolic and mitochondrial fractions during the incubation. Spontaneous cytochrome c release to cytosol was observed during 6-h incubation. However, TNF- α did not prevent this release significantly.

Discussion

In the present study, we have shown the cell-survival effect of TNF- α on rat seminiferous epithelium and the blocking of this survival effect by infliximab, a TNF- α antagonist. The two techniques used for

Figure 4 Western blotting images showing the expression of Bcl-xL, Bcl-w, Bad and Bax analyzed in total tubule homogenates from pooled stages IX–XII of the rat seminiferous epithelial cycle. The tubule segments were cultured in the presence or absence of different concentrations of TNF- α with and without infliximab for 21 h. The blots were stripped and subsequently reprobed with an antiactin monoclonal antibody for normalization of loading. The numbers refer to different treatments as follows: (0) 0 h, control; (1–8) 21-h incubation, (1–4) without infliximab and (5–8) with infliximab, (1, 5) control; (2, 6) TNF- α 1 ng/ml; (3, 7), TNF- α 10 ng/ml; (4, 8) TNF- α 100 ng/ml. The images are representative of three sets of independent experiments using different rats. No significant changes were observed in the expression of Bcl-xL, Bcl-w, Bad and Bax analyzed in total tubule homogenates from pooled stages IX–XII of the rat seminiferous epithelial cycle.

evaluation of apoptosis were *in situ* 3'-end labeling (ISEL) and cleaved caspase-3 immunohistochemistry. DNA fragmentation is a prominent and late feature in programmed cell death, and apoptotic cells can be identified and quantified with ISEL (21). The main intracellular effectors of apoptosis are a family of cysteine proteases called caspases (22). Caspase-3, a downstream caspase in the caspase cascade, is activated by proteolytic processing. With immunohistochemistry, positive cells for cleaved caspase-3 were easily distinguishable and were identified as germ cells on the basis of staining pattern and morphology. ISEL and immunohistochemistry were applied in combination with squash preparation of isolated seminiferous tubule segments from stage XII of the rat seminiferous epithelial cycle, as stage XII has been shown to be prone to spontaneous apoptosis (23). Both techniques gave similar results, as they clearly showed a cell-survival effect by TNF- α on rat seminiferous epithelium and the blocking of this survival effect by infliximab, which is in therapeutic use for RA and CD (12, 13). In addition, patients with psoriasis have experienced a clinical benefit from treatment with infliximab (24). Infliximab neutralizes the effect of TNF- α , but part of the effect in the treatment of CD might be mediated by the proapoptotic effect of infliximab on T lymphocytes in the gut mucosa (14). In our study, infliximab did not show any effect on the amount of apoptosis compared with control, but the ability of infliximab to block the prosurvival effect of TNF- α on the seminiferous epithelium might have clinical relevance when patients are receiving anti-inflammatory treatment with infliximab. In the mouse testis, TNF- α is produced in the pachytene spermatocytes and round spermatids, and the receptor TNFR1 has been identified in Sertoli cells (7). The localization of TNFR1 suggests that the effects of TNF- α on germ cells are paracrine rather

than direct. TNF- α simultaneously activates both apoptotic and cell-survival signals. The apoptotic signals are mediated through the death domain of TNFR1, leading to the activation of the caspase cascade. These apoptotic signals do not require active protein synthesis, whereas cell-survival effects do. The protein synthesis is mediated through the activation of NF- κ B (25), a transcription factor that regulates a multitude of genes, including many genes that promote cell survival, such as members of the inhibitors of apoptosis (IAP) family (c-IAP1 and c-IAP2) and the Bcl2 family (Bfl-1/A1 and Bcl-xL) (26–29). NF- κ B exists in an inactive cytoplasmic complex with an inhibitory protein, I κ B. Activation of TNFR1 leads to degradation of I κ B by I κ B kinases and release of the active NF- κ B complex, which translocates to the nucleus. We performed an experiment in which this translocation of the active complex was inhibited by SN50 (19). Inactivation of NF- κ B abolished the prosurvival effect of TNF- α . In fact, massive apoptosis occurred when seminiferous tubules were cultured with SN50, indicating the importance of NF- κ B as a mediator of prosurvival effects.

Proteins of the Bcl-2 family are important mediators of apoptotic signaling (22). The functions of Bcl-2 family proteins are regulated at several levels. Changes in subcellular localization, expression and post-translational modifications (such as phosphorylation and deamidation) of Bcl-2 family proteins tune their functions (30–33). We studied whether TNF- α stimulation has an impact on the expression of certain important Bcl-2 family members. We have earlier shown that stem cell factor (SCF) promotes cell survival during spermatogenesis by upregulating the prosurvival Bcl-2 family proteins, Bcl-w and Bcl-xL, and downregulating proapoptotic Bax (23). When they were analyzed in total seminiferous tubule homogenates, no alterations in

Figure 5 Western blotting results for cytochrome c, Bcl-xL, Bax and Fas ligand from subcellular fractions. Pooled stages IX–XII of the rat seminiferous epithelial cycle were cultured for 6 h in the presence or absence of TNF- α with and without infliximab; subsequently, subcellular fractions were extracted for Western blotting analyses. (A) Western blotting images showing the expression of cytochrome c, Bcl-xL, Bax and Fas ligand in cytosolic and mitochondrial fractions. The blots were stripped and subsequently reprobed with the next antibody. The numbers refer to different treatments as follows: (1) 0 h, control; (2) 6 h, control; (3) 6 h, TNF- α (10 ng/ml); (4) 6 h, infliximab; (5) 6 h, TNF- α (10 ng/ml) plus infliximab. Spontaneous cytochrome c release to cytosol was observed during 6-h incubation. During 6-h incubation, the amount of Bcl-xL decreased in the mitochondrial fraction compared with 0-h control. TNF- α stimulation increased the amount of Bcl-xL in the mitochondrial fraction. Infliximab blocked this increase. No changes were observed in the cytosolic fraction of Bcl-xL. Bax expression remained unchanged in both the cytosolic and mitochondrial fractions during the incubation. Monoclonal antiactin antibody was used for normalization of loading, and COX4 antibody was used to verify the purity of the cytosolic fraction, that is, the fraction not containing mitochondria. For cytosolic and mitochondrial samples, 8 and 2 μ g of protein were loaded respectively. The images are representative of three sets of independent experiments using different rats. (B) Quantitative analysis of the expression of cytochrome c and fas ligand in cytosol. No significant changes were seen during the 6-h incubation. The 6-h control is designated as 100%. Each bar represents the mean $\pm$ S.E.M. of three independent experiments. A.D.U., arbitrary densitometric units. (C) Quantitative analysis of the expression of Bax and Bcl-xL in mitochondria. The 0-h control is designated as 100%. Each bar represents the mean $\pm$ S.E.M. of three independent experiments. A.D.U., arbitrary densitometric units. * $P < 0.05$ (compared with 6-h control).

the levels of the Bcl-2 family proteins were found in the presence of TNF- α or infliximab. Since the subcellular localization is an important factor affecting the function of Bcl-2 family members, we studied whether TNF- α and/or infliximab have a regulatory effect on Bax and Bcl-xL at the subcellular level. During 6-h incubation, the amount of Bcl-xL decreased in the mitochondrial fraction, but TNF- α was able to prevent this decrease, as the level of Bcl-xL protein was restored in mitochondria at the level of control extracted before the start of the culture. Infliximab was able to abolish this effect of TNF- α . Bcl-xL is specifically targeted on the mitochondrial outer membrane (34), but it is also found in cytosol (35), whereas Bax is predominantly a cytosolic protein (35). Bcl-xL is localized to spermatocytes and spermatids in the adult rat testis (36), and Bcl-xL might account for the protective effects of TNF- α in these cells, as the apoptotic germ cells were mostly primary spermatocytes and spermatids. The mechanism of the protective effect of Bcl-xL on apoptosis has been studied intensively. Bcl-xL has been shown to prevent Bax-induced cytochrome c release from mitochondria (37). Bcl-xL dimerizes with Bax but also inhibits apoptosis independently of Bax (38–40). Bcl-xL has a structural similarity with pore-forming bacterial toxins, and it forms ion-conducting channels in lipid membranes (41, 42). Controversial reports have appeared on its ability to interact with Apaf-1 (43–46). In the presence of dATP and cytochrome c, Apaf-1 binds to caspase-9, leading to the activation of caspase-3 (47). The ability of Bcl-xL to inhibit apoptosis cannot be due solely to the inhibition of cytochrome c release from mitochondria, since a microinjection of cytochrome c in cytochrome c-insensitive MCF7F cells does not overcome the antiapoptotic effects of Bcl-xL (48). In our study, no changes were seen in the expression of Bcl-xL in the cytoplasm, and as Apaf-1 is a cytosolic protein, it is more probable that antiapoptotic Bcl-xL action takes place in mitochondria. However, localization of Bcl-xL to the outer mitochondrial membrane might still enable Bcl-xL to interact with Apaf-1. No changes were seen in the expression of Bax during 6-h culture in either the mitochondria or cytosol. Bax protein has a long half-life, possibly explaining the absence of TNF- α effect during a short culture period (49). TNF- α has been shown to induce Bcl-xL expression through NF- κ B activation in neuronal cells (50). Bax forms a dimer with Bcl-xL, preventing the death-repressor activity of this antiapoptotic protein (39). This might be one pathway of cell survival in the seminiferous epithelium, since Bcl-xL was clearly upregulated in mitochondria without any effect on Bax expression. The effect was not mediated through the release of cytochrome c, as no decrease was observed in the cytosolic cytochrome c content in the presence of TNF- α . There are several other apoptogenic factors besides the cytochrome c released from mitochondria, such as HtrA2/Omi, endonuclease G, Smac/DIABLO

and AIF, which might serve as targets of the prosurvival effect of TNF- α . Besides controlling the release of apoptogenic factors from intermembrane space, Bcl-xL can act on the voltage-dependent anion channel (VDAC) maintaining free metabolite exchange across the outer mitochondrial membrane during cellular stress (51). Spermatogenesis is highly dependent on hormonal regulation, and survival of germ cells is regulated by different hormones, growth factors and cytokines (52). We have earlier shown that follicle-stimulating hormone (FSH) protects germ cells from apoptosis, and this effect is mediated partially through the SCF/c-kit pathway (53, 54) and may at least partly be due to the inhibition of proapoptotic Bok gene expression (55). Furthermore, TNFR1 expression in Sertoli cells is increased by FSH (8), and TNF- α has been shown to inhibit FSH action on cultured Sertoli cells (56). TNF- α has been shown to inhibit apoptosis in human seminiferous tubules by a mechanism that did not appear to be associated with the NF- κ B pathway (10). TNF- α modulated the Fas system by down-regulating the expression of Fas ligand. In our study, Fas ligand expression was not affected by TNF- α , suggesting that the mechanism of action for promoting cell survival is different between rat and human seminiferous epithelium. All in all, we have shown that TNF- α promotes cell survival during spermatogenesis, and this effect can be blocked by infliximab. This finding is important, as infliximab is in therapeutic use for autoimmune disorders such as RA and CD. The prosurvival effect of TNF- α might be at least partly mediated by modulating the expression and subcellular localization of Bcl-2 family proteins, as Bcl-xL was found to be upregulated in mitochondrial membranes in the presence of TNF- α , and this upregulation was inhibited by infliximab. Further studies are needed to understand the exact mechanism of action of TNF- α on cell survival in the testis and to evaluate the present findings in terms of the clinical use of infliximab.

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