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Involvement of soluble Fas Ligand in germ cell apoptosis in testis of rats undergoing autoimmune orchitis

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ABSTRACT

Experimental autoimmune orchitis (EAO) is a model of chronic inflammation and infertility useful for studying immune and germ cell (GC) interactions. EAO is characterized by severe damage of seminiferous tubules (STs) with GCs that undergo apoptosis and sloughing. Based on previous results showing that Fas–Fas Ligand (L) system is one of the main mediators of apoptosis in EAO, in the present work we studied the involvement of Fas and the soluble form of FasL (sFasL) in GC death induction. EAO was induced in rats by immunization with testis homogenate and adjuvants; control (C) rats were injected with adjuvants; a group of non-immunized normal (N) rats was also studied. Activation of Fas employing an anti-Fas antibody decreased viability (trypan blue exclusion test) and induced apoptosis (TUNEL) of GCs from STs of N and EAO rats, an effect more pronounced on GCs from EAO STs. By Western blot we detected an increase in sFasL content in the testicular fluid of rats with severe EAO compared to N and C rats. By intratesticular injection of FasL conjugated to *Strep-Tag* molecule (FasL-*Strep*, BioTAGnology) and its immunofluorescent localization, we demonstrated that sFasL is able to enter the adluminal compartment of the STs. Moreover, FasL-*Strep* induced GC apoptosis in testicular fragments of N rats. By flow cytometry, we detected an increase in the number of membrane FasL-expressing CD4+ and CD8+ T cells in testis during EAO development but no expression of FasL by macrophages. Our results demonstrate that sFasL is locally produced in the chronically inflamed testis and that this molecule is able to enter the adluminal compartment of STs and induce apoptosis of Fas-bearing GCs.

1. Introduction

Although the testis is an immunoprivileged organ, infection and inflammation associated with subfertility or infertility may occur. In fact, the disruption of immune privilege is of particular concern due to the high prevalence of inflammatory reactions among infertile men [1].

Experimental autoimmune orchitis (EAO), a model of chronic testicular inflammation, has provided important insight into immunologically impaired spermatogenesis and infertility. We developed EAO in rats by active immunization with testicular antigens and adjuvants [2]. In this model, testicular damage is characterized by an increased number of interstitial macrophages and T cells, different degrees of germ cell (GC) degeneration and sloughing, aspermatogenesis and atrophy of seminiferous tubules (STs) [3–6].

Fas–Fas Ligand (L) system has been shown to mediate both apoptotic and inflammatory reactions. In different experimental models of testicular damage such as cryptorchidia, irradiation, toxicants, heat exposure and hormone suppression, Fas–FasL system appeared to be the major pathway of GC apoptosis [7–10]. Fas (APO-1/CD95) is a type I-membrane protein that belongs to the tumor necrosis factor (TNF) receptor family and is a receptor for FasL, a type II-transmembrane protein also belonging to the TNF family. The Fas cell surface receptor mediates apoptotic signals upon engagement with FasL [11]. The membrane-bound (m) FasL (40 kDa) may be converted to soluble form (s) FasL (26 kDa) by the action of some matrix metalloproteases and by some members of the "a disintegrin and transmembrane metalloprotease" (ADAM) family [12,13].

In the normal testis, sFasL is produced and its content increases after exposure to toxicants [14]. In rats with EAO, the presence of apoptotic spermatocytes and spermatids expressing Fas, FasL or co-expressing both molecules in the damaged STs suggests that cell death occurs through a mechanism involving Fas–FasL system in an autocrine and/or paracrine way [4]. We hypothesize that infiltrating immune cells expressing FasL contribute to the apoptosis of Fas+ GCs. The aim of the present work

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was to determine whether sFasL is produced locally in the inflamed testis and to analyze its involvement in GC apoptosis induction. In order to evaluate the putative contribution of inflammatory interstitial cells to sFasL production we studied FasL expression by macrophages and T cells.

2. Materials and methods

2.1. Animals

Male adult *Sprague–Dawley* rats were kept at 22 °C on a 12 h light, 12 h dark schedule and fed standard food pellets and water *ad libitum*. Animal handling and experimentation were done in accordance with the EU Policy on Animal Welfare (EUPAW) with the approval of the CICUAL, Animal Committee of the School of Medicine of the University of Buenos Aires.

2.2. Immunization schedule

Rats of the EAO group ($n = 42$) were immunized under anesthesia (100 mg/kg ketamine and 5 mg/kg xylazine) with testicular homogenate (TH) prepared as previously described [2]. Briefly, rat testes were decapsulated, an equal volume of saline solution was added and they were disrupted in an Omni mixer for 30 s. The final TH concentration was 500 mg/ml. 0.4 ml of TH emulsified with 0.4 ml of complete Freund's adjuvant (CFA) (Sigma–Aldrich, St. Louis, MO, USA) was injected intradermally into footpads and at multiple sites near ganglionar regions. EAO rats were injected three times with 200 mg of TH/dose/rat, at 14 day intervals. The first two immunizations were followed by an i.v. injection of 0.5 ml of *Bordetella pertussis* (Bp) (strain 10536; Instituto Malbrán, Buenos Aires, Argentina) containing 10^{10} microorganisms and the third by an i.p. injection of 5×10^9 microorganisms. Rats in control (C) group ($n = 36$) were injected with an emulsion of saline solution and CFA, and Bp in the same conditions as the EAO group. EAO and C rats were killed at different time periods (50–60 and 70–80 days) after the first immunization. We also studied a group of nonimmunized normal (N) rats ($n = 49$) killed at the same time as rats from the other groups. Rats were euthanized with CO_2 and weighed, and testes were removed and weighed. One testis was processed for histopathology and the other was processed as described below.

2.3. Intratesticular Fas Ligand injection

To perform intratesticular injection we employed a novel bioactive FasL reagent named FasL-*Strep* (BioTAGnology, St Louis, MO, USA). FasL reagent contains a *Strep-Tag*® molecule fused to the three monomers of the extracellular domain of FasL. Rats ($n = 7$ rats/group) were injected under ketamine/xylazine anesthesia; 60 µl of murine FasL-*Strep* (3.3 µg/ml) were injected subalbuginea in the cephalic pole of the right testis with an ultrafine 30-gauge as previously described [15]. The same volume of saline solution was injected in the left testis. After 6 h rats were killed, the right testis was quickly frozen to immunolocalize FasL-*Strep* and the other testis was fixed in Bouin's solution and embedded in paraffin for histopathology.

2.4. Histopathology

Testes were fixed in Bouin's solution and embedded in paraffin. Histopathology was analyzed in transversal sections obtained from the poles and equatorial areas of testis and stained with hematoxylin–eosin.

2.5. Collection of testicular fluid

Testicular fluid (TF) was collected from rats killed on days 50 ($n = 6$ rats/group) and 80 ($n = 8$ rats/group) as previously described [16]. Drained fluid was collected after 16 h at 4 °C in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 10 µg/ml aprotinin, Sigma–Aldrich). TF was stored at −70 °C until use.

2.6. Isolation and culture of germ cells

Testes from N ($n = 6$) and EAO ($n = 7$) rats killed 50 days after the first immunization were decapsulated and digested with type II collagenase (0.3 mg/ml, Worthington Biochemicals Corporation, Freehold, NJ, USA) in phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA) for 15 min at 34 °C in a Dubnoff shaking water bath. After adding PBS, STs were allowed to settle and then washed three times with Dulbecco's Modified Eagle's Medium-nutrient mixture F12 (1:1; D-MEM/F12, 12500-039, Gibco BRL, Rockville, MD, USA). After mechanical dispersion of the STs with a Pasteur pipette, tissue debris was eliminated by pressing the cell suspension against a fine stainless steel screen. Isolated GCs (10^5 cells/well) were plated into a 96-well culture plate in DMEM/F12 medium supplemented with L-glutamine (2 mM; G-8540, Sigma–Aldrich), insulin-transferrin-selenium-A supplement (1×; ITS-A, 51300-044, Gibco BRL), sodium DL-lactic acid (1 mM; L-4263, Sigma–Aldrich) and antibiotic–antimycotic solution (1×; 5240–096, Gibco BRL) (DMEM/F12-S). Plates were incubated in the presence or absence of a monoclonal anti-human Fas antibody able to induce apoptosis (MAB142, R&D Systems, Minneapolis, USA) for 18 h at 34 °C in a humidified atmosphere with 5% CO_2. GC viability was assessed by MTS Cell Titer Cell Proliferation Assay.

2.7. Isolation and culture of seminiferous tubule segments

Testes removed from N ($n = 3$) and EAO ($n = 3$) rats killed 50 days after the first immunization were decapsulated and the seminiferous tubule segments (STS) microdissected under a transillumination stereomicroscope as previously described [17]. The STS (2 mm in length) isolated were transferred to a 96-well culture plate in D-MEM/F12-S medium (200 µl/well). Plates were incubated in the presence or absence of anti-Fas antibody (MAB142, R&D Systems) for 18 h at 34 °C in a humidified atmosphere with 5% CO_2. After the culture period, STS were squashed in order to obtain GCs and fixed as previously described [18]. Apoptosis was determined by the TdT-mediated dUTP nick end labeling (TUNEL) technique.

2.8. Isolation and culture of testis fragments

Testis fragments were obtained and cultured as described [19]. Briefly, testes removed from N rats ($n = 3$) were decapsulated and carefully dissected with scissors into 3 mm^3 fragments. In each well of a 24-well culture plate (BD Frankling Lakes, NJ, USA), two fragments were placed onto a 0.4 µm pore size PET insert (BD Frankling Lakes) and cultured in DMEM/F12 medium supplemented with L-glutamine (2 mM; G-8540, Sigma–Aldrich), insulin-transferrin-selenium-A supplement (1×; ITS-A, 51300-044, Gibco BRL) and antibiotic–antimycotic solution (1×; 5240-096, Gibco BRL) in the presence or absence of murine FasL-*Strep* (400 ng/ml, BioTAGnology) for 18 h at 34 °C in a humidified atmosphere with 5% CO_2. After the culture period, testes fragments were fixed in Bouin's solution and embedded in paraffin, and apoptosis was evaluated in 4 µm thick sections by TUNEL technique. Architecture, viability and cell functionality is maintained in the testicular fragments for 2 days culture [19].

2.9. Isolation of testicular interstitial cells

Interstitial cells were obtained from rats killed on days 50 (n = 7 rats/group) and 80 (n = 7 rats/group) as previously described [5]. Briefly, decapsulated testes were incubated with type I collagenase (0.3 mg/ml; Worthington Biochemical Corporation) at 34 °C for 15 min. Collagenase was inactivated by adding ice cold PBS and STs were allowed to settle; the supernatant was washed with PBS and red blood cells were depleted by osmotic lysis with ammonium chloride (160 mM NH_4Cl, 170 mM Tris–HCl, pH 7.2). Interstitial cells were washed, centrifuged and counted in a Neubauer chamber by the trypan blue exclusion method. Interstitial cells were immuno-labeled to study the expression of mFasL in macrophages and T cells.

2.10. Western blotting

Testicular fluid (TF) was diluted in ice cold RIPA buffer (1:100 v/v) (1.5 ml buffer/g of tissue) (50 mM Tris, 150 mM NaCl, 0.5% sodium dodecyl sulfate (SDS), 2 mM EDTA, 0.1% sodium desoxycholate, 1% NP-40, pH 7.4) to determine protein concentration by the Lowry method (Bio-Rad DC Protein Assay; Bio-Rad, Hercules, CA, USA). Samples were dissolved with sample buffer (500 μM Tris–HCl, pH 6.8, 10% SDS, 30% glycerol, 0.5% bromophenol blue) containing 0.5% β-mercaptoethanol, boiled for 5 min and immediately placed on ice. 50 μg of protein of TF was resolved in 14% SDS–polyacrylamide gel electrophoresis (PAGE) at 120 V for 90 min. Proteins were electroblotted at 66 mA overnight. Molecular weight of immunoreactive bands was determined by comparison to a ladder of prestained protein standards with a molecular weight range of 250–10 kDa (Precision Plus Protein Standards All Blue, Bio-Rad) applied to a line in each gel. Protein transference and equal loading were monitored by staining membranes with Ponceau red. Then, membranes were blocked with blocking solution [5% (w/v) of non-fat dry milk in TBST buffer: 10 mM Tris, 154 mM NaCl, 0.1% Tween-20 (v/v), pH 7.5] for 90 min. Blots were probed overnight with mouse monoclonal antibody against the extracellular domain of FasL of rat origin (2 μg/ml) (sc-73970, Santa Cruz Biotechnology, CA, USA). After six washes (5 min each) in TBST buffer, membranes were incubated with an anti-mouse biotinylated antibody rat adsorbed (0.08 μg/ml, BA-2001, Vector Laboratories, Burlingame, CA, USA). Primary and secondary antibodies were diluted in blocking buffer solution. The reaction was enhanced with horseradish–streptavidin–peroxidase conjugates (Chemicon International Inc., Temecula, CA, USA) and chemiluminescence was used to detect the horseradish–peroxidase-labeled protein. The bands were visualized in a G: Box Syngene system for imaging fluorescence and densitometrically quantified using Gene Tools software.

2.11. Immunofluorescence

Cryostat testis sections (5–7 μm) obtained from rats injected with FasL-*Strep* were fixed with acetone for 10 min at 4 °C. After blocking with 3% normal horse serum in PBS for 30 min at room temperature (RT), sections were incubated overnight with a monoclonal anti-*Strep-Tag* antibody (*Strep*MAB-Classic, 4 μg/ml, BioTAGnology) diluted in PBS for 1 h at RT in a humidified chamber. An anti-mouse secondary antibody made in horse (10 μg/ml, Vector Laboratories) was applied for 30 min at RT. After three washes in PBS, sections were blocked with 3% normal goat serum in 3% BSA in PBS for 30 min at RT. After three washes in PBS, sections were incubated with an anti-claudin-11 antibody (4 μg/ml, Santa Cruz Biotechnology) diluted in 3% normal goat serum in 3% BSA at 4 °C. Then, sections were incubated with a goat anti-rabbit antibody (Rhodamine, 20 μg/ml Chemicon Int.) diluted in 5% normal rabbit serum for 45 min at RT. Finally, sections were mounted with a medium containing 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

2.12. Assessment of germ cell viability (MTS)

After the culture period, GC viability was evaluated by MTS Cell Titer Cell Proliferation Assay (G5421, Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. This assay is a colorimetric method to determine the number of viable cells. MTS is bioreduced by dehydrogenase enzymes found in metabolically active cells into a formazan product soluble in the cell culture medium. The quantity of formazan product is directly proportional to the number of living cells in the culture. Optical density (OD) was read at a wavelength of 490 nm in a microplate reader.

2.13. Assessment of apoptosis

The squashed preparations and testicular fragment sections were irradiated in a microwave oven (370 W for 5 min) in 10 mM sodium citrate buffer, pH 6.0 and permeabilized with 0.1% Triton X-100 (T-9284, Sigma–Aldrich) in 0.1% sodium citrate for 5 min at 4 °C. Non-specific labeling was prevented by incubating preparations with blocking solution (5% blocking reagent; 11 096 176 001, Roche Molecular Biochemicals GmbH, Mannheim, Germany) in 150 mM NaCl and 100 mM maleic acid, pH 7.5 for 30 min at RT. After 10 min incubation with terminal deoxynucleotidyl transferase (TdT) buffer (11 966 006 001 Roche) containing 1.25 mg/ml BSA, apoptotic DNA was 3-end labeled with digoxigenin-11-dideoxy-uridine triphosphate (4 μM Dig-ddUTP; 11 363 905 910, Roche) by incubation with TdT (0.18U/ml TdT; 33 335 740 01, Roche) in TdT buffer for 1 h at 37 °C. As assay control, the TdT enzyme was replaced with the same volume of TdT buffer. Preparations were then incubated with blocking solution (2% blocking reagent in 150 mM NaCl and 100 mM maleic acid, pH 7.5) for 30 min at RT, followed by detection of Dig-dd-UTP with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000; 11 093 274 910, Roche) incubated for 2 h at RT. Squashed preparations and testicular fragment sections were rinsed and equilibrated in alkaline phosphatase buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgSO_4$, pH 9.5) containing 1 mM levamisole (L-9756, Sigma–Aldrich). Then, alkaline phosphatase substrates, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, 11 697 471 001, Roche) were added for 60 min. This reaction was stopped by washing preparations with Tris–EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Squashes and testicular fragment sections were light counterstained with eosin, dehydrated and mounted.

2.14. Flow cytometric analysis

To study FasL expression on T cell subsets, interstitial cells (1×10^6) were incubated with hamster anti-FasL antibody (20 μg/ml) for 30 min at 4 °C, washed with PBS and incubated with anti-hamster-APC antibody (12 μg/ml) for 30 min at 4 °C. After washing with PBS, cells were incubated with mouse anti-CD3-PE-Cy5 antibody (5 μg/ml) and mouse anti-CD4-FITC antibody (5 μg/ml) or mouse anti-CD3-PE-Cy5 antibody and mouse anti-CD8-FITC antibody (10 μg/ml) for 30 min at 4 °C. To analyze intracellular FasL expression, cells were fixed with 2% paraformaldehyde in PBS for 10 min at 4 °C and permeabilized with 0.1% saponin in PBS for 10 min at 4 °C before staining as previously described [20].

To study FasL expression on macrophages, interstitial cells (1×10^6) were incubated with a hamster anti-FasL antibody for 30 min at 4 °C. After washing with PBS, cells were blocked with 10% goat normal serum in PBS for 30 min at RT, washed with 1%

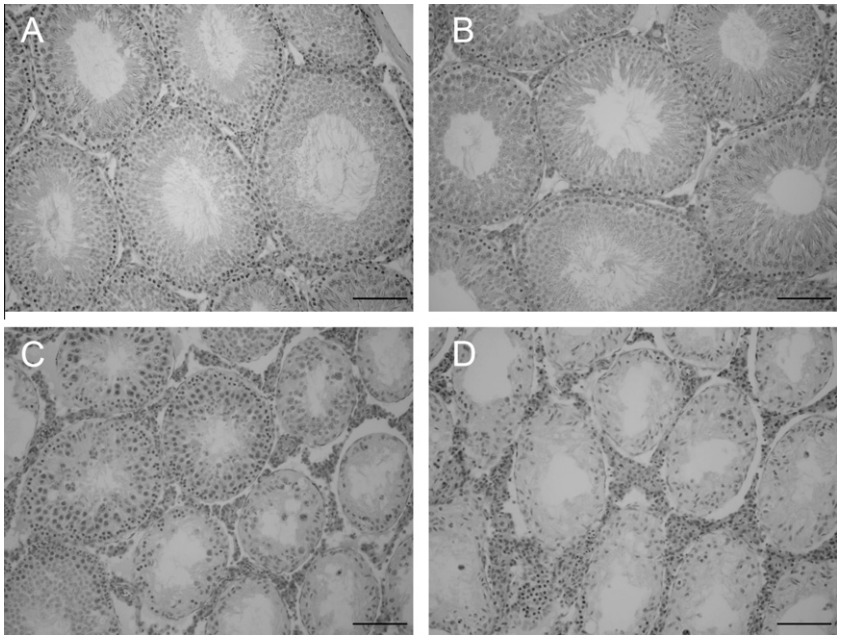

Fig. 1. Testicular histopathology. Histopathology of testis sections from normal (A), control (B) and EAO (C and D) rats. Foci of seminiferous tubules (STs) with germ cells (GCs) sloughing intermingled with normal STs and interstitial lymphomononuclear cell infiltrates in the testis of a rat with focal EAO (C). Severe and extensive damage of STs with increased interstitial cell density in the testis of a rat with severe EAO (D). Note GC degeneration and sloughing in STs of EAO rats. Scale bars indicate 100 μm.

BSA in PBS and incubated with goat anti-hamster-APC antibody for 30 min at 4 °C. Finally, cells were incubated with mouse anti-CD11b-FITC antibody (0.5 μg/ml) for 40 min at 26 °C. The appropriate control isotypes were used. All antibodies were purchased from BD Bioscience (BD Bioscience, San Diego, CA, USA), except for anti-hamster-APC antibody purchased from Jackson ImmunoResearch (West Grove, PA, USA) and anti-CD11b-FITC antibody purchased from Serotec Laboratories (Serotec, Raleigh, NC, USA). A BD FACSAria II cytometer was used and 30.000 events on CD3+ and CD11b+ cell gates were acquired. The absolute number of positive cells per testis was calculated from percentages obtained by flow cytometric analysis and total number of interstitial cells.

3. Results

3.1. Histopathology

As we previously described [2], 50 days after the first immunization the testis of EAO group of rats presented a focal orchitis characterized by mild lymphomononuclear cell infiltrate and several foci of damaged STs with degenerating GCs and sloughing intermingled with normal STs (focal EAO) (Fig. 1C). From day 80 on, we observed severe and extensive damage of most of the STs in which only spermatogonia and Sertoli cells remained attached to the tubular wall (severe EAO) (Fig. 1D). Although areas of dense lymphomononuclear infiltrates might be present in the interstitium, immune cells were never observed inside the ST. No testicular damage was observed in the testis of N or C (Fig. 1A and B) rats at any time studied.

3.2. Activation of Fas and germ cell apoptosis

We have previously shown by immunohistochemistry that the number of Fas^+ apoptotic GCs increases in EAO rats from 50 days onwards and correlates with the degree of testicular damage [4]. In order to functionally determine Fas involvement in GC apoptosis, we first studied the effect of an anti-Fas antibody on GCs from N and EAO rats. The Fas antibody is able to activate Fas, inducing

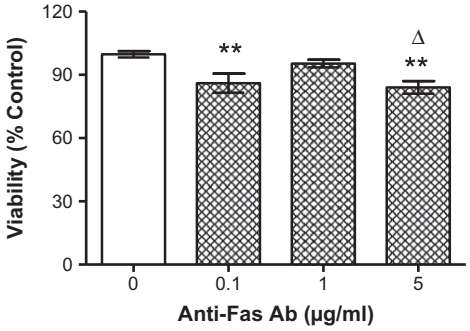

Fig. 2. Effect of anti Fas Antibody on viability of Normal Germ Cells. Germ cells (GCs) obtained from normal rats were incubated with or without anti-Fas antibody (Ab) for 18 h and viability was evaluated by MTS assay. The value of optical density measured after incubating GCs without the Ab was considered 100%. Each column represents the mean ± SEM $n = 8–10$ wells from 6 to 7 separate experiments, Newman–Keuls multiple comparison test. $^{**}p < 0.01$ vs medium and $^{\triangle}p < 0.05$ vs 1 μg/ml anti-Fas Ab.

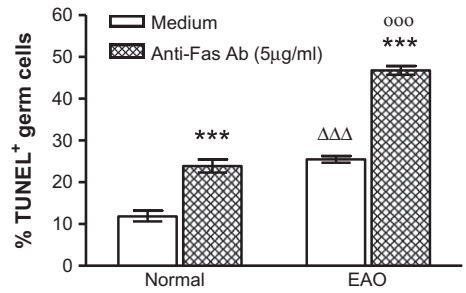

Fig. 3. Effect of anti Fas Antibody on Germ Cell apoptosis. Seminiferous tubule segments obtained from normal and EAO rats killed 50 days after the first immunization were incubated with or without anti-Fas antibody (Ab) for 18 h and apoptosis was evaluated by TUNEL technique. In each experiment, seminiferous tubule segments were individually squashed and $TUNEL^+$ germ cells counted. Each column represents the percentage ± CL of $TUNEL^+$ germ cells of the total number of cells counted ($n = 2500–11,000$ cells/group from three independent experiments), Chi^2 test. $^{***}p < 0.001$ vs respective medium; $^{\triangle\triangle\triangle}p < 0.001$ vs normal cells incubated with medium; $^{ooo}p < 0.001$ vs normal cells incubated with anti-Fas Ab.

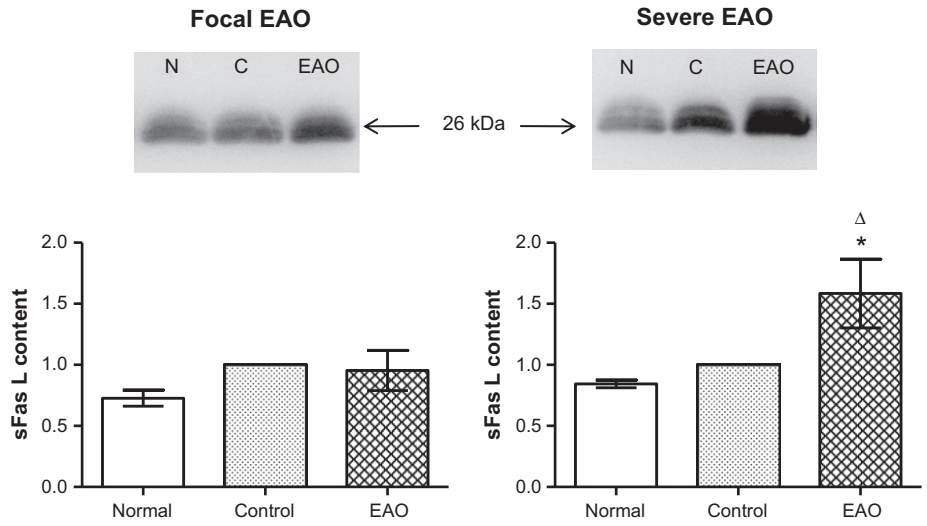

Fig. 4. Soluble Fas Ligand in testicular fluid. Testicular fluid was obtained from normal (N), control (C), focal and severe EAO rats and soluble (s) Fas Ligand (L) content was evaluated by Western blot. Each column represents the mean ± SEM of arbitrary unit of densitometry. Data from EAO and N were compared to data from C rats, arbitrary set at 1. $n = 6–8$ rats/group, Newman–Keuls multiple comparison test. $^{*}p < 0.05$ vs C; $^{\triangle}p < 0.05$ vs N.

biological effects on cells [21]. Anti-Fas antibody reduced viability of N GCs (Fig. 2) and increased GC death assessed by trypan blue exclusion test (% of dead cells, medium: 23.09 ± (0.90 – 0.87), Ab Fas (5 μg/ml): 34.75 ± (3.02 – 2.88) $p < 0.001$, Chi^2 test $n = 10{,}000$ total cells counted from two independent experiments). Anti-Fas antibody also increased the percentage of dead cells from EAO rats, an effect more pronounced compared to the effect on cells from N rats (% of dead cells, medium: 39.35 ± (1.39 – 1.35), Ab Fas (5 μg/ml): 53.70 ± (1.43 – 1.43), Chi^2 test $n = 10{,}000$ total cells counted from two independent experiments).

Similarly, we also demonstrated that anti-Fas antibody significantly increased the percentage of TUNEL$^+$ GCs obtained from STS of N and EAO rats. Fas activation in GCs from rats with orchitis resulted in a higher percentage of TUNEL$^+$ cells compared to Fas activation in N GCs (Fig. 3).

3.3. Soluble Fas Ligand production

Since our results show that GCs are sensitive to Fas-induced apoptosis, we then analyzed the presence of sFasL in the TF by Western blot. We observed that testis from N, C and EAO rats produced sFasL (26 kDa). As shown in Fig. 4, testicular sFasL content was similar in N and C rats compared to rats with focal EAO; however it significantly increased in testis of EAO rats during severe orchitis. As housekeeping proteins cannot be evaluated in the TF to monitor equal loading, we determined total protein content in

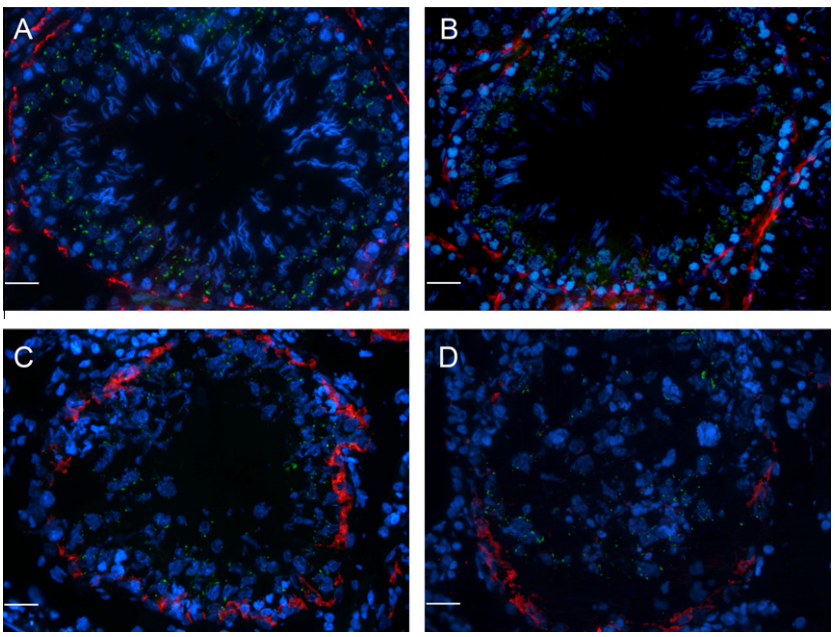

Fig. 5. Immunolocalization of FasL-*Strep*. The cephalic pole of the testis of normal (N), control (C) and EAO rats killed on days 50–60 (focal EAO) was injected subalbuginea with a Fas Ligand (L) molecule conjugated to *Strep-Tag*® (FasL-*Strep*). Double immunofluorescence was performed to detect FasL-*Strep* (green) and Claudin-11 (red) protein in N (A), C (B) and EAO testes with different degree of damage (C and D). Detection of FasL-*Strep* (green) in the adluminal area of seminiferous tubules in N (A), C (B) and EAO (C and D) rats. Note the more adluminal localization of FasL-*Strep* as severity of the damage increase (C vs D). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar indicates 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

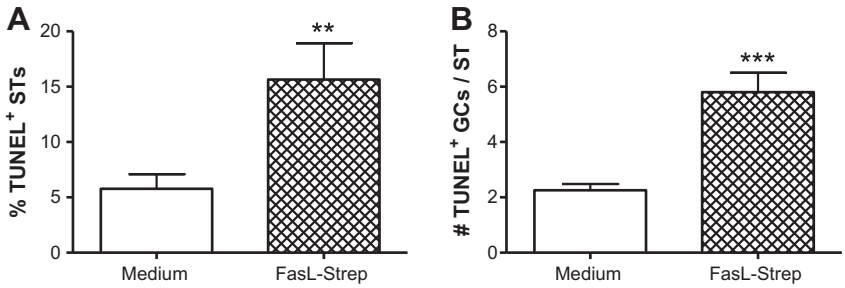

Fig. 6. Effect of FasL-Strep on Germ Cell apoptosis. Testicular fragments obtained from normal rats were incubated with or without murine FasL-*Strep* for 18 h and apoptosis was evaluated by TUNEL technique. (A) Each column represents the mean ± SEM of the percentage of TUNEL$^+$ seminiferous tubules (STs) (n = 15–25 non-consecutive sections of testicular fragments obtained from three rats, 430 STs were counted in each condition). (B) Each column represents the mean ± SEM of the number of TUNEL$^+$ germ cells (GCs) per TUNEL$^+$ ST. Non-parametric Mann–Whitney Test. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs medium.

Red Ponceau stained membranes. Total protein content was similar in all groups studied (Arbitrary units of densitometry, mean ± SEM, focal EAO: Normal: 69.826 ± 2.088, Control: 67.053 ± 0.883, EAO: 65.635 ± 2.784; severe EAO: Normal: 66.560 ± 0.889, Control: 64.094 ± 1.154, EAO: 64.475 ± 0.884, n = 6–8 rats/group).

3.4. Permeability of blood-testis barrier to soluble Fas Ligand

In order to evaluate whether sFasL is able to go through the blood-testis barrier (BTB) and reach the adluminal compartment of STs, we injected via subalbuginea a FasL molecule conjugated to *Strep-Tag*® (FasL-*Strep*) in the testis of N, C and EAO rats; then we identified and localized it with an anti-*Strep-Tag*® antibody. BTB was identified with an anti-claudin-11 antibody. In the three groups of rats studied, FasL-*Strep* appeared as a compact dotted image in the adluminal compartment of STs; pachitene spermatocytes identified by its nuclear morphology were the main cells that bound FasL-*Strep*. In N and C rats, FasL-*Strep* was observed along the basal area of the adluminal compartment (Fig. 5A and B) while in rats with focal EAO FasL-*Strep* was distributed more irregularly close to the sloughed GCs localized in the lumen of STs (Fig. 5C and D).

3.5. Germ cell apoptosis induced by FasL-Strep

Given that Fas activation triggers GC apoptosis and that sFasL is able to enter the adluminal compartment of the STs, we functionally determine the apoptotic effect of this molecule in testis fragments of N rats. As shown in Fig. 6, FasL-*Strep* significantly increased the percentage of TUNEL$^+$ STs containing apoptotic GCs. Also, the number of apoptotic GCs per ST increased after FasL-*Strep* treatment compared to the control condition.

3.6. Membrane Fas Ligand expression by immune cells

In order to identify putative cellular sources of sFasL in N and inflamed testis, we studied by flow cytometry the expression of FasL on macrophages and on CD4+ and CD8+ T cell subsets during EAO development. Interstitial cells were non-permeabilized or fixed and permeabilized to investigate cell surface expression or total expression of FasL, respectively. We observed an increased number of CD4+ and CD8+ T cells expressing FasL in testis of EAO compared to N and C rats at both times studied. Although both T cell subsets were found to express FasL throughout EAO development, we detected a higher number of CD8+ cells than of CD4+ cells (Fig. 7). No

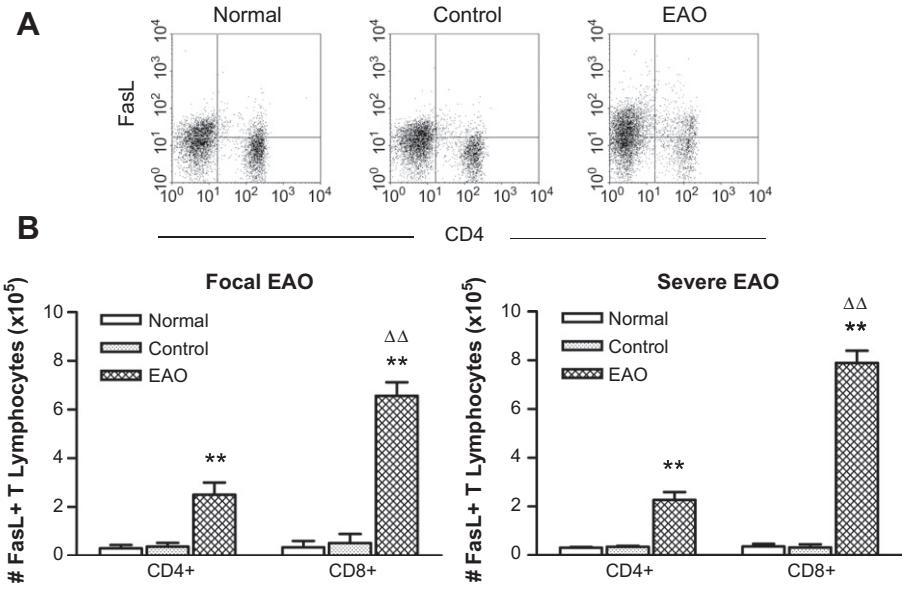

Fig. 7. Expression of membrane-bound Fas Ligand on infiltrated T cells. (A) Flow cytometric representative dot plots of membrane-bound (m) Fas Ligand (L) expression in CD4+ T cells from testis of normal (N), control (C) and severe EAO rats. Isolated testicular interstitial cells were stained for CD3 (PE-Cy5), CD4 (FITC) or CD8 (FITC) and FasL (APC) and analyzed by flow cytometry. Analysis gates were set on T cell populations by scatter properties and CD3 expression. (B) Absolute number of CD4+ and CD8+ T cells expressing mFasL in testes of N, C and EAO rats killed on days 50–60 (focal EAO) and 70–80 (severe EAO) after the first immunization. Data are representative of staining of 7 rats/group/time, non-parametric Kruskal–Wallis one-way ANOVA. $^{**}p < 0.01$ vs respective N and C; $^{\Delta\Delta}p < 0.01$ CD8+ vs respective CD4+.

differences in the percentage of positive cells or fluorescence intensity of FasL were detected when permeabilized or non-permeabilized T cells were assessed (data not shown), indicating that FasL is expressed only as mFasL.

No staining of FasL was detected in permeabilized and non-permeabilized macrophages of rats with EAO (Supplementary Material Fig. 1S).

4. Discussion

In EAO, the main target of the immunological attack is GCs that undergo apoptosis and sloughing. Previous results based on the increased number of GCs expressing Fas and FasL and on the detection of Fas on apoptotic GCs (mainly spermatocytes and spermatids) suggest that Fas–FasL system is one of the main mediators of GC death [4]. In the present work, an *in vitro* assay was developed in order to functionally demonstrate the susceptibility of GC to the Fas-dependent apoptosis. Results showing that anti-Fas antibody reduced viability and increased GC death indicate that the activation of Fas triggers apoptosis by signaling via FasL. The higher percentage of dead cells in EAO rats than in N rats could be explained by the enhanced expression of Fas on GCs, probably induced by the inflammatory testicular microenvironment.

Like other members of the TNF family, membrane-bound FasL (mFasL) can be processed by proteolytic shedding to an active soluble form [12]. We detected sFasL in testicular fluid of N, C and EAO rats; its content was significantly higher in testis of rats with severe orchitis. In addition to the increase in sFasL content, intratesticular injection experiments showed that FasL-*Strep* has the ability to enter STs. Moreover, FasL-*Strep* was able to induce GC apoptosis. The impairment of BTB in rats undergoing autoimmune orchitis, as we recently described [22], might facilitate sFasL passage into the adluminal compartment where it has the capacity to induce GC apoptosis by binding to its receptor. Molecules with similar molecular weight, such as IL-1 and GM-CSF, have been reported to penetrate into the adluminal compartment [23,24].

Previous results showing that the number of GCs expressing Fas, FasL or co-expressing both molecules increased in testis of rats with EAO suggests that GC apoptosis could be mediated by an autocrine and/or paracrine mechanism [4]. Testicular content of sFasL in rats with focal EAO is similar to that detected in N and C rats, however we cannot rule out sFasL participation in GC death during this phase of the disease; the higher number of GCs expressing Fas may sensitize [4] the testis to sFasL action. Intratesticular content of sFasL increases to its highest level in the severe stage of the disease concomitantly with the increased number of Fas+ GCs [4]. During severe orchitis when interactions between Fas and FasL bearing GCs are maximally disturbed, apoptosis could be primarily mediated by sFasL. Similarly, sFasL has been proposed to mediate apoptotic GC death in response to testicular exposure to MEHP, a toxicant of Sertoli cells [14].

Our previous results on phenotypic and quantitative analysis of testis-infiltrating immune cells in EAO rats showed an increased number of macrophages, dendritic cells and CD4+ Th1 and Th17 and CD8+ T cells [5,20,25]. Among others factors these cells secrete pro-inflammatory cytokines such as TNF-α, IL-6 and nitric oxide involved in the orchestration of inflammatory response and also in inducing GC apoptosis as we previously reported [26–28]. Numerous reports have shown that T cells release active sFasL after cell activation [29,30]. In the present work, flow cytometric data showed that testicular T cells expressed mFasL. Moreover, a significant increase in the number of CD4+ and CD8+ T cells expressing mFasL was detected in testis of EAO compared to N and C rats. The lack of detectable differences between cell surface and total expression of FasL by T cells supports the notion that in T cells mFasL, but not intracellular preformed FasL, could be the sole source of the soluble form released into the extracellular milieu. We detected neither membrane nor intracellular FasL expression in testicular macrophages in contrast with other authors who found that activated human monocytes release high levels of sFasL from preformed FasL stored within the intracellular compartment [31].

During the development of autoimmune orchitis in rats, infiltrating immune cells are localized in the interstitium but not inside STs. The requirement for Fas–FasL interactions to induce apoptosis of GCs presents a topological problem regarding the accessibility of mFasL expressed by interstitial immune cells to react with its receptor expressed in GCs localized within the adluminal compartment beyond the BTB. The production of a sFasL form would allow this molecule to interact with the Fas-sensitive target cells inside STs. Since we previously reported FasL expression on Leydig cells, we considered that they may also represent another potential source of sFasL [4].

Metalloproteases of the ADAM family are involved in the ectodomain shedding of several pro-inflammatory mediators such as FasL, TNF-α, TNFR1, TNFR2, IL-6, TGF-α [13]. ADAM10 and 17 have been proposed to contribute to the pathogenesis of autoimmune disorders, i.e. multiple sclerosis [32]. In the testis ADAM10 and 17 are expressed by pre-puberal GCs and ADAM17 has been proposed as one of the molecules triggering GC apoptosis during the first wave of spermatogenesis [33]. However, its expression in adult rat testis has not yet been studied. We consider it of interest to evaluate the expression of ADAMS as potential proteases involved in FasL shedding during the development of orchitis. Unpublished data from our group showed selective ADAM17 expression on cells from testicular interstitium in rats with orchitis.

The activity of sFasL has been extensively debated in the literature. Reports both favor and discard its apoptotic effect. In this line, Powell et al. [34] showed by *in vitro* experiments that the apoptotic inducing activity of sFasL is dependent on the target cell population. Tanaka et al. [35] also reported that sFasL produced by activated human peripheral T cells retains its trimeric configuration and specifically induces cytolysis of Fas-expressing cells.

It has been proposed that sFasL is involved in the pathogenesis of various autoimmune diseases such as hepatitis, systemic lupus erythematosus and rheumatoid arthritis (RA). sFasL significantly increased in the serum of patients with these autoimmune diseases and in some cases its level dramatically decreased after treatment [36]. Similarly to present results in which the highest levels of sFasL were detected during the severe phase of orchitis, clinical data of Matsuno et al. [37] showed that the level of sFasL was higher in the synovial fluid of patients with severe RA than in those with mild RA.

Up to now the majority of investigations of the effect of Fas activation have focused on the regulation of apoptosis. However, a larger number of studies are now analyzing the role of Fas–FasL signaling pathway in the induction of inflammatory responses [38]. sFasL has also been reported to be capable of triggering non-apoptotic signaling pathways such as NF-κB-dependent inflammatory process [39–41]. We therefore hypothesize that EAO development may be driven by a combination of Fas–FasL mediated apoptosis of sensitive GCs and Fas–Fas stimulation of inflammatory responses.

The increase in sFasL content in the testicular microenvironment concomitant with the rise in the number of Fas expressing GCs [4] that occurs during chronic orchitis, points to sFasL as a key molecule involved in triggering GC death in this phase of EAO characterized by disturbed Fas–FasL expressing cell–cell interaction. This concept is supported by the fact that activation of Fas via sFasL is able to trigger a death program in GCs resulting in

apoptosis. Also, the increased number of infiltrating CD4+ and CD8+ T cells expressing mFasL suggests that lymphocytes could be an important source of sFasL. Whether specific targeting of sFasL might provide effective strategies for controlling chronic testicular inflammation is a matter for future investigation.

5. Conclusion

We propose that interstitial sFasL produced at high levels during severe orchitis might go through the BTB and reach the adluminal compartment of STs where it has the ability to induce Fas-dependent GC apoptosis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2012.07.020.

References

[1] Schuppe HC, Meinhardt A, Allam JP, Bergmann M, Weidner W, Haidl G. Chronic orchitis: a neglected cause of male infertility? Andrologia 2008;40:84–91.
[2] Doncel GF, Di Paola JA, Lustig L. Sequential study of histopathology and cellular and humoral immune response during development of an autoimmune orchitis in Wistar rats. Am J Reprod Immunol 1989;20:44–51.
[3] Lustig L, Lourtau L, Perez R, Doncel GF. Phenotypic characterization of lymphocytic cell infiltrates into the testes of rats undergoing autoimmune orchitis. Int J Androl 1993;16:279–84.
[4] Theas S, Rival C, Lustig L. Germ cell apoptosis in autoimmune orchitis: involvement of the Fas–FasL system. Am J Reprod Immunol 2003;50:166–76.
[5] Rival C, Theas MS, Suescun MO, Jacobo P, Guazzone V, van Rooijen N, et al. Functional and phenotypic characteristics of testicular macrophages in experimental autoimmune orchitis. J Pathol 2008;215:108–17.
[6] Jacobo P, Guazzone VA, Jarazo-Dietrich S, Theas MS, Lustig L. Differential changes in CD4+ and CD8+ effector and regulatory T lymphocyte subsets in the testis of rats undergoing autoimmune orchitis. J Reprod Immunol 2009;81:44–54.
[7] Ohta Y, Nishikawa A, Fukazawa Y, Urushitani H, Matsuzawa A, Nishina Y, et al. Apoptosis in adult mouse testis induced by experimental cryptorchidism. Acta Anat 1996;157:195–204.
[8] Lee J, Richburg JH, Shipp EB, Meistrich ML, Boekelheide K. The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. Endocrinology 1999;140:852–8.
[9] Blanco-Rodríguez J, Martínez-García C. Apoptosis precedes detachment of germ cells from the seminiferous epithelium after hormone suppression by short term oestradiol treatment of rats. Int J Androl 1998;21:109–15.
[10] Francavilla S, D'Abrizio P, Rucci N, Silvano G, Properzi G, Straface E, et al. Fas and Fas Ligand expression in fetal and adult human testis with normal or deranged spermatogenesis. J Clin Endocrinol Metab 2000;85:2692–700.
[11] Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991;66:233–43.
[12] Kayagaki N, Kawasaki A, Ebata T, Ohmoto H, Ikeda S, Inoue S, et al. Metalloproteinase-mediated release of human Fas Ligand. J Exp Med 1995;182:1777–83.
[13] Schulte M, Reiss K, Lettau M, Maretzky T, Ludwig A, Hartmann D, et al. ADAM10 regulates FasL cell surface expression and modulates FasL-induced cytotoxicity and activation-induced cell death. Cell Death Differ 2007;14:1040–9.
[14] Richburg JH, Nañez A, Gao H. Participation of the Fas-signaling system in the initiation of GC apoptosis in young rat testes after exposure to mono-(2-ethylhexyl) phthalate. Toxicol Appl Pharmacol 1999;160:271–8.
[15] Russell LD, Saxena NK, Weber JE. Intratesticular injection as a method to assess the potential toxicity of various agents and to study mechanisms of normal spermatogenesis. Gamete Res 1987;17:43–56.
[16] Sharpe RM, Cooper I, Doogan DG. Increase in Leydig cell responsiveness in the unilaterally cryptorchid rat testis and its relationship to the intratesticular levels of testosterone. J Endocrinol 1984;102:319–27.
[17] Parvinen M, Hecht NB. Identification of living spermatogenic cells of the mouse by transillumination-phase contrastmicroscopic technique for 'in situ' analyses of DNA polymerase activities. Histochemistry 1981;171:567–79.
[18] Henriksen H, Hakovirta M, Parvinen M. Testosterone inhibits and induces apoptosis in rat STs in a stage-specific manner: in situ quantification in squash preparations after administration of ethane dimethane sulfonate. Endocrinology 1995;136:3285–91.
[19] Roulet V, Denis H, Staub C, Le Tortorec A, Delaleu B, Satie AP, et al. Human testis in organotypic culture: application for basic or clinical research. Hum Reprod 2006;21:1564–75.
[20] Jacobo P, Pérez CV, Theas MS, Guazzone VA, Lustig L. CD4+ and CD8+ T cells producing Th1 and Th17 cytokines are involved in the pathogenesis of autoimmune orchitis. Reproduction 2011;141:249–58.
[21] Dechsupa S, Kothan S, Vergote J, Leger G, Martineau A, Berangeo S, et al. Quercetin, Siamois 1 and Siamois 2 induce apoptosis in human breast cancer MDA-mB-435 cells xenograft in vivo. Cancer Biol Ther 2007;6:56–61.
[22] Pérez C, Sobarzo C, Jacobo P, Jarazo Dietrich S, Theas MS, Denduchis B, et al. Impaired expression and distribution of adherens and gap junction proteins in the seminiferous tubules of rats undergoing autoimmune orchitis. Int J Androl 2011;34:e566–77.
[23] McLay RN, Banks WA, Kastin AJ. Granulocyte macrophage-colony stimulating factor crosses the blood-testis barrier in mice. Biol Reprod 1997;57:822–6.
[24] Plotkin SR, Banks WA, Maness LM, Kastin AJ. Differential transport of rat and human interleukin-1alpha across the blood–brain barrier and blood–testis barrier in rats. Brain Res 2000;881:57–61.
[25] Rival C, Lustig L, Iosub R, Guazzone VA, Schneider E, Meinhardt A, et al. Identification of a dendritic cell population in normal testis and in chronically inflamed testis of rats with autoimmune orchitis. Cell Tissue Res 2006:311–8.
[26] Theas MS, Rival C, Jarazo-Dietrich S, Jacobo P, Guazzone VA, Lustig L. Tumour necrosis factor-alpha released by testicular macrophages induces apoptosis of germ cells in autoimmune orchitis. Hum Reprod 2008;23:1865–72.
[27] Rival C, Theas MS, Guazzone VA, Lustig L. Interleukin-6 and IL-6 receptor cell expression in testis of rats with autoimmune orchitis. J Reprod Immunol 2006;70:43–58.
[28] Jarazo Dietrich S, Fass M, Pérez C, Lustig L, Theas MS. Participación del óxido nítrico liberado por los macrófagos testiculares en la apoptosis de las células germinales en un modelo de inflamación testicular en la rata. Medicina 2011;71(Suppl. III):222.
[29] Suda T, Okazaki T, Naito Y, Yokota T, Arai N, Ozaki S, et al. Expression of the Fas Ligand in cells of T cell lineage. J Immunol 1995;154:3806–13.
[30] Zhang X, Brunner T, Carter L, Dutton RW, Rogers P, Bradley L, et al. Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. J Exp Med 1997;185:1837–49.
[31] Kiener PA, Davis PM, Rankin BM, Klebanoff SJ, Ledbetter JA, Starling GC, et al. Human monocytic cells contain high levels of intracellular Fas Ligand: rapid release following cellular activation. J Immunol 1997;159:1594–8.
[32] Kieseier BC, Pischel H, Neuen-Jacob E, Tourtellotte WW, Hartung HP. ADAM-10 and ADAM-17 in the inflamed human CNS. Glia 2003;42:398–405.
[33] Lizama C, Rojas-Benítez D, Antonelli M, Ludwig A, Bustamante-Marín X, Brouwer-Visser J, et al. TACE/ADAM17 is involved in germ cell apoptosis during rat spermatogenesis. Reproduction 2010;140:305–17.
[34] Powell WC, Fingleton B, Wilson CL, Boothby M, Matrisian LM. The metalloproteinase matrilysin proteolytically generates active soluble Fas Ligand and potentiates epithelial cell apoptosis. Curr Biol 1999;9:1441–7.
[35] Tanaka M, Suda T, Takahashi T, Nagata S. Expression of the functional soluble form of human Fas Ligand in activated lymphocytes. EMBO J 1995;14:1129–35.
[36] Li NL, Nie H, Yu QW, Zhang JY, Ma AL, Shen BH, et al. Role of soluble Fas Ligand in autoimmune diseases. World J Gastroenterol 2004;10:3151–6.
[37] Matsuno H, Yudoh K, Watanabe Y, Nakazawa F, Aono H, Kimura T. Stromelysin-1 (MMP-3) in synovial fluid of patients with rheumatoid arthritis has potential to cleave membrane bound Fas Ligand. J Rheumatol 2001;28:22–8.
[38] Chakour R, Allenbach C, Desgranges F, Charmoy M, Mauel J, Garcia I, et al. A new function of the Fas–FasL pathway in macrophage activation. J Leukoc Biol 2009;86:81–90.
[39] Ahn JH, Park SM, Cho HS, Lee MS, Yoon JB, Vilcek J, et al. Non-apoptotic signaling pathways activated by soluble Fas Ligand in serum-starved human fibroblasts. Mitogen-activated protein kinases and NF-kappaB-dependent gene expression. J Biol Chem 2001;276:47100–6.
[40] Xiao S, Jodo S, Sung SS, Marshak-Rothstein A, Ju ST. A novel signaling mechanism for soluble CD95 ligand. Synergy with anti-CD95 monoclonal antibodies for apoptosis and NF-kappaB nuclear translocation. J Biol Chem 2002;277:50907–13.
[41] O' Reilly LA, Tai L, Lee L, Kruse EA, Grabow S, Fairlie WD, et al. Membrane-bound Fas Ligand only is essential for Fas-induced apoptosis. Nature 2009;461:659–63.