Journal of Cellular Physiology

Involvement of TACE/ADAM17 and ADAM10 in Etoposide-Induced Apoptosis of Germ Cells in Rat Spermatogenesis

CARLOS LIZAMA,[1] DIEGO ROJAS-BENITEZ,[1] MARCELO ANTONELLI,[2] ANDREAS LUDWIG,[3] AND RICARDO D. MORENO[1]*

[1]*Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Chile*

[2]*Facultad de Medicina, ICBM, Universidad de Chile, Chile*

[3]*Institute for Pharmacology and Toxicology, RWTH Aachen University, Aachen, Germany*

Germ cell apoptosis is important to regulate sperm production in the mammalian testis, but the molecular mechanisms underlying apoptosis are still poorly understood. We have recently shown that in vitro, etoposide induces upregulation of TACE/ADAM17 and ADAM10, two membrane-bound extracellular metalloproteases. Here we show that in vivo these enzymes are involved in etoposide-, but not in heat shock-, induced apoptosis in rat spermatogenesis. Germ cell apoptosis induced by DNA damage was associated with an increase in protein levels and cell surface localization of TACE/ADAM17 and ADAM10. On the contrary, apoptosis of germ cells induced by heat stress, another cell death stimulus, did not change levels or localization of these proteins. Pharmacological in vivo inhibition of TACE/ADAM17 and ADAM10 prevents etoposide-induced germ cell apoptosis. Finally, Gleevec (STI571) a pharmacological inhibitor of p73, a master gene controlling apoptosis induced by etoposide, prevented the increase of TACE/ADAM17 levels. Our results strongly suggest that TACE/ADAM17 participates in in vivo apoptosis of male germ cells induced by DNA damage.

J. Cell. Physiol. 227: 829–838, 2012.

Sperm production relies on physiological and environmental factors, which may attenuate or even totally suppress testicular function. Germ cell apoptosis have been shown to play an important role in controlling sperm output in many species, and in humans it has been related to infertility (Feng et al., 1999; Weikert et al., 2004; Ji et al., 2009). In fact, it has been postulated that a substantial decline in world sperm production is caused by environmental toxicants that induce germ cell apoptosis (Sinawat, 2000). In the same way, modern life style and use of underwear has been proposed to produce an elevated scrotal temperature that may cause germ cell apoptosis, akin to transient (43°C for 15 min) heat stress in testis (Hikim et al., 2003; Jung and Schuppe, 2007). Germ cells undergoing meiosis (named spermatocytes) are highly sensitive to DNA damage and other insults such as heat shock, ionizing radiation, growth factor deprivation, and impact of chemotherapeutic agents (Russell et al., 2004; Meachem et al., 2005; Bieber et al., 2006; Lizama et al., 2009). In this way, we have recently shown that etoposide, a topoisomerase-II inhibitor, which induces DNA damage, induces a massive apoptosis of spermatocytes in 21-day-old rats, 24 h after treatment (Ortiz et al., 2009). Etoposide induces DNA breaks and then promotes the activation of several key proteins; among them is p73, a transcription factor that controls the expression of several genes, which ultimately will lead to apoptosis (Melino et al., 2003; Ramadan et al., 2005; Codelia et al., 2010). Normally, p73 is constantly degraded, but upon DNA damage, the tyrosine kinase c-Abl phosphorylates and stabilizes p73 (Melino et al., 2003). In this context, pharmacological inhibition of c-Abl with the tyrosine kinase inhibitor STI 571 (Gleevec, imanitib) has been shown to be an excellent tool to study the role of p73 under in vivo and in vitro conditions (Alvarez et al., 2008; Cancino et al., 2008). p73 is expressed in the testis and is upregulated after etoposide treatment, and pharmacological inhibition of c-Abl (using STI571) prevents germ cell apoptosis (Codelia et al., 2010).

Proteins belonging to the family of transmembrane metalloproteases known as "a disintegrin and metalloprotease" (ADAM) proteins are key components in protein ectodomain shedding of many receptors and signaling molecules (Huovila et al., 2005). ADAMs play a key role in diverse biological processes, such as fertilization, myogenesis, neurogenesis, heart development, and endothelial permeability, mainly regulating paracrine/juxtacrine cell-to-cell communication (Seals and Courtneidge, 2003; Blobel, 2005). ADAM proteases are type I transmembrane proteins of approximately 70–90 kDa (mature proteins; the unprocessed precursors are about 20 kDa larger due to their prodomain). The simplest mode of ADAM action is the constitutive shedding of a membrane substrate by cleaving a site in its juxtamembrane region (Seals and Courtneidge, 2003; Blobel, 2005; Huovila et al., 2005). The tumor necrosis factor-alpha (TNF-alpha) convertase (TACE or ADAM17) was the first member of this family for which a role in ectodomain shedding was found. However, other members of this protein family, such as ADAM10 or ADAM9 have been shown to have important roles

Additional supplementary information may be found in the online version of this article.

Contract grant sponsor: Chilean Research Council (FONDECYT); Contract grant number: 1070360.

*Correspondence to: Dr. Ricardo D. Moreno, Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda 340, Santiago, Chile. E-mail: rmoreno@bio.puc.cl

Received 4 January 2011; Accepted 8 April 2011

Published online in Wiley Online Library (wileyonlinelibrary.com), 18 April 2011. DOI: 10.1002/jcp.22795

in development, paracrine/autocrine signaling, and/or differentiation (Liu et al., 2006; Sung et al., 2006; Schulte et al., 2007; Murphy, 2008; Mezyk-Kopec et al., 2009). The mechanism underlying ADAMs protein regulation is a hot topic, since it has consequences for a wide range of biological process, including cancer. Interestingly, in human skin cancer cell lines, UV radiation induces transactivation of EGFR through shedding of amphiregulin, which is dependent on activation of ADAM9 and TACE/ADAM17 (Singh et al., 2009). UV induces DNA damage, such as formation of cyclobutane pyrimidine dimers, and single-strand breaks (Gruber et al., 2007; Beissert and Loser, 2008). We have recently shown that etoposide induces upregulation of TACE/ADAM17 and ADAM10 in the GC-1 and GC-2 cell lines, which are derived from mouse male germ cells (Lizama et al., 2011). Even more, pharmacological inhibition of these proteases, prevents apoptosis induced by etoposide, suggesting their active participation after genotoxic stimulus. TACE/ADAM17 and ADAM10 are expressed in pubertal rat testis, and pharmacological inhibition of TACE/ADAM17, but not ADAM10 prevents constitutive apoptosis (Lizama et al., 2010). Therefore, it remains to be established if in vivo TACE/ADAM17 participates only in constitutive germ cell apoptosis or if it is also activated after other death stimuli, such as etoposide or heat stress.

The results in the present work show that DNA damage of germ cells (induced by the anti-cancer drug etoposide), but not heat shock, promotes upregulation of TACE/ADAM17 and ADAM10, in 20-day-old rat testes. In addition, we show that pharmacological inhibition of these proteases prevents germ cell apoptosis induced by etoposide. Finally, we showed that Gleevec, a pharmacological inhibitor of p73, prevents the upregulation of TACE/ADAM17, but not ADAM10, after etoposide treatment. Our results uncover a new strategy of ADAMs metalloproteases regulation, as well as a new element in apoptosis induced by etoposide.

Materials and Methods

Animals

Male Sprague–Dawley rat of 21-days old were acquired from the Animal Facility of our Faculty. The rats were housed under a 12L:12D cycle and provided with water and rat chow ad libitum. The rats were killed by cervical dislocation after exposure to CO_2 for 30 sec. Investigations were conducted in accordance with the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* published by the Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, First Edition, 1988. All animal protocols were endorsed by the Chilean National Fund of Science and Technology (FONDECYT).

Chemicals and antibodies

Rabbit polyclonal antibodies against intracellular c-kit and TACE/ADAM17 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat polyclonal against the extracellular domain of c-kit (ACK2) was purchased from Millipore (Billerica, MA). Monoclonal mouse antibody against β-actin was purchased from Sigma (St. Louis, MO) and against ADAM9 was purchased from BD (Fremont, CA). Mouse monoclonal antibody against ADAM10 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-mouse UltraVision Detection Systems were obtained from LabVision (Fremont, CA). Etoposide and TAPI-0 were purchased from Merck (Darmstat, Germany). The GI254023X and GW280264X inhibitors were synthesized as described before (Ludwig et al., 2005; Schulte et al., 2007). Gleevec (Novartis Pharma, Basel, Switzerland) Etoposide, TAPI-0, GI254023X, or GW280264X were first dissolved in DMSO and stored a 4°C. Working solutions were made by diluting the stock solution in the proper volume of phosphate buffered saline (PBS, see below).

Intratesticular injections

Pubertal rats were anesthetized with ketamine:xilacine (10 and 75 mg/kg) i.m. The testes were exteriorized through a low midline incision. Ten microliters of a solution containing 100 nM TAPI-0, 100 μM Etoposide, 100 μM GI254023X or GW280264X, in PBS, were injected in the testes via a 30G needle. For c-Abl inhibition we used STI571(Gleevec) (Novartis Pharma, Basel, Switzerland) dissolved in NaCl 0.9% at a final concentration of 50 mg/ml. The concentrations used in this study were based in our previous in vivo studies (Ortiz et al., 2009; Codelia et al., 2010; Lizama et al., 2010). Following drug delivery, the testes were returned to the peritoneal cavity, and the incision was closed. In each experiment one testis was used for histology and the other for biochemical assays. As a control, PBS was injected into the testes.

Apoptosis induced by heat stress

Twenty-day-old rats were anesthetized with ketamine:xilacine (10 and 75 mg/kg) i.m., and the lower half of the torso of each animal was submerged in a thermostatically controlled water bath at 43°C (treated) for 15 min, after which time the animals were dried and returned to their cages. Rats were killed 24 h after treatment by cervical dislocation, and the levels of ADAM17, ADAM9, and ADAM10 were analyzed by western blot, flow cytometry, and RT-PCR (Rockett et al., 2001; Vera et al., 2004).

TUNEL analysis

Apoptotic fragmentation of DNA in histological sections of rat testes was evaluated by TUNEL analysis (Dead End System; Promega, Madison, WI). Samples were observed under phase contrast and fluorescence microscopy (Optiphot-2, Nikon, Japan) by using filters for wavelengths at 460–500 nm (excitation) and 510–560 nm (barrier). Micrographs were taken with a digital camera (CoolPix 4500, Nikon, Japan). TUNEL-positive germ cells were quantified in each tissue section by counting the number of TUNEL-positive cells in each round seminiferous tubule. The apoptotic index was calculated as the average number of TUNEL positive cells per seminiferous tubule cross-section as described before (Moreno et al., 2006; Codelia et al., 2008). Three testicular histological sections were taken per rat (three rats for each experimental treatment), and a minimum of 100 randomly selected tubules were counted in each tissue section (a total of 900 tubules were recorded per treatment). The data represent the mean ± SD.

Immunohistochemistry

ADAM proteins were assayed in testes fixed in Bouin's solution and included in paraffin. Sections were counterstained with peryodic acid-Schiff (PAS) and hematoxylin. The samples were first treated with 3% H_2O_2 for 5 min, then, to prevent unspecific binding, a standard protein block System (Ultra V block, LabVision, Fremont, VA) was applied for 5 min. Primary antibody against the extracellular domain of c-kit (2.5 μg/ml) or the intracellular domain of c-kit (2 μg/ml) was applied and incubated overnight at 4°C in a humidified chamber after being washed twice for 5 min in a Tris–HCl buffer, pH 7.6 with 0.3 M NaCl and 0.1% Tween 20. Biotinylated secondary antibody, streptavidin–biotinylated–peroxidase complex, amplification reagent (biotinyl tyramide) and peroxidase-conjugated streptavidin were applied step by step for 15 min each. Afterwards, incubation slides were washed twice in a buffer for 3 min each. Finally, substrate–chromogen solution consisting of concentrated Tris–HCl and 0.8% H_2O_2 (substrate) and 3, 3-diaminobenzidine tetrahydrochloride (DAB) solutions (chromogen) were applied for 5 min and washed in distilled water. Samples were observed under a phase contrast microscope (Optiphot-2, Nikon, Japan) and photographed with a digital

camera (CoolPix 4500, Nikon, Japan). Negative controls were performed using a pre-immmune serum or without the primary antibody.

Seminiferous tubules isolation and cell suspension preparation

Prepubertal male rats of 21-day-old were killed by cervical dislocation, and both testes were extracted, decapsulated, and digested with 0.5 mg/ml collagenase I (Sigma) and 0.4 mg/ml DNase (Sigma) for 15 min at 30°C in a modified Krebs–Henseleit buffer (KHB) medium containing 0.141 g/L magnesium sulfate (Anhydrous), 0.16 NaH_2PO_4, 0.35 g/L KCl, and 6.9 g/L NaCl supplemented with 5 mM L-lactate (Sigma, St. Louis) (KHB–lactate) and 0.5 mg/ml collagenase I. The collagenase causes the tubule walls to release the germ and Sertoli cells. Seminiferous tubules were washed three times in KHB–lactate medium, and cells were mechanically disintegrated by continuous pipetting in KHB solution containing 0.4 mg/ml DNase using a syringe with a 21G needle. The cell suspension was filtered through a nylon membrane of 250 and 70 μm (Small Parts) and subsequently washed once in KHB–lactate medium (Bellve et al., 1993; Lizama et al., 2007).

Protein extraction and western blot

Protein extraction was performed by homogenizing isolated seminiferous tubules in buffer A (1% Triton X-100, NaCl 1 M, EDTA 1 mM, PMSF 10 mg/ml, Tris–HCl 20 mM pH 7.0) and then centrifuged for 10 min at 9,300*g*. Protein quantification in the supernatant was performed by the Bradford assay (Bradford, 1976). The samples were run on a 12% polyacrylamide gel (SDS-PAGE) under reducing and denaturing conditions, and then transferred to nitrocellulose at 30 V overnight or 100 V for 1.5 h. Nitrocellulose was blocked with 2% BSA in PBS, pH 7.4, and then incubated overnight at 4°C with anti-ADAM17 (0.5 μg/ml), ADAM9 (0.25 μg/ml), anti-ADAM10 (0.2 μg/ml), or anti-β-actin (0.9 μg/ml) antibodies. After extensive washing with PBS plus 0.05% Tween 20 (PBS-Tween), the membrane was incubated with a secondary antibody conjugated to peroxidase (KPL, Gaithersburg, Maryland) diluted 1:3,000 in PBS-BSA for 1 h at room temperature.

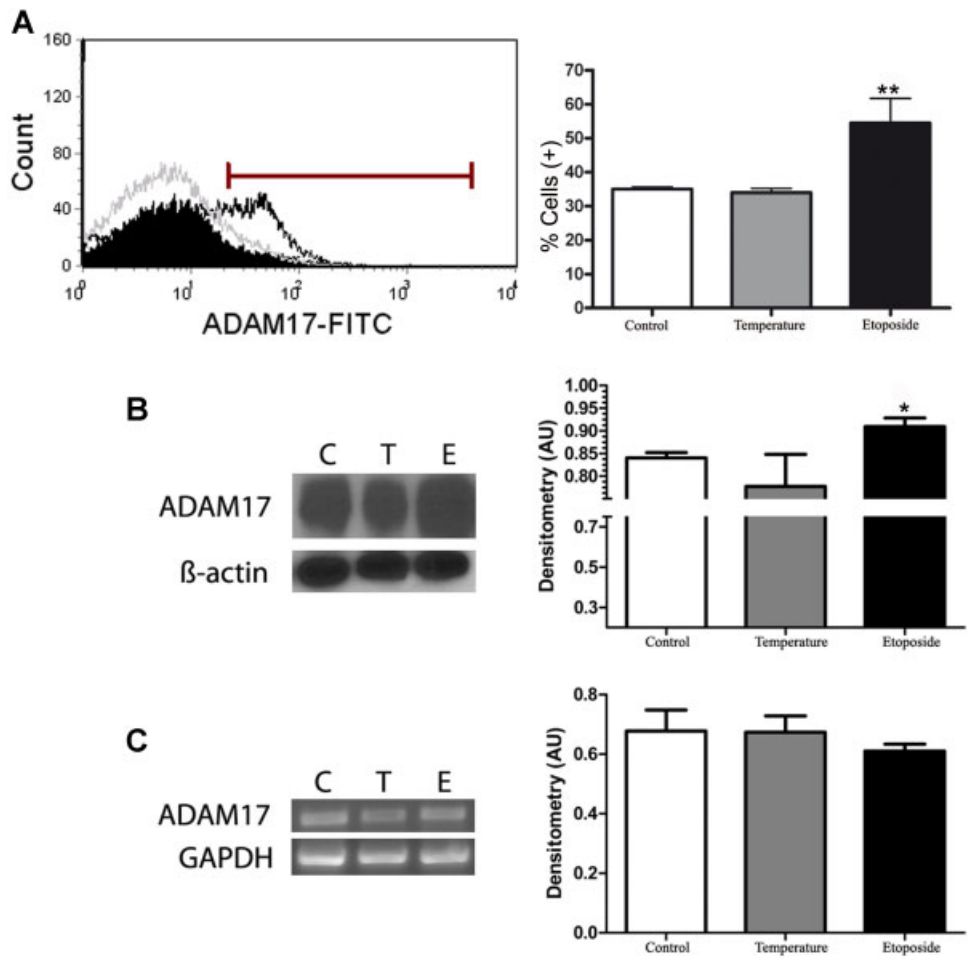

Fig. 1. Etoposide, but not heat shock, increases TACE/ADAM17 levels. Testes of 21-day-old rats were either injected with 200 μM etoposide or treated with heat shock, and the levels of ADAM17 were evaluated 24 h after treatment. (A) As evidenced by flow cytometry, the level of ADAM17, at the cell surface, was higher than control after etoposide treatment (black area and black line, respectively). Heat shock (gray line) was similar to control. Note that the gray line is over the background (black area) indicating that no positive cells were found in this case. (B) The levels of ADAM17 protein increased significantly after etoposide (E), but not after heat shock treatment (T) as compared with control (C). (C) mRNA levels of ADAM17 were similar in control and after both treatments. (*$P < 0.05$, n = 3) β-actin was used as a loading control.

Protein bands were revealed using the Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Flow cytometry

ADAMs surface detection. Cells from seminiferous tubules (isolated as state above) were fixed for 10 min in 2% paraformaldehyde and then washed in PBS. Cells were blocked 1 h in 3% PBS–BSA at room temperature. The primary antibody against TACE/ADAM17 (2 μg/ml), ADAM9 (2 μg/ml), or ADAM10 (2 μg/ml), were added diluted in blocking solution and left to incubate overnight. The next day cells were washed once with PBS and dissolved in blocking solution with the corresponding secondary FITC antibody (1:100, Zymed, Carlsbad, CA) and incubated for 1 h. Cells were then sedimented, washed, and resuspended in PBS. As controls, one autofluorescence sample, one sample with only primary antibody and one sample with only secondary antibody were analyzed. The samples were then analyzed within 10 min of buffer addition in a Coulter Epics XL cytometer; 10,000 gated events were acquired. Data was analyzed using the FCS express V2.0 software (De Novo Software, Los Angeles, CA).

Apoptosis and cell cycle analysis. To analyze cell cycles, the individual cells in KHB solution were pelletted and then fixed in 70% ethanol overnight. As described by Riccardi (Riccardi and Nicoletti, 2006) on the day of analysis the cells were sedimented and washed once with Phosphate Buffered Saline (PBS). The pellet was then dissolved in a cell cycle buffer containing 0.1% sodium citrate, 0.3% Triton X-100 (both Sigma-Aldrich Co.), 50 μg/ml propidium iodide, and 50 μg/ml RNase A (both Invitrogen Corporation) dissolved in distilled water. The samples were then analyzed within 10 min of buffer addition in a Coulter Epics XL cytometer; 10,000 gated events were acquired. Data was analyzed using the FCS express V2.0 software (De Novo Software, Los Angeles, CA).

RT-PCR

Total RNA of decapsulated testes was isolated using TRIzol-Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Total RNA was quantified, and after confirmation of its integrity, cDNA was generated from 1 μg of RNA using random primers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). The cDNA obtained was amplified by polymerase chain reaction (PCR) in 30 cycles using Taq polymerase (Fermentas) in 50 μl of the incubation mixture. Several primer sets were used to obtain the PCR products of ADAM9 forward 5′-CAGACTGCTGTGA-GAGAAG-3′ and reverse 5′-CATTCCTGCAGTTCCACCA-

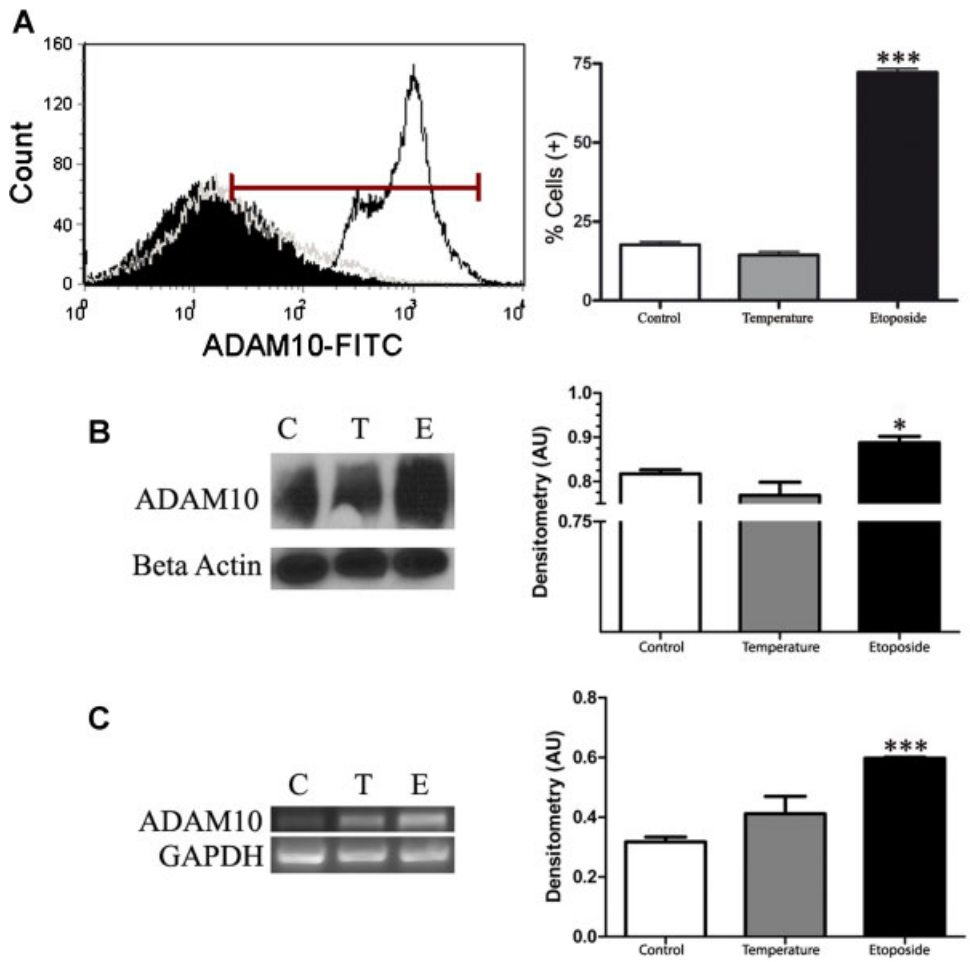

Fig. 2. Etoposide, but not heat shock, increases ADAM10 levels. Testes of 21-day-old rats were injected with 200 μM etoposide or treated with heat shock, and the levels of ADAM10 were evaluated 24 h after treatment. (A) As evidenced by flow cytometry, the level of ADAM10, at the cell surface, was higher than control after etoposide treatment (black area and black line, respectively). Heat shock (gray line) was similar to control. Note that the gray line is superimposed on the background (black area) indicating that no positive cells were found in this case. (B) The levels of ADAM10 protein increased significantly after etoposide (E), but not after heat shock (T) treatment as compared with control (C). (C) Etoposide, but not heat shock significantly increased the mRNA levels of ADAM10. (*$P < 0.05$, n = 3) β-actin was used as a loading control.

3′; ADAM10 forward 5′-CCTACGAATGAAGAGGGAC-3′ and reverse 5′-ATCACAGCTTCTCGTGTTCC-3′ ADAM17 forward 5′-GTTGGTGAGCCTGACTCTA-3′ and reverse 5′-CCTCTTGTGGAGACTTGA-3′ GAPDH forward 5′-TCCACCACCCTGTTGCTGTA-3′ and reverse 5′-ACCACAGTCCATGCCATCAC-3′ in the same conditions as previously described. Aliquots of the PCR products were run in a 1% agarose gel and then stained with 0.1 μg/ml ethidium bromide. Bands obtained were analyzed measuring the pixels with Adobe® Photoshop 7.0 (Adobe System Incorporated, San José, CA), and normalized by GAPDH mRNA level.

Statistical Analysis

For mean comparisons, we used analysis of variance (ANOVA). When the ANOVA test showed statistical differences, the Student–Newman–Keuls (SNK) test was used to discriminate between groups. The Student's *t*-test was used for comparison of frequencies. Statistical significance was defined as $P < 0.05$ (Sokal, 1995). Each experiment was performed in triplicate using at least three different animals.

Results

We have previously shown that a single intratesticular injection of 100 μM etoposide in 21-day-old rats induces apoptosis of meiotic spermatocytes, and in vitro this drug induces upregulation of ADAM10 and TACE/ADAM17 (Codelia et al., 2010; Lizama et al., 2010). Male Sprague–Dawley rats of 21 days old were chosen because they have almost undetectable levels of apoptosis and in this condition germ cell death is readily detectable (Rodriguez et al., 1997; Moreno et al., 2006; Codelia et al., 2008). Administration in vivo of etoposide induced upregulation of TACE/ADAM17 in a concentration-dependent manner (Supplemental Fig. 1). Administration in vivo of 100 μM etoposide led to a significant increase in the level of TACE/ADAM17 at the cell surface, as evaluated by flow cytometry (Fig. 1A, black line). Etoposide induced an upregulation of TACE/ADAM17 at the protein level, whereas mRNA levels

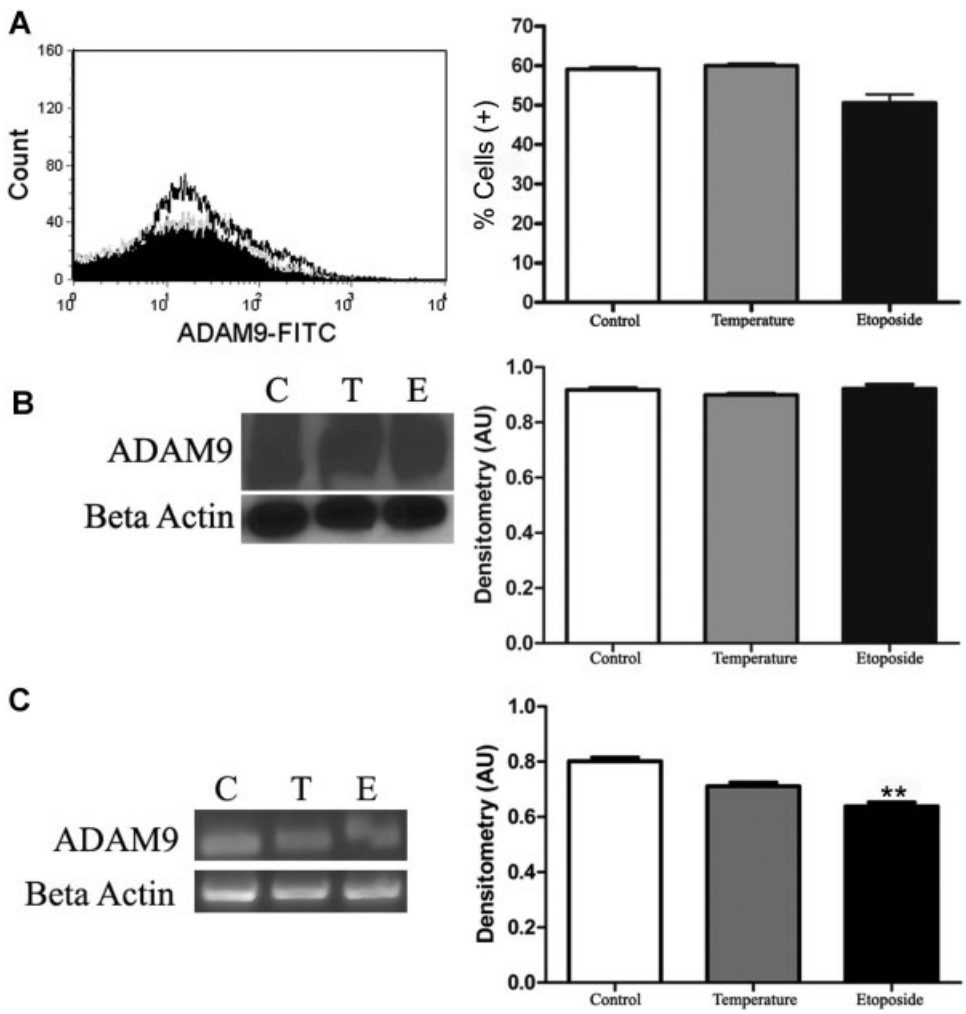

Fig. 3. ADAM9 levels are not affected by etoposide or heat shock. Testes of 21-day-old rats were either injected with 200 μM etoposide or treated with heat shock, and the levels of ADAM9 were evaluated 24 h after treatment. (A) As evidenced by flow cytometry, the levels of ADAM9, at the cell surface, after etoposide (black line) or heat shock (gray line) were similar to control (black area). Note that the gray and black lines are superimposed on the background (black area) indicating that no positive cells were found in this case. (B) The levels of ADAM9 protein did not change after treatment with etoposide (E) or heat shock (T) as compared with control (C). (C) The mRNA levels of ADAM9 remain the same after etoposide or heat shock treatment. β-actin was used as a loading control.

remained similar to the controls (injected with PBS) (Fig. 1B–C). The percentage of cells showing ADAM10 at the cell surface was significantly higher in those from etoposide treated testes as compared to controls (Fig. 2A). In addition, the protein levels, as well as the mRNA levels of ADAM10 significantly increased after etoposide treatment (Fig. 2B–C). Contrary to etoposide, heat shock treatment did not affect the levels of TACE/ADAM17 or ADAM10, as evaluated by cell surface localization (flow cytometry, gray line), protein level (western blotting) or mRNA levels (RT-PCR) (Figs. 1 and 2). Interestingly, cell surface localization, protein and mRNA levels of ADAM9, another member of this family, did not change after heat shock, and only its mRNA levels were significantly reduced after etoposide treatment (Fig. 3). Thus, our results suggest that etoposide regulates the protein levels and cellular localization of TACE/ADAM17 and ADAM10 in rat testis.

Next, we studied if pharmacological inhibitors of TACE/ADAM17 and ADAM10 could prevent *in vivo* germ cell apoptosis induced by etoposide. Two different inhibitors for ADAMs had been described before this work, GI254023X and GW280264X, which specifically block the activity of ADAM10 and TACE/ADAM17, respectively (Ludwig et al., 2005; Schulte et al., 2007). We injected these inhibitors independently in the testes of 21-day-old rats along with etoposide and apoptosis was evaluated by using the TUNEL technique. DNA fragmentation, as evaluated by TUNEL, showed a significant increase after etoposide treatment (Fig. 4A, B). Intratesticular injection of GI254023X or GW280264X (10 μl of a 50 μM solution) along with etoposide, significantly prevented germ cell apoptosis to the same extent, as compared to those testes injected with etoposide alone (Fig. 4A, B). Interestingly there was not difference between control and those testes treated with etoposide plus GI254023X or GW280264X, which means that inhibitors were able to totally rescue apoptosis. TAPI-0, another inhibitor of TACE/ADAM17, prevented the increase in TUNEL-positive cells after etoposide treatment (Fig. 5A). In mitotic cells, cell cycle analysis is a useful tool to test whether cells undergo cell death by measuring sub-G0/G1 group. We have previously used this same experimental approach to determine apoptosis after heat shock in 20-day-old rats (Lizama et al., 2009). Results showed that 10 μl of 100 nM TAPI-0 solution (0.457 ng/testis) did not have any effect on germ cell apoptosis in 21-day-old rat testes (Fig. 5A, B). Etoposide alone caused a significant increase in the percentage of sub-G0/G1 cells, which was prevented in the presence of TAPI-0 (0.457 ng/testis) (Fig. 5A, B). Therefore, these results suggest

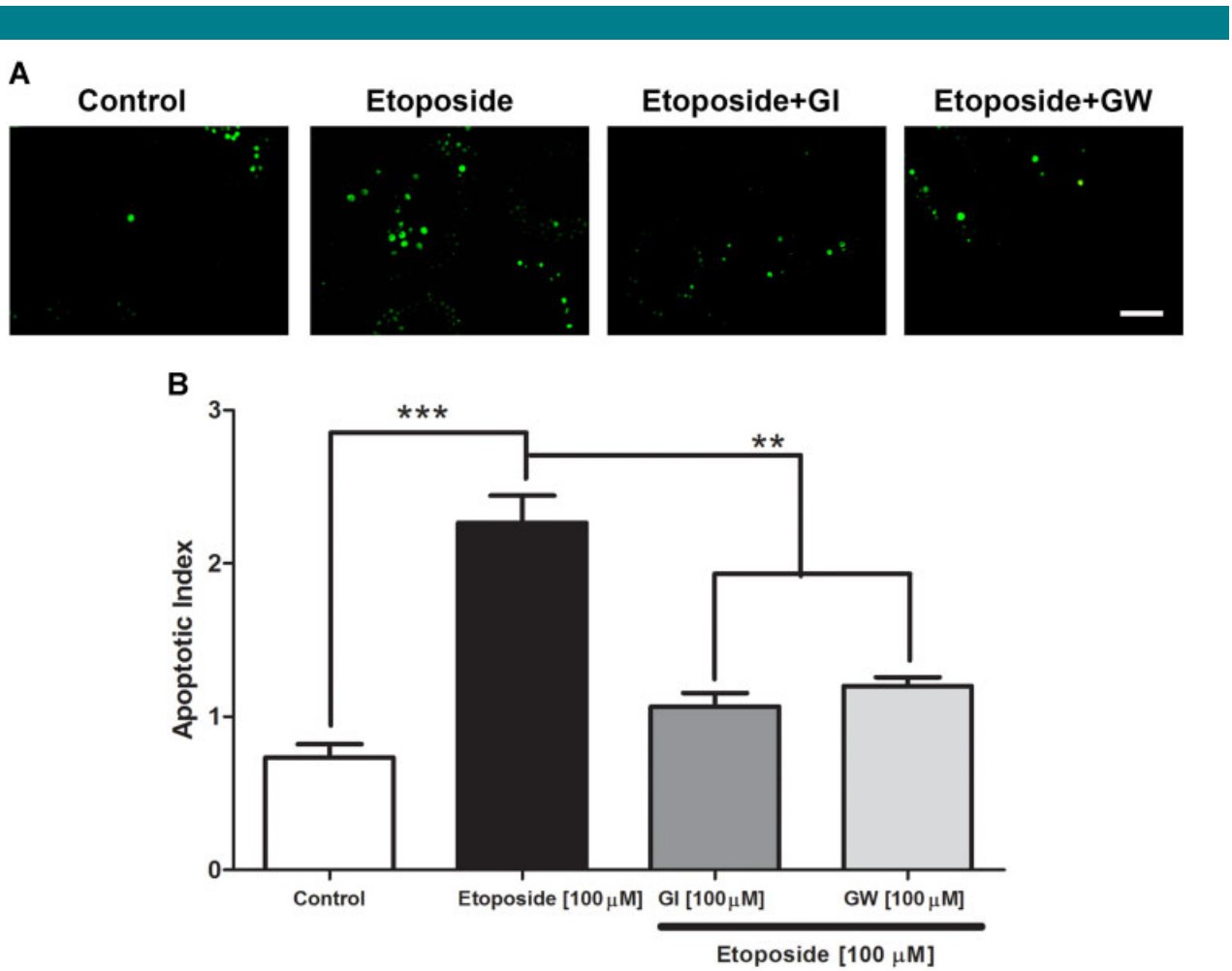

Fig. 4. Pharmacological inhibitors of TACE/ADAM17 and ADAM10 prevent the apoptosis induced by etoposide in prepubertal rat testes. (A) Sections of seminiferous tubules showing TUNEL (+) cells in control and after treatments. (B) The apoptotic index (TUNEL (+) cells per seminiferous tubules) increased after etoposide treatment. However, the number of TUNEL (+) was significantly reduced when etoposide was co-injected with GI254023X (GI) or GW280264X (GW) $^{**}P < 0.01$, $^{***}P < 0.001$. In each case at least 300 tubules cells were recorded using three different rats (n = 3), bar 100 μm.

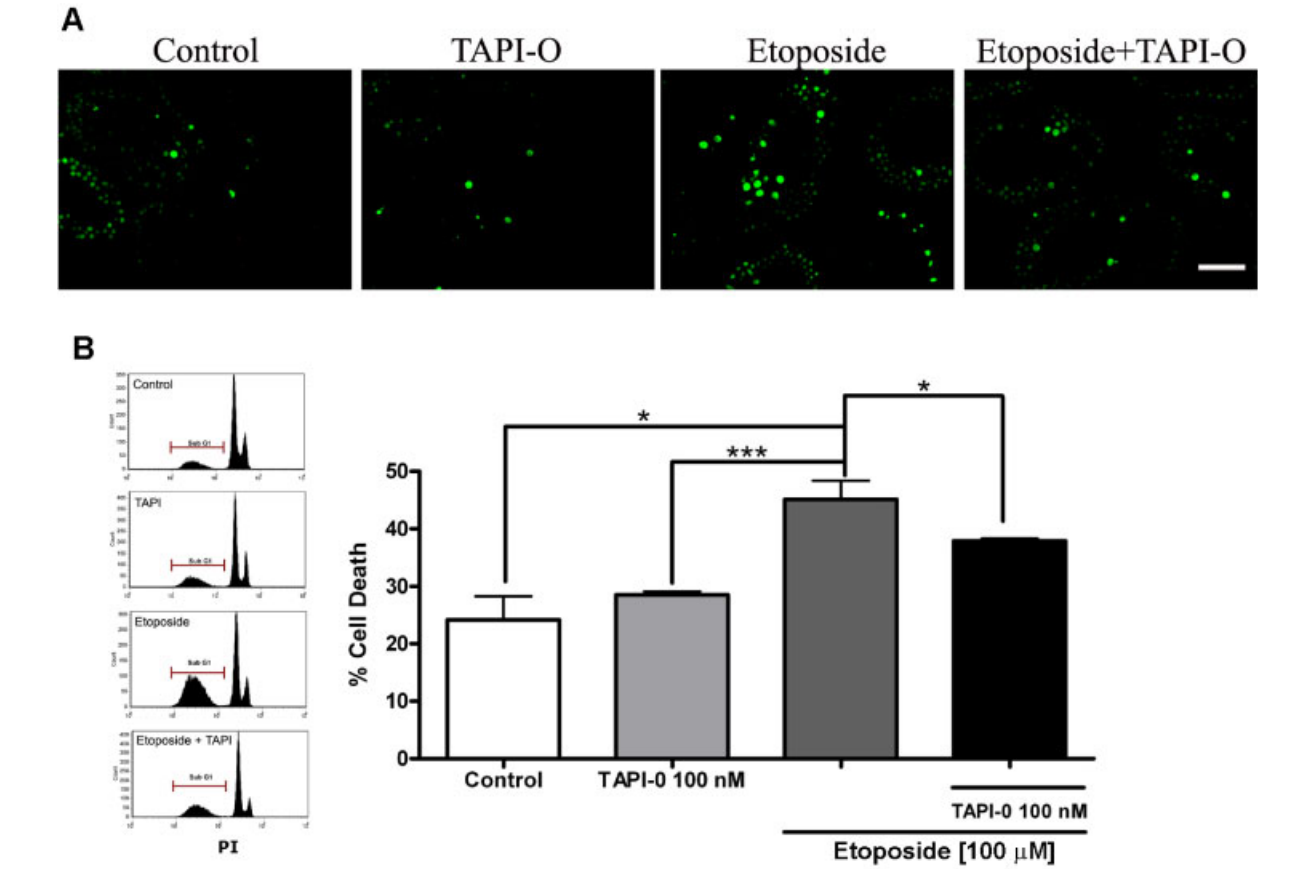

Fig. 5. TAPI-0 prevents etoposide-induced apoptosis of germ cells. Testes of 21-day-old rats were injected with 100 μM etoposide alone or in combination with 100 nM of TAPI-0. (A) Histological examination showed that TAPI-0 alone did not change the number of apoptotic germ cells (arrows) as compared with control. Etoposide induced a massive increase in the number of apoptotic cells that was prevented by co-injection of TAPI-0. Bar 100 μm. (B) Twenty one-day-old rats showed a similar percentage of cells in subG1/G0 as those testes injected with TAPI-0. Etoposide significantly increased the percentage of cells in sub-G1/G0 (apoptotic) cell. Co-injection of etoposide and TAPI-0 showed a significant lower percentage of germ cells in apoptosis as compared with etoposide alone. ($^{*}P < 0.05$; $^{***}P < 0.001$, n = 3).

that TACE/ADAM17 and ADAM10 participate in male germ cell apoptosis induced by etoposide.

A well-known substrate of TACE/ADAM17 is the tyrosine kinase receptor c-kit, which is a survival factor in spermatogenesis (Sakata et al., 2003; Bedell and Mahakali Zama, 2004; Cruz et al., 2004). Using specific antibodies against the extracellular or intracellular domain, we have shown that most germ cells undergoing constitutive apoptosis lack the extracellular domain of c-kit (Lizama et al., 2010). Apoptotic germ cells are easily recognized by their pyknotic appearance in paraffin slides (Fig. 6A, arrowheads). The antibody ACK2 recognizes only the extracellular domain of c-kit and labeled many spermatogonia and spermatocytes (Fig. 6A). Apoptotic (pyknotic) cells were mostly devoid of this label (Fig. 6A, arrowheads and insert). Another antibody recognizes only the intracellular domain of c-kit, and it labeled spermatocytes and spermatogonia, but most of the apoptotic cells were devoid of any label (Fig. 6B, arrowheads and insert). Quantification of the labeling showed that about 10% of apoptotic cells were immunoreactive to the antibody directed against the intracellular or extracellular domain of c-kit (Fig. 6A, B).

Since we have previously shown that the module c-Abl/p73 is activated after etoposide treatment in prepubertal rat testes, we evaluated if TACE/ADAM17 and ADAM10 upregulation is a downstream response in this pathway. Because p73 activation relies on its c-Abl-dependent phosphorylation, we used Gleevec (STI571), a pharmacological inhibitor of c-Abl. Under our conditions, 1 mM Gleevec (STI571), co-injected along with etoposide, did not change the protein levels of ADAM10, as compared with testes treated with etoposide alone (Fig. 7A). On the other hand, Gleevec co-injected with etoposide significantly prevented the increase of TACE/ADAM17 protein levels, as compared with testes treated with etoposide alone (Fig. 7B). Gleevec alone did not modify the apoptosis rate (Codelia et al., 2010). These results suggest that the upregulation of TACE/ADAM17 protein levels is related to c-Abl tyrosine kinase activity.

Discussion

The effect of etoposide has been well characterized in different in vitro and in vivo models. We have previously shown a contribution of TACE/ADAM17 and ADAM10 in etoposide-induced apoptosis using two germ cell-like cell lines (Lizama et al., 2011). In the present work we show that pharmacological inhibition of ADAM10 and TACE/ADAM17 significantly prevents the increase in TUNEL (+) cells after etoposide treatment. As a second approach we used TAPI-O, a widely used pharmacological inhibitor of TACE/ADAM17, which

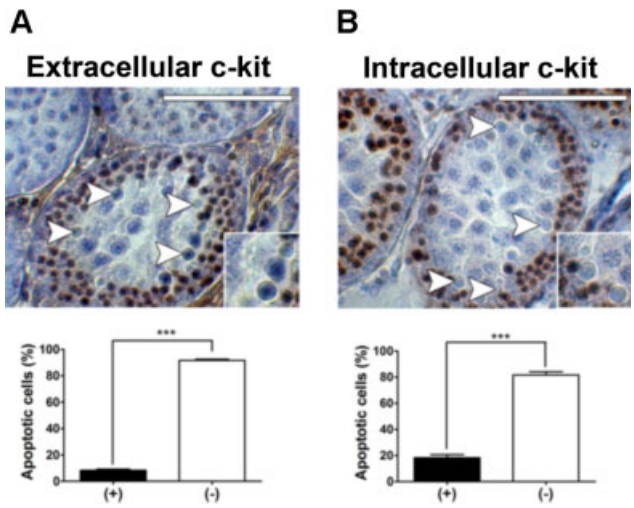

Fig. 6. Absence of c-kit in apoptotic germ cells after etoposide treatment. Etoposide was intratesticularly injected in 21-day-old rats and 24 h later the presence of the extracellular (A) and intracellular domain of c-kit was detected by immunohistochemistry B) intracellular. Apoptotic germ cells were clearly observed as pyknotic cells lacking staining with either antibody (A, B, arrowheads). The graphs show the quantification of apoptotic germ cells lacking (−, with bars) or showing (+, black bars) staining with the respective antibody. $^{***}p < 0.001$, a total of 300 cells were recorded from three different rats (n = 3). Bar 100 μm.

prevents the increase in the percentage of sub-G1 cells after etoposide treatment. Thus, two different methods (TUNEL and flow cytometry) and three different inhibitors, indicate that TACE/ADAM17 and ADAM10 are involved in germ cell apoptosis induced after etoposide treatment, which is similar to our previous in vitro results.

In this work we showed that in vivo etoposide administration induces upregulation of ADAM10 and TACE/ADAM17 protein

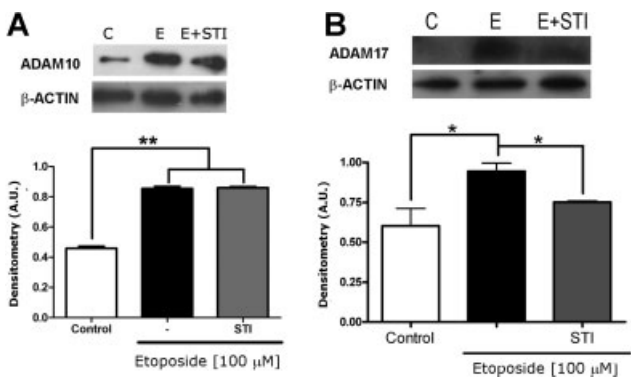

Fig. 7. Pharmacological inhibition of p73 prevents the increase of TACE/ADAM17 induced by etoposide. (A) Immunoblotting and densitometric analysis of ADAM10 in control (C), etoposide-treated testes (E) and etoposide plus STI (a p73/c-Abl inhibitor) (E + STI). Protein levels of ADAM10 are similar between etoposide and etoposide plus STI. (B) Immunoblotting and densitometric analysis of ADAM17 in control (C), etoposide-treated testes (E) and etoposide plus STI (a p73/c-Abl inhibitor) (E + STI). It is clear that etoposide causes a significant increase in the levels of ADAM17, which is prevented by STI. $^{*}P < 0.05$, $^{**}P < 0.01$, n = 3. β-actin was used as a loading control.

levels, at the cell surface as well, as determined by western blotting and flow cytometry, respectively. After etoposide treatment, an increase in the mRNA levels of ADAM10 was observed, whereas that of TACE/ADAM17 and ADAM9 mRNA remained unchanged, as compared with untreated controls. This is the first report to show that etoposide regulates ADAM10 and TACE/ADAM17 levels in vivo, suggesting that these changes could be related to an increase in the shedding activities of these two proteases at the cell surface (Fig. 8). We hypothesized that the increase of ADAM10 and TACE/ADAM17 levels at the surface of those cells affected by etoposide treatment (spermatocytes), promotes extracellular domain shedding of important receptors related to maintaining the balance of cell survival and cell death signaling (Fig. 8). The simplest explanation for our results is that the increase of TACE/ADAM17 and ADAM10 at the cell surface, probably as a consequence of their upregulation at the protein level, enhances ectodomain shedding of pro survival molecules (Maretzky et al., 2010). In an attempt to evaluate in vivo the shedding activity of TACE/ADAM17 and/or ADAM10, we assayed the presence of the extra- and intra-cellular domain of c-kit using two different antibodies directed against these domains. In our previous work we showed that germ cells undergoing apoptosis lack the extracellular domain of c-kit, but the intracellular domain remain inside apoptotic cells, suggesting the involvement of TACE/ADAM17 sheddase activity (Lizama et al., 2010). In the present work, we show that apoptotic cells are devoid of the extracellular and intracellular domain of c-kit. Even though it is speculative, one possibility is that upon shedding of the extracellular domain, the remaining part in the cell is rapidly internalized by the cell and degraded by ubiquitination or through the lysosomal pathway. Another possibility is that etoposide impairs mRNA synthesis or stability and thus compromises c-kit half-life and decreases its levels after treatment. Further experiments will be needed to properly answer these questions.

Our data point out that TACE/ADAM17 and ADAM10 are activated after etoposide treatment (upregulation and cell surface localization), but they are not activated by heat shock. Thus, it seems that in terms of ADAM protein activation, the mechanisms underlying heat shock and etoposide induced apoptosis are different. This difference could be related to the specific apoptotic mechanisms elicited in each injury. Germ cell apoptosis induced by heat shock seems to be related to the activation of the p38 MAPK and stress signals, opposite to etoposide that induces apoptosis by increasing DNA fragmentation and activating downstream pathways related to this injury (Bromberg et al., 2003; Ishii et al., 2005; Beissert and Loser, 2008; Lizama et al., 2009). In this context, we have previously shown that the tyrosine kinase c-Abl is activated upon etoposide treatment in the 21-day-old rat testis, and that this activation is important in order to induce germ cell apoptosis (Codelia et al., 2010). We show here that STI, a pharmacological inhibitor of c-Abl was able to prevent the upregulation of TACE/ADAM17, but not ADAM10, suggesting different control mechanisms between these two enzymes. Since the mRNA levels of TACE/ADAM17 are not affected by etoposide treatment, it is possible that c-Abl could be regulating this protein at the post-transcriptional level. In fact, it has been shown that c-Abl induces activation of PKC delta, which along with p38 MAPK and extracellular regulated kinase (ERK1/2) induces phosphorylation of TACE/ADAM17 and extracellular shedding of different substrates (Blass et al., 2002; Tang et al., 2002). On the other hand, ADAM10 seems to be regulated at the transcriptional level by a mechanism different from c-Abl/p73 in etoposide-treated cells. In this way, etoposide-induced upregulation of ADAM10 mRNA levels are observed under in vivo and in vitro conditions, but upregulation of TACE/ADAM17 mRNA is only observed in vitro (Lizama et al.,

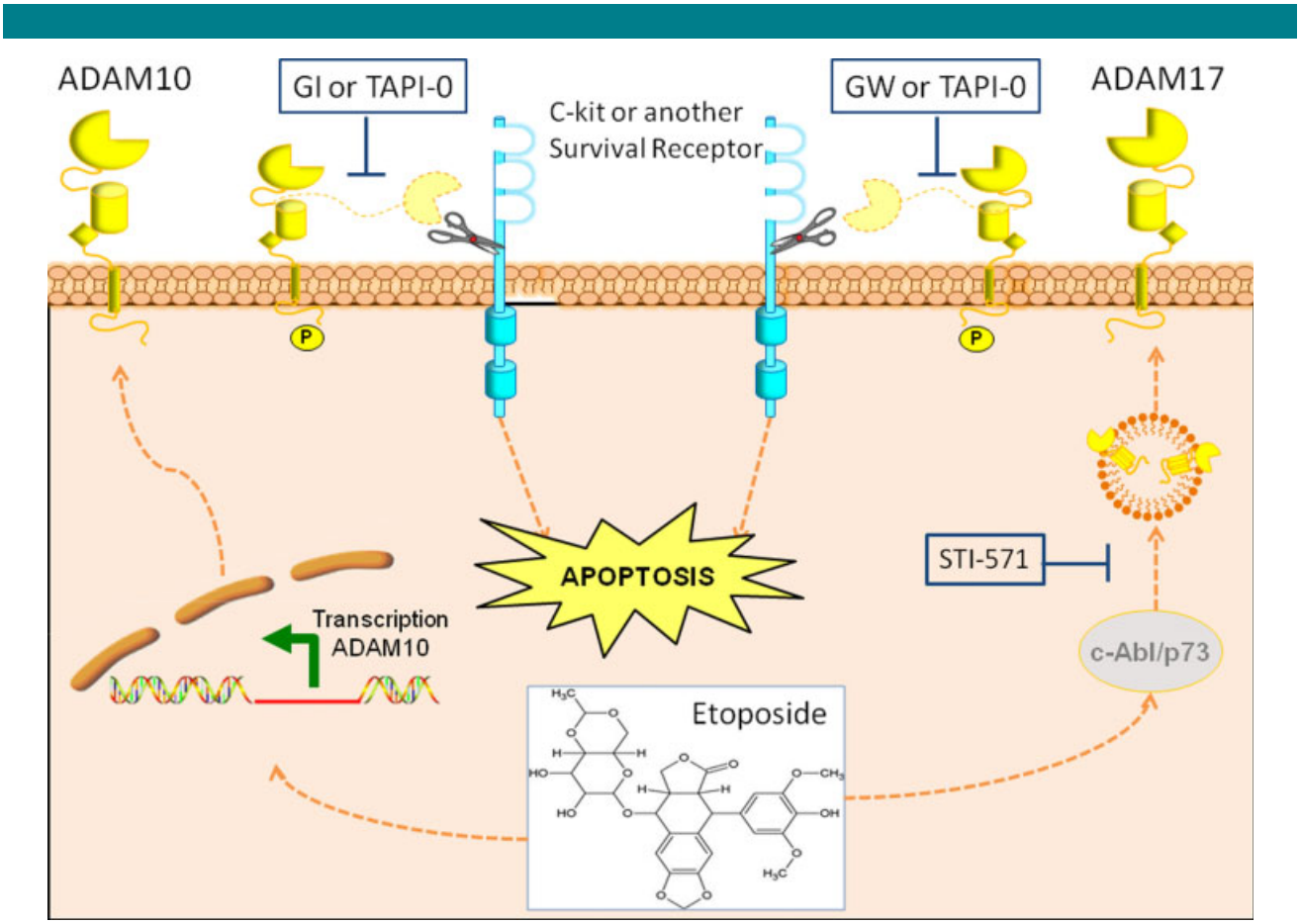

Fig. 8. Etoposide regulates the levels and localization of ADAM17 and ADAM10 at the cell surface. Etoposide induces increases of ADAM10 and ADAM17 protein levels in two different ways. First, it induces an increase in the mRNA levels of ADAM10, which in turn increases ADAM10 protein levels. On the contrary, etoposide upregulates ADAM17 protein levels directly, probably by protein stabilization and/or increases in the translation rate. Since upregulation of ADAM17 is blocked by STI, the protein kinase c-Abl is likely involved in this process. ADAM10 and ADAM17, at the cell surface, will shed the extracellular domain of c-kit or other receptors involved in survival signaling, which will lead to apoptosis.

2011). Therefore, it seems that the mechanism elicited by etoposide in vitro and in vivo does not always overlap. Since upregulation of TACE/ADAM17 mRNA was observed with cell lines derived from mouse spermatocytes (GC-1 and GC-2), it could be that this difference is related to the fact that biological responses by cell lines are not completely comparable to their in vivo counterpart.

Our data show that etoposide induces expression of extracellular proteases in normal viable germ cells. It is known that etoposide, as well as other anti-cancer drugs, induces a sever impairment in spermatogenesis, and some reports show that chemotherapy in adolescent males may have a profound effect on fertility during adulthood. Since ADAM proteases have been shown to be involved in the release of membrane-bound signaling molecules, it is possible that etoposide treatment promotes this situation in rat testes. On the other hand, upregulation of ADAM proteins could also lead to changes in cell motility and/or cell-cell interaction; since their disintegrin domain also has a role in cell adhesion. Therefore, etoposide could impair testicular function through the induction of germ cell apoptosis, but it might also change the levels of soluble signaling molecules.

Acknowledgments

This work was financed by a grant from the Chilean Research Council (FONDECYT, 1070360) to RDM. AL is supported in part by IZKF Biomat and SFB 542, project A12. The authors thank Mr. Jurriaan Brouwer-Visser for his excellent assistance in English grammar.

Literature Cited

Alvarez AR, Klein A, Castro J, Cancino GI, Amigo J, Mosqueira M, Vargas LM, Yevenes LF, Bronfman FC, Zanlungo S. 2008. Imatinib therapy blocks cerebellar apoptosis and improves neurological symptoms in a mouse model of Niemann-Pick type C disease. Faseb J 22:3617–3627.

Bedell MA, Mahakali Zama A. 2004. Genetic analysis of Kit ligand functions during mouse spermatogenesis. J Androl 25:188–199.

Beissert S, Loser K. 2008. Molecular and cellular mechanisms of photocarcinogenesis. Photochem Photobiol 84:29–34.

Bellve AR, Zheng W, Martinova YS. 1993. Recovery, capacitation, acrosome reaction, and fractionation of sperm. Methods Enzymol 225:113–136.

Bieber AM, Marcon L, Hales BF, Robaire B. 2006. Effects of chemotherapeutic agents for testicular cancer on the male rat reproductive system, spermatozoa, and fertility. J Androl 27:189–200.

Blass M, Kronfeld I, Kazimirsky G, Blumberg PM, Brodie C. 2002. Tyrosine phosphorylation of protein kinase Cdelta is essential for its apoptotic effect in response to etoposide. Mol Cell Biol 22:182–195.

Blobel CP. 2005. ADAMs: Key components in EGFR signalling and development. Nat Rev Mol Cell Biol 6:32–43.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Bromberg KD, Burgin AB, Osheroff N. 2003. A two-drug model for etoposide action against human topoisomerase IIalpha. J Biol Chem 278:7406–7412.

Cancino GI, Toledo EM, Leal NR, Hernandez DE, Yevenes LF, Inestrosa NC, Alvarez AR. 2008. STI571 prevents apoptosis, tau phosphorylation and behavioural impairments induced by Alzheimer's beta-amyloid deposits. Brain 131:2425–2442.

Codelia VA, Cisterna M, Alvarez AR, Moreno RD. 2010. p73 participates in male germ cells apoptosis induced by etoposide. Mol Hum Reprod 16:734–742.

Codelia VA, Cisternas P, Moreno RD. 2008. Relevance of caspase activity during apoptosis in pubertal rat spermatogenesis. Mol Reprod Dev 75:881–889.
Cruz AC, Frank BT, Edwards ST, Dazin PF, Peschon JJ, Fang KC. 2004. Tumor necrosis factor-alpha-converting enzyme controls surface expression of c-Kit and survival of embryonic stem cell-derived mast cells. J Biol Chem 279:5612–5620.
Feng HL, Sandlow JI, Sparks AE, Sandra A, Zheng LJ. 1999. Decreased expression of the c-kit receptor is associated with increased apoptosis in subfertile human testes. Fertil Steril 71:85–89.
Gruber F, Zamolo G, Kastelan M, Massari LP, Cabrijan L, Peharda V, Batinac T. 2007. Photocarcinogenesis–molecular mechanisms. Collegium Antropologicum 31:101–106.
Hikim AP, Lue Y, Yamamoto CM, Vera Y, Rodriguez S, Yen PH, Soeng K, Wang C, Swerdloff RS. 2003. Key apoptotic pathways for heat-induced programmed germ cell death in the testis. Endocrinology 144:3167–3175.
Huovila AP, Turner AJ, Pelto-Huikko M, Karkkainen I, Ortiz RM. 2005. Shedding light on ADAM metalloproteinases. Trends Biochem Sci 30:413–422.
Ishii T, Matsuki S, Iuchi Y, Okada F, Toyosaki S, Tomita Y, Ikeda Y, Fujii J. 2005. Accelerated impairment of spermatogenic cells in SOD1-knockout mice under heat stress. Free Radic Res 39:697–705.
Ji G, Gu A, Hu F, Wang S, Liang J, Xia Y, Lu C, Song L, Fu G, Wang X. 2009. Polymorphisms in cell death pathway genes are associated with altered sperm apoptosis and poor semen quality. Hum Reprod (Oxford, England) 24:2439–2446.
Jung A, Schuppe HC. 2007. Influence of genital heat stress on semen quality in humans. Andrologia 39:203–215.
Liu PC, Liu X, Li Y, Covington M, Wynn R, Huber R, Hillman M, Yang G, Ellis D, Marando C, Katiyar K, Bradley J, Abremski K, Stow M, Rupar M, Zhuo J, Li YL, Lin Q, Burns D, Xu M, Zhang C, Qian DQ, He C, Sharief V, Weng L, Agrios C, Shi E, Metcalf B, Newton R, Friedman S, Yao W, Scherle P, Hollis G, Burn TC. 2006. Identification of ADAM10 as a major source of HER2 ectodomain sheddase activity in HER2 overexpressing breast cancer cells. Cancer Biol Ther 5:657–664.
Lizama C, Alfaro I, Reyes JG, Moreno RD. 2007. Up-regulation of CD95 (Apo-1/Fas) is associated with spermatocyte apoptosis during the first round of spermatogenesis in the rat. Apoptosis 12:499–512.
Lizama C, Lagos CF, Lagos-Cabre R, Cantuarias L, Rivera F, Huenchunir P, Perez-Acle T, Carrion F, Moreno RD. 2009. Calpain inhibitors prevent p38 MAPK activation and germ cell apoptosis after heat stress in pubertal rat testes. J Cell Physiol 221:296–305.
Lizama C, Ludwig A, Moreno RD. 2011. Etoposide induces apoptosis and upregulation of TACE/ADAM17 and ADAM10 in an in vitro male germ cell line model. Biochim Biophys Acta 1813:120–128.
Lizama C, Rojas-Benitez D, Antonelli M, Ludwig A, Bustamante-Marin X, Brouwer-Visser J, Moreno RD. 2010. TACE/ADAM17 is involved in germ cell apoptosis during rat spermatogenesis. Reproduction (Cambridge, England) 140:305–317.
Ludwig A, Hundhausen C, Lambert MH, Broadway N, Andrews RC, Bickett DM, Leesnitzer MA, Becherer JD. 2005. Metalloproteinase inhibitors for the disintegrin-like metalloproteinases ADAM10 and ADAM17 that differentially block constitutive and phorbol ester-inducible shedding of cell surface molecules. Comb Chem High Throughput Screen 8:161–171.
Maretzky T, Zhou W, Huang XY, Blobel CP. 2010. A transforming Src mutant increases the bioavailability of EGFR ligands via stimulation of the cell-surface metalloproteinase ADAM17. Oncogene 30:611–630.
Meachem SJ, Ruwanpura SM, Ziolkowski J, Ague JM, Skinner MK, Loveland KL. 2005. Developmentally distinct *in vivo* effects of FSH on proliferation and apoptosis during testis maturation. J Endocrinol 186:429–446.
Melino G, Lu X, Gasco M, Crook T, Knight RA. 2003. Functional regulation of p73 and p63: Development and cancer. Trends Biochem Sci 28:663–670.
Mezyk-Kopec R, Bzowska M, Stalinska K, Chelmicki T, Podkalicki M, Jucha J, Kowalczyk K, Mak P, Bereta J. 2009. Identification of ADAM10 as a major TNF sheddase in ADAM17-deficient fibroblasts. Cytokine 46:309–315.
Moreno RD, Lizama C, Urzua N, Vergara SP, Reyes JG. 2006. Caspase activation throughout the first wave of spermatogenesis in the rat. Cell Tissue Res 325:533–540.
Murphy G. 2008. The ADAMs: Signalling scissors in the tumour microenvironment. Nat Rev Cancer 8:929–941.
Ortiz RJ, Lizama C, Codelia VA, Moreno RD. 2009. A molecular evaluation of germ cell death induced by etoposide in pubertal rat testes. Mol Hum Reprod 15:363–371.
Ramadan S, Terrinoni A, Catani MV, Sayan AE, Knight RA, Mueller M, Krammer PH, Melino G, Candi E. 2005. p73 induces apoptosis by different mechanisms. Biochem Biophys Res Commun 331:713–717.
Riccardi C, Nicoletti I. 2006. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 1:1458–1461.
Rockett JC, Mapp FL, Garges JB, Luft JC, Mori C, Dix DJ. 2001. Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. Biol Reprod 65:229–239.
Rodriguez I, Ody C, Araki K, Garcia I, Vassalli P. 1997. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. Embo J 16:2262–2270.
Russell LB, Hunsicker PR, Kerley M, Pyle A, Saxton AM. 2004. Etoposide exposure during male mouse pachytene has complex effects on crossing-over and causes nondisjunction. Mutation research 565:61–77.
Sakata S, Sakamaki K, Watanabe K, Nakamura N, Toyokuni S, Nishimune Y, Mori C, Yonehara S. 2003. Involvement of death receptor Fas in germ cell degeneration in gonads of Kit-deficient Wv/Wv mutant mice. Cell Death Differ 10:676–686.
Schulte M, Reiss K, Lettau M, Maretzky T, Ludwig A, Hartmann D, de Strooper B, Janssen O, Saftig P. 2007. ADAM10 regulates FasL cell surface expression and modulates FasL-induced cytotoxicity and activation-induced cell death. Cell Death Differ 14:1040–1049.
Seals DF, Courtneidge SA. 2003. The ADAMs family of metalloproteases: Multidomain proteins with multiple functions. Genes Dev 17:7–30.
Sinawat S. 2000. The environmental impact on male fertility. J Med Assoc Thai 83: 880–885.
Singh B, Schneider M, Knyazev P, Ullrich A. 2009. UV-induced EGFR signal transactivation is dependent on proligand shedding by activated metalloproteases in skin cancer cell lines. Int J Cancer 124:531–539.
Sokal RR. 1995. Biometry: The principles and practice of statistic in biological research. New York: W.H. Freeman. 887 p.
Sung SY, Kubo H, Shigemura K, Arnold RS, Logani S, Wang R, Konaka H, Nakagawa M, Mousses S, Amin M, Anderson C, Johnstone P, Petros JA, Marshall FF, Zhau HE, Chung LW. 2006. Oxidative stress induces ADAM9 protein expression in human prostate cancer cells. Cancer Res 66:9519–9526.
Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, Kidd VJ, Mak TW, Ingram AJ. 2002. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem 277:12710–12717.
Vera Y, Diaz-Romero M, Rodriguez S, Lue Y, Wang C, Swerdloff RS, Sinha Hikim AP. 2004. Mitochondria-dependent pathway is involved in heat-induced male germ cell death: Lessons from mutant mice. Biol Reprod 70:1534–1540.
Weikert S, Schrader M, Muller M, Krause H, Miller K. 2004. Expression of the apoptosis inhibitor survivin in testicular tissue of infertile patients. Int J Androl 27:161–165.