Biochimica et Biophysica Acta 1813 (2011) 120–128

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Etoposide induces apoptosis and upregulation of TACE/ADAM17 and ADAM10 in an *in vitro* male germ cell line model

Carlos Lizama [a], Andreas Ludwig [b], Ricardo D. Moreno [c,*]

[a] Departamento de Fisiología, Pontificia Universidad Católica de Chile, Santiago, Chile
[b] Institute for Pharmacology and Toxicology, RWTH Aachen University, Aachen, Germany
[c] Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda 340, Santiago, Chile

ARTICLE INFO

Article history:
Received 21 April 2010
Received in revised form 28 July 2010
Accepted 11 August 2010
Available online 25 August 2010

Keywords:
Apoptosis
Testis
Spermatogenesis
Etoposide
TACE

ABSTRACT

Etoposide is a widely used anticancer drug in the treatment of different tumors. Etoposide is known to activate a wide range of intracellular signals, which may in turn induce cellular responses other than apoptosis. ADAM10 and TACE/ADAM17 belong to a family of transmembrane extracellular metalloproteinases involved in paracrine/juxtacrine regulation of many signaling pathways. The aim of this work was to evaluate if etoposide induces upregulation of ADAM10 or TACE/ADAM17 in two cell lines (GC-1 and GC-2) derived from male germ cells. Results showed that etoposide induced apoptosis in a dose–response manner in both GC-1 and GC-2 cells. Apoptosis started to increase 6 h after etoposide addition in GC-2 cells, whereas the same was observed 18 h after addition to the GC-1 cells. Protein and mRNA levels of ADAM10 and TACE/ADAM17 increased 18 h after etoposide was removed from the GC-1 cells. In GC-2 cells, the protein levels of both proteins increased 12 h after etoposide was removed. ADAM10 mRNA increased after 3 h and then steadily decreased up to 12 h after removal, whereas TACE/ADAM17 mRNA decreased after etoposide removal. Finally, apoptosis was prevented in GC-1 and GC-2 cells by the addition of pharmacological inhibitors of ADAM10 and TACE/ADAM17 to the culture medium of etoposide-treated cells. Our results show for the first time that etoposide upregulates ADAM10 and TACE/ADAM17 mRNA and protein levels. In addition, we also show that ADAM10 and TACE/ADAM17 have a role in etoposide-induced apoptosis.

1. Introduction

Etoposide (VP-16) is a podophyllotoxin semi-derivative agent used in a variety of chemotherapeutic treatments. Previous reports have proposed that etoposide's activity is mediated by its interaction with topoisomerase II, an ATP-dependent nuclear enzyme that regulates DNA topology by transiently breaking and joining double-stranded DNA [1]. It has been shown that etoposide induces apoptosis, both p53-dependent and -independent, in several cultured cancer cell lines and is widely used in different protocols for testicular cancer [2]. In addition, etoposide induces a number of intracellular responses such as activation of protein kinase C delta (PKCδ), increase in reactive oxygen species (ROS) and activation of p38 MAPK [3–6]. Thus, it is possible that etoposide triggers several cellular responses, besides apoptosis, particularly in those cancer cells resistant to this drug or in non-cancerous viable cells. In addition, it has been shown that etoposide regulates the levels of matrix metalloproteinase 2 (MMP2) and activates the transforming growth factor-β1/p38/Smad3 signaling pathway in bone marrow stromal cells, but these responses seem not to be related with its pro-apoptotic activity [7,8].

Several signaling molecules, such as epidermal growth factor (EGF) and fibroblast growth factor (FGF), are synthesized as transmembrane proteins; then, upon specific cellular signals, the extracellular domains of these proteins are shed from the cell surface and their biological functions are executed in target cells [9]. A family of transmembrane metalloproteinases known as "a disintegrin and metalloprotease" (ADAM) proteins is a key component in protein ectodomain shedding [10]. ADAMs play a role in diverse biological processes mainly by regulating paracrine/juxtacrine cell-to-cell communication [9,11]. ADAMs are type 1 transmembrane proteins of approximately 70 to 90 kDa. They feature a common modular ectodomain structure, and a prodomain that is cleaved off in the trans-Golgi network by protein convertases. ADAM10 and the TNF-alpha convertase (TACE or ADAM17) are the most studied since they have relevant roles in the shedding of the ectodomain of several membrane-bound EGFR ligands such as neuregulin, EGF and developmental regulatory proteins such as the NOTCH receptor and pro-TNF-alpha [9,11].

In a given cell type certain ADAMs may participate in constitutive shedding while others only in stimulated shedding; an example of the latter is the shedding of ErbB ligands mediated by phosphorylation of

* Corresponding author. Fax: +56 2 222 5515.
E-mail address: rmoreno@bio.puc.cl (R.D. Moreno).

0167-4889/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2010.08.003

TACE/ADAM17 and induced by transforming growth factor beta (TGF-β) [12]. In addition, ADAM10 and TACE/ADAM17 can be induced to shed different ligands when cells are treated with phorbol 12-myristate 13-acetate (PMA), suggesting a role for protein kinase C in the activation of these enzymes [10]. The mechanism underlying ADAM protein activation is still far from elucidated. Some studies have suggested that TACE/ADAM17 activity could involve the activation of p38 MAPK, extracellular regulated kinases (ERK1/2) and PKC [4,6]. In fact, TACE/ADAM17 has been shown to be phosphorylated by ERK at threonine 735, which is associated with protein kinase C-regulated TrkA cleavage [13].

Etoposide, along with cisplatin and bleomicyn, are drugs widely used in the treatment of testicular cancer. We have shown that a single dosis of etoposide in pubertal rats induces an increase in the apoptosis of spermatocytes, along with p53 stabilization [14]. In order to better study the mechanisms underlying etoposide action upon germ cells, we used an *in vitro* model of germ cells with two established cell lines: GC-1 spg and GC-2 spc, which have been successfully used before as germ cell models [15,16].

In this work we show that etoposide induces apoptosis and upregulation of ADAM10 and TACE/ADAM17 in GC-1 and GC-2 cells. In addition, we show that pharmacological inhibitors of ADAM10 and TACE/ADAM17 prevent the apoptosis induced by etoposide.

2. Materials and methods

2.1. Cell lines

Germ cell-1 spg (GC-1) and germ cell-2 spc (GC-2) were purchased from the American Type Culture Collection (Rockville, MD). GC-1 was originally created by Hofmann et al. to study the process of germ cell differentiation [17,18]. Preleptotene spermatocytes from 6-week-old BALB/c mice were transformed using the SV40 virus's large T antigen. GC-1 cells have been characterized as being in a stage between spermatocytes and spermatogonia. GC-2 was obtained by cotransfecting with SV40 virus' large T antigen and a plasmid carrying a mutant form of the gene encoding p53 (trp53), a transcription factor involved in cell cycle regulation [17]. Previous studies have shown that when grown at 37 °C they express several markers characteristic of meiotic spermatocytes. Thus, these two cell lines make an appropriate *in vitro* model for spermatogenesis.

GC-1 and GC-2 were cultured at 37 °C in a 5% CO_2 atmosphere, in Dulbecco modified Eagles media (DMEM; Life Technologies, Carlsbad, CA) containing 4.5 g/L glucose, L-glutamine, 110 mg/L sodium pyruvate, pyridoxine hydrochloride, and supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin. Cells were grown up to 60-70% of confluence and then etoposide (50–100 μM) alone or in addition to ADAM inhibitors were administered and apoptosis was evaluated at 0 (Control), 3, 6, 12, 24 and 36 h after drug treatment.

2.2. Chemicals and antibodies

Etoposide and TAPI-0 were purchased from Merck (Darmstadt, Germany). Rabbit polyclonal antibodies against TACE/ADAM17 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal mouse antibody against β-actin was purchased from Sigma (St Louis, MO) and the 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). The GI254023X and GW280264X inhibitors were synthesized as described before [19,20]. 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 cell culture medium.

2.3. Protein extraction and Western blot

Protein extraction was performed by homogenizing cells 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 centrifuging for 10 min at 9300*g*. 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. The nitrocellulose membrane was blocked with 4% non-fat milk in PBS, pH 7.4, and then incubated overnight at 4 °C with anti-ADAM17 (0.5 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. Protein bands were revealed using the Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL). Bands obtained were analyzed by measuring the pixels with Adobe® Photoshop 7.0 (Adobe System Incorporated, USA), and normalizing to β-actin levels.

2.4. TUNEL analysis

Apoptotic fragmentation of DNA was evaluated by TUNEL analysis (Dead End System; Promega, Madison, Wis.). Cells were grown onto coverslips and then fixed with 1% paraformaldehyde for 15 min. The assay was performed according to the manufacturer´s protocol. 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).

2.5. Apoptosis and cell cycle analysis

To analyze cell cycles, the cell suspension in KHB solution was pelleted and fixed in ice-cold 70% ethanol overnight. As described by Riccardi [21], on the day of analysis the cells were pelleted and washed once with 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 ten minutes of buffer addition in a BD FACS Canto Flow Cytometer; 10,000 gated events were acquired. All date were analyzed with software FCS express V2.0 (De Novo Software, Los Angeles, CA).

2.6. 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 a polymerase chain reaction (PCR) of 30 cycles using Taq polymerase (Fermentas) in 50 μl of incubation mixture. Several primer sets were used to obtain the PCR products: ADAM10 forward 5'-CCTACGAATGAAGAGGGAC-3' and reverse 5'-A TCACAGCTTCTCGTGTTCC-3'; ADAM17 forward 5'-GTTGGTGAGCCTGACTCTA-3' and reverse 5'-CCTCTTGTGGA-GACTTGA-3'; GAPDH forward 5'-TCCACCACCCTGTTGCTGTA-3' and reverse 5'-ACCACAGTCCATGCCATCAC-3' under the same aforementioned conditions. 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 by measuring the pixels with Adobe® Photoshop CS3 (Adobe System Incorporated, USA), and normalizing to GAPDH mRNA levels.

2.7. Statistics

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 $p<0.05$ [22].

3. Results

First, we characterized the response of GC-1 and GC-2 cells to etoposide. Cells were cultured with 100 μM etoposide and apoptosis was analyzed at different time points. GC-1 cells cultured for 18 h with etoposide showed many round structures resembling apoptotic bodies, and incubation for longer periods of time showed clear evidence of cell detriment (Fig. 1A). TUNEL analysis showed positive staining in many cells, suggesting apoptosis induction after etoposide treatment (Fig. 1A, insert). In order to better evaluate apoptosis we analyzed cell cycles at different points of time, and determined the percentage of cells at the subG1 stage, which is a well-recognized parameter of apoptosis (Fig. 1B).To avoid individual variation between experiments, results were expressed as the ratio of change in relation to control cells (without etoposide). Results showed that from 3 to 12 h of incubation, there was a slight but non-significant increase in apoptosis. However, 18 h after etoposide addition, apoptosis increased almost four times, and then up to seven times in cells treated for 36 h, an effect that was concentration-dependent (Fig 1C, D). Etoposide-induced apoptosis in GC-2 cells showed a

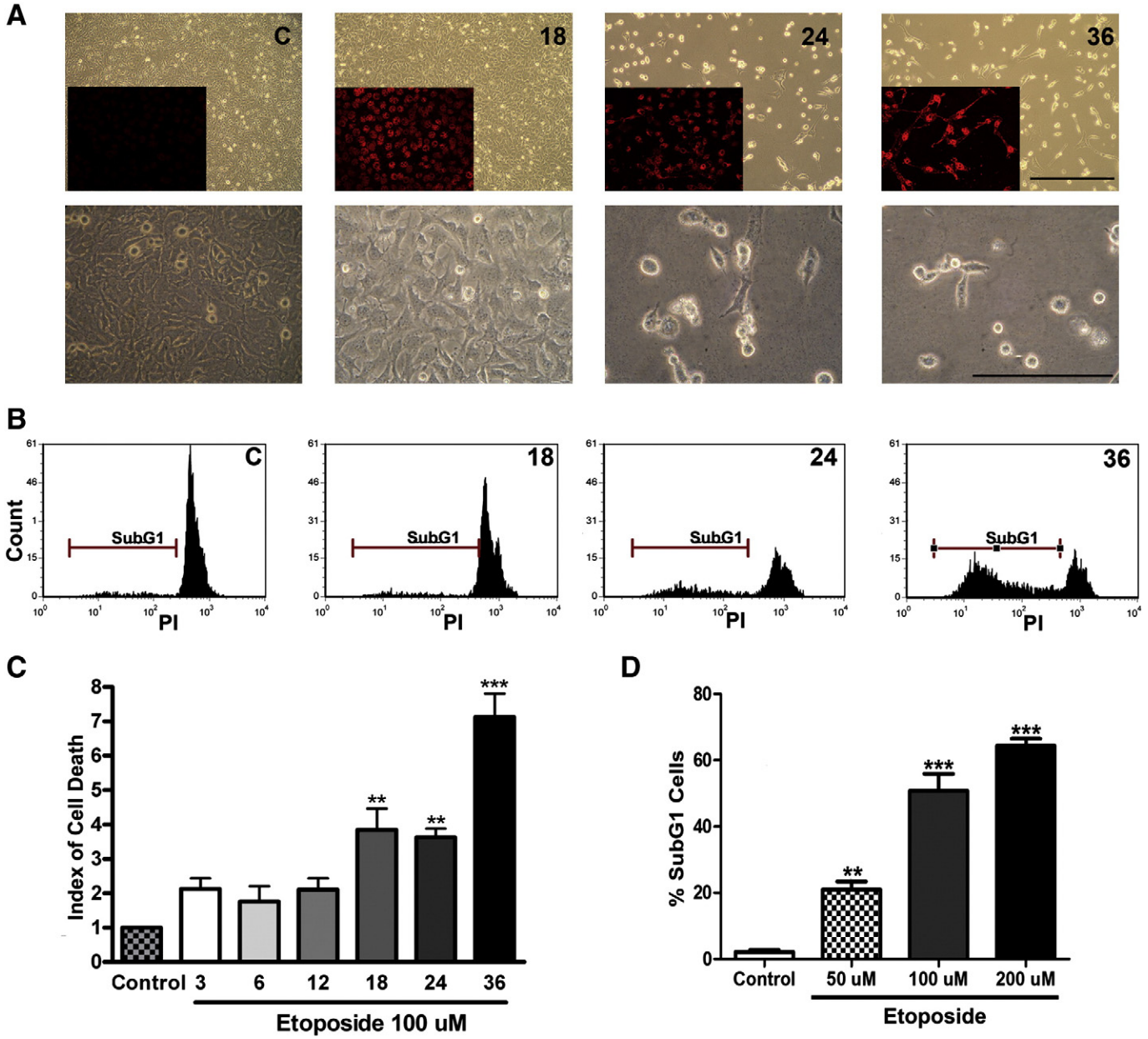

Fig. 1. Etoposide induces apoptosis in GC-1 cells. (A) Detachment and morphology changes of GC-1 cells after incubation with 100 μM etoposide at different time points. The inserts show TUNEL staining of cells at the same time points indicated in the contrast phase microphotograph. (B) Flow cytometry analysis of GC-1 incubated with 100 μM etoposide at different time points. The horizontal bar shows the gate of subG1 (apoptotic) cells. (C) The graph shows the ratio of change in apoptosis, relative to the starting point ($t=0$), at different time points of GC-1 cells treated with 100 μM etoposide, (D) Graph showing the percentage of the subG1 (apoptotic) population of GC-1 cell cultured for 24 h with different concentrations of etoposide (**$p<0.01$, ***$p<0.001$). Bars 50 μm.

significant increase after 6 h of incubation, up to almost four times 12 h after drug addition (Fig. 2A–C). We did not perform analyses at longer periods of time since only a few cells looked viable and remained attached to the culture dish. This effect was also concentration-dependent (Fig. 2D). It has been shown that GC-2 cells express a temperature-sensitive mutant of p53: the mutant p53 protein is fully active at 32 °C, only partially active at 37 °C, and completely inactive at 39 °C. We thought that this condition might influence etoposide-induced apoptosis. However, we observed that apoptosis induced by etoposide was equally effective either at 32 °C or 37 °C (data not shown). Since GC-1 cells were grown at 37 °C we decided to perform all experiments at 37 °C with both cell lines. Thus, etoposide induced apoptosis in both cell lines, although at different time points.

Next, we evaluated protein and mRNA levels of ADAM10 and TACE/ADAM17 in GC-1 and GC-2 cells at different time points after etoposide incubation. Results showed that in GC-1 cells cultured with 100 μM etoposide, mRNA levels of ADAM10 and TACE/ADAM17 peaked 12 h after etoposide addition, whereas protein levels (the mature forms of both enzymes) significantly increased after 18 h (Fig. 3A, B). On the other hand, mRNA levels of ADAM10 in GC-2 cells significantly increased 6 h after etoposide addition and then steadily declined (Fig. 4A, B). TACE/ADAM17 mRNA levels slightly declined from zero to 9 h after treatment (Fig. 4A). ADAM10 and TACE/ADAM17 protein levels in GC-2 cells increased slightly but significantly 12 h after etoposide addition (Fig. 4A, B).

Finally, we studied if TACE/ADAM17 was indeed associated with etoposide-induced apoptosis in GC-1 and GC-2 cells. To this end, we incubated GC-1 cells for 24 h and GC-2 cells for 12 h in the presence of 100 μM etoposide alone or in combination with 300 nM TAPI-0a well-known specific TACE/ADAM17 inhibitor. Results showed that TAPI-0 alone did not induce apoptosis related morphological changes in GC-1 or GC-2 cells (Figs. 5A, 6A). However, when GC-1 cells were treated with 100 μM etoposide in combination with 300 nM TAPI-0, the majority of cells had unaltered (viable) morphology after 24 h of incubation, and the rate of apoptosis was significantly lower than

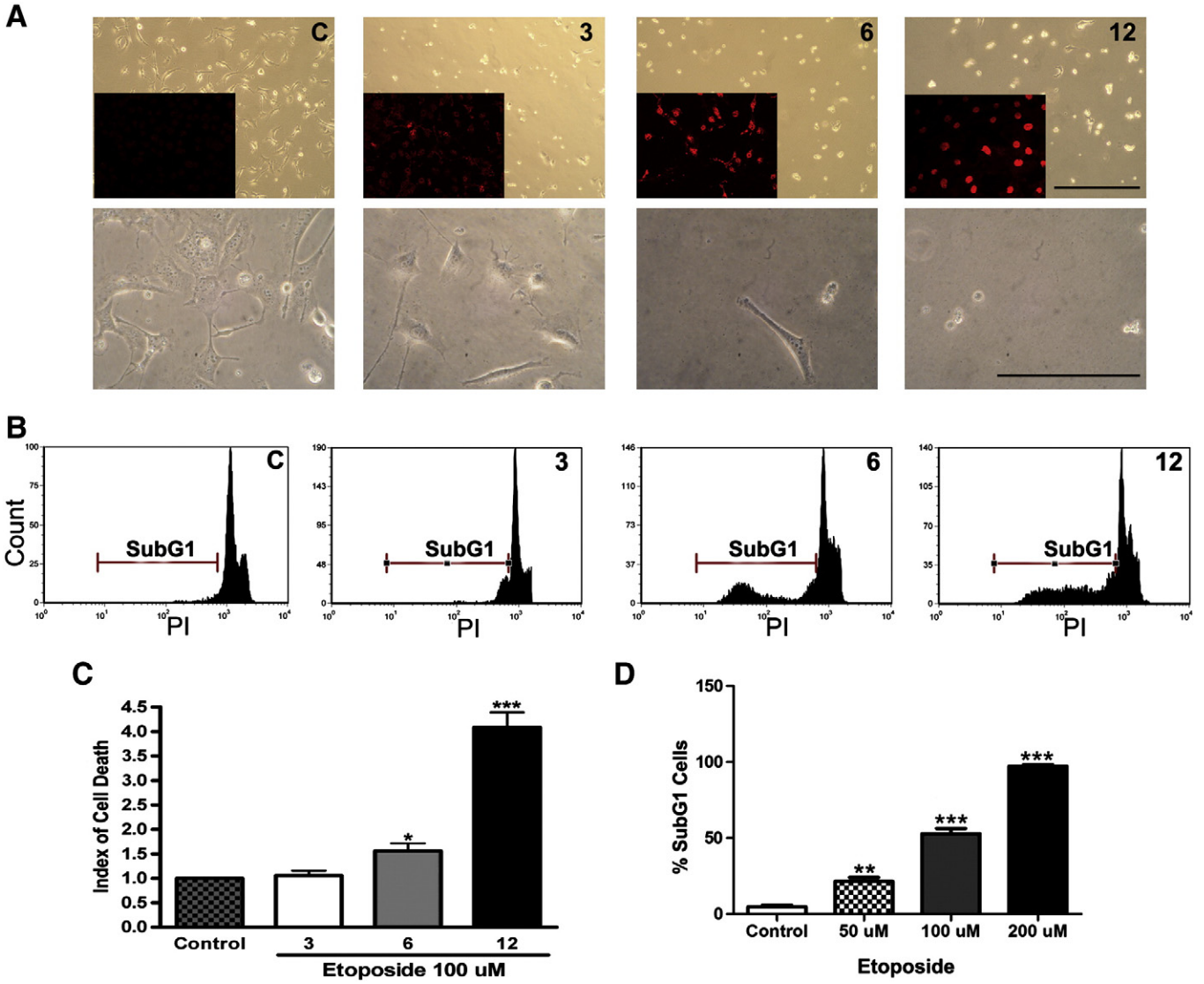

Fig. 2. Etoposide induces apoptosis in GC-2 cells. (A) Detachment and morphology changes of GC-2 cells after incubation with 100 μM etoposide at different time points. The inserts show TUNEL staining of cells at the same time points indicated in the contrast phase microphotograph. (B) Flow cytometry analysis of GC-2 incubated with 100 μM etoposide at different time points. The horizontal bar shows the gate of subG1 (apoptotic) cells. (C) The graph shows the ratio of change in apoptosis, relative to the starting point (t = 0), at different time points of GC-2 cells treated with 100 μM etoposide. (D) Graph showing the percentage of the subG1 (apoptotic) population of GC-2 cells cultured for 12 h with different concentrations of etoposide (**$p<0.01$, ***$p<0.001$). Bars 50 μm.

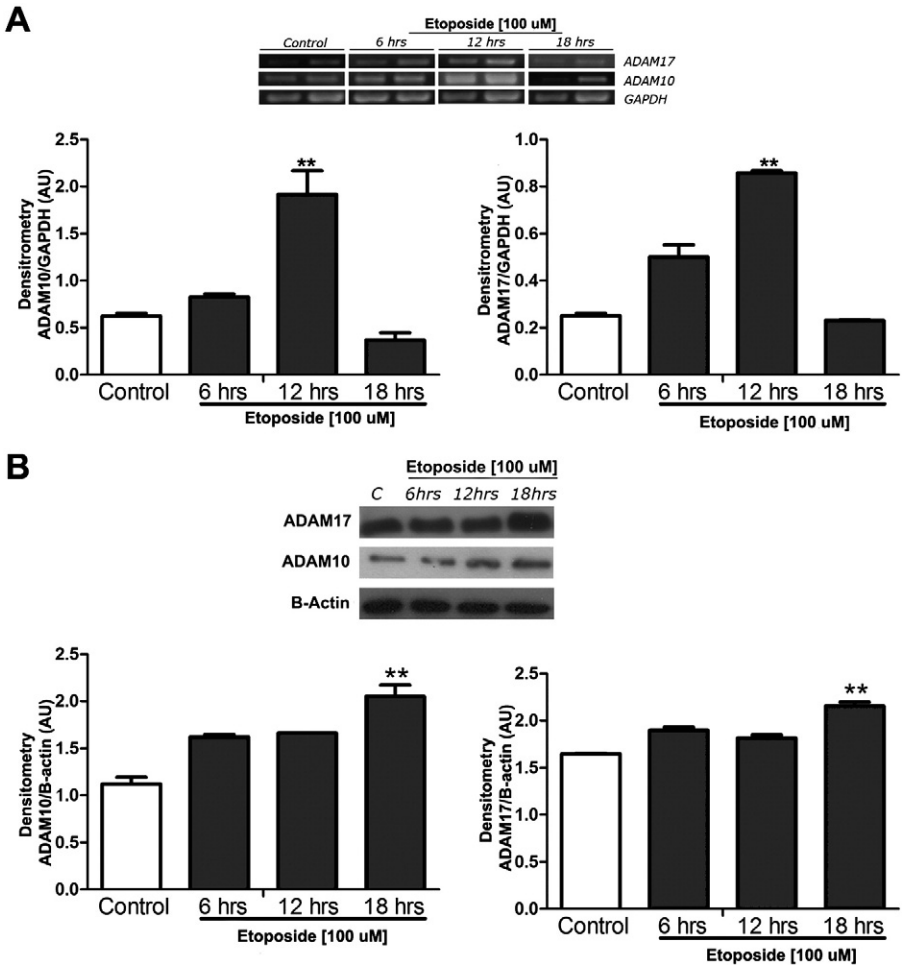

Fig. 3. Etoposide induces the expression of ADAM10 and TACE/ADAM17 in GC-1 cells. GC-1 cells were incubated with 100 μM etoposide for 18 h and subsequently mRNA and protein levels were measured at different time points. (A) RT-PCRs of ADAM10 and TACE/ADAM17 at the indicated time points after etoposide incubation. GADPH was used as a loading control. The figure shows two independent experiments. The graph shows the average intensities of ADAM10 and TACE/ADAM17 bands relative to GADPH, at the indicated time points. Both mRNAs significantly increased 12 h after incubation with etoposide. (B) Western blots of ADAM10 and TACE/ADAM17 of GC-1 cells at different time points following 100 μM etoposide incubation. The Western blots show the mature forms of both proteins. The graph shows the intensity of each band relative to β-actin, as a loading control. Both proteins significantly increased 18 h after treatment (**$p<0.01$, $n=3$).

those incubated with etoposide alone (Fig. 5A, B). In order to discriminate between the relative contribution of ADAM10 and TACE/ADAM17, we used other pharmacological inhibitors. Two different inhibitors for ADAMs had been described prior to this work: compound GW280264X blocks both ADAM10 and TACE/ADAM17 with comparable IC50 values, whereas compound GI254023X has 100-fold higher specificity for ADAM10 [19,23]. These inhibitors have been used to study the role of TACE/ADAM7 and ADAM10 in other systems [19,20,24]. We found that 5 μM GI254023X significantly prevented apoptosis induced by etoposide, and that 5 μM GW280264X showed an even higher inhibition of apoptosis than GI254023X in GC-1 cells (Figs. 5D). When GC-2 cells were treated with etoposide in combination with 300 nM TAPI-0, only a few attached cells were observed after 12 h of culture, a similar situation as with etoposide alone (Fig. 6A). Flow cytometry analysis confirmed that TAPI-0 did not significantly change the apoptosis rate in GC-2 cells in combination with 100 μM etoposide (Fig. 6B, C). Interestingly, 5 μM of GW280264X and GI254023X significantly decreased etoposide-induced apoptosis to a similar extent (Fig. 6D). Therefore, our results show that ADAM10 and TACE/ADAM17 in GC-1, but only ADAM10 in GC-2 cells, appear to be involved in etoposide-induced apoptosis.

4. Discussion

Matrix metalloproteinases play a key role in many biological processes, including cancer. Here we show that etoposide induces upregulation of two metalloproteinases: ADAM10 and TACE/ADAM17. Even more, we showed that ADAM10, and more importantly TACE/ADAM17, are components of the machinery triggered by etoposide to induce apoptosis in germ cells.

It has been shown that etoposide induces apoptosis mainly in spermatocytes in pubertal rat testes [14,25,26]. Here we show that etoposide induces apoptosis in GC-1 and GC-2 cell lines, which have been used previously as a germ cell model to test apoptosis after genotoxic stimulus [15]. Our results clearly showed that, in GC-1 cells upregulation of protein levels of ADAM10 and TACE/ADAM17 (18 h after removal of etoposide) was coincident with the increase in apoptosis observed in this cell line. On the contrary, ADAM10 and TACE/ADAM17 protein levels were upregulated 12 h after incubation in GC-2 cells, even though apoptosis was observed after 6 h of etoposide incubation. Thus, the time course of apoptosis is coincident with the upregulation of both metalloproteinases after etoposide treatment. Despite the fact that both cells lines were derived from germ cells, they behave differently in response to etoposide treatment. GC-1 cells show

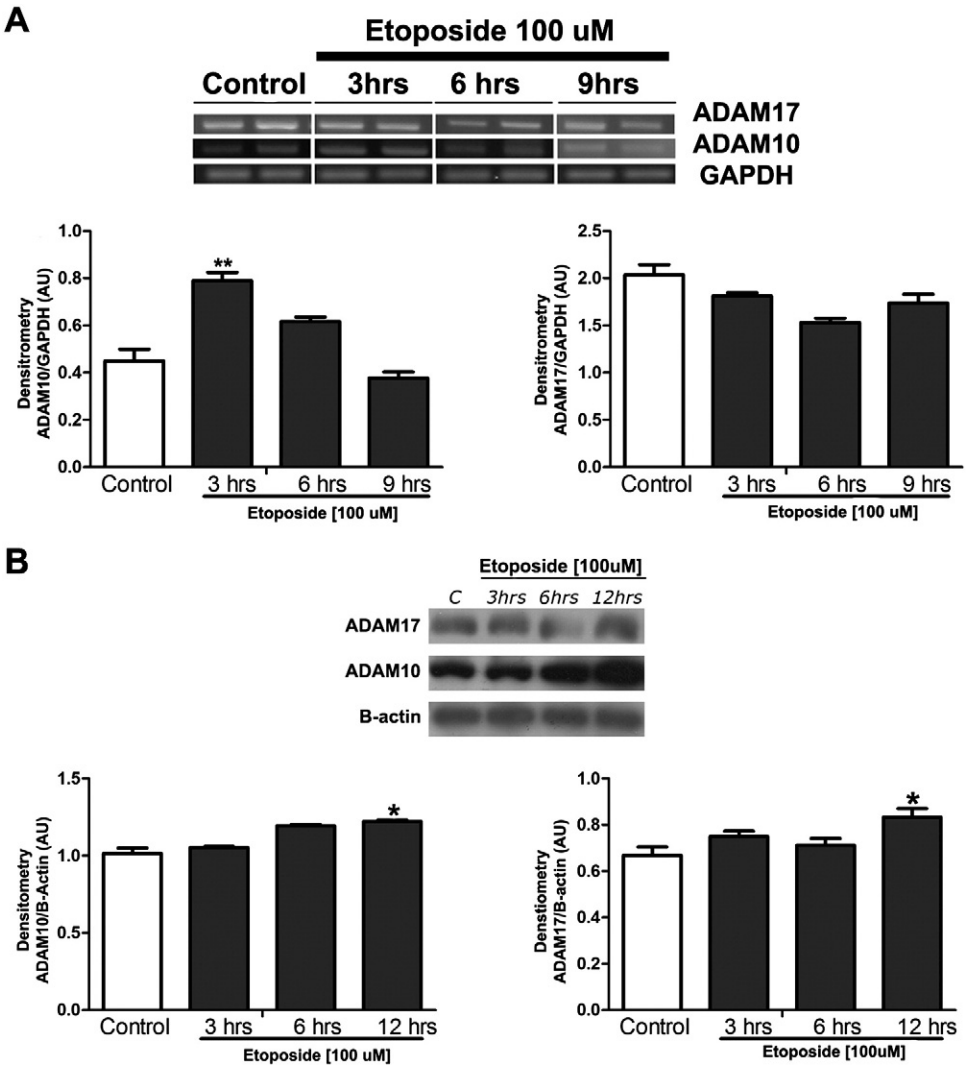

Fig. 4. ADAM10 and TACE/ADAM17 protein and mRNA levels in etoposide-treated GC-2 cells. GC-2 cells were incubated with 100 μM etoposide for 12 h and subsequently the mRNA and protein levels were measured at different time points. (A) RT-PCRs of ADAM10 and TACE/ADAM17 at the indicated time points after etoposide treatment. GADPH was used as a loading control. The figure shows two independent experiments. The graph shows the intensities of ADAM10 and TACE/ADAM17 bands relative to GADPH, at the indicated time points. (B) Western blots of ADAM10 and TACE/ADAM17 of GC-2 cells at different time points following 100 μM etoposide incubation. Only mature forms were visualized by Western blots. The graph shows the intensity of each band relative to β-actin, as a loading control. Both proteins significantly increased 12 h after treatment ($^{**}p<0.01$, $n=3$).

characteristics of a stage between type B spermatogonia and primary spermatocytes, but GC-2 cells, which were derived from GC-1, express markers of primary spermatocytes. It is possible that these differences may be related to p53, which is mutated in GC-2 cells [17,18]. This observation is partially supported by the fact that under our conditions (culture at 37 °C) p53 is not fully active, but is fully active in GC-1 cells. Thus, p53 could change the responsiveness to etoposide and could induce the upregulation of ADAM proteins. In fact, we have shown that etoposide induced germ cell apoptosis *in vivo* and *in vitro* (using GC-2 cells) is dependent upon p73 activation, a closely related member of p53 [27]. Thus, it seems that p73, along with p53, are important regulators of germ cell apoptosis. It remains to be shown whether p53 and/or p73 can regulate ADAMs gene expression in physiological conditions or after genotoxic treatment.

We show here that pharmacological inhibition of TACE/ADAM17 (using TAPI-0) was able to prevent apoptosis induced by etoposide in GC-1, but not in GC-2 cells. TAPI-0 inhibits TACE/ADAM17-dependent shedding of different membrane bound proteins. However, it has also been shown that extracellular shedding in cells harboring an inactivating mutation for TACE/ADAM17 is prevented by TAPI-0, suggesting that it could inhibit other related metalloproteinases, probably ADAM10 [28]. In this work we used two other ADAM inhibitors, which have been previously shown to be useful to elucidate ADAM-dependent ectodomain shedding [19,20]. The compound GW280264X blocks both ADAM10 and ADAM17 with comparable IC50 values, whereas compound GI254023X has 100-fold higher specificity towards ADAM10. We found that GI254023X, which inhibits ADAM10, significantly prevents etoposide-induced apoptosis in both cells lines, suggesting a role for ADAM10 in this process. However, GW280264X prevented etoposide-induced apoptosis in GC-1 to an even higher extent than GI254023X, which suggests that TACE/ADAM17 has an important role in apoptosis in this cell line. Thus, our results that ADAM10 and ADAM17 are important in etoposide induced apoptosis in GC-1 and GC-2 cell lines. This is the first report showing that apoptosis induced by a genotoxic stimulus is mediated by an ADAM metalloproteinase.

Our results show that etoposide induces a transient upregulation of TACE/ADAM17 and ADAM10 mRNA in both cell lines. These results

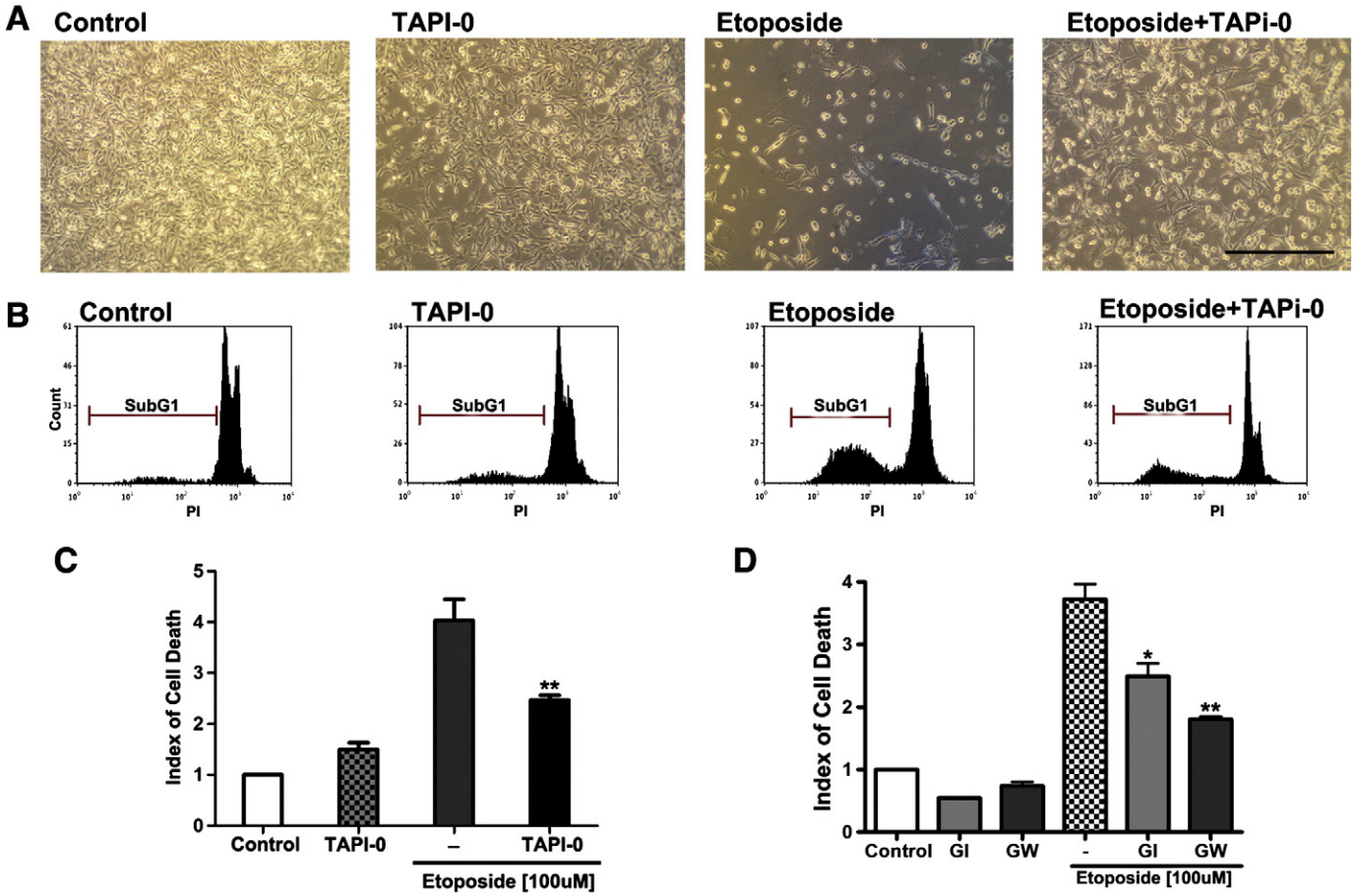

Fig. 5. Pharmacological inhibitors of ADAM10 and TACE/ADAM17 prevent etoposide-induced apoptosis in GC-1 cells. GC-1 cells were incubated with 100 μM etoposide for 24 h, with or without 300 nM TAPI-0. (A) Incubation with TAPI-0 alone did not induce detachment or morphological changes. Cultures treated with etoposide in combination with TAPI-0 showed an increased number of cells compared to the cultures with etoposide alone. (B) Cell cycle analysis of GC-1 cells under different conditions. Apoptotic cells are readily recognized as subG1 cells, horizontal bar in each graph. (C) Apoptosis was evaluated as the ratio of changes in subG1 cells, relative to control cells (without etoposide). TAPI-0 alone did not have any effect upon cell survival; however, it was able to significantly prevent apoptosis in cells treated with etoposide. (D) Effects of specific metalloprotease inhibitors GW280264X (GW, equally specific for ADAM17 and ADAM10) and GI254023X (GI, 100-fold more specific for ADAM10) on etoposide-induced apoptosis ($^{**}p<0.01$, $n=3$). Bars 50 μm.

suggest that mRNA levels of both metalloproteinases are tightly regulated. Hence, it is possible that GC-1 cells regulate the protein levels of both metalloproteinases at the mRNA level. In GC-2 cells the same seems to be true for TACE/ADAM17, but the mRNA levels of ADAM10 slightly decline during the studied period. It has been shown that ADAM10 and ADAM17 can be upregulated at the protein level by activation of protein kinase C or 38 MAP kinase, extracellular regulated kinase (ERK1/2), PKC and an increase in the concentration of free intracellular calcium [4,6,29,30]. In particular, it has been shown that p38 MAP kinase binds and interacts with the cytoplasmic domain of TACE and phosphorylates it on Thr(735), which is required for TACE-mediated ectodomain shedding [30]. Interestingly, it seems that p38 MAP kinase and PKC interact with different domains with the cytoplasmic domain of TACE/ADAM17 in order to promote L-selectin shedding from mouse leucocytes [29]. On the other hand, ADAM10 protein levels could be increased by Ca(2+) influx as well as be stimulated by PMA under some conditions, suggesting that PKC activation might be involved in ADAM10-mediated proteolysis [10,31,32]. It has been shown that along with topoisomerase II inhibition and DNA breaks promotion, etoposide induces activation of PKC, p38 MAP kinase, and an increase of reactive oxygen species and of the free intracellular calcium concentration [5,6,33,34]. Thus it is possible that activation of PKC, ROS or p38 MAP kinase in etoposide-treated cells induces upregulation of TACE/ADAM17 and ADAM10 at the protein level.

In conclusion, in this work we describe TACE/ADAM17 and ADAM10 as new elements in the molecular pathway elicited by etoposide to induce apoptosis in GC-1 and GC-2 cells. Etoposide, alone or in combination with other anticancer drugs is widely used in the treatment of several cancers, and it has several unwanted side effects. GC-1 and GC-2 cell lines are a model of normal (not cancer) male germ cells and the reported effect in this work might be similar to *in vivo* conditions. It is possible that upregulation of metalloproteinases such as ADAM17 or ADAM10 may be causing unwanted secondary effects in normal viable cells in patients undergoing chemotherapy. Thus, it remains to be determined whether etoposide elicits upregulation of TACE/ADAM17 and ADAM10 in cancer cell lines as well or only in normal viable germ cells.

Acknowledgments

Part of this work was financed by a grant from the Chilean Research Council (FONDECYT, 1070360) to RDM. CL is a PhD fellow from the National Commission for Scientific Research and Technology (CONICYT).

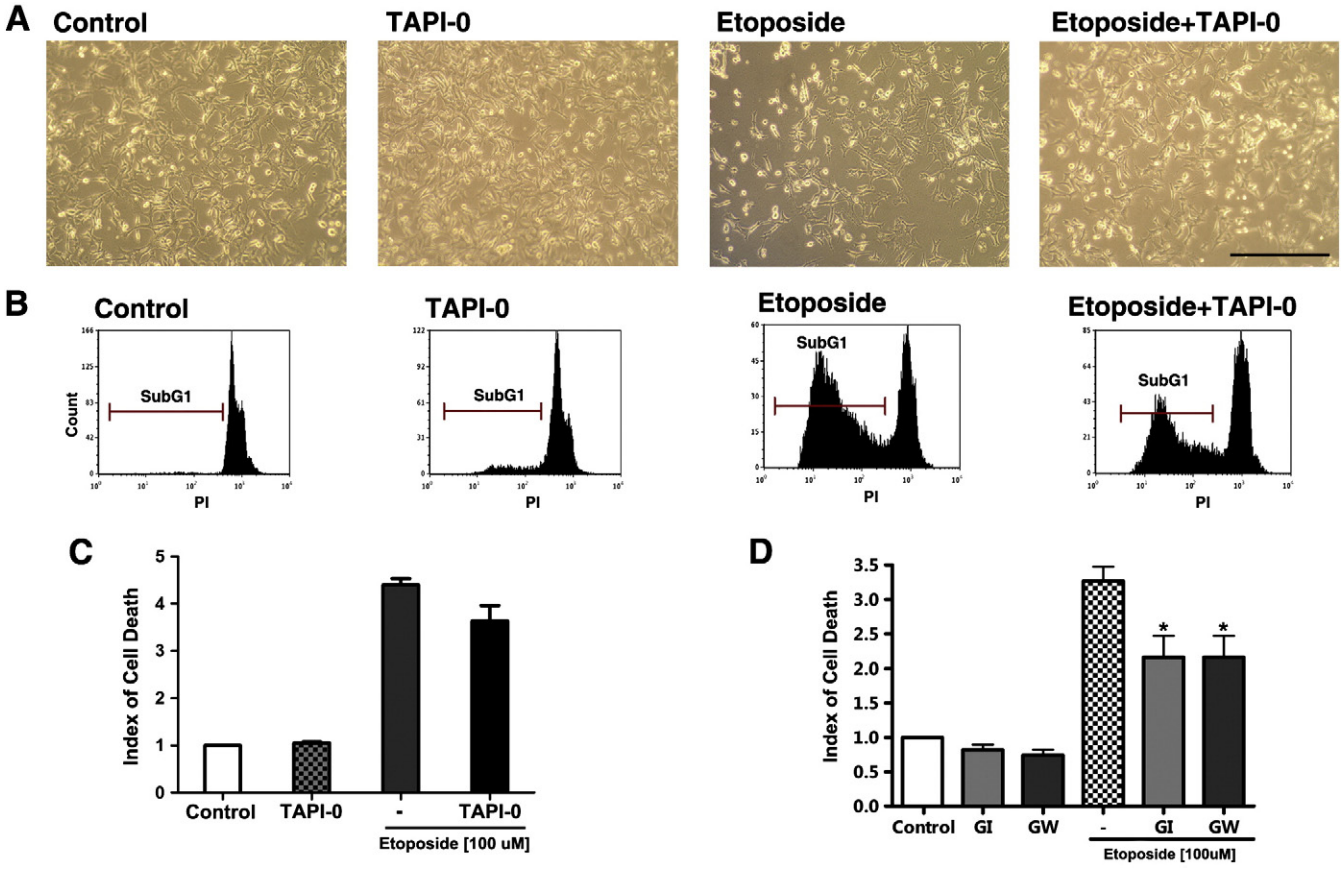

Fig. 6. Pharmacological inhibitors of ADAM10 and TACE/ADAM17 prevent etoposide-induced apoptosis in GC-2 cells. GC-2 cells were incubated with 100 μM etoposide for 12 h, with or without 300 nM TAPI-0. (A) GC-2 cells treated with etoposide plus TAPI-0 or with TAPI-0 alone showed a similar low number of cells after 12 h of culture. (B) Cell cycle analysis of GC-2 cells in different conditions. Apoptotic cells are easily recognized as subG1 cells, horizontal bar in each graph. (C) Apoptosis was evaluated as the ratio of changes in subG1 cells, relative to control cells (without etoposide). TAPI-0 did not modify the apoptosis rate and was not able to prevent apoptosis in cells treated with etoposide. (D) Effect of specific metalloproteinase inhibitors GW280264X (GW, equally specific for ADAM17 and ADAM10) and GI254023X (GI, 100-fold more specific for ADAM10) on etoposide-induced apoptosis ($^{**}p<0.01$, $n=3$). Bars 50 μm.

References

[1] K.D. Bromberg, A.B. Burgin, N. Osheroff, A two-drug model for etoposide action against human topoisomerase IIalpha, J. Biol. Chem. 278 (2003) 7406–7412.

[2] C.M. Sturgeon, M.J. Duffy, U.H. Stenman, H. Lilja, N. Brunner, D.W. Chan, R. Babaian, R.C. Bast Jr., B. Dowell, F.J. Esteva, C. Haglund, N. Harbeck, D.F. Hayes, M. Holten-Andersen, G.G. Klee, R. Lamerz, L.H. Looijenga, R. Molina, H.J. Nielsen, H. Rittenhouse, A. Semjonow, M. Shih Ie, P. Sibley, G. Soletormos, C. Stephan, L. Sokoll, B.R. Hoffman, E.P. Diamandis, National Academy of Clinical Biochemistry laboratory medicine practice guidelines for use of tumor markers in testicular, prostate, colorectal, breast, and ovarian cancers, Clin. Chem. 54 (2008) e11–e79.

[3] N.O. Karpinich, M. Tafani, R.J. Rothman, M.A. Russo, J.L. Farber, The course of etoposide-induced apoptosis from damage to DNA and p53 activation to mitochondrial release of cytochrome c, J. Biol. Chem. 277 (2002) 16547–16552.

[4] D. Tang, D. Wu, A. Hirao, J.M. Lahti, L. Liu, B. Mazza, V.J. Kidd, T.W. Mak, A.J. Ingram, ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53, J. Biol. Chem. 277 (2002) 12710–12717.

[5] B.J. Kim, S.W. Ryu, B.J. Song, JNK- and p38 kinase-mediated phosphorylation of Bax leads to its activation and mitochondrial translocation and to apoptosis of human hepatoma HepG2 cells, J. Biol. Chem. 281 (2006) 21256–21265.

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

[7] S.D. Clutter, J. Fortney, L.F. Gibson, MMP-2 is required for bone marrow stromal cell support of pro-B-cell chemotaxis, Exp. Hematol. 33 (2005) 1192–1200.

[8] L. Wang, S. Clutter, J. Benincosa, J. Fortney, L.F. Gibson, Activation of transforming growth factor-beta1/p38/Smad3 signaling in stromal cells requires reactive oxygen species-mediated MMP-2 activity during bone marrow damage, Stem Cells 23 (2005) 1122–1134 (Dayton, Ohio).

[9] C.P. Blobel, ADAMs: key components in EGFR signalling and development, Nat. Rev. Mol. Cell Biol. 6 (2005) 32–43.

[10] A.P. Huovila, A.J. Turner, M. Pelto-Huikko, I. Karkkainen, R.M. Ortiz, Shedding light on ADAM metalloproteinases, Trends Biochem. Sci. 30 (2005) 413–422.

[11] D.F. Seals, S.A. Courtneidge, The ADAMs family of metalloproteases: multidomain proteins with multiple functions, Genes Dev. 17 (2003) 7–30.

[12] S.E. Wang, B. Xiang, M. Guix, M.G. Olivares, J. Parker, C.H. Chung, A. Pandiella, C.L. Arteaga, Transforming growth factor beta engages TACE and ErbB3 to activate phosphatidylinositol-3 kinase/Akt in ErbB2-overexpressing breast cancer and desensitizes cells to trastuzumab, Mol. Cell. Biol. 28 (2008) 5605–5620.

[13] E. Diaz-Rodriguez, J.C. Montero, A. Esparis-Ogando, L. Yuste, A. Pandiella, Extracellular signal-regulated kinase phosphorylates tumor necrosis factor alpha-converting enzyme at threonine 735: a potential role in regulated shedding, Mol. Biol. Cell 13 (2002) 2031–2044.

[14] R.J. Ortiz, C. Lizama, V.A. Codelia, R.D. Moreno, A molecular evaluation of germ cell death induced by etoposide in pubertal rat testes, Mol. Hum. Reprod. 15 (2009) 363–371.

[15] Y. Chandrasekaran, C.M. McKee, Y. Ye, J.H. Richburg, Influence of TRP53 status on FAS membrane localization, CFLAR (c-FLIP) ubiquitinylation, and sensitivity of GC-2spd (ts) cells to undergo FAS-mediated apoptosis, Biol. Reprod. 74 (2006) 560–568.

[16] Y. Chandrasekaran, J.H. Richburg, The p53 protein influences the sensitivity of testicular germ cells to mono-(2-ethylhexyl) phthalate-induced apoptosis by increasing the membrane levels of Fas and DR5 and decreasing the intracellular amount of c-FLIP, Biol. Reprod. 72 (2005) 206–213.

[17] M.C. Hofmann, R.A. Hess, E. Goldberg, J.L. Millan, Immortalized germ cells undergo meiosis in vitro, Proc. Natl Acad. Sci. USA 91 (1994) 5533–5537.

[18] M.C. Hofmann, S. Narisawa, R.A. Hess, J.L. Millan, Immortalization of germ cells and somatic testicular cells using the SV40 large T antigen, Exp. Cell Res. 201 (1992) 417–435.

[19] A. Ludwig, C. Hundhausen, M.H. Lambert, N. Broadway, R.C. Andrews, D.M. Bickett, M.A. Leesnitzer, J.D. Becherer, 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 (2005) 161–171.

[20] M. Schulte, K. Reiss, M. Lettau, T. Maretzky, A. Ludwig, D. Hartmann, B. de Strooper, O. Janssen, P. Saftig, ADAM10 regulates FasL cell surface expression and modulates FasL-induced cytotoxicity and activation-induced cell death, Cell Death Differ. 14 (2007) 1040–1049.

[21] C. Riccardi, I. Nicoletti, Analysis of apoptosis by propidium iodide staining and flow cytometry, Nat. Protoc. 1 (2006) 1458–1461.
[22] R.R. Sokal, F.J. Rohlf, Biometry : the principles and practice of statistic in biological research W, H. Freeman, New York, 1995.
[23] C. Hundhausen, D. Misztela, T.A. Berkhout, N. Broadway, P. Saftig, K. Reiss, D. Hartmann, F. Fahrenholz, R. Postina, V. Matthews, K.J. Kallen, S. Rose-John, A. Ludwig, The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion, Blood 102 (2003) 1186–1195.
[24] J. Pruessmeyer, A. Ludwig, The good, the bad and the ugly substrates for ADAM10 and ADAM17 in brain pathology, inflammation and cancer, Semin. Cell Dev. Biol. 20 (2009) 164–174.
[25] A.M. Bieber, L. Marcon, B.F. Hales, B. Robaire, Effects of chemotherapeutic agents for testicular cancer on the male rat reproductive system, spermatozoa, and fertility, J. Androl. 27 (2006) 189–200.
[26] T. Stumpp, E. Sasso-Cerri, E. Freymuller, S.M. Miraglia, Apoptosis and testicular alterations in albino rats treated with etoposide during the prepubertal phase, Anat. Rec. 279 (2004) 611–622.
[27] V.A. Codelia, M. Cisterna, A.R. Alvarez, R.D. Moreno, p73 participates in male germ cells apoptosis induced by etoposide, Mol. Hum. Reprod. (2010).
[28] M.P. Sanderson, C.A. Abbott, H. Tada, M. Seno, P.J. Dempsey, A.J. Dunbar, Hydrogen peroxide and endothelin-1 are novel activators of betacellulin ectodomain shedding, J. Cell. Biochem. 99 (2006) 609–623.
[29] D.J. Killock, A. Ivetic, The cytoplasmic domains of TNFalpha-converting enzyme (TACE/ADAM17) and L-selectin are regulated differently by p38 MAPK and PKC to promote ectodomain shedding, Biochem. J. 428 (2010) 293–304.
[30] P. Xu, R. Derynck, Direct activation of TACE-mediated ectodomain shedding by p38 MAP kinase regulates EGF receptor-dependent cell proliferation, Mol. Cell 37 (2010) 551–566.
[31] K. Horiuchi, T. Kimura, T. Miyamoto, K. Miyamoto, H. Akiyama, H. Takaishi, H. Morioka, T. Nakamura, Y. Okada, C.P. Blobel, Y. Toyama, Conditional inactivation of TACE by a Sox9 promoter leads to osteoporosis and increased granulopoiesis via dysregulation of IL-17 and G-CSF, J. Immunol. 182 (2009) 2093–2101.
[32] K. Horiuchi, S. Le Gall, M. Schulte, T. Yamaguchi, K. Reiss, G. Murphy, Y. Toyama, D. Hartmann, P. Saftig, C.P. Blobel, Substrate selectivity of epidermal growth factor-receptor ligand sheddases and their regulation by phorbol esters and calcium influx, Mol. Biol. Cell 18 (2007) 176–188.
[33] A. Ganguly, B. Das, A. Roy, N. Sen, S.B. Dasgupta, S. Mukhopadhayay, H.K. Majumder, Betulinic acid, a catalytic inhibitor of topoisomerase I, inhibits reactive oxygen species-mediated apoptotic topoisomerase I-DNA cleavable complex formation in prostate cancer cells but does not affect the process of cell death, Cancer Res. 67 (2007) 11848–11858.
[34] R. Gomel, C. Xiang, S. Finniss, H.K. Lee, W. Lu, H. Okhrimenko, C. Brodie, The localization of protein kinase Cdelta in different subcellular sites affects its proapoptotic and antiapoptotic functions and the activation of distinct downstream signaling pathways, Mol. Cancer Res. 5 (2007) 627–639.