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FLIP is expressed in mouse testis and protects germ cells from apoptosis

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Abstract

Apoptosis control in adult testis is crucial to achieve normal spermatogenesis. In this study c-FLIP, an apoptosis-modulating protein, was investigated. In Western blot and immunohistochemical analyses, the 55 KDa c-FLIP long isoform (c-FLIP$_L$) was found to be expressed strongly in spermatocytes and spermatids, at low levels in spermatogonia and at almost undetectable levels in Sertoli cells. This expression pattern was confirmed by Northern blot analyses. Further experiments carried out on GC-1spg germ cell line revealed that reducing c-FLIP$_L$ expression increases Fas-dependent apoptosis. Conversely, restoring c-FLIP$_L$ expression reduces this response to control levels. Caspase-10 expression was found to match c-FLIP$_L$ expression pattern; further, caspase-10 activation upon anti-Fas treatment inversely correlated with c-FLIP$_L$ expression. Finally, TUNEL staining of seminiferous tubules incubated with anti-Fas antibody showed that apoptosis occurs mostly in basally located germ cells, indicating that such cells, expressing low levels of c-FLIP$_L$, are sensitive to Fas-mediated apoptosis.

These data indicate for the first time that c-FLIP$_L$ might control germ cell apoptosis and caspase activity in the adult testis.
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Keywords: caspases; fas; spermatogenesis

Abbreviations: c-FLIP, cellular-flice-inhibitory-protein; c-FLIP$_L$, 55 KDa c-FLIP long isoform; c-FLIP$_S$, FLIP short isoform; DEDs, death effector domains; FADD, fas-associated death domain protein; AU, arbitrary units; BIM, bisindolylmaleimide VIII; FU, fluorimetric units; PKC, protein kinase C

Introduction

During establishment of spermatogenesis at the puberal age, an early germ cells apoptotic wave occurs likely aimed to remove abnormal germ cells and to maintain a proper cell number ratio between maturing germ cells and Sertoli cells. [1,2] As a result, up to 75% spermatogenic cells are eliminated during this maturation.[3,4] It has been shown that the intracellular balance of BclxL and Bax proteins is crucial for this early apoptotic wave; in fact, sterility has been observed in Bax knock-out mice and in transgenic mice overexpressing BclxL, likely because of spermatogenic cells overproduction.[5] These data demonstrate that apoptosis control is crucial to achieve normal mature spermatogenesis. Fas/FasL are known regulators of this process[6] likely by inducing germ-cell apoptosis.[7] Under particular conditions (namely, criptorchid-, heated-, drug-treated-, irradiated-, or hormone-deprived-testes) the Fas–FasL system has been shown to be the major inducer of germ-cell apoptosis.[8–13]

Fas–FasL expression in the testis has been reported by various Authors. Fas has been shown to be expressed in both germ cells[11,14] and in Sertoli cells,[15,16] while FasL expression is more controversial mainly because of the lack of reliable specific antibodies. In fact some Authors report its expression in germ cells,[15,17] while others claim it is present in Sertoli cells.[7,14,18,19] FasL has been reported to trigger germ-cell apoptosis,[6,7] even though the fine molecular events involved are still largely unknown.

While apotosis is a massive event in the first puberal spermatogenesis wave, in the adult testis, cells become more resistant and apoptosis mainly affects spermatogonia.[5,20,21] The reason still remains to be clarified: according to some authors, spermatogonia exceeding the supportive capacity of Sertoli cells are eliminated to prevent seminiferous tubule overcrowding;[1] others suggest that spermatogonia elimination may represent an early selection of abnormal cells before the onset of meiosis.[5] The present study investigated molecular mechanisms conferring either susceptibility or resistance to apoptotic stimuli respectively, in spermatogonia or other germ-cell types.

In different cellular systems, c-FLIP protein (also known as FLAME-1, CASPER, CLARP, I-FLICE, CASH, MRIT and Usurpin) has been shown to modulate the response to apoptotic stimuli.[22–27] Several studies show that it can prevent Fas-mediated apoptosis. In fact, while high c-FLIP levels reduce Fas-dependent arpoptosis in lymphocytes and melanoma,[28–30] c-FLIP downregulation makes lymphocytes more sensitive to Fas and affects self-tolerance.[31] Further, in dendritic cells, c-FLIP levels directly correlate with protection from Fas-mediated apoptosis.[32] Although c-FLIP is reported to be strongly expressed in adult human testis,[22] its role in controlling testis apoptosis has never been investigated.

c-FLIP expression is known to be regulated by RNA alternative splicing leading to different protein isoforms.[30]

c-FLIP long isoform (c-FLIP$_L$) has two 'death effector domains' (DEDs) and one caspase-like domain, structurally resembling caspase-8 and -10, but lacking proteolytic activity.[22–24] c-FLIP$_S$ isoform (c-FLIP short) is smaller, lacks the caspase-like domain but still possesses two DEDs.[25,30] Additional splice variants lacking part of the caspase-like domain have also been described.[33] Different mechanisms have been reported for c-FLIP action.[34] In fact, c-FLIP isoforms may specifically interact with target proteins, namely Fas-associated death domain protein (FADD) and caspase-8/-10, via a DED–DED interaction, thus interfering with caspase recruitment and activation. In addition, c-FLIP$_L$ caspase-like domain was shown to heterodimerize with the corresponding domain of caspase-8/-10.[30] However, other authors have not observed interaction with FADD and caspase-8 prior to triggering of death receptors.[23,24,35] In all these conditions, caspases cascade activation is prevented, thus inhibiting apoptosis in numerous cell types.

The specific aims of the present study were (i) to elucidate the role of c-FLIP$_L$ in the mouse testis (ii) to investigate the correlation with caspase activity, and (iii) to clarify the possible involvement of c-FLIP$_L$ in the protection from Fas-induced apoptosis in adult germ cells.

Results

c-FLIP expression in mouse testis

c-FLIP expression in the male gonad has been shown in adult human testis,[22,30] while no data are available on its cell-specific localization. To explore this point, germ cells from mouse testis were separated into homogeneous populations and analyzed in Northern blot experiments. A representative experiment is shown in Figure 1a. Five c-FLIP transcripts were identified in total adult mouse testis at 46–60 days from birth, as well as in purified pachytene spermatocytes and round spermatids. While four transcripts are common to humans,[30] the fifth one found in the mouse may represent an additional transcript or a degradation product. Three transcripts were found to be expressed at lower level in spermatogonia while Sertoli cells did not show any detectable c-FLIP expression.

Western blot analysis showed that both pachytene spermatocytes and round spermatids express the 55 kDa c-FLIP$_L$ isoform, whereas the expression was weak in spermatogonia and almost undetectable in Sertoli cells (Figure 1b). In spermatogonia two additional bands are evident, which may represent additional isoforms or degradation products. These results allowed us to conclude that both mouse pachytene spermatocytes and round spermatids express c-FLIP$_L$ at high levels while spermatogonia express it at low levels.

Northern and Western blot analyses were also carried out on the immortalized germ cell line GC-1spg, which corresponds to a stage between type B spermatogonia and preleptotene spermatocytes.[36] Similar to freshly isolated cell populations, c-FLIP RNA was found in GC-1spg cell line (Figure 1a) and the 55 kDa c-FLIP$_L$ protein was detected at levels comparable to those found in spermatocytes (Figure 1b).

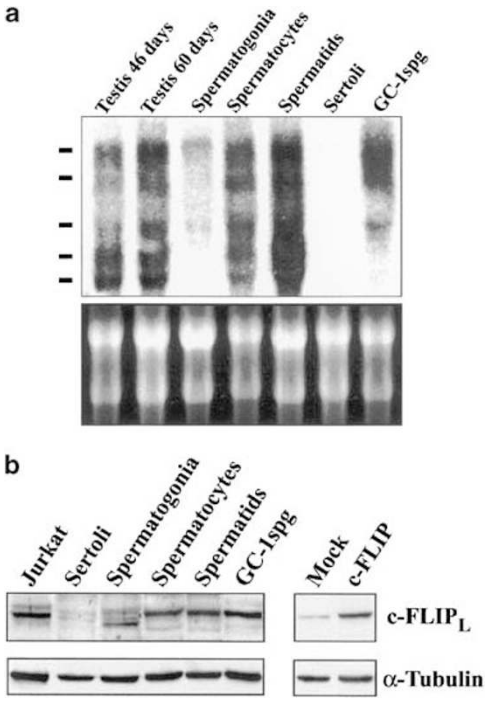

Figure 1 (**a**) c-FLIP Northern blot analysis in total RNA (20 μg/lane) extracted from whole testes at 46 and 60 days after birth, from homogeneous testicular cell populations and from GC-1spg cell line (upper panel). The integrity and equal loading of RNA was ascertained by ethidium bromide staining of the gel before transfer (lower panel). Data are representative of three independent experiments. (**b**) c-FLIP$_L$ Western blot analysis in whole cell extracts from purified testis cell populations and from GC-1spg cell line (50 μg/lane). Jurkat cells and c-FLIP-transfected cells were used as positive controls (upper panels). α-tubulin staining was also carried out to show similar loading (lower panels). Data are representative of three independent experiments

c-FLIP$_L$ immunohistochemical localization in adult mouse testis

c-FLIP$_L$ localization was then examined by immunohistochemistry in mouse testes from young adults (Figure 2a). c-FLIP$_L$-specific staining was observed in the cytoplasm of pachytene spermatocytes (arrow). In addition, round spermatids showed a strong immunoreactivity in the acrosomal region (arrowhead). By contrast, Sertoli cells, peritubular myoid cells and spermatozoa appeared negative while some spermatogonia were weakly stained. Further immunohistochemical analysis of the epididymis confirmed that spermatozoa lack c-FLIP$_L$-specific staining (Figure 2c).

Taken together these results indicate that c-FLIP$_L$ is expressed at low levels in spermatogonia and at high levels in spermatocytes and spermatids, while it is not detectable in Sertoli cells and spermatozoa. Since c-FLIP$_L$ plays a key role in controlling apoptosis in other cell systems, we hypothesized a similar function in differentiating germ cells. In order to test this hypothesis, expression of the caspases known to be targets of c-FLIP$_L$ action and Fas-mediated apoptosis were investigated.

Caspase-8 and -10 expression in mouse testis

c-FLIP is known to inhibit caspase-8 and -10 recruitment to Fas and TNFR-1 death receptors in different tissues, by direct

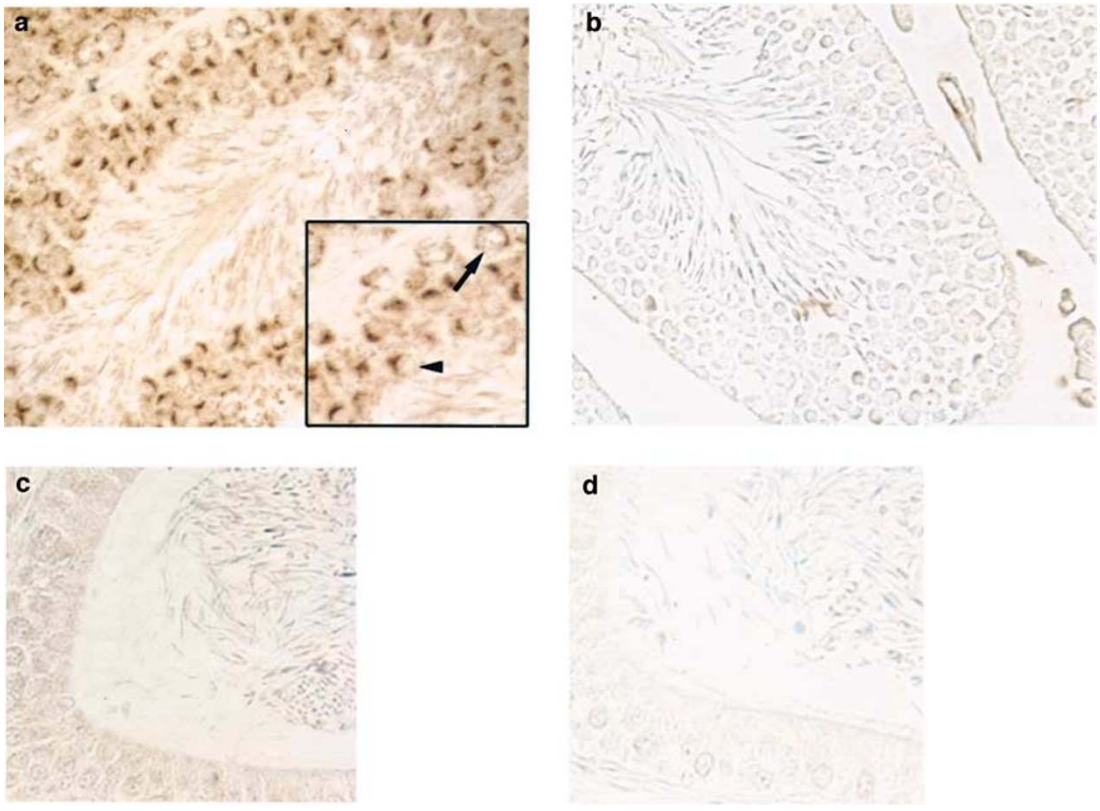

Figure 2 Immunohistochemical localization of c-FLIP$_L$ in mouse testis (**a**, **b**) and epididymis (**c**, **d**) at 46 days after birth. Panels **a** and **c** show sections stained with anti-c-FLIP$_L$ antibody. In the inset, representing a portion at higher magnification ($\times$ 750), the arrow indicates a pachytene spermatocyte and the arrowhead indicates a round spermatid. Panels **b** and **d** represent sections stained with an isotype antibody, as negative controls. Magnification $\times$ 400.

competition,[37] but its function and its targets in the testis are poorly investigated.[38–40] Thus, the expression pattern of such caspases in mouse testis and in GC-1spg germ-cell line was investigated. Western blot analysis showed that pachytene spermatocytes, round spermatids and GC-1spg cells strongly express caspase-10 proenzyme, while spermatogonia express it at low levels and Sertoli cells express it at almost undetectable levels (Figure 3a). Interestingly, Western blot analysis of caspase-8 showed that neither freshly isolated germ cells nor GC-1spg cells nor Sertoli cells express the proenzyme at significant levels (Figure 3b).

These data show that caspase-10 expression pattern is cell specific and matches that of c-FLIP$_L$ in mouse testis. In agreement with other cell types, this finding may suggest caspase-10 as a possible target of c-FLIP$_L$ action in testis, further supporting a possible role for c-FLIP$_L$ in controlling germ-cell apoptosis.

Fas-induced apoptosis and caspase activity in mouse testis

The hypothesis that c-FLIP$_L$ might modulate Fas-mediated apoptosis in germ cells was investigated by (i) downregulating c-FLIP$_L$ expression with bisindolylmaleimide VIII (BIM) or with antisense-specific oligonucleotides and (ii) subsequently restoring the expression by transfecting these cells with a c-FLIP-expression vector. Transfection as well as antisense oligonucleotides experiments were carried out on GC-1spg germ cell line, since primary germ cells are known to undergo a dramatic viability drop in culture.[41,42] On the other hand, GC-1spg cell line expresses both c-FLIP$_L$ and caspase-10 at levels comparable to primary pachytene spermatocytes (Figures 1b and 3a). Therefore, it likely represents a good experimental system under this respect.

Several bisindolylmaleimide derivatives, originally described as inhibitors of protein kinase C (PKC), are known to facilitate Fas-mediated apoptosis in different cell lines substantially.[43] In dendritic cells this effect was related to c-FLIP$_L$-specific downregulation.[32] We found that GC-1spg cells express c-FLIP$_L$ (Figure 1b) as well as Fas (data not shown); therefore, we investigated whether BIM downregulates c-FLIP$_L$ and whether, under these conditions, Fas-dependent apoptosis is increased. BIM treatment (0–50 μM) reduced c-FLIP$_L$ protein expression up to 40%, dose-dependently after 16 h treatment. Figure 4a reports one representative experiment and the densitometric quantification averaged from three independent experiments. The effect on c-FLIP$_L$ expression was BIM-specific; in fact, a different PKC inhibitor, namely staurosporine, did

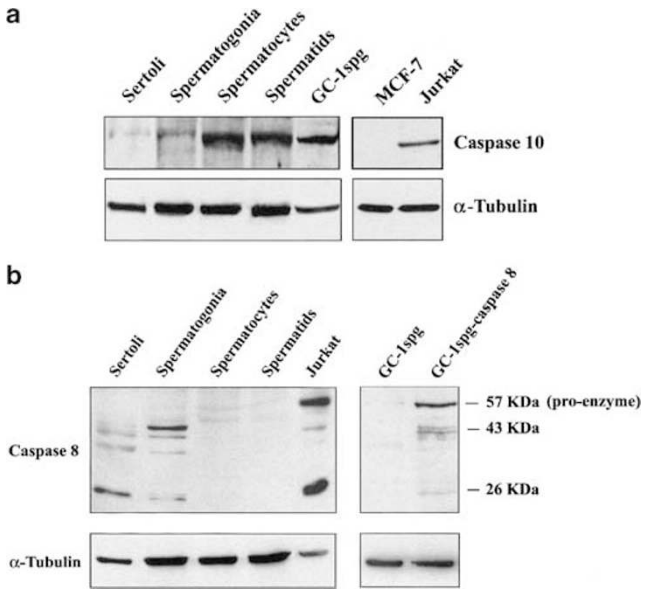

Figure 3 (**a**) Caspase-10 Western blot analysis in whole cell extracts from homogeneous testicular cell populations and from GC-1spg cell line (50 μg/lane). Positive control (Jurkat cell lysate) and negative control (MCF-7 cell lysate) are also shown (upper panels). α-tubulin staining was also carried out to show similar loading (lower panels). Data are representative of three independent experiments. (**b**) Caspase-8 Western blot analysis in whole cell extracts from homogeneous testicular cell populations and from GC-1spg cell line (50 μg/lane). Jurkat cells and caspase-8-transfected cells were used as positive controls (upper panels). α-tubulin staining was also carried out to show similar loading (lower panels). Data are representative of three independent experiments

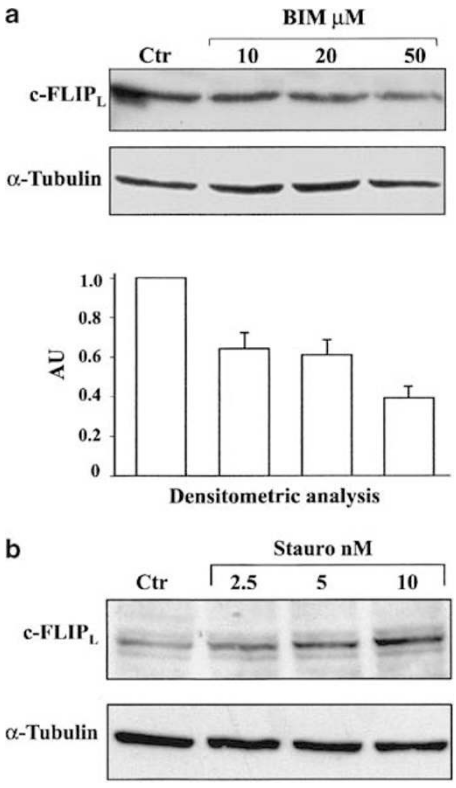

Figure 4 (**a**) c-FLIP$_L$ Western blot analysis in whole cell extracts (50 μg/lane) from GC-1spg cell line, treated with BIM at the indicated doses. Quantification reported in the histogram was achieved by densitometry normalized *versus* α-tubulin content, and represents the average $\pm$ s.e.m. of three independent experiments. (**b**) c-FLIP$_L$ Western blot analysis in whole cell extracts (50 μg/lane) from GC-1spg cell line, treated with staurosporine (Stauro) at the indicated doses. Data are representative of three independent experiments

not down regulate c-FLIP$_L$ expression (Figure 4b). GC-1spg cells were then treated with BIM (20 μM), or with anti-Fas (1 μg/ml), or with a combination of BIM and anti-Fas (20 μM and 1 μg/ml, respectively). The number of apoptotic and nonviable cells, evaluated by flow cytometer quantification of propidium iodide incorporation, was found to be significantly increased by the combined treatment (41%), when compared with anti-Fas alone (11%) or BIM alone (22%) (Figure 5). These results indicated that GC-1spg cells show a markedly higher apoptotic response to anti-Fas treatment when c-FLIP$_L$ expression is reduced by BIM treatment.

Under these experimental conditions, caspases activity was also investigated by measuring DEVD-AFC cleavage; DEVD-AFC is reported to be a substrate for different caspases. BIM-treated cells, expressing lower levels of c-FLIP$_L$, show a markedly increased caspase activity in response to anti-Fas treatment (712% *versus* control), as compared with cells expressing normal levels of c-FLIP$_L$, which show very low caspase activation in response to anti-Fas treatment (103% *versus* control; Figure 6a). Moreover, anti-Fas stimulation failed to increase caspase activity in staurosporine-treated cells. Similar to GC-1spg cell line, BIM-treated pachytene spermatocytes display increased caspase activity in response to anti-Fas (386% *versus* control), as shown in Figure 6b.

Caspase-8 activity was analyzed by IETD-AFC cleavage fluorescence assay in GC-1spg cell line. As shown in Figure 6c, it was found to be markedly lower under control conditions

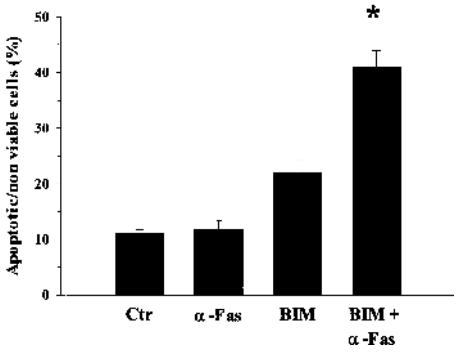

Figure 5 Fas-induced apoptosis of GC-1spg germ cells. The histogram shows the percentage of apoptotic and nonviable cells compared to total population, revealed by propidium iodide staining. Data are expressed as the mean $\pm$ s.e.m. of three independent experiments. Asterisk indicates significant difference ($P<0.05$) as compared to anti-Fas antibody alone or BIM alone.

as compared to DEVD-AFC cleavage shown in Figure 6a (i.e. 50 *versus* 284 FU). Moreover, caspase-8 activity was not modulated by anti-Fas and BIM incubation. This finding correlates with Western blotting experiments showing irrelevant expression levels of procaspase-8 in GC-1spg cells (Figure 3b).

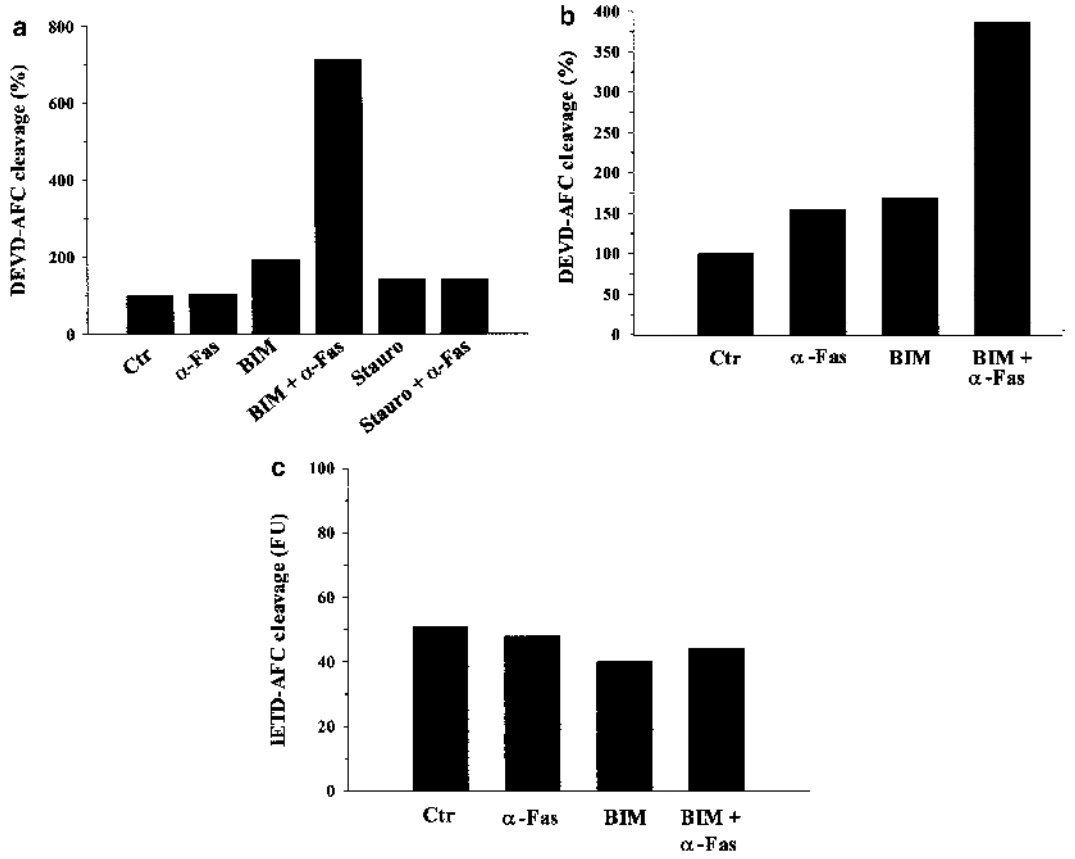

Figure 6 (**a**) Caspase activity evaluated by DEVD-AFC cleavage, in cytosolic extracts of GC-1spg cells treated as indicated. Results are reported as % *versus* control (control absolute fluorescence value was 284 FU). The panel is representative of three independent experiments. Stauro: staurosporine. (**b**) Caspase activity evaluated by DEVD-AFC cleavage, in cytosolic extracts of pachytene spermatocytes treated as indicated. Results are reported as % *versus* control (control absolute fluorescence value was 90 FU). Results are representative of three independent experiments. (**c**) Caspase-8 activity evaluated by IETD-AFC cleavage, in cytosolic extracts of GC-1spg cells treated as indicated. Results are reported as absolute values of FU and are representative of three independent experiments

To further verify whether c-FLIP$_L$ mediates the response to anti-Fas treatment, its expression was restored in BIM-treated GC-1spg cells by transfection with a c-FLIP expression vector. In mock-transfected cells, anti-Fas treatment did not significantly change caspases activity *versus* control (246 *versus* 284 FU), while anti-Fas combined with BIM substantially increased DEVD-AFC cleavage as compared with anti-Fas alone (1529 *versus* 246 FU) or BIM alone (1529 *versus* 570 FU) as reported in Figure 7a. This result was expected from the experiment reported in Figure 6a. Conversely, in c-FLIP-transfected cells, the combined treatment with anti-Fas and BIM did not significantly change DEVD-AFC cleavage *versus* control (Figure 7a). Similar results were observed for caspase-10-specific activity measured by AEVD-AFC cleavage (Figure 7b).

These results show that Fas-mediated caspase activation, namely caspase-10, is specifically controlled by c-FLIP$_L$ expression in GC-1spg cells.

To confirm c-FLIP$_L$ role in Fas-mediated caspase activation, specific antisense morpholino-oligonucleotide against mouse c-FLIP$_L$ mRNA was used to specifically inhibit c-FLIP$_L$ translation, in GC-1spg cells.[44] Western blot analysis (Figure 8a) showed that a specific c-FLIP$_L$ downregulation, corresponding to about 40% inhibition by densitometric analysis (Figure 8b), was achieved in antisense-treated cells as compared to control oligonucleotide-treated cells, while control oligonucleotide failed to modulate c-FLIP$_L$ expression as compared to untreated cells. Subsequently, GC-1spg cells, previously incubated with antisense or control oligonucleotides, were treated with anti-Fas antibody. As shown in Figure 8c, anti-Fas-induced DEVD-AFC cleavage was increased in antisense oligo-treated cells, as compared to control oligo-treated cells (230 *versus* 140 FU).

Taken together, these data demonstrate that c-FLIP$_L$-specific inhibition leads to an increased Fas-mediated caspase activation.

Fas-dependent apoptosis in seminiferous tubules

The biological role of c-FLIP$_L$ in protecting from Fas-mediated apoptosis was then investigated in an organ–culture system. Seminiferous tubules from adult testes were isolated and cultured in small fragments (about 1 mm length). In this model, the classical tissue architecture is maintained and spermatogonia are the basal cells, characteristically located at the periphery. After 16 h culture with anti-Fas antibody (5 μg/ml),[7]

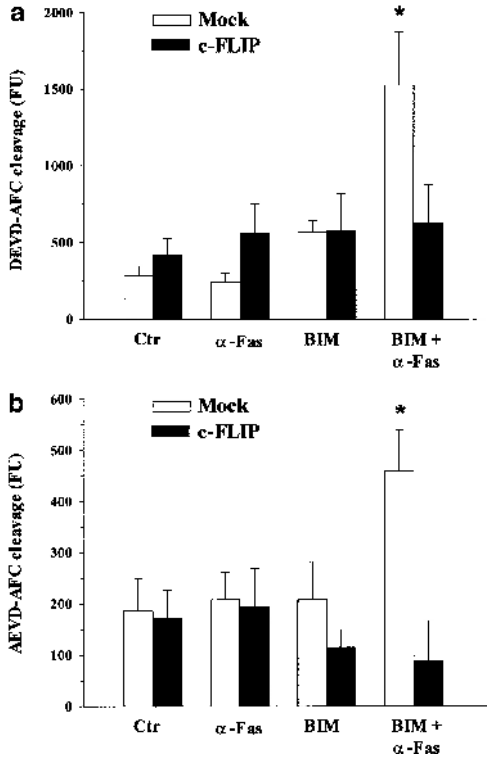

Figure 7 (**a**) Caspase activity evaluated by DEVD-AFC cleavage, in cytosolic extracts from mock-transfected and c-FLIP-transfected GC-1spg cells. Data represent the mean of FU absolute values of three independent experiments and are expressed as average ± s.e.m. Asterisk indicates statistically significant difference *versus* control. (**b**) Caspase-10 activity evaluated by AEVD-AFC cleavage, in cytosolic extracts from mock-transfected and c-FLIP-transfected GC-1spg cells. Data represent the mean of FU absolute values of three independent experiments and are expressed as average ± s.e.m. Asterisk indicates statistically significant difference *versus* control

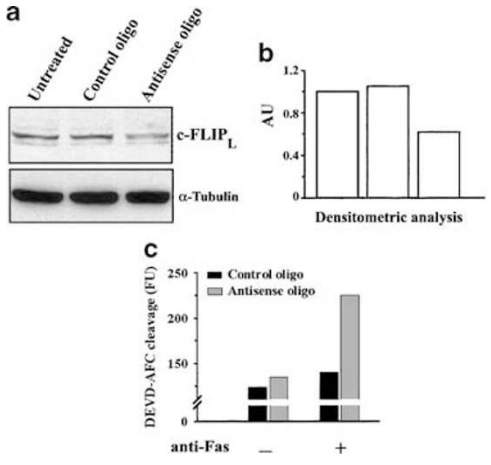

Figure 8 GC-1spg cell treatment with antisense oligo anti-c-FLIP. (**a**) c-FLIP$_L$ Western blot analysis in GC-1spg cells incubated with antisense or control oligo, or untreated. α-tubulin staining was also carried out to show similar loading. The panel is a representative of three independent experiments; (**b**) Densitometric analysis of the experiment shown in panel a. (**c**) Caspase activity evaluated by DEVD-AFC cleavage

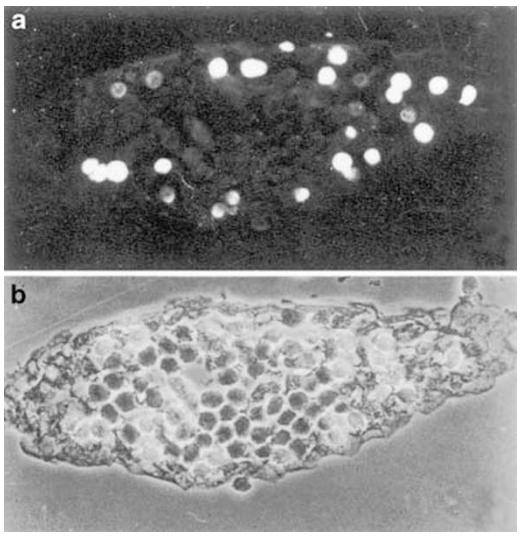

Figure 9 TUNEL staining of anti-Fas antibody-treated seminiferous tubules. Apoptosis in sections of seminiferous tubules treated with anti-Fas antibody was detected by TUNEL staining. The same microscopic field was pictured by phase contrast and fluorescence microscopy (**b** and **a**, respectively). Magnification $\times$ 300.

apoptosis was revealed by TUNEL staining on paraffin sections. Apoptosis was found to occur mostly in spermatogonia, while spermatocytes and spermatids, located more centrally, appeared to be protected (Figure 9). These data confirm that spermatogonia are more sensitive to anti-Fas treatment; in fact Fas-mediated apoptosis occurs in seminiferous tubules at the site where mostly spermatogonia are located. According to Western blot, Northern blot and immunohistochemistry analyses reported above, this experiment indicates that, in the adult testis, cells with low c-FLIP$_L$ expression levels undergo Fas-mediated apoptosis.

Discussion

Apoptotic processes occur in mammalian testis during development as well as in adulthood, and are known to play an important role in regulating spermatogenesis;[4,7] therefore, signals leading to apoptosis are carefully controlled to avoid inappropriate cell loss. Several studies have investigated the Fas/FasL system in mouse testis,[17,45,46] and how it controls germ-cell apoptosis.[6,7] However, mechanisms conferring either susceptibility or resistance to apoptotic stimuli, in spermatogonia or in other germ cell types, respectively, are still largely unknown.

Experiments carried out in the current study consistently showed that, in mouse primary spermatocytes as well as in the murine germ-cell line GC-1spg, anti-Fas-induced caspase activation is very weak or absent while in other studies only high doses (5 μg/ml) of anti-Fas effectively induced apoptosis.[7] These observations prompted us to hypothesize that a mechanism controlling Fas-mediated death might be present in germ cells. Previous studies showed that c-FLIP, a Fas-pathway regulator, is strongly expressed in human testis.[22,30]

Therefore, we investigated (i) whether c-FLIP is expressed in mouse testis and (ii) whether it controls Fas-dependent apoptosis of germ cells. c-FLIP$_L$ was found to be expressed in mouse testis, namely in differentiating germ cells, while it was undetectable in Sertoli cells and spermatozoa and expressed at low levels in spermatogonia. Northern blot experiments showed three faint c-FLIP transcripts in spermatogonia. Conversely, pachytene spermatocytes and round spermatids showed high levels of five c-FLIP transcripts. It is noteworthy that different transcripts of the same gene in mitotic and meiotic germ cells have already been observed in other studies.[47] Western blot and immunohistochemical analyses confirmed that c-FLIP$_L$ is not detectable in Sertoli cells, while it is expressed at low levels in spermatogonia and, at higher levels, in pachytene spermatocytes and round spermatids.

c-FLIP is known to interfere with caspase-8 and -10 function in other cell types directly; we observed, for the first time, that caspase-8 is almost absent while caspase-10 protein is significantly expressed in mouse testis, with a cell-specific expression pattern perfectly matching that of c-FLIP$_L$. Such finding suggested that caspase-10, as in other cell types, might be the inhibited target of c-FLIP$_L$ in differentiating germ cells. This hypothesis was experimentally tested in GC-1spg germ cell line; in these cells, specific c-FLIP$_L$ downregulation achieved by either BIM or c-FLIP-antisense oligonucleotides treatment led to a marked increase of caspase activity (namely caspase-10 activity) and apoptosis in response to anti-Fas antibody. Moreover, c-FLIP transfection in BIM-treated cells restored their resistance to anti-Fas treatment. All together, these data allowed us to conclude that c-FLIP$_L$ levels directly control Fas-dependent apoptosis in these cells. Similarly, in pachytene spermatocytes, Fas-induced caspase activation was found to be dependent on c-FLIP$_L$ levels. As we reported previously,[41] in culture spermatids undergo a high viability drop and similarly spermatogonia rapidly die (data not shown), making it difficult to study the sensititivity of such cell types to Fas-induced caspase activation. Hence, spermatogonia and spermatids Fas-induced apoptosis was only investigated in the seminiferous tubules organ culture model, which resembles more closely the physiological environment. Such experiments showed higher Fas-dependent apoptosis in spermatogonia, which express low levels of c-FLIP$_L$, as compared to other germ cells with high c-FLIP$_L$ expression, indicating c-FLIP$_L$ as a key molecule protecting germ cells from Fas-mediated apoptosis.

These results could help elucidating the molecular mechanisms controlling germ-cell apoptosis and might explain, at least in part, why apoptosis *in vivo* occurs mainly in spermatogonia in adulthood. In conclusion, these data (i) demonstrate a cell-specific expression pattern of c-FLIP$_L$, in the mouse testis, (ii) indicate its role in controlling Fas-mediated apoptosis in germ cells *in vitro* and in seminiferous tubules explants and (iii) suggest a specific role of c-FLIP$_L$ in protecting specific germ-cell populations from apoptosis.

These data open new perspectives to the understanding of testicular pathologies where germ-cell content is compromised. The identification of the specific mechanisms controlling germ-cell apoptosis could have special relevance for the understanding of some aspects of male infertility.

Materials and Methods

Cell cultures

Spermatogonia-enriched germ cells were collected by sequential collagenase A-hyaluronidase-trypsin treatment of testes from 7- to 8-day-old CD1 mice as previously described.[48] In order to remove contaminating somatic cells the suspension was preplated for 5 h on 10 cm tissue dishes with minimum Eagle's medium (MEM; Gibco BRL, Paisley, Scotland) supplemented with sodium lactate, sodium pyruvate and 10% FCS in a 5% CO_2 atmosphere at 32°C. Floating germ cells were then collected and replated. Floating spermatogonia were collected after 16 h for Western and Northern blotting analyses.

For meiotic germ-cell separation, seminiferous tubules from mice (30-day-old) were digested twice in Hanks' balanced salt solution (HBSS; Gibco BRL) containing 0.1% type XI-collagenase+10 μg/ml DNase (Roche Diagnostics, Mannheim, Germany). Pachytene spermatocytes and round spermatids were obtained from the unfractionated single-cell suspension as previously described.[49,50] Homogeneity of cell populations ranged between 80 and 85% (pachytene spermatocytes) and between 90 and 95% (round spermatids) and was routinely monitored morphologically.

Germ-cell populations were plated at 32°C in serum-free MEM supplemented with sodium lactate and sodium pyruvate. After 16 h cells were collected and lysed for Western and Northern blotting.

Sertoli cells were prepared from 15-day-old CD1 mice as described previously.[51] After 3 days culture at 32°C in 5% CO_2, in serum-free (MEM), Sertoli cell monolayers were incubated at room temperature with 20 mM Tris-HCl buffer (pH 7.4) for 2 min to remove residual germ cells present in the culture.[52] After 4 days culture, Sertoli cell monolayers were processed for Western and Northern blotting as described below.

GC-1spg cell line (kindly provided by Prof. Carla Boitani from the Department of Histology and Medical Embryology, University of Rome 'La Sapienza') was cultured in Dulbecco's MEM (Gibco BRL) supplemented with sodium pyruvate and 10% fetal calf serum (FCS) and maintained in 5% CO_2 at 37°C.

Seminiferous tubules culture

Testes from young adult mice were microdissected on Petri dishes containing culture medium (MEM). Segments of seminiferous tubules (about 1 mm length) were isolated and transferred to culture plates containing the same medium either in the presence or in the absence of anti-Fas antibody (5 μg/ml). Samples were then incubated overnight at 32°C in serum-free conditions in a humidified atmosphere containing 5% CO_2. Such experimental conditions were carefully selected to achieve maximal cell targeting. We have found in preliminary observations (data not shown), according to other authors,[53] that the diffusion toward the lumen is maximal with 1–2 mm tubules fragments length. Apoptosis was revealed by TUNEL staining.

Western blotting

Total cell lysates were prepared with Cell Lysis Buffer (New England Biolabs, Beverly, MA, USA) containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin.

Protein concentration was determined by the micro-BCA method (Pierce, Tattenhall, Cheshire, UK). Equal amounts of proteins (50 μg) were subjected to SDS-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Hybond C, Amersham

Pharmacia Biotech, Buckinghamshire, UK). Equal loading was routinely checked by Ponceau red staining of the membranes and by α-tubulin staining. The nitrocellulose was saturated with nonfat dry milk. c-FLIP$_L$ was detected by incubating the membrane with an anti-c-FLIP$_L$ rabbit polyclonal IgG (Upstate Biotechnology, Lake Placid, NY, USA). Caspase-10 was detected by incubating the membrane with an anticaspase-10 rabbit polyclonal IgG (Santa Cruz Biotechnology, CA, USA), α-tubulin was detected with an anti-α-tubulin mouse monoclonal IgG (Sigma Chemical Co., St. Louis, MO, USA). Secondary antibodies were horseradish peroxidase conjugated (Zymed Laboratories Inc., South San Francisco, CA, USA) and detection was performed by chemioluminescence measurement (ECL, Amersham Pharmacia Biotech).

Northern blotting

Northern blotting was performed as previously described.[54] Briefly, total RNA was extracted by using TRIZOL reagent (Life Technologies, Milano, Italy) according to the manufacturer's instructions. RNA was subjected to electrophoresis through a 1% agarose gel containing formaldehyde and was transferred onto a nylon membrane (Schleicher & Schuell, Keene NH, USA). Filters were prehybridized at 42°C for 4 h. Hybridization was performed overnight in prehybridization buffer containing 1×10^6 cpm/ml of ^{32}P-labeled human c-FLIP cDNA.[22]

Immunohistochemical analysis

Testis and epididymis were fixed in Bouin solution, dehydrated and embedded in paraffin wax prior to sectioning. Serial sections (3 μm) were mounted on APES-(DAKO, Glostrup, Denmark) coated slides. Briefly, tissue sections were deparaffinized in xylene, rehydrated in graded ethanols and treated with methanol containing 0.3% H_2O_2 to block endogenous peroxidase activity. Slides, in citrate buffer, were microwaved for 10 min. After rinsing in PBS without Ca^{2+} and Mg^{2+} (pH 7.4), slides were incubated in 10% normal horse serum (NHS) for 30 min at room temperature. The latter and all subsequent reagents were diluted in PBS containing 0.1% bovine serum albumin (BSA), and incubations were performed in a sealed moisture chamber. NHS was removed and slides were incubated overnight at 4°C with rabbit polyclonal anti-c-FLIP$_L$ IgG (1 : 800). An isotype antibody was used to verify specific staining. Slides were then washed twice in PBS. Horse anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, USA) was used at 1 : 500 dilution. After 30 min incubation and two washes in PBS, avidin-biotin complex (Vector Laboratories, Inc.) was added for 30 min at room temperature. Slides were washed in PBS and the peroxidase reaction was performed using 0.06% diaminobenzidine (Sigma Chemical) and 0.01% hydrogen peroxide. After a final rinse, sections were dehydrated in ethanol, cleared in xylene and mounted in Permount.

Caspase activity assay

GC-1spg cells were incubated overnight with anti-Fas antibody 1 μg/ml (BD Pharmingen, San Diego, CA, USA) and BIM 20 μM (Vinci-Biochem Alexis, Firenze, Italy), alone or in combination, with staurosporin 5 nM (Sigma Chemical) alone or in combination with anti-Fas 1 μg/ml in culture medium containing 5% FCS.

Caspase activity was tested according to Deveraux *et al*.[55] Briefly, cells were suspended in caspase buffer (100 μl) (20 mM Hepes pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF) containing 50 μM of the fluorogenic substrate DEVD-AFC or AEVD-AFC or IETD-AFC (Calbiochem, San Diego, CA, USA). This suspension was then passed through a $21^{1/2}$G needle, left on ice for 15 min and centrifuged at 25 000 $\times$ *g* for 15 min. The supernatant was collected and centrifuged at 10 0000 $\times$ *g* at 4°C for 75 min. Protein concentration in the supernatant was determined by the micro-BCA method as described above and 30 μg of each sample as then assayed. In morpholino-oligos experiments, 15 μg of each sample was assayed. Caspase activity was allowed to proceed for 2 h at 37°C. Under these conditions caspase activity is proportional to the release of AFC from the substrate; fluorescence emission was measured with a Perkin-Elmer LS50 fluorometer.

Apoptosis assay by flow cytometry

GC-1spg cells were incubated overnight with anti-Fas (from BD Pharmingen, San Diego, CA, USA) and BIM (from Vinci-Biochem, Firenze, Italy), alone or in combination, in culture medium containing 5% FCS.

In order to measure apoptosis level, propidium iodide (PI) staining was performed. Cells were washed with PBS and 1 μg/ml PI (Sigma Chemicals) was added. Analysis was performed on a Coulter Epics XL flow cytometer (Beckman Coulter). Data were then analyzed with the WinMDI free software.

Transfection

Expression vector for c-FLIP, previously described,[35] was kindly provided by Dr. M Peter (from The Cancer Center University of Chicago). GC-1spg cells transfection was carried out in serum-free medium on 80% confluent cells in 35-mm plastic dishes using Lipofectamine-PLUS reagent (Life Technologies) according to the manufacturer's instructions. After transfection, medium was replaced with fresh culture medium. Transgene expression was evaluated for each experiment by Western blot 36 h after transfection.

Antisense oligonucelotides treatment

Incubation of GC-1spg cells was carried out in the presence of morpholino-oligonucleotide (1 μM) complementary to 25 bp of mouse FLIP mRNA sequence (antisense oligonucleotide) 5′-GCTCTGGGAACCACGA-GAAGCCAAC-3′[44] or standard control 5′-CCTCTTACCTCAGTTA-CAATTTATA-3′[44], according to the manufacturer's instructions (Gene Tools, Philomath, OR, USA). After 7 h, the medium was removed and replaced with fresh medium with or without anti-Fas antibody (1μg/μl) overnight.

TUNEL staining

TUNEL staining was performed usign Dead End™ Fluorometric TUNEL System (Promega, Madison WI, USA). Segments of seminiferous tubules were fixed in 4% paraformaldehyde and paraffin sections were prepared as described above. Tissue sections were then processed according to the manufacturer's instructions.

Statistical analysis

Statistical evaluation was performed according to *t*-test analysis and $P < 0.05$ was considered statistically significant.

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