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Bcl-2 and Bax regulation of apoptosis in germ cells during prenatal oogenesis in the mouse embryo

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Abstract

Apoptosis is the main cause of primordial germ cell and oocyte degeneration in the developing fetal ovary. In this study we examined by immunohistochemistry and immunoblotting the expression of the anti- and pro-apoptotic proteins Bcl-2 and Bax in primordial germ cells and fetal oocytes during pre natal oogenesis in the mouse embryo. While Bcl-2 and Bax were not detectable in primordial germ cells *in vivo*, both proteins were upregulated when they undergo apoptosis in culture. Treatment with the stem cell factor (SCF), a growth factor known to partially reduce primordial germ cell apoptosis, resulted in decreased Bax expression. Bcl-2 was barely detectable in oocytes entering into meiosis and its expression did not change during the stage of meiotic prophase I examined. On the contrary, high levels of Bax was expressed in degenerating oocytes while low levels of the protein was present in many apparently healthy oocytes between 15.5 days post coitum (d.p.c.) and birth, when Bax was down-regulated. Oocytes isolated from 15.5 days post coitum (d.p.c.) ovaries that progress through prophase I and undergo a wave of apoptosis at the stage of pachytene/diplotene *in vitro*, showed a pattern of Bax expression similar to the *in vivo* condition. Although the addition of SCF to the culture medium reduced significantly apoptosis in oocytes at the pachytene/diplotene stages, it was not possible to directly correlate this effect with the downregulation of Bax in the surviving oocytes. These findings indicate that whereas a balance between Bcl-2 and Bax might regulate apoptosis of proliferating primordial germ cells under a partial control by SCF, Bax-mediated apoptosis in meiotic oocytes may be due to intrinsic meiotic checkpoints which act to monitor aberrant DNA recombination rather than to a growth factor-dependent process. Elimination of supernumerary oocytes might be a subsequent apoptotic phenomenon controlled by the availability of growth factors such as SCF within the ovary.

Keywords: apoptosis; primordial germ cells; Bax; Bcl-2; SCF; oocytes

Abbreviations: d.p.c., days post coitum; SCF, stem cell factor; LIF, leukemia inhibitory factor; IGF, insulin growth factor; PGCs, primordial germ cell

Introduction

In mammals, oogenesis begins with primordial germ cell (PGC) formation and encompasses a series of cellular differentiation, from PGCs to oogonia (embryo-fetus), from oogonia to oocytes (fetus) and from oocytes to eggs (adult). Extensive degeneration of germ cells occurs during embryonic, fetal and early postnatal stages of oogenesis before follicle formation (reviewed in[1,2]). In the mouse embryo, early morphological studies have shown that cell death affects proliferating primordial germ cells or oogonia (12–13 days post coitum, d.p.c.) and mainly oocytes at the zygotene/pachytene stage of meiotic prophase (from 16.5 d.p.c. through birth).[3,4] There is now evidence that apoptosis is the process mainly responsible for the loss of germ cells in the developing ovary.[5–7] Moreover, recent data from our laboratory suggest that intrinsic meiotic checkpoints and defects in the expression of c-Kit receptor by oocytes and/or deficiency of its ligand stem cell factor (SCF), trigger germ cell apoptosis monitoring both the quality and the number of oocytes during the stages of meiotic prophase.[8]

In this study, we have investigated the expression of two key anti- or pro-apoptotic proteins such as Bcl-2 and Bax by embryonic germ cells *in vivo* and *in vitro* and whether the levels of these proteins in germ cells in culture can be positively or negatively regulated by the stem cell factor (SCF), a growth factor which partly prevents apoptosis both in primordial germ cells and oocytes.[5,8] The results are discussed in the view that Bax-dependent apoptosis is crucial either for PGC and oocyte degeneration during prenatal oogenesis.

Results

Expression of Bcl-2 and Bax in embryonic and early postnatal ovaries

Studies from our and other laboratories have shown that primordial germ cell and fetal oocyte degeneration occurs by apoptosis and that *in vitro* growth factors such as SCF, LIF and IGF are able to reduce apoptosis in such cells.[5–7,9] To identify anti- and pro-apoptotic genes involved in germ cell

apoptosis, we first studied the expression of Bcl-2 and Bax proteins in tissue sections of embryonic (12.5–19.5 d.p.c.) and early postnatal ovaries (1–4 days).

Bax staining was absent in 12.5–13.5 d.p.c. ovaries but intense in oocytes showing morphological features of degeneration at later stages (Figure 1). In ovaries between 15.5 d.p.c. and birth, Bax was expressed at lower level also in several oocytes that did not show morphological signs of degeneration (Figure 1). Little or no Bax expression was observed in diplotene-arrested oocytes in 3–4 day postnatal ovaries (data not shown). We were unable to detect Bcl-2 immunoreactivity in germ cells of the ovary sections in all stages examined.

The upregulation of Bax around 15.5–16.5 d.p.c. was confirmed by immunoblotting analysis of extracts of germ cells obtained from ovaries of embryos at different developmental stages (Figure 2).

Bcl-2 and Bax are upregulated in apoptotic PGCs in culture and SCF partly prevents Bax up regulation

PGCs obtained from 11.5–12.5 d.p.c. gonadal ridges and cultured for 16–18 h were examined for the expression of Bcl-2 and Bax and for apoptotic morphologies. Since SCF was previously shown to partially suppress apoptosis of PGCs,[5] PGCs were also cultured in the presence of 100 ng/ml SCF.

In accord with the *in vivo* results Bcl-2 was not detectable in freshly isolated PGCs while a few cells only resulted Bax positive (Figure 3A,C). At this time rare apoptotic morphologies were found ($<1\%$). After 16–18 h of culture most of the PGCs upregulated both Bcl-2 and Bax (Figure 3B,D). This upregulation of Bcl-2 and Bax occurred in parallel with a marked increase of the

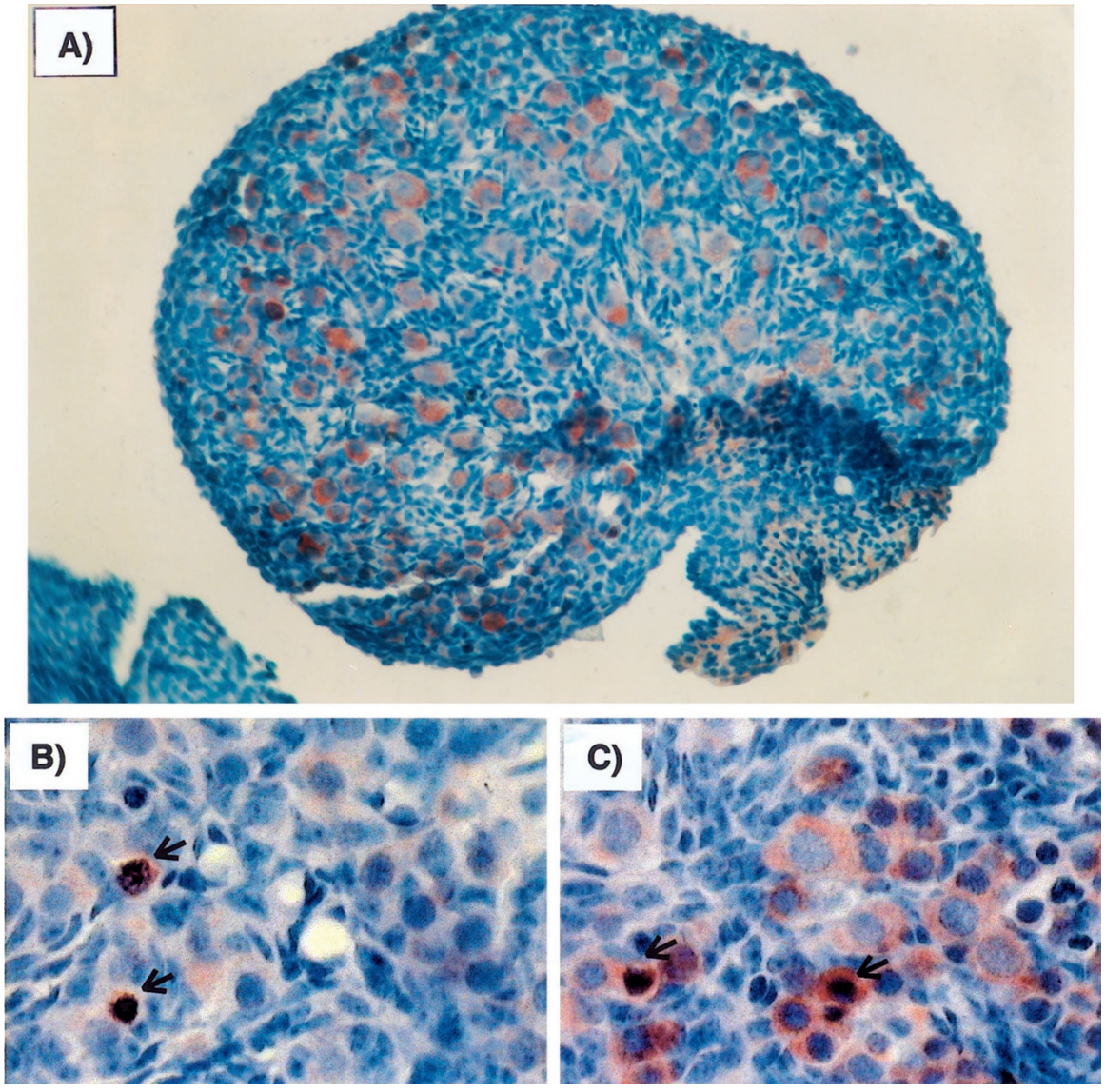

Figure 1 Paraffin section of a 16.5 d.p.c. ovary showing Bax immunostaining in several oocytes. Note stronger Bax staining generally associated with apoptotic morphologies (arrows); (**A**) approximately 280×; (**B**) and (**C**) approximately 600×

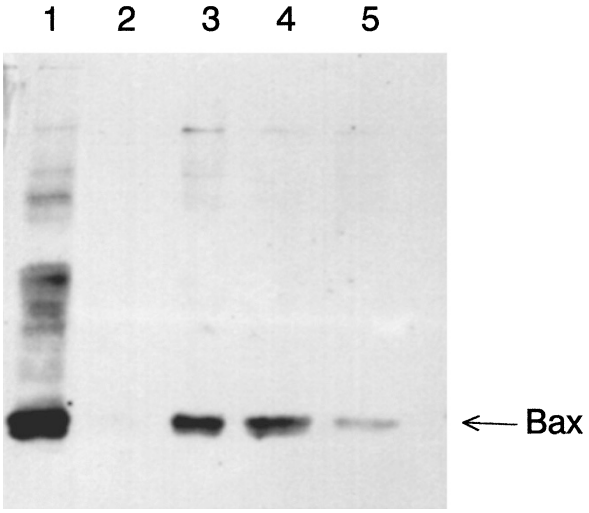

Figure 2 Bax immunoblotting in germ cells obtained from ovaries of different embryonic ages. Lane 1, spleen; lane 2, 12.5 d.p.c. PGCs; lane 3, 15.5 d.p.c. oocytes; lane 4, 16.5 d.p.c. oocytes; lane 5, perinatal oocytes

percentage of PGCs showing apoptotic features (60–70%) (Figure 4A). The frequency of PGCs with moderate or high Bax immunoreactivity after 16–18 h in culture was significantly lower in the presence of 100 ng/ml SCF in comparison with the control (Figure 4A). In accord with our previous observations,[5] at this time of culture the presence of SCF resulted also in a significant decrease of the percentage of apoptotic PGCs (Figure 4A).

Immunoblotting experiments confirmed these results. Both Bcl-2 and Bax were not detectable in freshly collected PGCs but were clearly upregulated following *in vitro* culture. The presence of SCF did not affect the expression of Bcl-2, but in two experiments out of three performed caused a significant reduction of Bax expression (Figure 4B,C).

Expression of Bcl-2 and Bax in cultured oocytes and the effect of SCF

Oocytes obtained from 15.5 d.p.c. ovaries were examined for the expression of Bcl-2 and Bax and for apoptotic morphologies during 1–4 days of culture. Since we have previously shown that SCF partially prevents apoptosis of oocytes which in culture reach the pachitene/diplotene stage of the meiotic prophase I,[8] oocytes were also cultured in the presence of 100 ng/ml SCF.

Bcl-2 immunopositivity was faint or absent in freshly isolated 15.5 d.p.c. oocytes (not shown) whereas Bax staining was intense in oocytes showing apoptotic features (2–5%) and expressed at low levels in oocytes with normal morphology (Figure 5A,B). During the culture, no evident changes in Bcl-2 positivity were observed (data not shown), while the number of Bax positive oocytes increased in parallel with that of oocytes showing apoptotic morphologies (Figures 5C,D, 6A,B and 7A,B). At the end of the culture period, the most part of the oocytes showed apoptotic features or advanced stages of degeneration resembling necrosis rather than opoptosis. These oocytes were Bax positive while the few oocytes with normal morphology were generally Bax negative (Figure 7C,D).

Immunoblotting showed that Bax signal was present in freshly isolated oocytes and did not change after 1–2 days of culture (Figure 6C). After 4 days of culture Bax expression was no more detectable (not shown).

As expected, since oocytes at leptotene/zygotene stages of meiotic prophase do not express the SCF receptor c-Kit,[10] the presence of SCF during the first 1–2 days of culture did not influence the oocyte apoptosis (Figure 6A). Accordingly, the analysis of Bax expression by immunohistochemistry (Figure 6B) and immunoblotting (Figure 6C), did not reveal any SCF effect on the expression of this protein. On the contrary, after 4 days of culture, when most of the oocytes reach the pachytene/diplotene stage and c-Kit is upregulated,[8] SCF appeared to reduce significantly the number of apoptotic oocytes (Figure 6A). The most part of surviving oocytes resulted Bax negative (Figure 7C,D). At this time, as for control oocytes (see above), immunoblotting analysis was unable to reveal any Bax signal (not shown).

Discussion

Recent studies have demonstrated that apoptosis is the main mechanism responsible for the massive germ cell loss during prenatal mouse oogenesis.[5–8] In addition, we have shown that growth factors such as SCF and LIF are important regulators of apoptosis in primordial germ cells.[5] Recently, we have provided evidence that intrinsic meiotic checkpoints and local gradient of SCF may be important determinants of oocyte apoptosis and control the extent to which apoptosis is induced or suppressed in such cells.[8] Accordingly, Morita *et al*.[9] showed that SCF alone or in combination with other growth factors promotes oocyte survival in ovary explants probably through a phosphatidylinositol-3′-kinase signaling pathway.

Among molecules central for the regulation of cell death in eukariotic cells are members of the Bcl-2 family of proteins. In this study we have investigated whether Bcl-2 and Bax are involved in the control of germ cell apoptosis during prenatal oogenesis and whether SCF could positively or negatively influence the expression of such proteins in germ cells. Previous studies demonstrated that the ablation of *bcl-2* leads to a formation of a smaller follicle store whereas a deficiency of *bax* results in the increased number of oocytes and primordial follicles and prolongation of functional lifespan of the ovary as well.[11–13] Moreover, transfection of PGCs with *bcl-2* reduces their degeneration in culture.[14] The importance of Bax in regulation of apoptosis induced by chemotherapic drugs in mature methaphase II mouse oocytes has also recently been demonstrated.[15]

Immunohistochemical analysis of ovary tissue sections showed no evidence for Bcl-2 protein expression in germ cells while Bax was expressed in most oocytes undergoing degeneration. Interestingly, moderate Bax positivity was also seen in several apparently healthy oocytes in the ovary between 15.5 d.p.c. and birth. The significance of

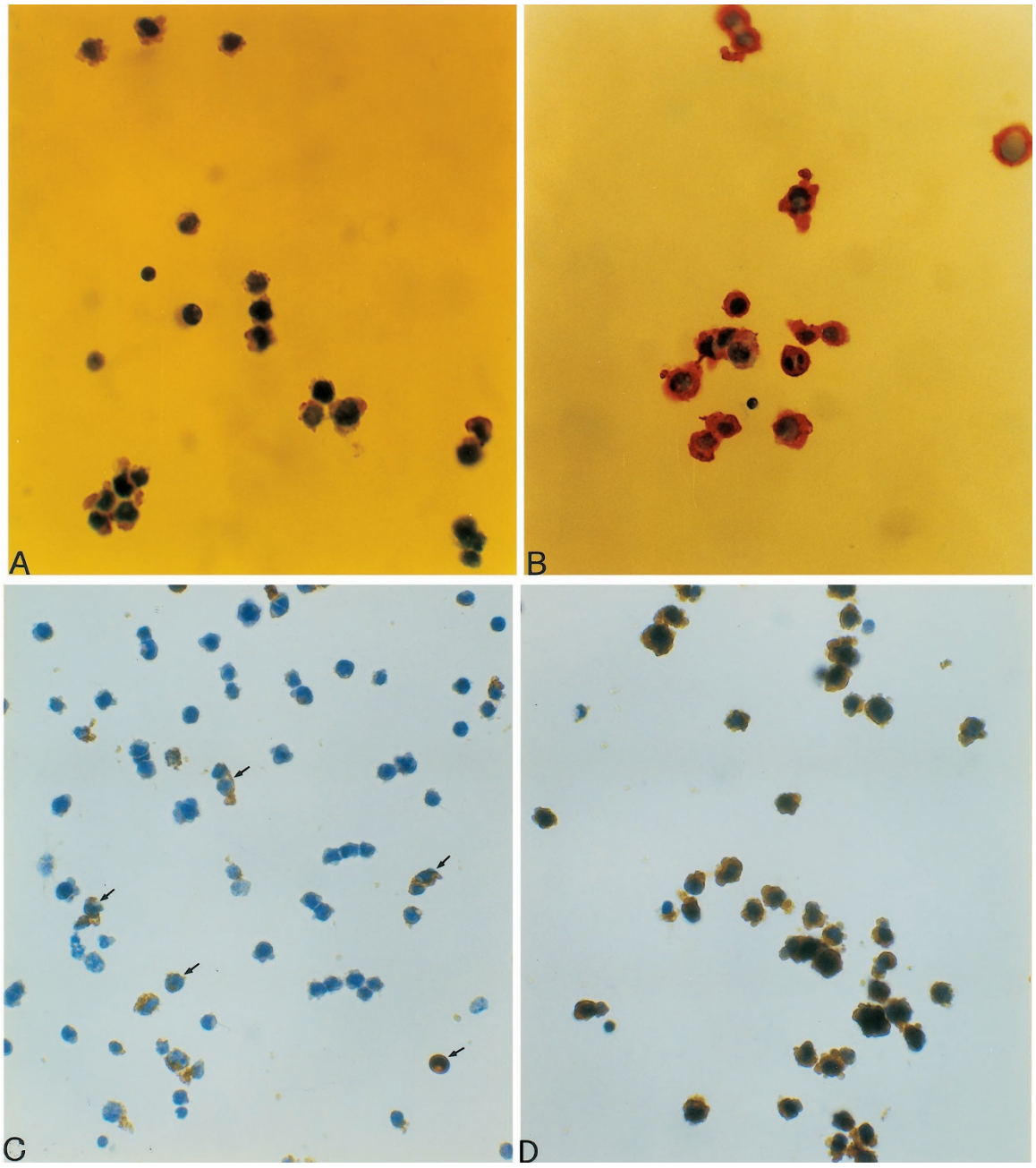

Figure 3 Bcl-2 and Bax immunostaining in isolated PGCs in culture. (**A**) Freshly isolated Bcl-2 negative PGCs; (**B**) Bcl-2 positive PGCs after 16–18 h of culture; arrows indicate apoptotic PGCs. Approximately 600×; (**C**) Freshly isolated PGCs immunostained for Bax; (**B**) PGCs immunostained for Bax after 16–18 h of culture. Note that while at the beginning of culture only a few PGCs showed Bax positivity (arrows), most of them were Bax immunopositivity after16–18 h of culture. Approximately 500×

Bax expression by such oocytes is intriguing. It was not possible to determine whether apoptosis is the fate of all oocytes expressing moderate levels of Bax or whether Bax is down regulated at later stages of development. It is interesting to note, however, that the period of Bax positivity parallels with the maximal loss of oocytes by apoptosis[8] and that Bax expression in oocytes is down-regulated around birth, when oocytes are arrested at the diplotene stage of the meiotic prophase. These observations suggest that the ability of the oocyte to balance the Bax apoptotic effect may be one of the mechanisms regulating the oocyte survival during prenatal oogenesis. In particular, it is possible to hypothesize that only oocytes which are able to maintain low and eventually to down regulate Bax at the end of the meiotic prophase can escape from apoptosis. The results of our *in vitro* studies support such an hypothesis and also give evidence about the involvement of Bax in PGC apoptosis.

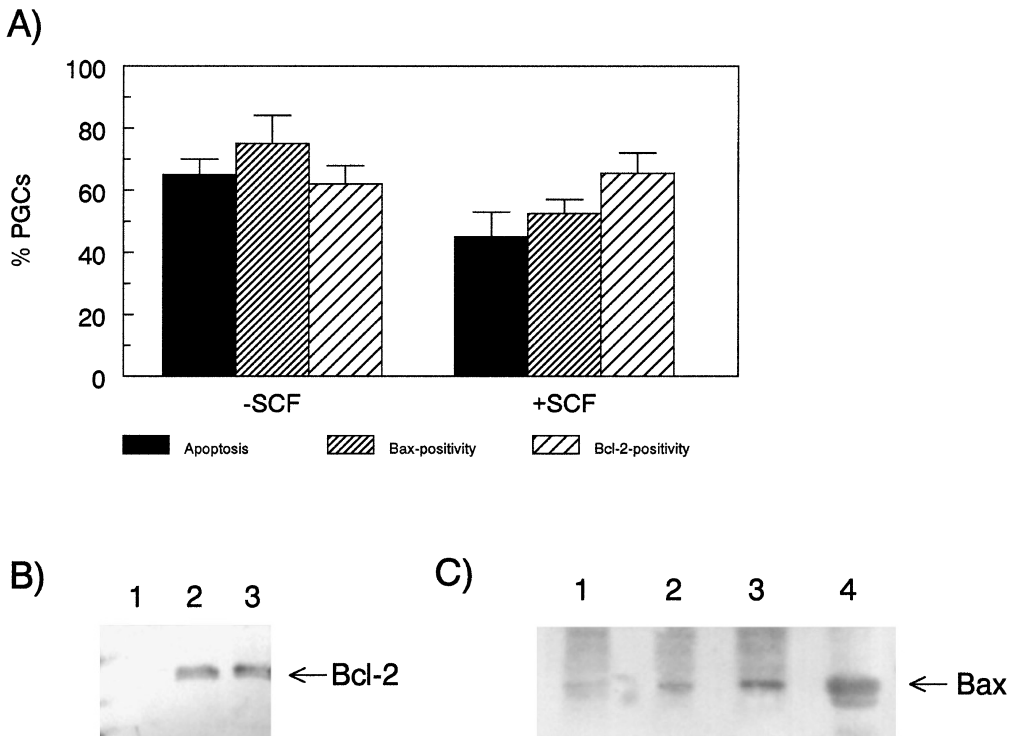

Figure 4 Apoptosis and Bax and Bcl-2 expression in PGCs cultured for 1 day in the presence or in the absence of 100 ng/ml SCF. (**A**) Percentage of apoptotic or of Bax and Bcl-2-immunopositive PGCs; results are given as the means ± S.E. of at least three different experiments; (**B**) and (**C**) Immunoblotting of Bcl-2 and Bax, respectively; (**B**) lane 1, freshly isolated PGCs; lane 2, PGCs after 16–18 h of culture in the presence of 100 ng/ml SCF; lane 3, PGCs after 16–18 h of culture without SCF; (**C**) lane 1, freshly isolated PGCs; lane 2, PGCs after 16–18 h of culture in the presence of 100 ng/ml SCF; lane 3, PGCs after 16–18 h of culture without SCF; lane 4, spleen

We found that both Bcl-2 and Bax were not detectable in freshly isolated PGCs. Most PGCs, however, express high levels of Bcl-2 and, at the same time, accumulate Bax in the cytoplasm while undergoing apoptosis following *in vitro* culture. The expression of Bcl-2 by PGCs in culture may be interpreted as a tentative to prevent apoptosis. This expression is likely to be independent by SCF and it is not sufficient to rescue PGCs probably due to the parallel accumulation of Bax or to the activation of other apoptotic pathways. While in hematopoietic cells SCF is able to prevent apoptosis by up regulating Bcl-2,[16] our results indicate that the anti-apoptotic effect of SCF on PGCs can be due, at least in part, to its ability to reduce the level of Bax rather than to act on Bcl-2. Although SCF can exert anti-apoptotic action also on oocytes in culture as they reach the pachytene/diplotene stage and the SCF receptor c-Kit is up regulated,[8] it was not possible to demonstrate that this effect was directly due to Bax downregulation. In fact, both apoptosis and Bax expression appear SCF-independent during the most part of meiotic prophase I when the SCF receptor c-Kit is expressed at very low level.[10] At the end of 4 days culture, when oocytes reach the pachytene/diplotene stage and c-Kit is up regulated,[8] the frequency of Bax positive oocytes was increased, even though we were unable to detect Bax expression by immunoblotting, in oocytes cultured either in the presence and in the absence of SCF. Moreover, immunohistochemistry showed that Bax was downregulated in most of the apparently healthy oocytes independently from the presence of SCF. The findings that the frequency of Bax positive oocytes increases during culture with or without SCF but that immunoblotting was unable to detect Bax signal after 4 days of culture appear contradictory. A possible explanation is that Bax may be degraded in the high number of oocytes in advanced stage of degeneration present after 4 days of culture and that it is down regulated in the surviving oocytes. Given these observations, we favor the hypothesis that SCF may increase oocytes survival without directly affecting Bax expression. Therefore we suggest that SCF is important to regulate the survival of the oocytes which have already passed the meiotic checkpoints monitoring aberrant DNA recombination during the first stages of meiotic prophase I. In this view, Bax-mediated apoptosis of oocytes may be an effector of intrinsic meiotic checkpoints controlling DNA recombination during prophase I rather than a growth factor-dependent process. In line with this hypothesis, Barlow *et al*,[17] showed recently that ATM protein, one of the proteins required for mitotic and meiotic checkpoints[18,19] is important for suppressing p53, p21 and Bax levels in the testis.

In conclusion, these results represent the first direct indication that key apoptotic genes such as *bcl-2* and *bax* are expressed and differentially regulated in germ cells during prenatal oogenesis. Moreover, we present evidence that over expression of Bax is related to PGC apoptosis in

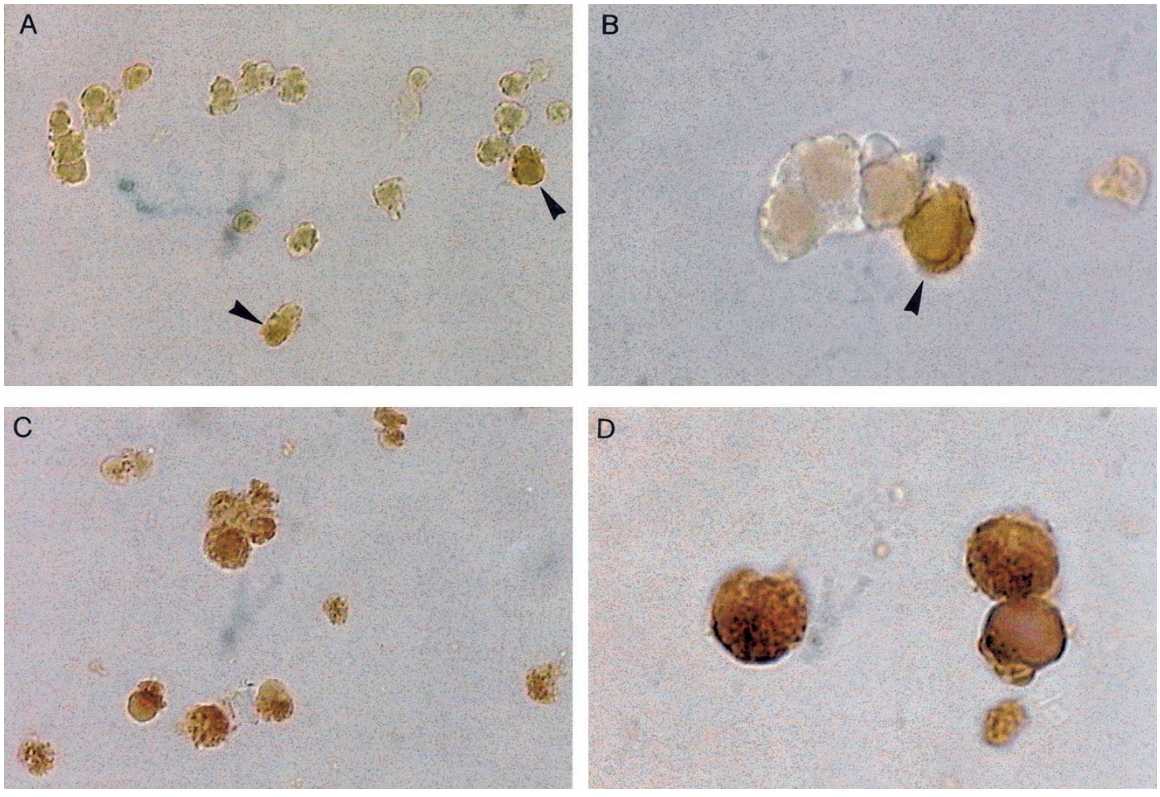

Figure 5 Bax immunostaining in 15.5 d.p.c. oocytes in culture. (**A**) and (**B**) freshly isolated oocytes; (**C**) and (**D**) oocytes after 4 days of culture. Note that while at the beginning of culture a few oocytes showed Bax positivity (arrows), most of them were Bax immunopositivity after 4 days of culture. (**A**) and (**C**) approximately 500×; (**B**) and (**D**) approximately 1000×

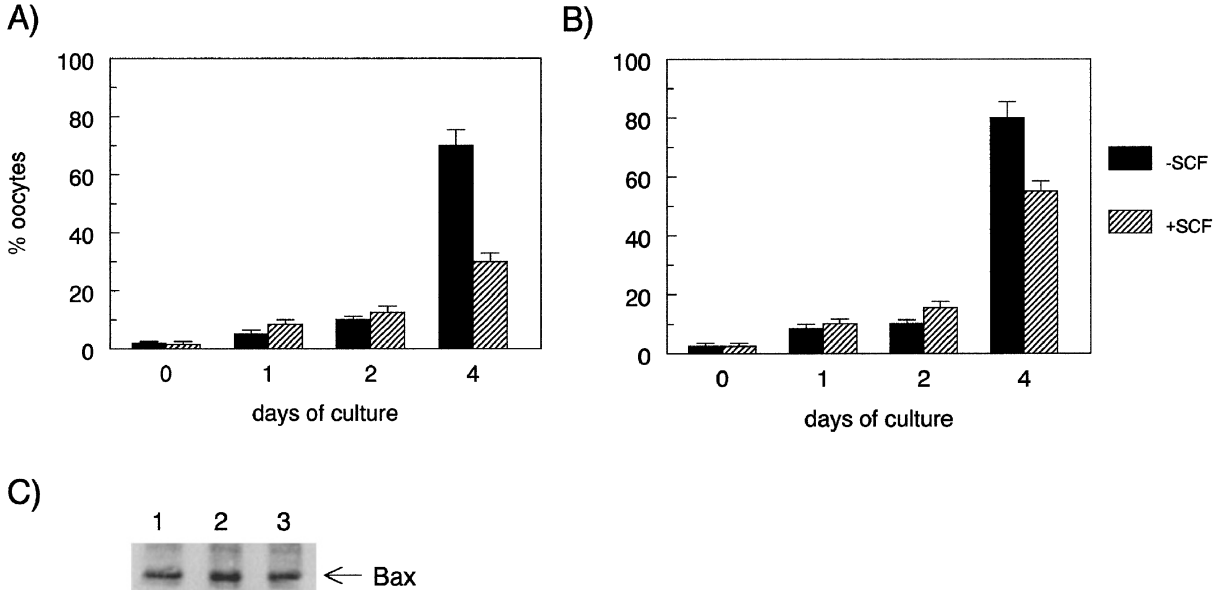

Figure 6 Percentage of apoptotic (**A**) and Bax-immunopositive oocytes (**B**) cultured in the absence or in the presence of 100 ng/ml SCF; results are given as the means ± S.E. of at least three different experiments; (**C**) Bax immunoblotting in 15.5 d.p.c. oocytes; lane 1, freshly isolated oocytes; lane 2, oocytes after 1 day of culture in the presence of 100 ng/ml SCF; lane 3, oocytes after 1 day of culture without SCF

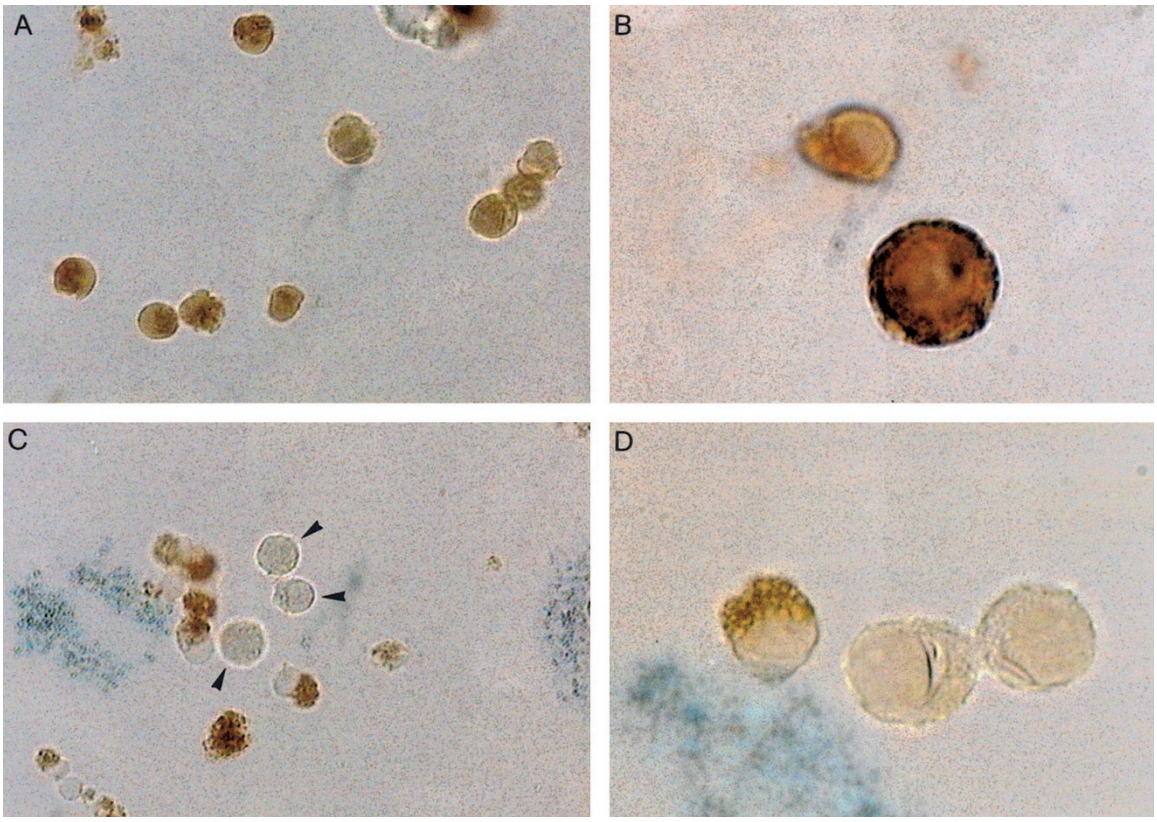

Figure 7 Bax immunostaining in 15.5 d.p.c. oocytes. (**A**) and (**B**), oocytes cultured for 4 days without SCF; (**C**) and (**D**), oocytes cultured for 4 days in the presence of 100 ng/ml SCF. In this latter condition the number of Bax negative oocytes (arrows) was increased. (**A**) and (**C**), approximately 500 ×; (**B**) and (**D**), approximately 1000 ×

culture and that the anti-apoptotic effect of SCF in these cells is due, at least in part, to its ability to reduce the level of Bax. Also in oocytes, apoptosis is associated with up regulation of Bax, however, in this case Bax-mediated apoptosis may be an effector of the intrinsic meiotic checkpoints controlling DNA recombination during prophase I. Further experiments are required to verify the relationship between meiotic checkpoint proteins, like those of the ATM family, and the regulation of Bax expression in oocytes.

Materials and Methods

Isolation and culture of germ cells

Primordial germ cells and oocytes were isolated by EDTA-mechanical treatment of 11.5–12.5 d.p.c. gonadal ridges and 15.5–19.5 d.p.c. ovaries, respectively, of CD-1 mouse embryos as described in De Felici and McLaren.[20] For immunoblotting PGCs purified by MiniMACS[21] were used. Cultures were incubated at 37°C in 5% CO_2 for the indicated times, in 0.5 ml of a modified MEM supplemented with 5% horse serum and 2.5% heat-inactivated fetal calf serum (hFCS, Flow) in a Falcon tube.[22] SCF (mouse recombinant) was purchased from Genzyme.

Identification of apoptotic germ cells

Apoptotic germ cells in suspension were recognized as reported in Pesce *et al*.[8] Briefly, cells were allowed to attach to poly-L-lysine coated slides, fixed with 4% paraformaldehyde for 10 min and after identification of germ cells by alkaline phosphatase staining, labeled with Hoechst 33258 (Calbiochem) (1 μg/ml, 10 min, room temperature) to identify typical morphological features of apoptosis (condensed nuclear chromatin, fragmented nuclei). Oocytes that after 3–4 days of culture become negative for alkaline phosphatase staining were easily recognizable on the basis of morphological criteria. After Hoechst labeling apoptotic cells were scored in a minimum of three fields (100 cells per field) under a 40× objective. Statistical analysis was performed by *t*-test; P values <0.05 were judged significant.

Immunostaining

Bcl-2 and Bax were identified in paraffin embedded tissue sections and in isolated germ cell suspensions. Bcl-2 was detected with a rat monoclonal IgG antibody from Santa Cruz (sc#578), Bax with a rabbit polyclonal antibody kindly provided by Dr. J Tilly (Harvard Medical School USA) or purchased from Santa Cruz (sc#526). Gonads were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate for 4–6 h and embedded in paraffin according to standard procedures. Cells were attached to poly-L-lysine coated slides, fixed in 4% paraformaldehyde for 15 min and washed in PBS with 5 mg/ml BSA for 2 h. Following dewaxing and rehydrating, tissue sections and cell

samples were treated with 0.1% Triton X-100 for 10 min, washed and incubated for 3 h with the primary antibody (1 : 100 anti Bcl-2, 1 : 2000 anti Bax). Biotinylated rabbit anti-rat IgG or goat anti-rabbit IgG (BIOSPA) were used as secondary antibodies. Lastly, peroxidase-conjugated streptavidin (BIOSPA) was used and the enzyme revealed by DAB or AEC as chromogen substrate. Control experiments were performed used the same primary antibodies following neutralization with specific blocking peptides (Santa Cruz) (tenfold excess of peptide in PBS for 2 h at room temperature). Immunopositive cells present in the cell suspension attached to the poly-L-lysine slides were scored in a minimum of three fields (100 cells per field) under a 40 $\times$ objective. In some experiments immunostaining of germ cells for Bcl-2 and Bax performed in tissue sections or in cell suspensions was followed by counterstaining with Mayers's hematoxylin solution.

Immunoblotting

Germ cell suspensions (approximately 10^5 cells) were centrifuged (15 min, 1200 r.p.m.) and resuspended in Laemmli sample buffer (10% glycerol, 2% SDS, 0.06 M Tris-HCl-pH 6.8, 5% β-mercaptoethanol, 0.02% bromophenol blue) on ice. Samples were sonicated and boiled before being submitted (about 50 μg/lane) to 12% SDS – PAGE. Proteins were transferred to nitrocellulose membranes, vacant sites blocked with 10% non-fat milk in PBS and 0.01% Tween-20 and the washed membranes incubated for 1 – 2 h at room temperature with the anti-mouse Bcl-2 (1 : 800 dilution) or the anti mouse Bax (1 : 1000 dilution) antibodies as previously described. Secondary antibodies and detection reagents were used according to the instructions of the enhanced chemioluminescent (ECL) kit (Amersham). Extracts from the spleen of adult mice were used as a positive control for the expression of Bcl-2 and Bax proteins. Experiments were repeated at least three times for each condition examined.

Acknowledgements

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