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Stem cell factor protects germ cells from apoptosis in vitro

Wei Yan, Janne Suominen and Jorma Toppari*

Departments of Physiology and Pediatrics, University of Turku, Kiinamyllynkatu 10, Turku, 20520, Finland
*Author for correspondence (e-mail: jorma.toppari@utu.fi)

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SUMMARY

Stem cell factor (SCF) plays an important role in migration, adhesion, proliferation, and survival of primordial germ cells and spermatogonia during testicular development. However, the function of SCF in the adult testis is poorly described. We have previously shown that, in the presence of SCF, there were more type A spermatogonia incorporating thymidine at stage XII of rat seminiferous tubules cultured in vitro than in the absence of SCF, implying that the increased DNA synthesis might result from enhanced survival of spermatogonia. To explore the potential pro-survival function of SCF during spermatogenesis, the seminiferous tubules from stage XII were cultured in the presence or absence of SCF (100 ng/ml) for 8, 24, 48, and 72 hours, respectively, and apoptosis was analyzed by DNA laddering and in situ 3′-end labeling (ISEL) staining. Surprisingly, not only spermatogonia, but also spermatocytes and spermatids, were protected from apoptosis in the presence of SCF. Apoptosis took place much later and was less severe in the SCF-treated tubules than in the controls. Based on previous studies showing that FSH prevents germ cells from undergoing apoptosis in vitro, and that SCF level is increased dramatically in response to FSH stimulation, we also tested if the pro-survival effect of FSH is mediated through SCF by using a function-blocking monoclonal antibody, ACK-2, to block SCF/c-kit interaction. After 24 hours of blockade, the protective effect of FSH was partially abolished, as manifested by DNA laddering and ISEL analyses. The present study demonstrates that SCF acts as an important survival factor for germ cells in the adult rat testis and FSH pro-survival effect on germ cells is mediated partially through the SCF/c-kit pathway.

Key words: Stem cell factor, c-kit, Apoptosis, DNA ladder, ISEL, Testis, Rat

INTRODUCTION

Apoptosis plays an essential role in balancing the ratio of germ cell number to Sertoli cell number during testicular development (Rodriguez et al., 1997). In the adult testis, apoptosis is a normal physiological process for eliminating germ cells with genetic defects or DNA damages caused by exposure to chemicals or irradiation (Matsui, 1998). Interestingly, germ cell apoptosis in the seminiferous epithelium occurs in a stage-specific fashion and also shows stage-specific responses to apoptosis-inducing factors, such as irradiation, chemotherapy drugs, etc. (Brinkworth et al., 1995; Henriksen et al., 1995a,b, 1996a,b; Sjoblom et al., 1998). The stage-specific pattern of apoptosis of germ cells has been speculated to result from the stage-specific actions of hormones, or growth factors that are involved in the regulation of spermatogenesis (Leibovitch and Buttyan, 1991; Henriksen et al., 1995a). In the testis, stem cell factor (SCF) is produced by Sertoli cells (Rossi et al., 1991; Manova et al., 1993; Hakovirta et al., 1999) and interacts with c-kit receptor, a trans-membrane tyrosine kinase receptor, on spermatogonia, spermatocytes and round spermatids (truncated form), as well as Leydig cells (Manova et al., 1990; Manova and Bacharova, 1991; Sorrentino et al., 1991; Yoshinaga et al., 1991; Rossi et al., 1992). During testicular development, SCF/c-kit interaction plays an important role in primordial germ cell migration and survival, and in spermatogonial adhesion, proliferation and survival (Matsui et al., 1991; Pesce et al., 1993; Yee et al., 1994; Packer et al., 1995). In the adult rat testis, SCF mRNA expression is high (Hakovirta et al., 1999), implying that it might play a role during spermatogenesis. However, the exact function of SCF in the adult testis remains poorly understood. Rat seminiferous tubules cultured in vitro have more thymidine-incorporating type A spermatogonia at stage XII when SCF is present, suggesting that the increased DNA synthesis might result from the enhanced survival of spermatogonia (Hakovirta et al., 1999). To explore the potential pro-survival function of SCF, we cultured the seminiferous tubules from stage XII of the epithelial cycle in the presence or absence of SCF and analyzed apoptosis by DNA laddering and in situ end-labeling (ISEL) staining.

By using similar methodology, a previous study (Henriksen et al., 1996a) showed that FSH prevented germ cells from undergoing apoptosis in vitro. On the basis of this study and our previous finding that SCF expression are highly dependent on FSH stimulation in the rat seminiferous tubules cultured in vitro (Yan et al., 1999), we also tested if the pro-survival effect of FSH is mediated through SCF/c-kit interaction by using a function-blocking anti-c-kit antibody, ACK-2. ACK-2 monoclonal antibody can block SCF function by binding to c-

kit receptor both in vitro and in vivo and its validity has been documented by several studies using mice (Yoshinaga et al., 1991; Packer et al., 1995; Tajima et al., 1994; Vincent et al., 1998). Given the highly conserved nature of c-kit receptor within rodents (Lyman and Jacobson, 1998), in the present study, we applied ACK-2 antibody to rats to block SCF/c-kit interaction and analyzed apoptosis of germ cells in the seminiferous tubules from stage XII cultured in the presence or absence of FSH by DNA laddering and ISEL analyses.

MATERIALS AND METHODS

Experimental animals

Sprague-Dawley rats at 2-3 months of age were used and they were housed in constant temperature (20°C) and light-dark cycle (light on 06.00-20.00) with free access to food and water.

All animal experiments were approved by the Turku University Committee on Ethics of Animal Experimentation.

Microdisection and tissue culture

Rats were killed by neck dislocation under CO_2 anesthesia and the testes were decapsulated. Seminiferous tubule segments from stage XII were isolated in Dulbecco's modified Eagle's medium/F12 medium (1:1) (DMEM/F12) (Gibco BRL, Paisley, Scotland, UK) supplemented with 15 mM HEPES, 1.25 g/l sodium bicarbonate, 10 mg/l gentamycin sulphate, 60 mg/l G-penicillin, 1 g/l BSA and 0.1 mM 3-isobutyl-1-methylxanthin (MIX) (Aldrich Chemie, Steinheim, Germany) under a stereomicroscope by transillumination-assisted microdisection technique as described previously (Toppari and Parvinen, 1985). Twenty pieces of 2-mm-long segments from stage XII were cultured in 100 µl of medium in the presence or absence of 100 ng/ml of recombinant mouse SCF (Genzyme Transgenics Corp., Cambridge, MA) at 34°C for 8, 24, 48, or 72 hours in a humidified atmosphere containing 5% CO_2 in air. For ACK-2 blocking experiments, tubule segments were cultured in medium containing vehicle (as control), recombinant human FSH (at 10 ng/ml, Org 32489, Organon, Oss, The Netherlands), FSH (10 ng/ml) + ACK-2 monoclonal antibody (at 5 µg/ml, kindly provide by Dr T. Kunisada, Department of Immunology, Faculty of Medicine, Tottori University, Japan), ACK-2, or mouse IgG (Zymed Laboratories, Inc., San Francisco, CA) for the same time and under the same culture condition as described above.

Non-radioactive DNA laddering

DNA was isolated from five pieces of 2 mm-long tubule segments by phenol/chloroform extraction after digestion in TES buffer (10 mM Tris-HCl, pH 7.6, 10 mM EDTA, 100 mM NaCl) containing 1% SDS, proteinase K (100 µg/ml, Boehringer Mannheim, Mannheim, Germany), and RNase A (10 µg/µl, Boehringer Mannheim) at 55°C for 30 minutes. The 3′-end labeling was performed in a 20 µl reaction volume containing 100 ng DNA, 4 µl 1× terminal transferase buffer (Promega, Madison, WI), 0.5 µl terminal deoxynucleotidyl transferase (20 U/µl, Promega), 0.1 µl DIG-11-ddUTP (10 nmol/µl, Boehringer Mannheim). The reaction mixture was incubated for 30 minutes at 37°C and then 1 µl 0.5 M EDTA, pH 8.0, was added to terminate the reaction. The reaction mixture was size-fractionated in a 1.6% agarose gel followed by overnight transfer to a nylon membrane in 10× SSC. The DNA was fixed by baking the membrane in an oven at 80°C for 1 hour followed by UV cross-linking. The membrane was incubated in 20 ml 1× blocking buffer (Boehringer Mannheim) containing 1 µl anti-DIG-AP antibody (Boehringer Mannheim) at RT for 1 hour followed by three TBST buffer washes (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.1% Tween-20) that were each 15 minutes. 1 ml chemiluminescent substrate CSPD (Boehringer Mannheim) was applied onto the blot. The blot was incubated at RT for 10 minutes and then sealed in a plastic bag. The blot was exposed to X-ray film (Fuji RX, Tokyo, Japan) for 5-10 minutes.

Modified squash preparation

One piece of 2 mm-long tubule segment was washed twice with PBS in a 0.5 ml microcentrifuge tube and then 100 µl 4% paraformaldehyde (PFA) was added. The segment was immediately transferred in 20 µl 4% PFA onto a poly-lysine-treated slide and gently covered with a coverslip. After 10-15 minutes of fixation, the slide was immersed in PBS for 10-30 minutes and the coverslip was carefully removed. The slide was washed once more with PBS for 10 minutes and then rinsed briefly with water followed by incubation in cold absolute ethanol for 2-5 minutes. The slide was air dried and kept at RT for further analysis.

In situ 3′-end labeling (ISEL)

The slides, on which cultured tubule segments were fixed by a modified squash preparation as described above, were incubated in 2× SSC at 80°C for 20 minutes followed by washing twice with water and once with Proteinase K buffer (20 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$) for 5 minutes each. The slides were then treated with proteinase K (10 µg/ml, Boehringer Mannheim) in proteinase K buffer at 37°C for 30 minutes. An aliquot of 20 µl of 3′-end labeling reaction mixture containing 4 µl 5×TdT buffer (Promega), 0.1 µl Dig-11-ddUTP (10 nmol/µl, Boehringer Mannheim), 0.2 µl dd-ATP (5 mM, Promega), 1 µl terminal deoxynucleotidyl transferase (Boehringer Mannheim) and 14.7 µl nuclease-free water (Promega) was applied to one 2-mm-long squashed segment. The slides were kept in a humidified box and incubated at 37°C for 1 hour. The slides were then washed three times with TBST for 10 minutes each. An anti-Dig-HRP monoclonal antibody (DAKO Corp., Glostrup, Denmark, 1:200 dilution in TBST containing 1% BSA,) was applied and the slides were incubated in a humidified box at RT for 1 hour followed by three 5 minute washes with TBST. Finally, the labeled cells were visualized by incubating the slides with DAB (Sigma, St Louis, MO, USA) for 0.5-2 minutes.

Quantitative analysis

The X-ray films of DNA laddering results were first scanned by a UMAX scanner (UMAX Inc., Fremont, CA) and a Binuscan® Photoperfect software package (Binuscan Inc., New York, NY). The images were saved as TIFF-type files (*.tif, Microsoft Co. and Aldus Co., New York, NY) and then quantified by TINA 2.0 densitometric analytical system (Raytest Isotopenmeβ gerate GmbH, Straubenhardt, Germany) according to the manufacturer's instructions.

For quantification of ISEL results, all ISEL-positive cells of the 2-mm-long tubule were counted without distinguishing cell types. For evaluation of spermatogonial apoptosis, only the ISEL-positive germ cells lying on the basement membrane of the tubule were counted because they were largely spermatogonia. The early apoptosis of spermatocytes and spermatids were determined according to the morphology of these cells. However, accurate recognition of the late apoptotic spermatocytes and spermatids was difficult because severe nuclear condensation, shrinkage, and collapse had changed cellular morphology. Nevertheless, the characteristic distribution of spermatocytes and spermatids pushed outside the squashed tubule during preparation allowed the late apoptotic spermatocytes and spermatids to be estimated. In the present study, the number of apoptotic spermatocytes and spermatids were evaluated by a semi-quantitative analysis. The following criteria were used for the semi-quantification: +, less than 50; ++, between 50-150; +++, more than 150 ISEL-positive cells in the region of spermatocytes, or spermatids in the squash preparation.

Replication of experiments and statistical analysis

All the experiments were repeated independently at least four times.

In DNA laddering analyses, the highest densitometric value was designated as 100% and other values were expressed as the percentages of the highest one. For ISEL staining, the apoptotic cell number was counted per 2 mm tubule segment. The values from all the experiments were pooled for the calculation of the means and their standard errors and for one way analysis of variance and Duncan's new multiple range test to determine the significant differences between different experimental groups by using StatView 4.51 statistic program (Abacus Concepts Inc., Berkeley, CA). The *P* values less than 0.05 were considered statistically significant.

RESULTS

Quantitative analysis of DNA fragmentation in the SCF-treated seminiferous tubule segments from stage XII

Seminiferous tubule segments from stage XII started to show a typical apoptosis pattern of DNA fragmentation after 8 hours of culture in the absence of SCF, whereas tubules cultured for the same period of time in the presence of SCF showed very little DNA fragmentation (Fig. 1A). After 24-hour culture, the SCF-treated tubules displayed much less DNA fragmentation than the untreated samples, which had extensive DNA fragmentation. After 48 hours of culture, both SCF-treated and untreated samples showed extensive DNA fragmentation. However, differences in the extent of fragmentation exhibited by these two groups remained significant (Fig. 1B, n=4, P<0.01 for 48 hours, 0.01<P<0.05 for 72 hours, respectively).

Quantitative analysis of apoptotic germ cells in the SCF-treated seminiferous tubules from stage XII

Sixteen pieces of 2-mm-long stage XII tubule segments were analyzed by the modified squash preparation and ISEL staining. ISEL-positive cells both outside and inside of the squashed tubules were counted under a microscope (Fig. 2A). Apoptotic cell number of the control samples almost doubled as compared with that of the SCF-treated tubules after 8 hours of culture. The differences in apoptotic cell numbers between the SCF-treated and controls became significant upon 24 hours of culture and remained such until 72 hours of culture (Fig. 2B, n=16, P<0.01).

The types of germ cells inside the tubule could not be accurately recognized because several different stages are present within any given preparation. However, the single layer of cells lying on the basement membrane of the tubule could be discerned to be mostly spermatogonia because it has the fewest overlapping layers of cells in the tubule. These cells were counted to evaluate the number of apoptotic spermatogonia (Fig. 3A). As shown in Fig. 3B, the spermatogonial apoptosis took place much earlier in the controls than in SCF-treated samples. For the SCF-treated samples, there was no significant difference in the number of apoptotic spermatogonia between 0 hours and 8 hours. However, approximately a 4-fold increase in the number of apoptotic spermatogonia was observed in the controls after 8 hours of culture. After 24 hours and 48 hours of culture, the apoptotic spermatogonia increased both in SCF-treated and in control samples. Nevertheless, the differences between the two groups were still significant (n=16, P<0.01). Upon 72 hours of culture, the number of apoptotic spermatogonia in SCF-treated samples was 5.5-fold higher than that at 8 hours of culture. But

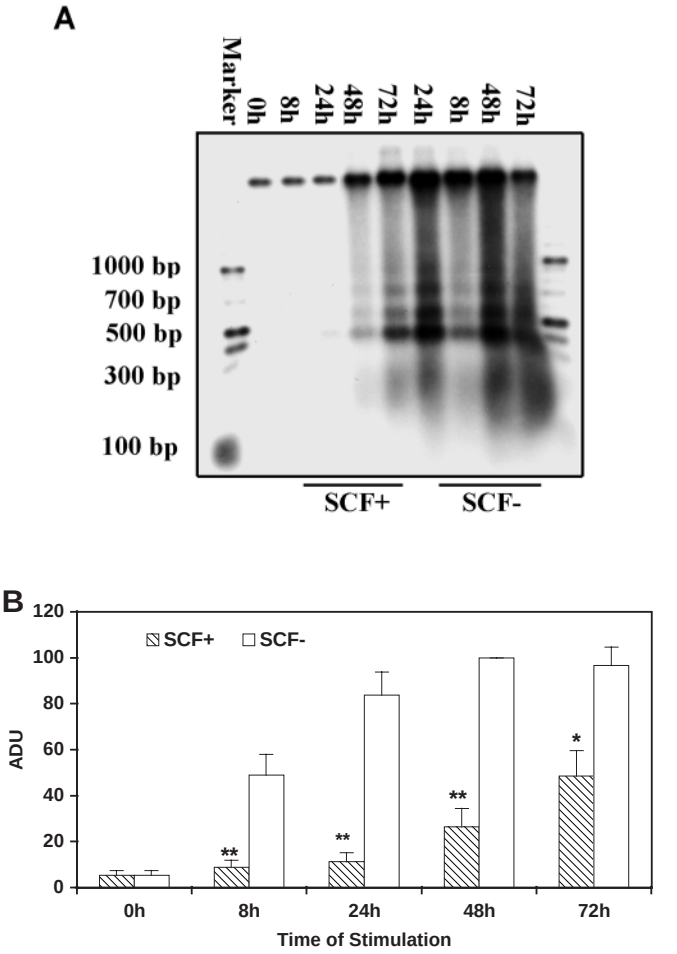

Fig. 1. DNA laddering analysis of the rat seminiferous tubule segments from stage XII of the epithelial cycle in the presence or absence of SCF in vitro. (A) A representative result of DNA laddering analysis. The time points and the treatments were marked on the top and bottom, respectively. (B) Quantitative analysis of DNA laddering results. The bands between 100-1,000 bp were quantified. Each bar represents the mean ± s.e.m. ADU: arbitrary densitometric unit (defined as percentage of the densitometric value of the control samples at 48 hour, which always gave the most severe DNA fragmentation); n=4, *P<0.05, **P<0.01 (compared with controls).

it was still 1.5-fold lower than that in the controls after 72 hours of culture (n=16, P<0.05).

There were more apoptotic spermatocytes and spermatids than apoptotic spermatogonia in the absence of SCF (Figs 2, 3). The apoptotic spermatocytes and spermatids (Fig. 4) were much less prevalent in the SCF-treated tubules than in the control samples at all time points (Table 1).

FSH protects germ cells from apoptosis

In the presence of FSH, DNA fragmentation was much less severe than that observed in the control sample at all time-points (Fig. 5). No significant differences were observed between FSH-treated and SCF-treated groups (P>0.05, n=4). In agreement with DNA laddering, quantitative analysis of ISEL staining demonstrated significant differences between FSH-treated and control samples at all time-points except for 8 hours of culture (Fig. 6, P<0.05, n=16). No significant

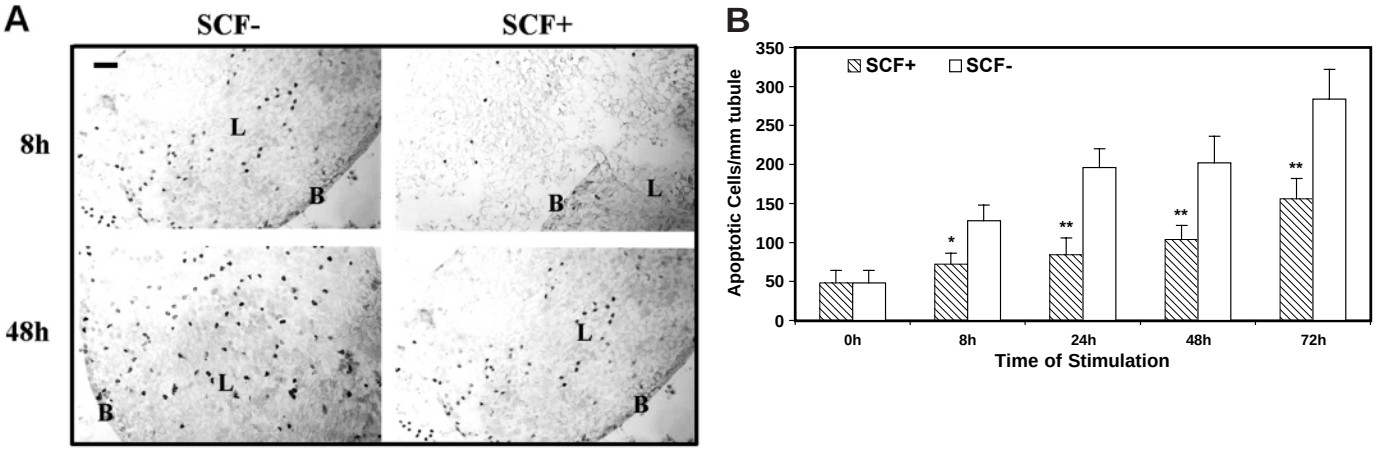

Fig. 2. ISEL staining of the rat seminiferous tubule segments cultured in the presence or absence of SCF (100 ng/ml) in vitro. (A) A microphotograph showing the ISEL positive cells at stage XII seminiferous tubules treated with SCF (SCF+) or vehicle (SCF-) for 8 hours and 48 hours. Bar, 10 μm. L, luminal side; B, basal side. (B) Quantitative analysis of 16 pieces of 2-mm-long segments from stage XII treated with SCF (SCF+) or vehicle (SCF–) for 8, 24, 48, and 72 hours. Each bar represents the mean ± s.e.m. of 16 samples from four times of independent experiments. n=16, *P<0.05, **P<0.01 (compared with controls).

differences between FSH-treated and SCF-treated samples were observed (P>0.05, n=16). Microscopic observation showed no difference in apoptotic cell types, which were mainly spermatids and spermatocytes (Fig. 6A).

Blockade of SCF function by ACK-2 abolished protective effects of FSH

When the function-blocking antibody, ACK-2, was applied to the SCF-treated samples, it dramatically reduced the pro-survival effects of SCF, showing the specificity and effectiveness of the antibody (Fig. 5). When ACK-2 alone, or mouse IgG, was applied to the control samples, no significant effects were observed (data not shown). However, when ACK-2 was applied to FSH-treated samples, FSH pro-survival effects were partially abolished at 24 hours and 48 hours of culture, as manifested by DNA laddering and ISEL staining (Figs 5, 6).

DISCUSSION

In situ 3′-end labeling (ISEL) has become a powerful tool for identifying and quantifying apoptotic cells. In the

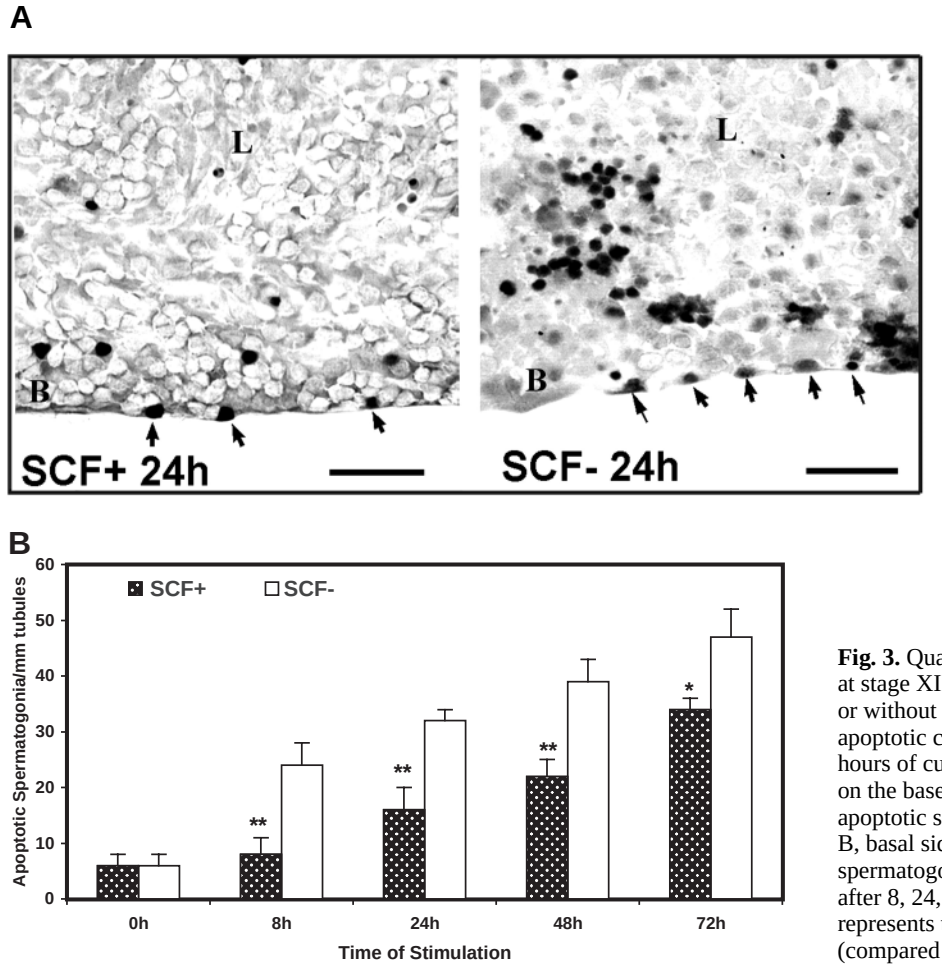

Fig. 3. Quantitative analysis of apoptotic spermatogonia at stage XII seminiferous tubule segments treated with or without SCF. (A) A microphotograph showing the apoptotic cells inside the seminiferous tubule after 24 hours of culture in vitro. Only ISEL-positive cells lying on the baseline of the tubule (arrows) were counted as apoptotic spermatogonia. Bars, 10 μm. L, luminal side; B, basal side. (B) Quantification of apoptotic spermatogonia from 16 pieces of 2-mm-long segments after 8, 24, 48, and 72 hours of culture. Each bar represents the mean ± s.e.m. n=16, *P<0.05, **P<0.01 (compared with controls).

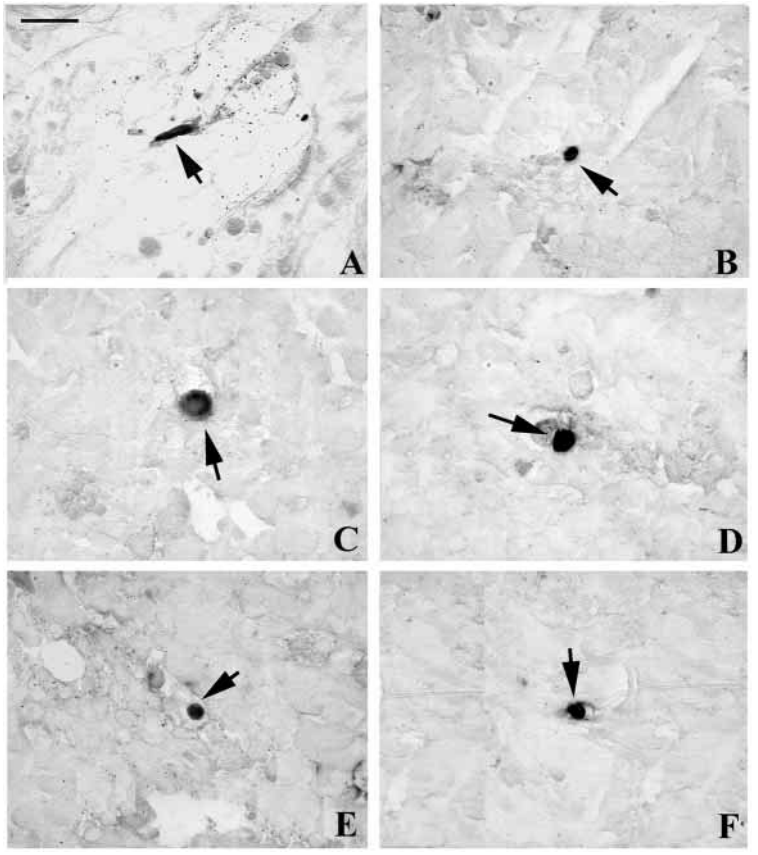

Fig. 4. Microphotographs showing the apoptotic spermatocytes and spermatids in the seminiferous tubule segments from stage XII cultured for 8 hours in the absence of added SCF. (A and B) An early and a late apoptotic step 12 spermatids (arrows), respectively. (C and D) An early and a late apoptotic pachytene spermatocytes (arrows), respectively. (E and F) An early and a late apoptotic zygotene spermatocytes (arrows), respectively. Bar, 0.2 μm.

present study, we applied this technique in combination with a modified squash preparation for isolated seminiferous tubule segments from stage XII of the epithelial cycle. ISEL can detect all cells with DNA breakage, including apoptotic and necrotic cells. The latter could be common in the in vitro tissue culture system. The internucleosomal cleavage of DNA is a prominent feature of apoptosis, which can be classically visualized by agarose gel electrophoresis and ethidium bromide staining as a discontinuous ladder of discrete 185-200 bp multimeric bands (Cohen and Duke, 1984). Necrotic cells display random DNA degradation and no discrete band pattern if separated on the gel. Therefore, in the present study, we also used the DNA laddering analysis to further validate our data from ISEL analysis. The slight differences between DNA laddering and ISEL staining results in the present study might just reflect the specificity of the two methods employed. To increase the sensitivity of DNA laddering, we incorporated digoxigenin-labeled ddUTP in the 3′-end labeling reaction followed by anti-digoxigenin antibody and chemiluminescent detection. This method gave similar sensitivity to radioactive methods (data not shown). By using this method, DNA fragmentation can be visualized from as little as 100 ng of starting DNA, corresponding to the DNA extracted from a 1 mm-long seminiferous tubule segment.

In the present study, we used similar culture conditions and SCF concentration (100 ng/ml) as in our previous study (Hakovirta et al., 1999; Yan et al., 1999) to explore the potential pro-survival effect of SCF on germ cells in vitro. SCF is synthesized by Sertoli cells both in vivo and in vitro since we can detect SCF mRNA from the isolated and cultured seminiferous tubules (Yan et al., 1999). However, the expression levels of SCF are much lower in vitro than in vivo because SCF expression is highly dependent on FSH stimulation. As we have shown in our earlier study (Yan et al., 1999), the SCF mRNA levels are 2-5-fold higher in the presence of FSH (10 ng/ml) than in the absence of FSH after 30 hours of culture. This suggests that SCF production in vitro is much less than in vivo due to a lack of FSH. Low levels of endogenous SCF are not sufficient for germ cell survival, which is consistent with our observation that many germ cells died as soon as 8 hours after being placed in culture. Thus, the effect of the added SCF could be assessed by observing the difference between the SCF-treated and control samples.

The present findings suggest that the enhanced thymidine incorporation by stage XII type A spermatogonia that occurs in the presence of SCF might be due to reduced apoptosis rather than more stem spermatogonia entering the cell cycle (Hakovirta et al., 1999). To our surprise, not only spermatogonia, but also spermatocytes and spermatids were protected from death by SCF. In principle, the added SCF could reach spermatocytes and spermatids either by passing through the cytoplasm of the Sertoli cells or diffusion through the lumen. It might be very difficult for the added SCF to rapidly diffuse from the lumen to spermatocytes and spermatids in vivo given that Sertoli cells produce seminiferous tubular fluid. However, in the in vitro culture system, the environment has been changed and thus the dynamics of seminiferous tubular fluid might be affected and the upstream pressure that SCF must overcome may be much lower than in in vivo condition. Moreover, the concentration of the added SCF that we used in the present study might be much higher than the physiological levels (no data available so far). The higher SCF concentration may produce higher diffusion pressure. Therefore, it is most likely that the added SCF

Table 1. Semi-quantitative analysis of apoptotic spermatocytes and spermatids in the presence (SCF+) or absence (SCF−) of SCF in the seminiferous tubule segments from stage XII

Incubation Time (hour)	SCF+		SCF−	
	Sp	Sd	Sp	Sd
0	+	+	+	+
8	+	+	++	+
24	+	+	++	++
48	+	+	+++	+++
72	++	++	+++	+++

Sp, Spermatocytes; Sd, Spermatids. +, less than 50; ++, between 50-150; +++, more than 150 ISEL-positive cells per 2-mm-long tubule segment in the region of spermatocytes, or spermatids of squash preparation.

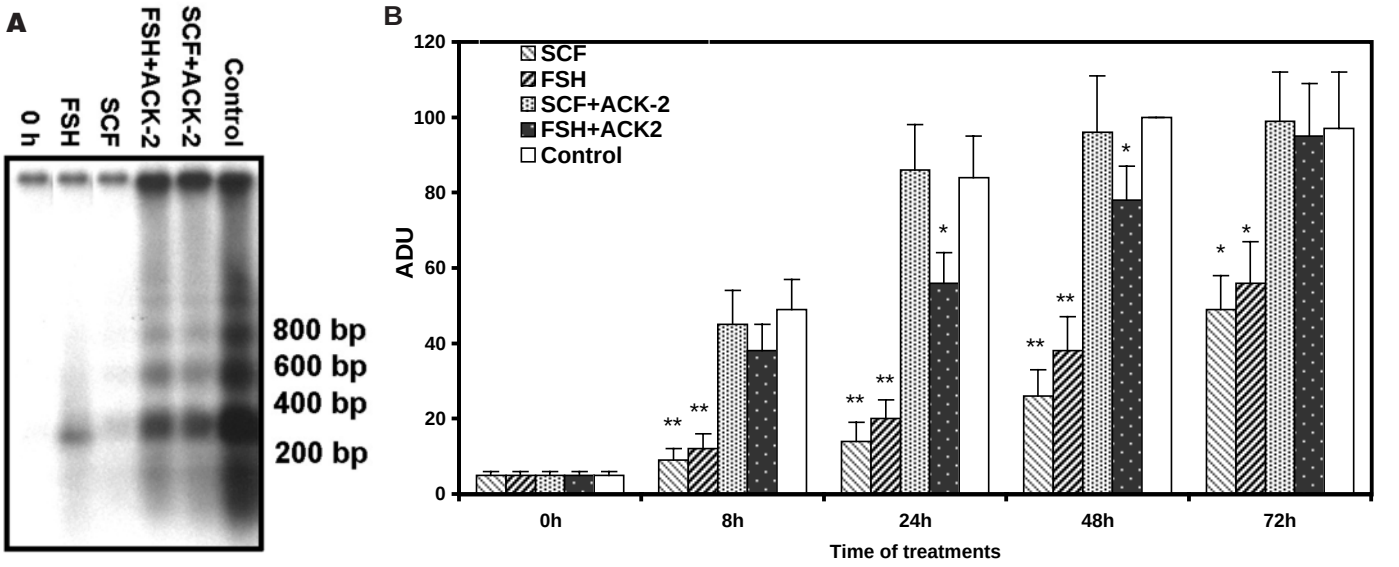

Fig. 5. DNA laddering analysis of stage XII seminiferous tubule segments treated with or without ACK-2 antibody in the presence or absence of FSH or SCF. (A) A representative DNA laddering showing apoptotic DNA fragmentation of the stage XII tubules after 24 hours of treatments. (B) Quantitative analysis of DNA laddering. The bands between 100-1,000 bp were quantified. Each bar represents the mean ± s.e.m. from four independent experiments. ADU: arbitrary densitometric unit; n=4, *P<0.05, **P<0.01 (compared with controls).

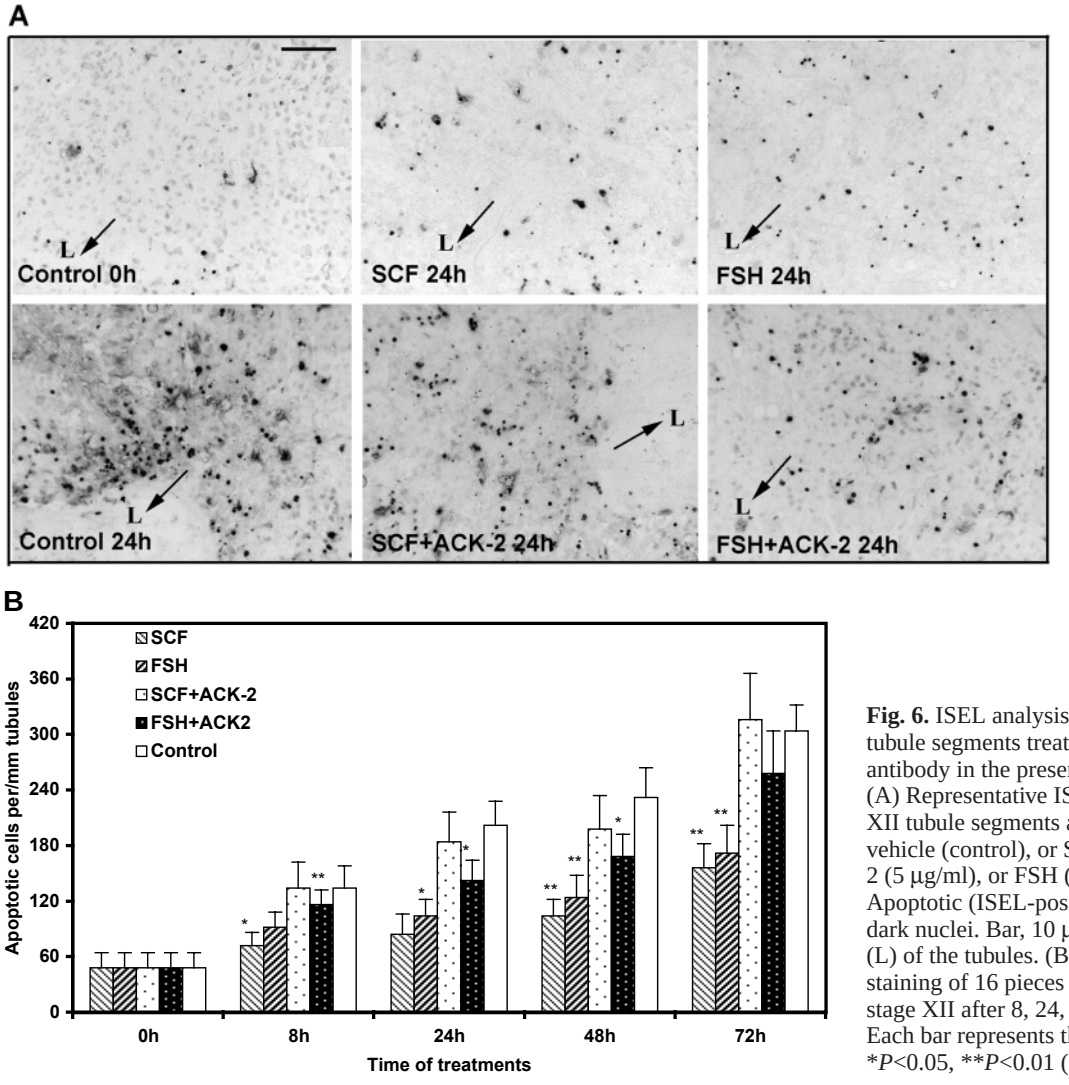

Fig. 6. ISEL analysis of stage XII seminiferous tubule segments treated with or without ACK-2 antibody in the presence or absence of FSH or SCF. (A) Representative ISEL staining results for stage XII tubule segments after 24 hours of treatment with vehicle (control), or SCF (100 ng/ml), or SCF+ACK-2 (5 μg/ml), or FSH (10 ng/ml), or FSH+ACK-2. Apoptotic (ISEL-positive) cells can be discerned as dark nuclei. Bar, 10 μm. Arrows point to the lumen (L) of the tubules. (B) Quantitative analysis of ISEL staining of 16 pieces of 2-mm-long segments from stage XII after 8, 24, 48, and 72 hours of treatments. Each bar represents the mean ± s.e.m. n=16, *P<0.05, **P<0.01 (compared with controls).

reached spermatocytes and spermatids by diffusion through the lumen. The protective effect of the added SCF was observed at 8 hours of culture, but it was less significant ($P<0.05$ at 8 hours, $P<0.01$ at others) than at other time points. This might reflect the progressive diffusion of SCF onto spermatocytes and spermatids.

Several lines of evidence suggest that the c-kit receptor is present not only on spermatogonia (Manova et al., 1990, 1993; Sorrentino et al., 1991), but also on spermatocytes and spermatids (Vincent et al., 1998; Albanesi et al., 1996; Ali et al., 1996). However, since c-kit receptor on spermatids is in a truncated form lacking extracellular, transmembrane, and part of intracellular domain (Rossi et al., 1992), the mechanism by which SCF interacts with this truncated form of c-kit remains unclear.

The pro-survival effect of SCF appeared to be mediated through interaction with c-kit receptors on spermatogonia, spermatocytes, and spermatids since ACK-2 antibody could totally abolish the pro-survival effect of SCF on all these cells. This effect was specific because the ACK-2 antibody abolished the protective effect of SCF and mouse non-immune IgG had no effect. More germ cells would undergo apoptosis due to the blockade of SCF/c-kit interaction when ACK was added to the control samples. However, no significant effect was observed when ACK-2 was applied to the controls (without added SCF). One possible explanation is that this effect is covered by the high background of germ cell death due to low SCF levels in the control samples under these in vitro culture conditions.

Several studies have shown that FSH upregulates SCF expression in both immature and mature testis (Rossi et al., 1993; Taylor et al., 1996; Yan et al., 1999). In the present study, the fact that anti-c-kit antibody partially abolished the pro-survival effect of FSH on germ cells strongly suggests that SCF/c-kit system is involved in the regulation of germ cell apoptosis. However, compared to the efficiency that ACK-2 antibody blocks the protective effect of SCF, the partial blockade of the FSH pro-survival effect implies that, besides SCF/c-kit system, there must be some other factor(s) involved in the FSH protective effects. Recent studies have found that activation of apoptosis machinery in germ cell involves the Fas/Fas ligand system (Lee et al., 1997; Wang et al., 1998; Woolveridge et al., 1999;). Fas ligand is produced by Sertoli cells (Bellgrau et al., 1995; French et al., 1996; Lee et al., 1997) and Fas receptor is localized on spermatocytes and spermatids, as well as Sertoli cells (Lee et al., 1997; Sugihara et al., 1997; Li et al., 1997). SCF/c-kit system has similar expression sites. The interrelation between SCF/c-kit system and Fas system in regulation of germ cell apoptosis during spermatogenesis remains an interesting topic for future studies.

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