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Stem Cell Factor Functions as a Survival Factor for Mature Leydig Cells and a Growth Factor for Precursor Leydig Cells after Ethylene Dimethane Sulfonate Treatment: Implication of a Role of the Stem Cell Factor/c-Kit System in Leydig Cell Development

SCF是睾丸產生睾固酮的來狄氏細胞的存活因子

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The significance of the interaction between Sertoli cell-produced stem cell factor (SCF) and its receptor, c-kit, on Leydig cells (LCs) during LC development and differentiation is unknown. In the present study, we investigated the potential role of the SCF/c-kit system in LC apoptosis and precursor LC proliferation after ethylene dimethane sulfonate (EDS) treatment in rats. A function-blocking anti-c-kit antibody, ACK-2, was used to block SCF/c-kit interaction at four time points, corresponding to the peak of LC apoptosis and three waves of proliferation of precursor LCs. Blockade of SCF/c-kit interaction by ACK-2 accelerated LC apoptosis and inhibited proliferation of precursor LCs during the first two waves of precursor LC proliferation around days 3–4 and day 10, but not the third wave of precursor LC proliferation around day 20 after EDS treatment. The data suggest that the soluble SCF might act as a survival factor for mature LCs and a growth factor for precursor LCs after EDS-induced LC depletion. This is also supported by a close correlation between the oscillating levels of soluble SCF mRNA and the profiles of LC apoptosis and regeneration. Since regeneration of the LC population after EDS treatment resembles the development of adult-type LCs during prepubertal life, the present findings imply that soluble SCF might participate in regulation of the formation of the LC population during testicular development. Our data also support a model in which delicate and reciprocal regulation exists between soluble SCF production by Sertoli cells, testosterone production by LCs, and pituitary gonadotropins.

Key Words: **stem cell factor; c-kit; testosterone; Leydig cell; testis.**

INTRODUCTION

In the rat testis, stem cell factor (SCF) is produced by Sertoli cells and it interacts with its receptor, c-kit, on germ cells and Leydig cells (LCs). This SCF/c-kit interaction plays important roles in germ cell proliferation, differentiation, and apoptosis during germ cell development (for review see Loveland and Schlatt, 1997). However, the role of SCF/c-kit interaction in LC development and differentiation is unknown despite the fact that LCs express c-kit. Sertoli cells produce two forms of SCF, soluble SCF and membrane-associated SCF (Rossi *et al.*, 1991, 1994). Two alternatively spliced SCF transcripts encode two cell-associated SCF protein products, KL-1 and KL-2. The KL-2 protein lacks the major proteolytic cleavage site for the generation of soluble SCF, thus representing a more stable cell-associated form of SCF. Previously it was thought that the soluble SCF arises from alternative splicing of exon 6 followed by protease cleavage (Flanagan *et al.*, 1991; Galli *et al.*, 1994). However, this concept has been challenged by a recent study showing that ablation of exon 6 using a homologous recombination technique did not abolish the production of soluble SCF, indicating that soluble SCF can

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be produced from KL-2 by proteolytic cleavage at sites other than that encoded by exon 6 *in vivo* (Tajima *et al.*, 1998). Both forms can interact with the SCF receptor, c-kit, present on spermatogonia, spermatocytes, and spermatids (Anderson *et al.*, 1990; Langley *et al.*, 1992). It has been shown that the membrane-associated form of SCF is more important than the soluble form as regards germ cell proliferation and survival (Tajima *et al.*, 1991). However, the role of the soluble form is largely unknown. The ratio of these two forms of SCF appears to be tissue-specific (Huang *et al.*, 1992). It has been noticed that the ratio changes during testicular injury (Allard *et al.*, 1996; Blanchard *et al.*, 1998). The soluble SCF is also thought to be necessary for distant target cells, such as LCs (Loveland and Schlatt, 1997). Blocking SCF function by means of an anti-c-kit monoclonal antibody, ACK-2, results in depletion of proliferating type A spermatogonia *in vivo* (Yoshinaga *et al.*, 1991), delay of spermatid maturation (Vincent *et al.*, 1998), and enhanced apoptosis of all types of germ cells *in vitro* (Yan *et al.*, 2000), but no significant effect on LC function, even though transient elevation of testosterone levels has been observed (Yoshinaga *et al.*, 1991).

Ethylene dimethane sulfonate (EDS) selectively induces apoptosis of mature LCs in the rat (Morris *et al.*, 1986). After EDS administration, mesenchymal-like precursor Leydig cells start to proliferate and further differentiate into mature LCs (Teerds *et al.*, 1988). Several studies have shown that the regeneration of LCs can take place in the absence of LH and other pituitary hormones (for review see Teerds, 1996). It is strongly suggested that locally produced growth factors may be involved in the regulation of LC repopulation. However, the nature of these factors still remains unknown. The proliferation and differentiation of precursor LCs into mature LCs after EDS administration can be used as a model for investigating regulation of the development of adult-type LCs before puberty, due to the many similarities between these two processes (for review see Teerds, 1996).

In the present study, we employed EDS-treated rats and a function-blocking anti-c-kit antibody, ACK-2, to study the potential role of the SCF/c-kit system in LC development and differentiation. By monitoring LC apoptosis and precursor LC proliferation at various time points after EDS administration using TUNEL staining and immunohistochemical detection of *in vivo*-incorporated BrdU, respectively, we obtained a detailed time course of mature LC apoptosis and precursor LC proliferation. By means of Northern hybridization and quantitative RT-PCR, an interesting correlation between the changes in soluble SCF mRNA levels and LC apoptosis and proliferative activity of precursor LCs was observed, which strongly implicated the involvement of this factor in LC apoptosis and precursor LC proliferation. We then administered ACK-2 antibody at the time points corresponding to the peak of LC apoptosis and three waves of precursor LC proliferation, to see how blockade of SCF/c-kit interaction affects these processes.

MATERIALS AND METHODS

Animals and Treatments

Adult male rats of the Sprague–Dawley strain, ages 60–70 days, were used. The rats were housed under controlled conditions of light (14 h light, 10 h of darkness) and temperature (21–22°C), with free access to water and food.

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

The animals were injected ip with a single dose of EDS (75 mg/kg BW). EDS was synthesized as previously described (Jackson and Jackson, 1984) and dissolved in dimethyl sulfoxide:water (1:3, vol/vol). Control animals received injection of vehicle. Rats (three per group) were killed by cervical dislocation under CO_2 anesthesia on days 1, 2, 3, 4, 7, 10, 15, 20, 30, and 40 after EDS administration. One hour before sacrifice, the rats received a single ip injection of BrdU (50 mg/kg BW) dissolved in physiological saline. Blood was collected for hormone measurements. One testis was fixed in freshly prepared 4% paraformaldehyde at 4°C overnight and embedded in paraffin for TUNEL staining and immunohistochemical detection of incorporated BrdU. Half of the other testis was snap frozen in liquid N_2 and then stored at −70°C for isolation of RNA, and the other half was used for LC isolation.

A separate experiment was conducted to study the effect of blockade of SCF/c-kit interaction on LC apoptosis and regeneration by using a function-blocking anti-c-kit antibody, ACK-2, which was generously provided by Dr. T. Kunisada (Department of Immunology, Faculty of Medicine, Tottori University, Japan). To determine the optimal dose at which the interaction of SCF/c-kit can be effectively blocked, a dose–response experiment was performed by injecting ACK-2 antibody iv at 0.5–5.0 mg/kg BW in physiological saline, and induction of spermatogonial apoptosis was monitored by *in situ* 3′-end labeling (TUNEL) staining. A dose of 3.5 mg/kg BW was chosen for the following experiments since a maximal number of TUNEL-positive spermatogonia was observed at a dose between 3.0 and 5.0 mg/kg B.W. (data not shown). The time schedules for administering ACK-2 antibody are shown in Fig. 1. Rats were divided into three groups according to the schedules with 12 rats in each: 3 treated with EDS + ACK-2, 3 treated with EDS only, and 6 controls including 3 treated with EDS + a monoclonal anti-β-actin antibody (ICN Biomedicals, Inc., Aurora, OH) and 3 treated with vehicle. In schedule 1, the EDS + ACK-2 group received an ACK-2 injection every 2 days during days 0–4 after EDS administration (Fig. 1, schedule 1). In schedules 2 and 3, the EDS + ACK-2 group received an ACK-2 injection every 2 days during days 7–11 and 20–24, respectively, after EDS treatment (Fig. 1, schedules 2 and 3). BrdU injection and tissue collection were performed as described above.

Hormone Measurements

Serum testosterone (T) concentrations were measured from diethyl ether extracts by RIA, as described previously (Huhtaniemi *et al.*, 1985). Serum LH levels were measured using a supersensitive immunofluorometric assay, based on the Delfia principle (Wallac Oy, Turku, Finland), as described previously (Haavisto *et al.*, 1993). The sensitivity of the assay was 0.75 pg/tube, the intraassay coefficient of variation (CV) was 7%, and the interassay CV 10%. The results were expressed in terms of the NIDDK (Bethesda, MD) reference preparation LH-RP-2. Serum FSH levels were determined by a double-antibody RIA method (Kolho *et al.*, 1988), using kits supplied by NIDDK. The sensitivity of the assay was 0.15 ng/tube,

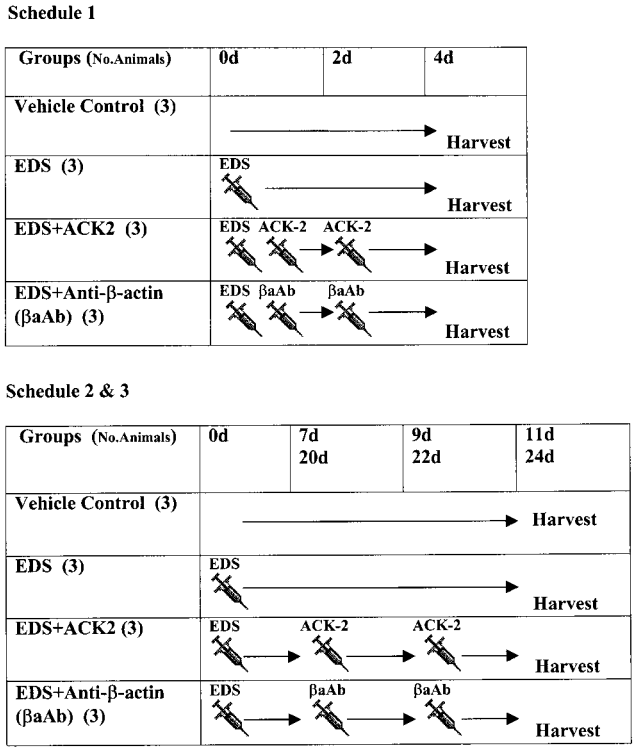

FIG. 1. Schematic presentation of the experimental design. A single dose of EDS (75 mg/kg BW) was injected ip on day 0 and ACK-2 antibody (3.5 mg/kg BW) was injected iv every 48 h during days 0–4, 7–11, and 20–24 after EDS treatment. Vehicles and an unrelated antibody, a monoclonal anti-β-actin antibody (βaAb), were injected at 3.5 mg/kg BW in the control groups.

the intraassay CV was <8%, and the interassay CV was <15%. The results were expressed in terms of reference preparation FSH-RP-2.

TUNEL Staining of Apoptotic LCs

Two consecutive sections (5 μm thick) were cut from each paraffin block, one for TUNEL staining and the other for periodic acid-Schiff (PAS)–hematoxylin staining. After rehydration, the sections were incubated in 2× SSC at 80°C for 20 min followed by two washes with water and one with proteinase K buffer (20 mM Tris–HCl, pH 7.4, 2 mM $CaCl_2$) for 5 min each. The slides were then treated with proteinase K (10 μg/ml; Boehringer Mannheim GmbH, Mannheim, Germany) in proteinase K buffer at 37°C for 30 min. 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 cross section. The slides were kept in a humidified box and incubated at 37°C for 1 h followed by three washes with TBST buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 0.1% Tween 20) for 10 min each. A 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 room temperature for 1 h followed by three washes with TBST for 5 min each. Finally, the labeled cells were visualized by use of 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) for 0.5–2 min.

Immunohistochemical Detection of Incorporated BrdU in Proliferating Interstitial Cells

Two 5-μm-thick consecutive sections were cut from each sample and mounted on poly-lysine-coated slides. One section was used for immunohistochemical staining of BrdU and the other for PAS–hematoxylin staining.

After dewaxing and rehydration, using xylene and a series of ethanol dilutions, the slides were washed twice in TBS buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl) for 5 min each followed by microwave antigen retrieval at 700 W for 15 min in 10 mM sodium citrate solution, pH 6.0. After two washes with TBS, an aliquot of 50 μl of blocking solution (TBS containing 1% BSA, 3% fetal calf serum, and 3% normal horse serum) was applied and the sections were incubated for 1 h at room temperature. After blocking, an aliquot of 50 μl mouse monoclonal anti-BrdU antibody (1:200 diluted in TBS containing 1% BSA) was applied and the sections were incubated at 4°C overnight. Incubation with secondary antibody and visualization of BrdU-positive cells were carried out by using a Vectastain Elite kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Antibody neutralized with a 100-fold excess of BrdU was used in control sections for monitoring the specificity of the immunostaining.

Isolation of Precursor LCs

The procedure used to isolate the LCs and precursor LCs was carried out essentially as described previously (Abney and Zhai, 1998). Briefly, dispersed interstitial cells were separated from the tubular compartment by filtration through surgical gauze and washed twice in DMEM/F12 by centrifugation at 800*g* to precipitate the cells and remove collagenase. The cells were suspended in 55% Percoll and centrifuged at 20,000*g* for 60 min at 4°C in a Beckman JA-20 fixed angle rotor. A gradient was thereby generated in which cells banded in the region of their isodensity. A gradient containing density marker beads was centrifuged simultaneously to provide a guide for fractionation. A gradient fraction containing precursor LCs was removed from the Percoll gradient between densities of 1.064 and 1.070 g/ml, and a gradient fraction enriched in mature LCs was collected from a density region heavier than 1.070 g/ml. Each fraction was diluted with DMEM/F12 and centrifuged at 800*g* to remove the Percoll while pelleting the cells. The purity of the precursor LCs and mature LCs isolated by this procedure has been reported previously to be 90 and 93%, respectively, based on 3β-hydroxysteroid dehydrogenase histochemical staining (Shan and Hardy, 1992).

Cell Proliferation ELISA

Precursor and mature LCs isolated from rats at days 3, 10, and 20 after EDS treatment were cultured in Hepes-buffered Waymouth's medium supplemented with 9% heat-inactivated horse serum (Life Technologies, Inc., Paisley, Scotland, UK) and 4.5% fetal calf serum (Biochler, Wilts, UK) containing penicillin (10,000 units/L) and streptomycin (50 mg/L), at a density of 10^4 cells/well/100 μl in 96-well plates for 24 h at 37°C in an atmosphere of 5% CO_2 in air. The medium was then changed and the cells were incubated in

fresh medium containing vehicle (control), recombinant mouse SCF (100 ng/ml; Genzyme Transgenics Corp., Cambridge, MA), ACK-2 (5 μg/ml) + SCF, or mouse IgG (5 μg/ml; Zymed Laboratories, Inc., San Francisco, CA) + SCF for 12 h and then an aliquot of 10 μl of BrdU solution (100 μM) was added to each well and incubation was continued for another 12 h. Colorimetric ELISA was then performed to detect BrdU incorporation according to the instructions of the kit manufacturer (Boehringer Mannheim). For each time point, cells from three treated and three untreated animals were analyzed in triplicate. The absorbance of the samples was measured in an ELISA reader (Wallac Oy) at 370 nm. A wavelength of 495 nm was used for reference.

Quantitative Analysis of BrdU Contents in DNA from Isolated LCs

DNA was extracted from precursor and mature LCs isolated from rats at days 3, 10, and 20 after EDS treatment by using a conventional phenol/chloroform method. After denaturation, 100 ng of DNA was blotted onto a nylon membrane (Hybond-N; Amersham, Aylesbury, UK) using a vacuum slot-blot apparatus (Schleicher & Schuell, Dassel, Germany). After UV cross-linking, the membranes were subjected to chemiluminescent immunodetection of BrdU using the same monoclonal anti-BrdU antibody as was used in BrdU immunohistochemistry and an ECL system (Amersham, Buckinghamshire, UK) according to the supplier's instructions. To normalize loading differences, the membrane was then hybridized with a rat β-globin cDNA probe, which was generated by PCR using a primer pair (5′-CCA ATC TGC TCA CAC AGG ATA GAG AGG GCA GG-3′; 5′-CCT TGA GGC TGT CCA AGT GAT TCA GGC CAT CG-3′) and labeled with [^{32}P]dCTP by the random priming method.

Quantitative Analysis of BrdU Immunohistochemical Staining of Precursor Leydig Cells

The number of BrdU-positive cells in the interstitium (except occasionally positive myoid cells) in one cross section was counted under a microscope. The slides were then scanned by using a UMAX scanner (UMAX, Inc., Fremont, CA) and a Binuscan Photoperfect software package (Binuscan, Inc., New York, NY) and the images of the sections were subjected to a quantitative image analysis program, Tina (version 2.04; Raytest Isotopenmeß gerate GmbH, Straubenhardt, Germany), to calculate the area of the cross sections according to the manufacturer's instructions. Proliferative activity was represented by the number of BrdU-positive precursor LCs per square millimeter of cross section.

Northern Blot Analysis

Total RNA extraction and Northern blot hybridization of SCF mRNA were performed as described earlier (Hakovirta *et al.*, 1999).

Quantitative RT-PCR for SCF

First-strand complementary DNA was synthesized using 1 μg total RNA in the presence of 12.5 U of avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI) and 100 pmol of random primer (Promega). After reverse transcription (RT) reaction, 2 μl of the incubation mixture was used for the subsequent PCR. SCF-specific primers were designed to encompass exon 6 so that the two forms of SCF could be distinguished. The sense primer, 5′-ACTTGGATTCTCACTTGCATTTATC-3′, and the antisense primer, 5′-CTTCCAGTATAAGGCTCCAAAAGC-3′, correspond to nt 199–223 and nt 874–897, respectively, of the rat SCF cDNA sequence (Martin *et al.*, 1990). The expected sizes of the PCR products were 699 bp for the soluble SCF and 613 bp for the membrane SCF.

To monitor equal PCR amplification efficiency, an internal control, a 395-bp fragment of the L19 ribosomal protein gene (Chan *et al.*, 1987), was coamplified with SCF, using a sense primer, 5′-GAAATCGCCAATGCCAACTC-3′, and an antisense primer, 5′-TCTTAGACCTGCGAGCCTCA-3′. The PCR volume was 50 μl containing 50 pmol of each primer, dNTP mixture (250 μmol/L) containing 50 μmol of digoxigenin–dUTP/L (Boehringer Mannheim), and 5 U of Dynazyme-DNA polymerase in 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Triton X-100, pH 8.8) (Finnzymes, Espoo, Finland). The PCR was performed under the following conditions: 94°C for 1 min (4 min for the first cycle), 53°C for 1 min, 72°C for 1 min (10 min for the last cycle). Ten to 40 cycles of PCR were tested to find the exponential phase for both SCF and L19. Twenty cycles were chosen for further analysis because at this time both SCF and L19 were in the exponential phase (data not shown). After 20 cycles of PCR, the reaction mixture was loaded onto a 1.6% agarose gel and separated at 100 V for 30 min followed by blotting onto a nylon membrane (Hybond-N; Amersham) with 10× SSC. The membrane was incubated in 1× blocking solution (Boehringer Mannheim) containing anti-Dig$^+$–AP antibody (1:10,000 dilution) for 30 min and then chemiluminescent detection by CSPD (Boehringer Mannheim) was performed according to the manufacturer's instructions. Finally, the PCR products were exposed to X-ray film for further quantitative analysis.

Quantitative Analysis of RT-PCR and Northern Hybridization

The X-ray films of the RT-PCR and Northern blotting results were first scanned by using a UMAX scanner (UMAX Inc.) and a Binuscan Photoperfect software package (Binuscan, Inc.). The images were saved as TIFF-type files (*.tif; Microsoft Co. and Aldus Co., New York, NY) and then quantified by using a Tina 2.0 densitometric analytical system (Raytest Isotopenmeß gerate GmbH) according to the manufacturer's instructions. For RT-PCR results, the densitometric values were first normalized to L19 values, the highest one was designated 100%, and the others were expressed as percentages of this. For Northern blotting results, similarly, after normalization to the densitometric values of 28S rRNA, the highest densitometric value was designated 100%, and the other values were expressed as percentages of this.

Immunohistochemical Detection of Kit Receptor Protein on Precursor LCs

Immunohistochemical detection of Kit protein was performed as described for detection of BrdU except for the primary antibody used, which was rabbit anti-Kit polyclonal antibody M-14 (Santa Cruz, Santa Cruz, CA).

Statistical Analysis

Values from three to four samples receiving the same treatment were pooled for calculation of their means and standard errors (SEMs) and for one-way analysis of variance and Duncan's new multiple range test, to determine significant differences between different experimental groups by using StatView 4.51 statistic program (Abacus Concepts, Inc., Berkeley, CA). Probability values less than 0.05 were considered statistically significant.

RESULTS

Hormone Profiles after EDS Treatment

In the present study, more time points after EDS treatment have been analyzed compared with previous studies (Henriksen *et al.*, 1995; Tena-Sempere *et al.*, 1997). Detailed hormone profiles (testosterone, LH, and FSH) after EDS treatment are shown in Fig. 2. Serum LH levels increased significantly within 48 h compared with controls and peaked on days 10–20. Thereafter, LH levels declined and they returned to control levels on day 40 after EDS treatment (Fig. 2, upper graph). Serum T levels decreased to undetectable within 48 h and remained as such until day 10 after EDS treatment. Serum T levels started to increase from day 20 onward and nearly reached control levels on day 40 (Fig. 2, middle). Serum FSH levels were significantly elevated during days 2–20 and they decreased afterward to control levels by day 40 after EDS treatment (Fig. 2, bottom).

Time Course of LC Apoptosis after EDS Administration

As shown in Fig. 3, apoptotic LCs induced by EDS on days 1–4 were visualized by TUNEL staining. Most LCs started to be TUNEL-positive within 24 h and the number of TUNEL-positive LCs peaked at around 48 h after EDS treatment. On days 3 and 4, only a few late-stage apoptotic LCs and apoptotic blasts absorbed into the capillaries were present in the interstitium. No TUNEL-positive interstitial cells were seen on day 7 after EDS treatment (data not shown).

Blockade of SCF/c-Kit Interaction by ACK-2 Accelerates LC Apoptosis Induced by EDS

Depletion of mature LCs took place within 4 days and LC apoptosis peaked at day 2 after EDS treatment. Therefore, we applied ACK-2 during the first 4 days after EDS treatment to see if this would affect LC apoptosis. As shown in Fig. 3, LC apoptosis was much more severe in the group receiving EDS and ACK-2 simultaneously (EDS + ACK-2) than in the EDS-only groups on day 1. Upon day 2, the interstitium of the testis was similar to that of the EDS-only group on day 4. Only apoptotic blasts inside the capillaries, rather than dispersed apoptotic LCs *in situ*, were observed in the interstitium. Thus, the testicular interstitium appeared to be emptier in the EDS + ACK-2 group than in the EDS-only group on day 2, indicating faster depletion of LCs after EDS treatment in the presence of ACK-2.

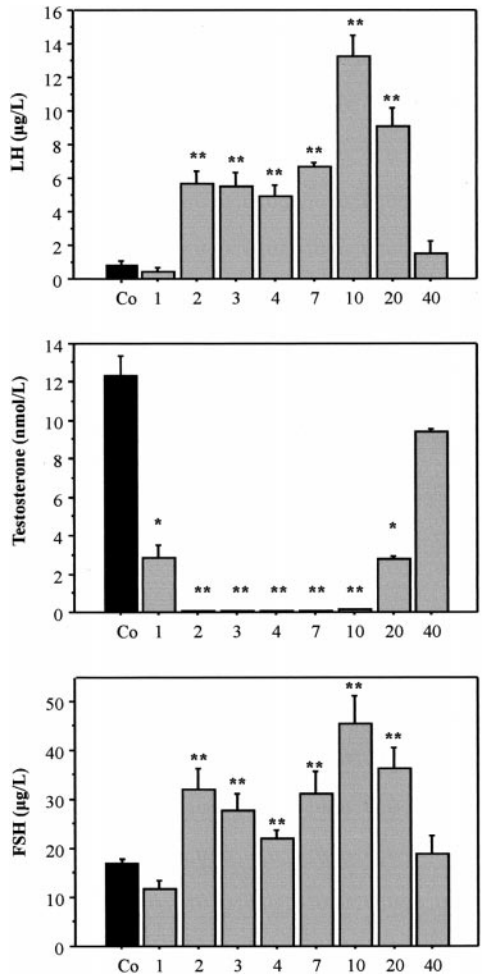

FIG. 2. Hormonal profiles of EDS-treated rats. Values of the controls (Co) at all time points (days 1–40) were pooled. LH levels after EDS treatment are shown in the upper graph. Serum testosterone and FSH levels are indicated in the middle and lower graph, respectively. Data are represented as means ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$, compared with controls.

Time Course of Precursor LC Proliferation

Immunodetection of incorporated BrdU in proliferating cells enabled us to monitor precursor LC proliferation after depletion of mature LCs by means of EDS (Fig. 4A). Quantitative analysis of BrdU-positive new LCs revealed three peaks of BrdU incorporation after EDS treatment (Fig. 4B). The first peak appeared on days 3 and 4, when mature LCs were almost completely depleted. The second peak was

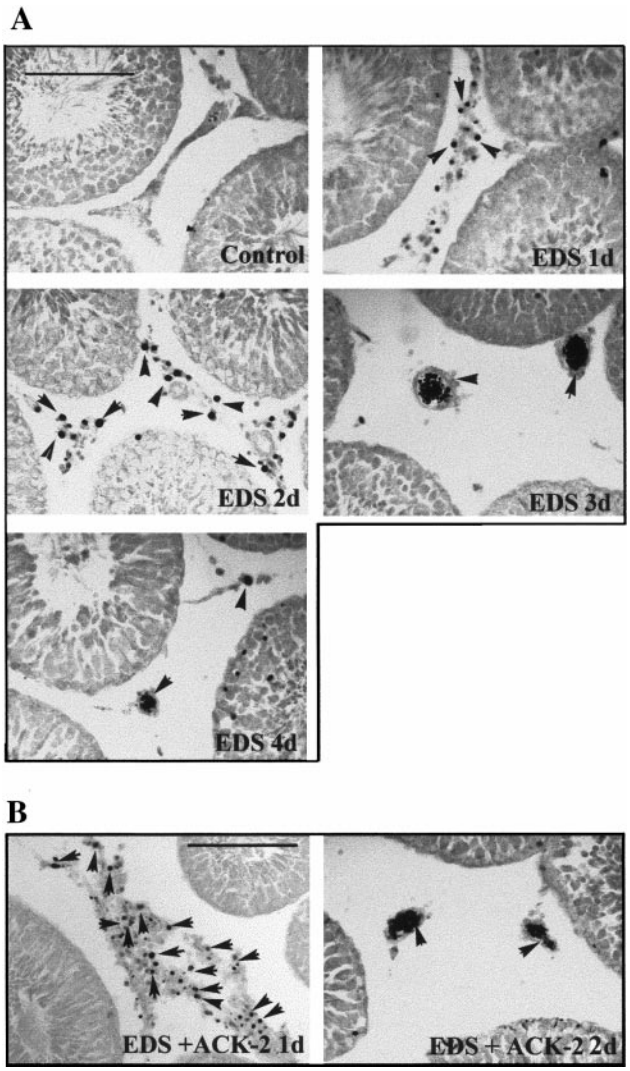

FIG. 3. TUNEL staining results showing LC apoptosis in the first 4 days after EDS treatment (A) and the effect of ACK-2 injection during the first 2 days on LC apoptosis (B). Arrows point to TUNEL-positive LCs or apoptotic blasts. Bar, 100 μm and all images are of the same magnification.

observed around day 10, when serum T concentrations had declined to undetectable levels (Fig. 2) and germ cells were extensively undergoing apoptosis (data not shown). The third peak was around day 20, when testosterone levels had increased significantly in comparison to those on days 2–10 (Fig. 2). Morphological quantification of the proliferative activity of precursor LCs was further validated by DNA BrdU content assay, which detected the amount of BrdU incorporated into DNA during proliferation of precursor LCs. Three peaks of BrdU incorporation were observed at similar time points (Figs. 4C and 4D).

Blockade of SCF/c-Kit Interaction by ACK-2 Inhibits the First Two Waves but Not the Third Wave of Proliferation of Precursor LCs

ACK-2 was generated by immunizing rats with murine mast cells, and it can antagonize murine c-kit (Yoshinaga *et al.*, 1991). It has been shown that ACK-2 can also act as an antagonist of rat c-kit (Yan *et al.*, 2000). In the present study, ACK-2 antibody was administered iv on days 1–4, 7–11, and 21–24 after EDS treatment, the time points corresponding to the three peaks of precursor LC proliferation. Effectiveness was monitored by observing significantly reduced numbers of proliferating spermatogonia (BrdU immunohistochemical staining) and increased numbers of apoptotic spermatogonia (TUNEL staining) in the seminiferous epithelium (data not shown). To exclude possible artifact caused by the mouse monoclonal antibody used, we employed a similar type of mouse monoclonal anti-β-actin antibody as a control. No effect on the proliferation of precursor LCs in the EDS-treated rats was observed when this irrelevant antibody was injected at a similar dose at all the three time points (Fig. 5). A severe reduction in the number of BrdU-positive precursor LCs in the EDS + ACK2 group was revealed, compared with the EDS-only group at days 1–4 and 7–11 (Fig. 5, $P < 0.05$, $n = 3$). During the first two waves of precursor LC proliferation (days 1–4 and 7–11), the number of BrdU-positive LCs in the EDS + ACK-2 group was only half of that in the EDS-only group. Surprisingly, no significant difference in the proliferative activity of precursor LCs was observed between these two groups during the third wave of precursor LC proliferation (Fig. 5, days 20–24).

SCF Stimulates Proliferation of Precursor LCs and Mature LCs Inhibit This Stimulatory Effect

Primary cell culture and colorimetric cell proliferation ELISA were employed to analyze the effect of recombinant SCF on the proliferation of precursor LCs and mature LCs isolated from the EDS-treated rats on days 3, 10, and 20. After 24 h culture, the isolated cells were attached and BrdU was incorporated into the proliferating cells during the last 12 h of the following 24 h culture in the presence of SCF, ACK-2 + SCF, mouse IgG + SCF, or corresponding vehicle controls. Interestingly, a three- to fourfold increase of BrdU incorporation was observed in the presence of SCF at each time point in the enriched precursor LCs, while no effect was found in mature LCs under the same culture conditions (Fig. 6). ACK-2 or mouse IgG alone did not show significant effect on precursor LC proliferation (data not shown). When the cells were preincubated with ACK-2 (5 μg/ml) for 4 h followed by SCF (100 ng/ml) stimulation for 24 h, no significant effect on the proliferative activity of precursor LCs was found comparing to the precursor LC-only group. However, when cells were pretreated with mouse IgG followed by SCF stimulation, the stimulatory effect of SCF was maintained at all three time points. Surprisingly, the stimulatory effect of SCF was almost totally abolished when precursor LCs were cocultured with

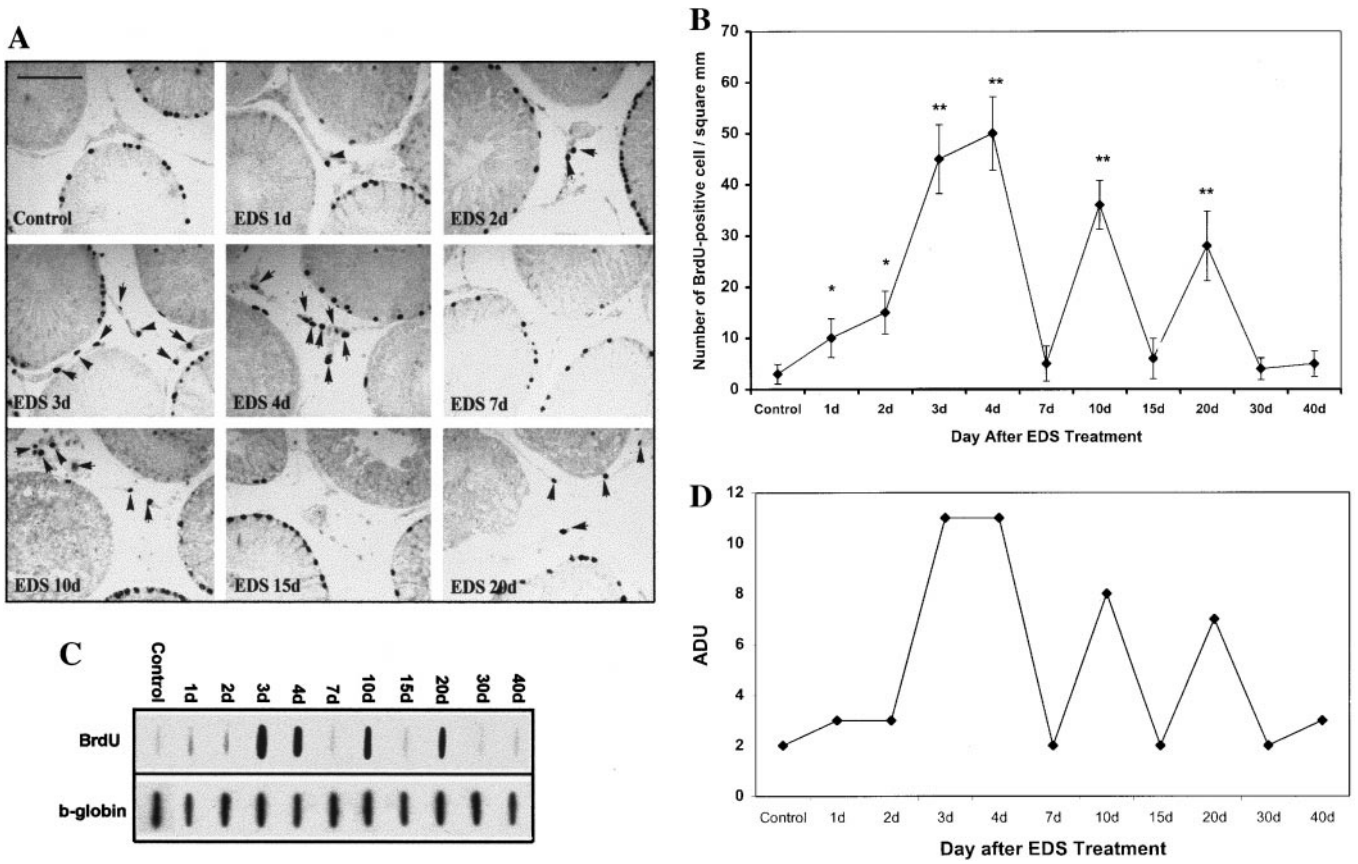

FIG. 4. Visualization of proliferating precursor LCs by immunohistochemical detection of incorporated BrdU and quantification of proliferative activity of precursor LCs during LC regeneration after depletion by means of EDS. (A) Photomicrographs of immunohistochemical detection of BrdU incorporated into proliferating precursor LCs and germ cells. Arrows point to BrdU-positive precursor LCs. Bar, 100 μm and all images are of the same magnification. (B) Quantitative analysis of proliferative activity of precursor LCs during the whole regeneration process. Proliferative activity is represented by the number of BrdU-positive precursor LCs per square millimeter cross section. Data are represented as means ± SEM ($n = 15$). $^*P < 0.05$, $^{**}P < 0.01$, compared with the controls. (C) A representative result of DNA BrdU content assay. DNA was isolated from purified LCs and an aliquot of 100 ng of DNA was blotted onto a nylon membrane and subjected to immunodetection using an anti-BrdU antibody and an ECL system. The evenness of blotting was monitored by hybridization with a PCR-generated β-globin cDNA probe labeled with [^{32}P]dCTP. (D) Quantitative analysis of DNA BrdU content assay. Data are representative of three independent experiments. ADU, arbitrary densitometric unit.

mature LCs at a ratio of 1:1 (Fig. 6). Interestingly, no significant effect on precursor LC proliferation was observed when the ratio of precursor LCs/mature LCs was higher than 1:1 (data not shown).

Blockade of SCF/c-Kit Interaction Affects Testosterone Production by Regenerated LCs

Administration of ACK-2 for 4 days to normal adult rats did not cause significant changes in testosterone levels even though massive spermatogonial apoptosis was induced. The observation is consistent with that of a previous report (Yoshinaga *et al.*, 1991). Regenerated LCs started to produce testosterone around day 20 after EDS treatment. Hormone levels in the EDS + ACK-2 group and the EDS-only group were measured to see if ACK-2 administration affects T production by newly formed LCs. As shown in Fig. 7, serum T levels in the EDS + ACK-2 group were significantly higher than those in the EDS-only group (Fig. 7, bottom; $P < 0.01$, $n = 3$). Consistently, LH levels in the EDS + ACK-2 group were significantly lower that those in the EDS-only group (Fig. 7, top; $P < 0.05$, $n = 3$). No significant difference was observed in FSH levels between these two groups during the three periods of ACK-2 treatment and FSH levels in both groups were all higher than those in the control groups (data not shown).

Precursor LCs Express Kit

It has long been known that mature LCs express c-kit protein. To see if precursor LCs are also c-kit-positive,

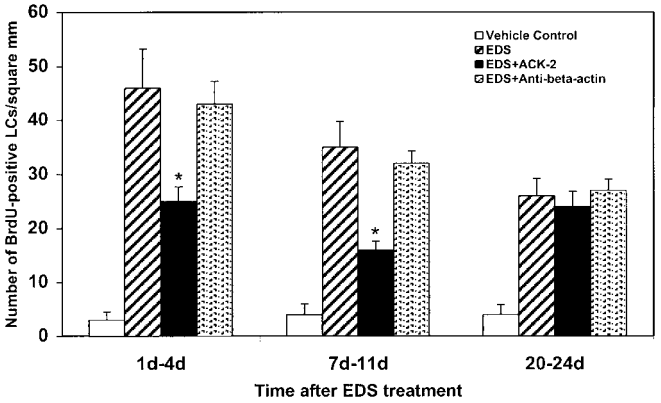

FIG. 5. Quantitative analysis of the effect of blockade of SCF/c-kit interaction on the proliferative activity of precursor LCs after EDS treatment. Proliferative activity is represented by the number of BrdU-positive precursor LCs per square millimeter of cross section, as described for Fig. 4. The rats that received injection of vehicle displayed sporadic and a few BrdU-positive interstitial cells at all three time points. As a control, a mouse monoclonal anti-β-actin antibody was injected at dose and time points similar to ACK-2. Data are presented as means ± SEM of 15 sections from 3 rats of each group. $^*P < 0.05$ compared with the EDS group.

immunohistochemical staining using a rabbit anti-c-kit polyclonal antibody, M-14, was carried out using testis sections from rats at days 4, 7, and 20 after EDS treatment (Figs. 8A–8C). Kit protein was exclusively localized to the cytoplasmic membrane of spermatogonia, precursor LCs, and regenerated (differentiated) LCs. Preabsorbed antibody was used as a control and no specific staining was observed (Fig. 8D)

Down-regulated SCF mRNA Levels Correlate with LC Apoptosis after EDS Treatment

To see how SCF levels are regulated during LC depletion and regeneration after EDS treatment, both Northern blotting and semiquantitative RT-PCR were performed. An oscillating pattern of SCF mRNA levels during these processes was observed (Fig. 9). After EDS administration Northern hybridization showed a significant reduction in SCF mRNA levels within 48 h in comparison with the controls (Figs. 9A and 9B). Since Northern blotting cannot distinguish the two forms of SCF mRNAs (named KL1 and KL2), a semiquantitative RT-PCR system was set up to monitor their changes. RT-PCR analysis revealed that it was KL1, not KL2, that was significantly down-regulated on days 1–2 after EDS treatment (Figs. 9C and 9D).

Up-regulated KL1 mRNA Levels Correlate with the Proliferative Activity of Precursor LCs

A fivefold increase in SCF mRNA levels on days 3 and 4 and two- to threefold increases on days 10 and 20 after EDS treatment were observed in Northern hybridization analysis in comparison with controls (Figs. 9A and 9B). The results were further confirmed by quantitative RT-PCR analysis (Figs. 9C and 9D). On days 3 and 4, KL1 mRNA levels were three- to fourfold higher than in the controls. On days 10 and 20, a threefold increase in KL1 mRNA

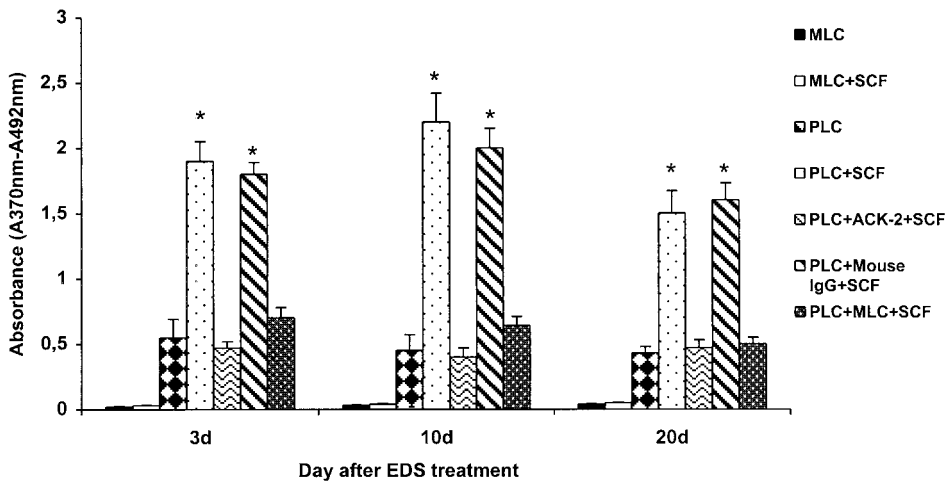

FIG. 6. Effect of SCF on the proliferation of precursor LCs *in vitro*. Precursor (PLC) and mature (MLC) LCs isolated from rats at days 3, 10, and 20 after EDS treatment were cultured at a density of 10^4 cells/well/100 μl medium in 96-well plates for 24 h at 37°C. The medium was then changed and the cells were incubated for 12 h in fresh medium containing vehicle (control), recombinant mouse SCF (100 ng/ml), ACK-2 (5 μg/ml) + SCF, or mouse IgG (5 μg/ml) + SCF. An aliquot of 10 μl of BrdU solution (100 μM) was then added to each well and incubation was continued for another 12 h. Colorimetric ELISA was then performed according to the instructions of the supplier. $^*P < 0.01$ ($n = 9$) compared with PLC groups.

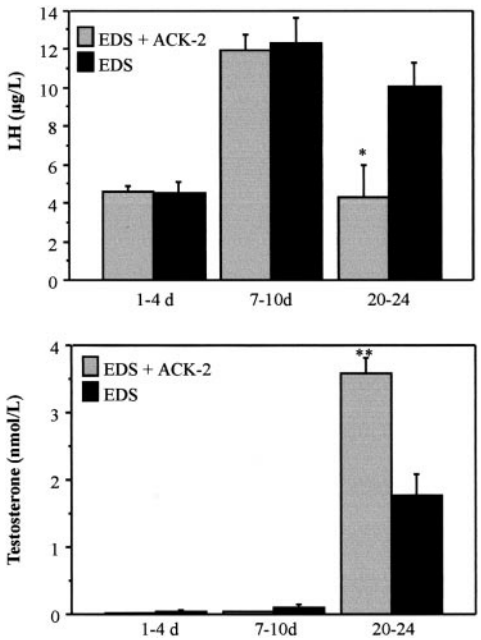

FIG. 7. Effect of blockade of SCF/c-kit interaction on testosterone production by regenerated LCs. ACK-2 antibody was injected during days 1–4, 7–10, and 20–24 after EDS treatment, corresponding to the three proliferation peaks of precursor LCs. Serum LH and T levels were measured. Data are presented as means ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$, compared with the EDS-only group.

levels was detected. No significant variations in KL-2 mRNA levels were detected 3–40 days after EDS treatment.

DISCUSSION

The membrane-associated form of SCF appears to be more important for primordial germ cell migration, adhesion, survival, and proliferation, since some types of *Sl* mutant mice produce soluble SCF, but still display impaired germ cell development and spermatogenesis (for review see Loveland and Schlatt, 1997). However, the physiological role of soluble SCF during testicular development and spermatogenesis is largely unknown although both soluble and membrane SCF are highly expressed within the testis and both can interact with c-kit (Anderson *et al.*, 1990; Langley *et al.*, 1992; Allard *et al.*, 1996; Blanchard *et al.*, 1998). Given that c-kit is located on LCs in addition to spermatogonia and spermatocytes and soluble SCF could be secreted and reach the interstitium, it is plausible to assume that soluble SCF might be involved in LC development and/or differentiation. In the present study, revealing the time points corresponding to the peak of mature LC apoptosis and three waves of precursor LC proliferation after EDS treatment using TUNEL staining and immunohistochemical detection of incorporated BrdU enabled us to block SCF/c-kit interaction with ACK-2 antibody during these specific periods and to see the effects.

Several groups of investigators have verified that EDS depletes LCs through apoptosis (Tapanainen *et al.*, 1993;

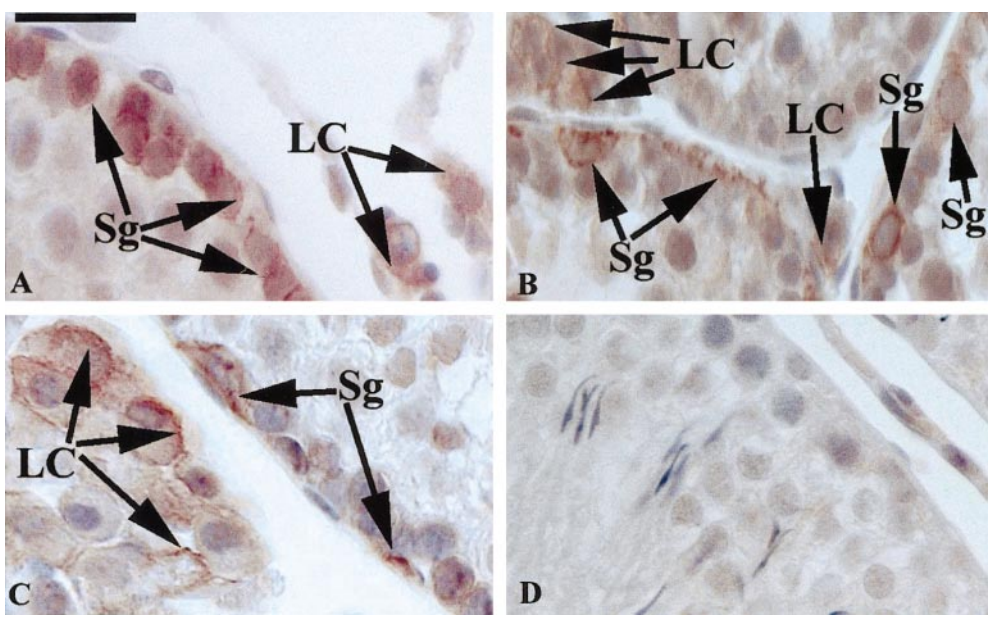

FIG. 8. Immunohistochemical detection of c-kit on precursor Leydig cells. Cross sections of rat testis on days 4 (A), 7 (B), and 20 (C) after EDS treatment were stained by using M-14 (1:200 dilution). Specific staining appears to be on the cytoplasmic membrane of precursor or mature Leydig cells (LC) and spermatogonia (Sg). Sections stained with preabsorbed antibody were used as control (D). Bar, 50 μm.

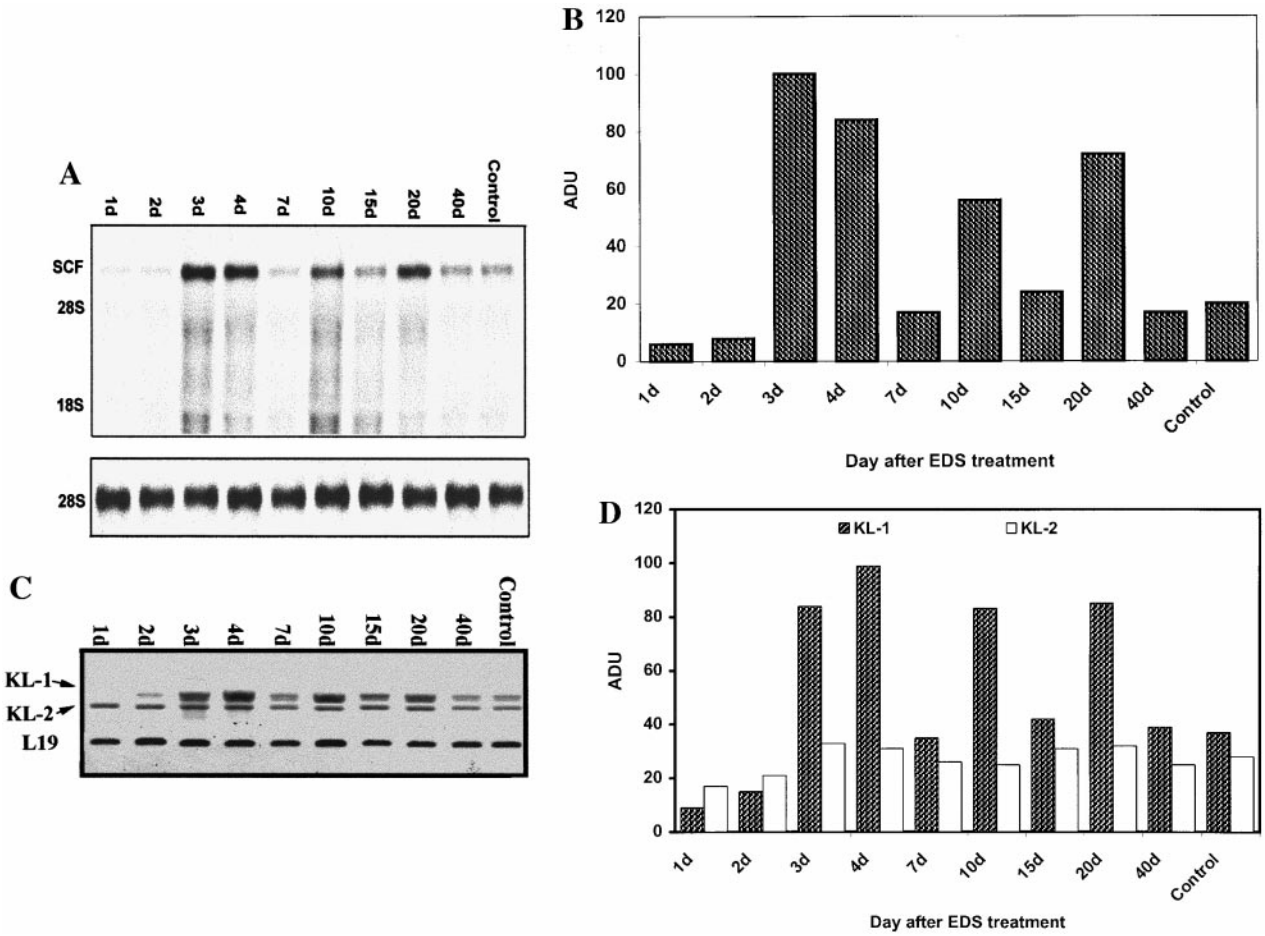

FIG. 9. Testicular SCF mRNA levels during LC apoptosis and regeneration after EDS treatment. (A) A representative Northern hybridization result. Ten micrograms of total RNA was loaded in each lane and hybridized with an SCF-specific riboprobe labeled with [^{32}P]UTP. The evenness of loading was monitored by rehybridization with a 28S cDNA probe. (B) Quantitative analysis of Northern blotting results. Data are representative of three independent experiments. ADU, arbitrary densitometric unit. (C) A representative quantitative RT-PCR result showing changes in the levels of the two forms of SCF mRNAs (KL-1 and KL-2) after EDS treatment. One microgram of total RNA was reverse-transcribed followed by PCR amplification using a pair of primers encompassing exon 6. The sizes of KL1 and KL2 appeared to be 699 and 613 bp, respectively. (D) Quantitative analysis of RT-PCR results. Data are representative of three independent experiments. ADU, arbitrary densitometric unit.

Henriksen *et al.*, 1995; Taylor *et al.*, 1998). However, the mechanism by which EDS triggers LC apoptosis remains unclear. After EDS-mediated depletion, LC repopulation can occur from interstitial mesenchymal-like precursor cells (Teerds, 1996). The identity of the repopulating LCs has been very well characterized by checking various structural and functional markers, in a recent study (Teerds *et al.*, 1999). Given the fact that the regeneration of LCs from precursor cells after EDS treatment is independent of LH and other pituitary hormones, it has been speculated that locally produced growth factors might play an important role (for review see Teerds, 1996). However, the nature of these factors remains unknown. In the present study, the finding that blockade of SCF/c-kit interaction during days 1–2 after EDS treatment could accelerate mature LC apoptosis, and that the levels of KL-1 mRNA, which represents the major source of soluble SCF, were significantly suppressed during the first 2 days after EDS treatment, suggests that soluble SCF might be involved in the apoptosis of mature LCs induced by EDS. However, no induction of LC apoptosis after administration of ACK-2 to adult male mice was shown in a previous study (Yoshinaga *et al.*, 1991) or by our own observations (data not shown). This might reflect the fact that the presence or absence of other factors might be required for induction of LC apoptosis and blockade of the prosurvival effect of SCF alone is not enough to trigger this process. Alternatively, it is also possible that iv injection of ACK-2 did not totally block soluble SCF/c-kit

interaction and failed to generate a situation similar to that brought about by EDS in suppressing SCF expression and thus could not induce LC apoptosis.

Interestingly, immediately after LCs underwent apoptosis, precursor LCs started to proliferate and this proliferation first peaked on days 3–4, when the apoptotic LCs were not yet completely diminished. Blockade of SCF/c-kit interaction significantly inhibited the proliferative activity of precursor LCs during the first wave of proliferation. Similarly, this phenomenon was also observed in the second wave of precursor LC proliferation around day 10. It is noteworthy that a striking elevation in KL-1 mRNA levels correlated with these two peaks of proliferation. These findings strongly suggest that soluble SCF might be able to stimulate proliferation of precursor LCs during the first two waves of proliferation. Using primary culture in conjunction with cell proliferation ELISA, we analyzed the effect of recombinant SCF on the proliferative activity of precursor LCs and mature LCs isolated at days 3, 10, and 20 after EDS treatment. The stimulatory effect of SCF on the proliferation of precursor LCs rather than mature LCs *in vitro* corroborated our finding that administration of ACK-2 inhibited the proliferation of precursor LCs after EDS treatment *in vivo*. Abolishment of this effect by preincubation with ACK-2 further proved that this effect was mediated through SCF/c-kit interaction. The fact that ACK-2 administration on days 1–4 and 7–11 after EDS treatment could not completely abolish the proliferation of precursor LCs suggests that either some other factors were involved in the process as well or this antibody injection-based method can only partially and never totally block the function of soluble SCF. The latter is likely, because in the present study spermatogonia in the seminiferous epithelium were not completely depleted but were significantly reduced in number 4 days after ACK-2 treatment (data not shown).

Oscillation of KL-1 mRNA levels during LC death and recovery is of great interest, since it implies that it is the soluble form of SCF that is involved and strictly regulated in these two specific cellular events induced by EDS. The mechanisms behind the oscillating levels of KL-1 mRNA seem to be complex, since both T and FSH concentrations changed dramatically during the regeneration process after EDS treatment. One possibility could be that T might be able to suppress SCF expression normally and when T levels decline to certain levels as a result of LC depletion, the suppressive effect is removed and consequently, KL-1 mRNA levels are up-regulated. However, the unaffected levels of KL-1 mRNA on days 7 and 15 do not support this hypothesis, since at these time points T levels were undetectable. Therefore, SCF mRNA levels may not be directly regulated by T even though the involvement of T regulation cannot be completely excluded. This is also supported by the results of two previous studies showing no effects of T on SCF gene expression *in vitro* (Taylor *et al.*, 1996; Yan *et al.*, 1999). During the whole regeneration period, FSH levels in the EDS-treated groups remained higher than in the controls and the fluctuation of FSH levels correlated very well with that of KL-1 mRNA levels. On the basis of previous studies showing that SCF gene expression is mainly regulated by FSH through the cAMP/PKA pathway (Rossi *et al.*, 1991, 1994; Taylor *et al.*, 1996; Yan *et al.*, 1999), it is plausible to suggest that the oscillation of KL-1 mRNA levels results from FSH regulation. The fact that FSH levels were higher in the EDS-treated rats than in the controls is also documented in two previous studies (Henriksen *et al.*, 1995; Tena-Sempere *et al.*, 1997). An intriguing question arising from these observations is how FSH levels became elevated after EDS treatment. One possibility might be that the increased FSH levels were a direct result of the decreased T levels via stimulation of the GnRH pathway (Plant, 1986). Another possibility might be that EDS could suppress some factors, such as inhibin and follistatin (Halvorson and DeCherney, 1996), which can negatively regulate FSH, and result in increased FSH levels. However, our recent data indicated that inhibin levels, in fact, were elevated, rather than inhibited, in EDS-treated rats (Tena-Sempere *et al.*, 1999). Thus, it will be interesting to see how follistatin expression is regulated in EDS-treated animals. The suppressive effects of the factors involved might be T-dependent or T-independent. All these hypotheses remain to be clarified in future studies.

To our surprise, the third wave of precursor LC proliferation was not significantly affected by ACK-2 administration. However, the proliferative activity of precursor LCs isolated at day 20 after EDS treatment was found to be elevated in the presence of SCF when they were cultured *in vitro*. The discrepancy appeared to be caused by the inhibitory effect of mature LCs on the proliferation of precursor LCs, as shown in our primary cell culture experiment. A comparable situation could be found around day 20 after EDS treatment, when an appreciable proportion of LCs had already differentiated and started to function. This is a very intriguing finding because it strongly suggests that mature LCs could suppress precursor LC proliferation probably by secreting some factor(s) that may antagonize the SCF/c-kit-mediated proliferation-stimulatory effect. This might be a mechanism by which mature Leydig cell numbers are controlled during prepubertal LC development and LC repopulation after depletion by means of EDS. Moreover, it is noteworthy that around day 20 the new LCs are already functional, as manifested by the significantly elevated levels of T in comparison with those on days 2–10. It is likely that the factor(s) that mature LCs may secrete to suppress precursor LC proliferation might be under the regulation of T.

The finding of a significant elevation of T levels in the ACK-2-treated group in comparison with the untreated group between days 21 and 24 after EDS treatment is consistent with the results of a previous study showing that administration of ACK-2 to adult male mice results in a transient elevation of T concentrations, although it was regarded as insignificant (Yoshinaga *et al.*, 1991). In the present study, the increased serum T levels in the EDS + ACK-2 group were accompanied by significantly decreased LH levels, compared with the EDS-only group, indicating

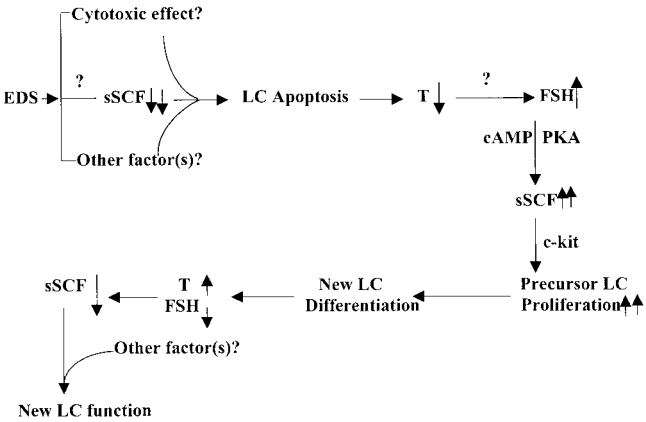

FIG. 10. Schematic presentation of a proposed mechanism by which SCF acts as a survival factor for mature LCs and a growth factor during precursor LC proliferation after EDS treatment. EDS may affect soluble SCF production by Sertoli cells and the severely reduced levels of soluble SCF fail to provide enough support for mature LC survival. Consequently, LCs undergo apoptosis. Activation of LC apoptosis may also involve other factors and the cytotoxic effect of EDS. Serum T levels decrease dramatically due to the massive death of mature LCs. This stimulates FSH, which in turn up-regulates soluble SCF levels via the cAMP/PKA pathway. The up-regulated soluble SCF reaches interstitial precursor LCs and interacts with c-kit on these cells to stimulate proliferation. After two waves of proliferation of precursor cells, these new LCs differentiate and start to produce T. The resumed level of T brings down FSH to control levels. Consequently, the production of soluble SCF in Sertoli cells declines to control levels and SCF/c-kit interaction starts to affect T production rather than proliferation.

that the increased levels of T were not artifacts. The physiological significance of elevated T levels after ACK-2 administration remains unclear. It might imply that SCF/c-kit interaction switches its function from stimulating proliferation to regulating steroidogenesis. One recent elegant study using a "knock-in" technique showed that LCs displayed hyperplasia and T levels appeared to be normal but LH levels were significantly elevated in transgenic mice bearing a mutation at the PI3 kinase binding site of c-kit receptor (Kissel *et al.*, 2000). In these mice the PI3 kinase/Akt kinase-mediated c-kit downstream signaling transduction pathway was totally abolished. The phenotype of LCs in the mutant mice suggests that the PI3 kinase/Akt kinase-mediated c-kit downstream signaling pathway might be involved in the negative control of LC proliferation and steroidogenesis. However, another independent group using similar strategy failed to show the phenotypes in LCs (Blume-Jensen *et al.*, 2000). Since activation of c-kit by SCF could in fact activate multiple signaling transduction pathways, the effect of other signaling pathways and the net effect of the interaction of these pathways on LC proliferation and T production would be of great interest for better interpreting the present findings and for gaining more insight into the physiological role of c-kit on LCs.

Figure 10 depicts a proposed mechanism by which soluble SCF supports mature LC survival and stimulates precursor LC proliferation after EDS treatment. It is suggested that EDS may directly and/or indirectly inhibit soluble SCF production by Sertoli cells and thus suppress its prosurvival effect on mature LCs. Consequently, LCs undergo apoptosis. As discussed earlier, it is most likely that activation of LC apoptosis also involves other factors and the cytotoxic effect of EDS. The dramatically decreased serum T level due to massive death of mature LCs stimulates FSH, which in turn up-regulates the levels of soluble SCF. The up-regulated soluble SCF reaches its targets, interstitial precursor LCs, and interacts with c-kit on these cells to stimulated proliferation. After two waves of proliferation, the new LCs differentiate and start to produce T. The resumed level of T brings FSH down to control levels. As a result, production of soluble SCF in Sertoli cells declines to control levels and SCF/c-kit interaction starts to affect T production rather than cell proliferation.

Taken together, the findings of the present study provide evidence, for the first time, that SCF functions as a survival factor for mature LCs and a growth factor during regeneration of the LC population from precursor LCs after LC depletion by means of EDS. Furthermore, SCF also participates in regulating T production by mature LCs. The proposed model provides new insight into the mechanisms of LC apoptosis and regeneration after EDS treatment, which might be applicable to the regulation of adult-type LC development in the prepubertal rat testis, given the fact that the regeneration process of precursor LCs after LC depletion by means of EDS mimics the development of adult-type LCs during prepubertal life.

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