Murine male germ cell apoptosis induced by busulfan treatment correlates with loss of c-kit-expression in a Fas/FasL- and p53-independent manner

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Abstract Male germ cell apoptosis has been extensively explored in rodents. In contrast, very little is known about the susceptibility of developing germ cells to apoptosis in response to busulfan treatment. Spontaneous apoptosis of germ cells is rarely observed in the adult mouse testis, but under the experimental conditions described here, busulfan-treated mice exhibited a marked increase in apoptosis and a decrease in testis weight. TdT-mediated dUTP-X nicked end labeling analysis indicates that at one week following busulfan treatment, apoptosis was confined mainly to spermatogonia, with lesser effects on spermatocytes. The percentage of apoptosis-positive tubules and the apoptotic cell index increased in a time-dependent manner. An immediate effect was observed in spermatogonia within one week of treatment, and in the following week, secondary effects were observed in spermatocytes. RT-PCR analysis showed that expression of the spermatogonia-specific markers c-kit and Stra 8 was reduced but that Gli I gene expression remained constant, which is indicative of primary apoptosis of differentiating type A spermatogonia. Three and four weeks after busulfan treatment, RAD51 and FasL expression decreased to nearly undetectable levels, indicating that meiotic spematocytes and post-meiotic cells, respectively, were lost. The period of germ cell depletion did not coincide with increased p53 or Fas/FasL expression in the busulfan-treated testis, although p110Rb phosphorylation and PCNA expression were inhibited. These data suggest that increased depletion of male germ cells in the busulfan-treated mouse is mediated by loss of c-kit/SCF signaling but not by p53- or Fas/FasL-dependent mechanisms. Spermatogonial stem cells may be protected from cell death by modulating cell cycle signaling such that E2F-dependent protein expression, which is critical for G1 phase progression, is inhibited.

Keywords: Spermatogonial stem cell; Cell cycle; Mouse testis; Busulfan; Apoptosis

1. Introduction

Germ cell apoptosis can be induced by pathological conditions such as heat stress, exposure to ionizing radiation or toxic substances, hormonal depletion, and loss of stem cell factor signaling [1–6]. Busulfan is used for the treatment of ovarian cancer and chronic myeloid leukemia [7]. However, in the mouse and rat testis, busulfan treatment results in marked spermatogenic cell death, with the exception of a small subpopulation of cells attached to the basal membrane of the seminiferous epithelium. The busulfan-induced depletion of spermatogenic germ cells is reversible and animals generally regain fertility, albeit to varying degrees, following the active re-proliferation of surviving germ cells [8–13]. Therefore, busulfan is also commonly used to examine spermatogonial stem cell function and to prepare a recipient testis for germ cell transplantation during treatment for male infertility.

Apoptotic signaling during male germ cell loss has been demonstrated for a limited number of genes, including Fas/FasL and p53. The Fas system is a well-known apoptotic signal transduction pathway in which a ligand–receptor interaction triggers the cell death pathway [14,15]. Previous studies reported the spontaneous apoptosis of testis germ cells in BALB/c and lpr/lpr mice [16,17]. Since lpr/lpr mice are deficient in Fas, these results indicate that Fas is not involved in spontaneous germ cell apoptosis, although it may be partially involved in cryptorchidism-induced apoptosis [17]. Furthermore, the tumor suppressor protein p53 is known to be involved in apoptosis in the testis [18,19], and the expression of p53 mRNA and protein in primary spermatocytes suggests that p53 plays a role in meiotic prophase. In irradiated testes, the expression of p53 is enhanced not only in spermatocytes but also in spermatogonia [5,20]. These results suggest that p53 is important in the regulation of apoptosis in spermatocytes and spermatogonia.

Chemotherapeutic drugs such as procarbazine and other alkylating agents produce prolonged and sometimes irreversible azoospermia in humans [21]. Chemotherapy with alkylating agents may trigger several cellular mechanisms in both

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germ cells and somatic cells. Cytotoxic effects are exerted via the transfer of the alkyl group(s) to various cellular constituents; however, DNA alkylation events within the nucleus probably constitute the major provocations that lead to cell death [22]. A previous study reported that round spermatids are highly sensitive to alkylating agents such as cyclophosphamide [23], but a repeat of this study using quantitative RT-PCR failed to demonstrate their hypersensitivity to alkylating agents. Since the precise molecular target of alkylating agents differs according to their chemical structure, it is possible that the biological effects and target(s) of busulfan are different from those of cyclophosphamide. Indeed, the mechanisms by which apoptosis is induced in male germ cells after chemotherapy are not fully understood. Following busulfan treatment, spermatogonial stem cells do not die, suggesting that they harbor factors for resistance to stressful stimuli. Thus, the purpose of this study was to define which cell types are more sensitive to stress induced by busulfan treatment and to investigate the molecular mechanism of cell death suppression in spermatogonial stem cells following busulfan treatment.

2. Materials and methods

2.1. Animals and busulfan treatment

One hundred twenty ICR male mice ranging in age from 8 to 12 weeks (30–40 g) were used. Eight-week-old ICR male mice received a single intraperitoneal injection of busulfan (40 mg/kg body weight) diluted in sesame oil as previously described [10,11]. The mice were housed in wire cages at 22 ± 1 °C under a 12-h light–dark cycle with 70% humidity and fed ad libitum. Animals were maintained and experiments were conducted in accordance with the Gyeongsang National University Guide for the Care and Use of Laboratory Animals.

2.2. TUNEL assay (TdT-mediated dUTP-X nicked end labeling)

Testes were fixed in 4% (w/v) paraformaldehyde in 0.01 M PBS (pH 7.4), washed in PBS, dehydrated in ethanol (70%, 90%, and 100%) and embedded in paraffin wax. Testicular sections (5 μm) were rehydrated (xylene 5 min; ethanol 100%, 95%, 70%, 2 min each) and washed in distilled water prior to TUNEL staining. The sections were incubated for 15 min with proteinase K (20 μg/mL) at room temperature and washed with PBS (1×), and endogenous peroxidase activity was blocked with 2% H_2O_2 for 5 min. Sections were re-washed three times with PBS (1×) and incubated for 60 min at 37 °C in a moist chamber with the TUNEL mix (0.3 U/μL calf thymus terminal deoxynucleotidyl transferase, 7 pmol/μL biotin dUTP, and 1 mM cobalt chloride in 1× reaction buffer in distilled water). After washing (four PBS baths of 5 min each at RT), the sections were saturated in 2% BSA for 10 min at RT. Sections were treated for 30 min at 37 °C in a moist chamber with a 1:20 dilution of ExtraAvidin peroxidase antibody. After three PBS washes, detection was performed with DAB [1.24 mg DAB, 25 μL of 3% $NiCl_2$, and 152 μL of 1 M Tris–HCl (pH 7.5) in 2 ml distilled water]. Slides were mounted in crystal mount (Biomeda, Foster City, CA).

2.3. RT-PCR analysis

Total RNA was isolated from testes with Trizol and RT-PCR was performed with the Gibco RT-PCR system. Denaturation was for 30 s at 94 °C, annealing for 30 s at 50–60 °C, and synthesis for 1 min at 72 °C. The primers for each germ cell-specific marker are shown in Table 1.

2.4. Laser scanning cytometry (LSC)

Stained cells were analyzed on a three-color fluorescence activated cell sorter (FACScan; Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) using CELL Quest software (Becton-Dickinson Immunocytometry Systems). Dead cells and monocytes were excluded by forward and side-scatter gating. An average of 20 000 events was acquired depending on the cytokine studied. Statistical markers were set using labeled isotype monoclonal antibody-blocked negative controls as a reference.

2.5. Apoptosis assay by flow cytometry

Detection of apoptotic cells was based on the principle of Annexin V binding to translocated plasma membrane phosphatidylserine (PS) as previously described [44,45]. Briefly, cells were washed, trypsinized, pelleted, resuspended in staining buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 5 mM $CaCl_2$) containing 1 μg/ml PI and 5 μl Annexin V (BD PharMingen), and incubated for 15 min. The cell mixture was then subjected to analysis by a flow cytometer (FACSCalibur, Becton-Dickinson). A minimum of 3×10^4 events was collected for each sample. Analysis of the multivariate data was performed with CELLQuest software (Becton-Dickinson).

2.6. Immunohistochemical analysis

Immunohistochemical staining was performed as previously described, with slight modifications [10]. Tissue for immunostaining was cleared in Histoclear for approximately 10 min and dehydrated in decreasing concentrations of ethanol. Immunohistochemistry was performed according to standard procedures provided by the manufacturer (Mouse, Rabbit and Rat UniTectTM Immunohistochemistry System, Oncogene Science, Inc.). Sections were placed in 3% peroxide in pure methanol and in 0.1% pepsin in 0.05 N HCl (pH 2.25) for 30 min each to reduce background staining. Sections were washed twice (5 min each) in TBS (0.05 M Tris–HCl, pH 7.4, and 0.85% NaCl), blocked with normal horse serum diluted in TBS (1:5; NHS–TBS), and incubated overnight with primary Rb (Pharmingen) and PCNA (Pharmingen) antibodies diluted 1:250 in NHS–TBS. One drop of horse serum was used as a negative control. Excess antibody was removed by washing twice for 5 min with TBS, and then biotinylated secondary IgG or IgG2a was added for 30 min, followed by washing with three changes of TBS for 5 min. Color was developed with a solution of 0.5% diaminobenzidine in 0.05 M Tris–HCl (pH 7.6) containing 0.01% hydrogen peroxide, after which sections were washed in water, dehydrated, and mounted with a coverslip.

2.7. Immunoblot analysis

Decapsulated testes from mice were snap-frozen in liquid nitrogen in lysis buffer [50 mM Tris · HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM α-D-octylglucoside, 1 mM EDTA, 14 mM mercaptoethanol, protease inhibitor cocktail (5 μl/0.1 g, Sigma), and 2 mM PMSF]. After sonication for 20 s, the lysates were cleared by centrifugation at 15 000 rpm for 15 min at 4 °C. Protein extracts were analyzed by 7.5% SDS-PAGE and electroblotted to PVDF membranes. Membranes were treated with antibodies to Rb (Pharmigen, 1:100) or PCNA (Pharmigen, 1:100) and FasL (Pharmigen, 1:100) followed by peroxidase-conjugated anti-mouse IgG secondary antibody (1:25 000, ImmunoTech). An anti-actin antibody (1:500; Chemicon International) was used to verify equal protein loading. Signals were visualized using an ECL kit (Amersham) and bands were quantified by densitometry and image analysis using Image J 1.23 (NIH image).

2.8. Data analysis

All experimental data are presented as means ± S.D. Each experiment was performed at least three times and subjected to statistical analysis. Representative experiments are presented in the Figures. For statistical analysis, one-way analysis of variance (ANOVA) was performed to determine whether there were differences among all groups ($P < 0.05$), and Fisher's post-test was performed to determine significance between pairs of groups. A P value below 0.05 was considered significant. Statistical tests were performed using StatView software version 5.0 (SAS Institute Inc, Cary, NC).

3. Results

3.1. Effects of busulfan treatment on testicular weights and histology

We initially investigated the effects of busulfan on the whole testis and on developing male germ cells, as visualized in sections of mouse testes. The body weight of both control and busulfan-treated mice increased steadily from 8 to 12 weeks. Testicular weight also increased steadily in control animals for the duration of the experiment. In contrast, testis weight

Table 1
Primers and conditions used for semi-quantitative RT-PCR

Primer name	Primer sequence	PCR conditions	Size (bp)	Ref./GenBank
c-kit	F: GCATCACCATCAAAAACGTG R: GATAGTCAGCGTCTCCTGGC	Tm: 58 °C 35 cycles	332	NM021099
GLI 1	F: ACTGGGGTGAGTTCCCTTCT R: GCTGAGCTTTGAGCTGTCCT	Tm: 61.5 °C 40 cycles	357	[27]
Odc	F: CATCCAAAGGCAAAGTTGGT R: GCCTGGCTCAGCTATGATTC	Tm: 60 °C 30 cycles	393	NM013614
Stra8	F: GCCAGAATGTATTCCGAGAA R: CTCACTCTTGTCCAGGAAAC	Tm: 57 °C 29 cycles	650	[43]
P53	F: GGAGTATTTGGACGACCG R: TCAGTCTGAGTCAGGCCC	Tm: 51 °C 35 cycles	350	NM011640
Prohibitin	F: GTGGCGTACAGGACATTGTG R: AGCTCTCGCTGGGTAATCAA	Tm: 57 °C 30 cycles	307	NM008831
SRF-1	F: TCCATTCAGCACCTTCAACA R: TCATCCAAATGGAAAGAGCC	Tm: 60 °C 30 cycles	304	[29]
Mak	F: TGGGAAGTATAATGGCCGAG R: TGCAGAAGAGCCCAGTACCT	Tm: 57 °C 30 cycles	326	NM008547
Iba 1	F: CGATGATCCCAAATACAGCA R: GACCAGTTGGCCTCTTGTGT	Tm: 60 °C 30 cycles	314	[31]
Bcl-2	F: TAAGCTGTCACAGAGGGGCT R: TGAAGAGTTCCTCCACCACC	Tm: 65 °C 30 cycles	344	NM009741
Bax	F: CGAGCTGATCAGAACCATCA R: GAAAAATGCCTTTCCCCTTC	Tm: 58 °C 30cycle	283	NM007527
RAD51	F: GTTACCATACAGTGGAGGCTGT R: GGTGATTACCACTGCGACAC	Tm: 60 °C 30 cycles	665	NM011234
TNFα R55	F: TGCTGCACCAAGTGCCACAAAG R: CACACGGTGTTCTGAGTCTCC	Tm: 57 °C 25cycles	325	[43]
PCNA	F: GCTGACATGGGACACTTA R: CTCAGGTACAAACTTGGTG	Tm: 59 °C 30cycles	160	NM011045
Fas	F: GAGAATTGCTGAAGACATGACAATCC R: GTAGTTTTCACTCCAGACATTGTCC	Tm: 67 °C 35 cycles	374	[43]
FasL	F: CTTAGCTTCTCTGGAGCAGTCAGCGTC R: CCTTCTTCTTTAGAGGGGTCAGTGGC	Tm: 70 °C 35 cycles	300	[43]
GAPDH	F: GTGAAGGTCGGTGTGAACGG R: GATGCAGGGATGATGTTCTG	Tm: 60 °C 30 cycles	620	NM008084

dropped in busulfan-treated mice, with this decrease commencing in week 1 and reaching its lowest point four weeks after busulfan injection (33% of control value) (Fig. 1A). Testicular weight progressively recovered thereafter to reach approximately 90% of control weight at 12 weeks. Four weeks after the injection of a single dose of busulfan, male germ cells were significantly reduced in number, and most of the spermatogenic cells except spermatogonia were absent (Fig. 1B). In contrast, at all time-points investigated, somatic cells, including Sertoli and Leydig cells, appeared normal in size, shape and localization, but their numbers were slightly reduced by busulfan treatment (data not shown).

3.2. *Identification of apoptotic cells by TUNEL assay following busulfan treatment*

We previously demonstrated that busulfan treatment induces apoptosis in male germ cells in a dose- and time-dependent manner [10,11]. Busulfan was administered at the rate of 40 mg/kg body weight to induce a maximal number of apoptotic cells while minimizing the number of necrotic cells. Testicular cell apoptosis and necrosis were analyzed by flow cytometry. Exposure of mice to a single dose of busulfan caused a significant increase in the percentage of apoptotic cells ($P < 0.05$) in a time-dependent manner, as assessed by Annexin V-FITC staining (Fig. 2B).

Very few TUNEL-positive cells were detected in the testes of control adult mice and most of these were spermatocytes. However, a marked increase in the frequency of TUNEL-positive cells was observed in testis sections from mice sacrificed one week after busulfan treatment (Fig. 2A, and week 1 of Fig. 2B). This effect peaked at week 2 and then decreased gradually, with the number of TUNEL-positive cells reaching normal control levels by four weeks (Fig. 2B). One week following busulfan treatment, most TUNEL-positive germ cells were spermatogonia and a few were spermatocytes (Fig. 2A, week 1). Two to three weeks post-treatment, a marked cell-specific increase in apoptosis was observed in meiotic spermatocytes (Fig. 2A, weeks 2 and 3). At four weeks, testes from

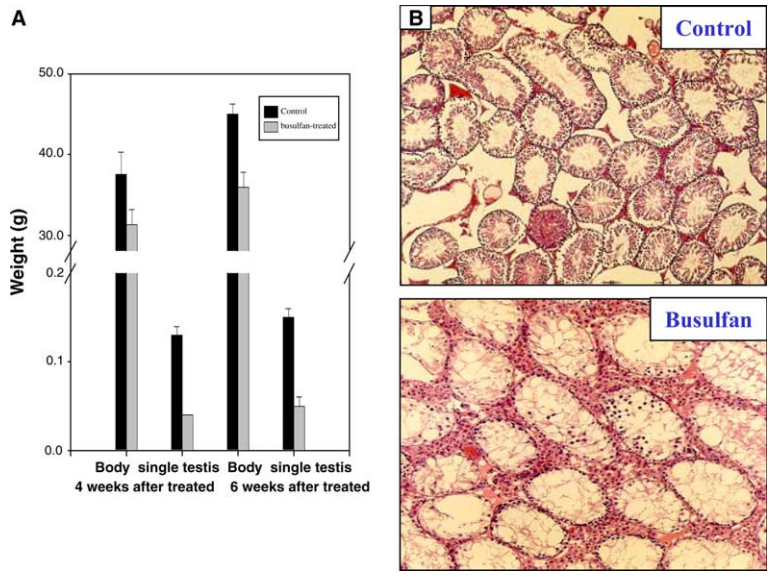

Fig. 1. Photomicrographs of cross-sections from mouse testis. (A) Body and testis weights four and six weeks after busulfan treatment. (B) Histological analysis of testis sections from 12-week-old control and busulfan-treated mice. Dewaxed paraffin sections were stained in hematoxylin and eosin. Four weeks after busulfan treatment, the testes of most busulfan-treated mice are depleted in germ cells although they contain somatic cells and spermatogonia. Magnification: 200×.

busulfan-treated mice showed a complete loss of differentiating germ cells, with the exception of some undifferentiated spermatogonial stem cells (Fig. 2A, week 4). During weeks 5 and 6, more differentiated germ cells began to appear, including pachytene cells and early spermatocytes (data not shown). This observation indicates that undifferentiated spermatogonial germ cells are more resistant than differentiated spermatogonia to busulfan toxicity and that the re-appearance of spermatocytes in busulfan-treated testes is due to the proliferation and differentiation of surviving spermatogonial stem cells.

3.3. RT-PCR analysis of male germ cell-specific marker genes

To determine which cell types are sensitive to busulfan-induced apoptosis, we performed RT-PCR for germ cell-specific markers (Table 1 and Fig. 3). At week 1 post-treatment, c-kit and Stra 8 expression was significantly decreased (Fig. 3A, panel a). However, Gli-1, which is more highly expressed in type B spermatogonia, was expressed at constant levels in the busulfan-treated testis (Fig. 3B). Since c-kit is involved in the survival of pre-meiotic germ cells, it is possible that apoptosis triggered by busulfan might be related to the decreased expression of this growth factor receptor. This would suggest that c-kit-expressing type A spermatogonia are more sensitive to busulfan-induced damage and that germ cell apoptosis is controlled in a cell type-specific fashion. One to two weeks after busulfan treatment, Rad51 mRNA decreased slightly, and by three weeks the level of transcript was significantly reduced (Fig. 3A, panel b, and Fig. 3B). However, the level of prohibitin mRNA increased from one to three weeks but decreased after four weeks (Fig. 3B). These results suggest that pachytene spermatocytes are also sensitive to busulfan and that apoptosis follows spermatogonial degeneration. The testicular expression of FasL, Iba I and TGF βRI mRNA remained constant for three weeks after busulfan treatment and then decreased at four weeks (Fig. 3B). This observation indicates that haploid germ cells had completely disappeared or died and that a third wave of apoptosis induced by busulfan involves post-meiotic spermatids.

3.4. Depletion of male germ cells is mediated by a FasL/Fas- and p53-independent pathway

It is well known that the p53 and FasL/Fas systems regulate apoptosis in germ cells [14,19]. To determine whether busulfan kills testicular germ cells through an apoptotic pathway, we performed Western blot analysis. As shown in Fig. 5, p53 and Bax protein expression was not affected by busulfan treatment. Next, we analyzed FasL and Fas protein expression levels after busulfan treatment (Fig. 5). FasL and Fas protein expression were not modified during weeks 1–3 despite the depletion of pre-meiotic germ cells that express Fas. Since the DNA-damaging agent busulfan did not result in an increased level of p53 and FasL/Fas mRNA and protein during the period of germ cell death, this result suggests that germ cell apoptosis can occur independently of p53 and FasL/Fas (Fig. 5).

3.5. Busulfan-induced hypophosphorylation of Rb prevents apoptosis of spermatogonial stem cells by inhibiting PCNA expression

Four to six weeks after busulfan injection, testis weights were significantly reduced and most spermatogenic cells were depleted. Approximately $1.5 \pm 0.8 \times 10^6$ ($n = 5$) cells per testis were recovered from busulfan-treated mice, which is about 3.5% of the cells in a normal testis ($4.3 \pm 0.9 \times 10^7$, $n = 5$). To identify the point in the cell cycle at which busulfan acts, we used flow cytometric analysis. Testicular cells were divided into three groups (1*n*, 2*n*, 4*n*) based on DNA content as assessed by PI labeling intensity (Fig. 4A). Diploid spermatogonia and somatic cells fell into the 2*n* group. Busulfan administration resulted in a gradual increase in the frequency of 2*n* cells, which corresponds to the G_0/G_1 phase (Fig. 4B), whereas the number of cells in S (1*n*) and/or G2 + M (4*n*) was significantly reduced depending on the time after busulfan treatment. At

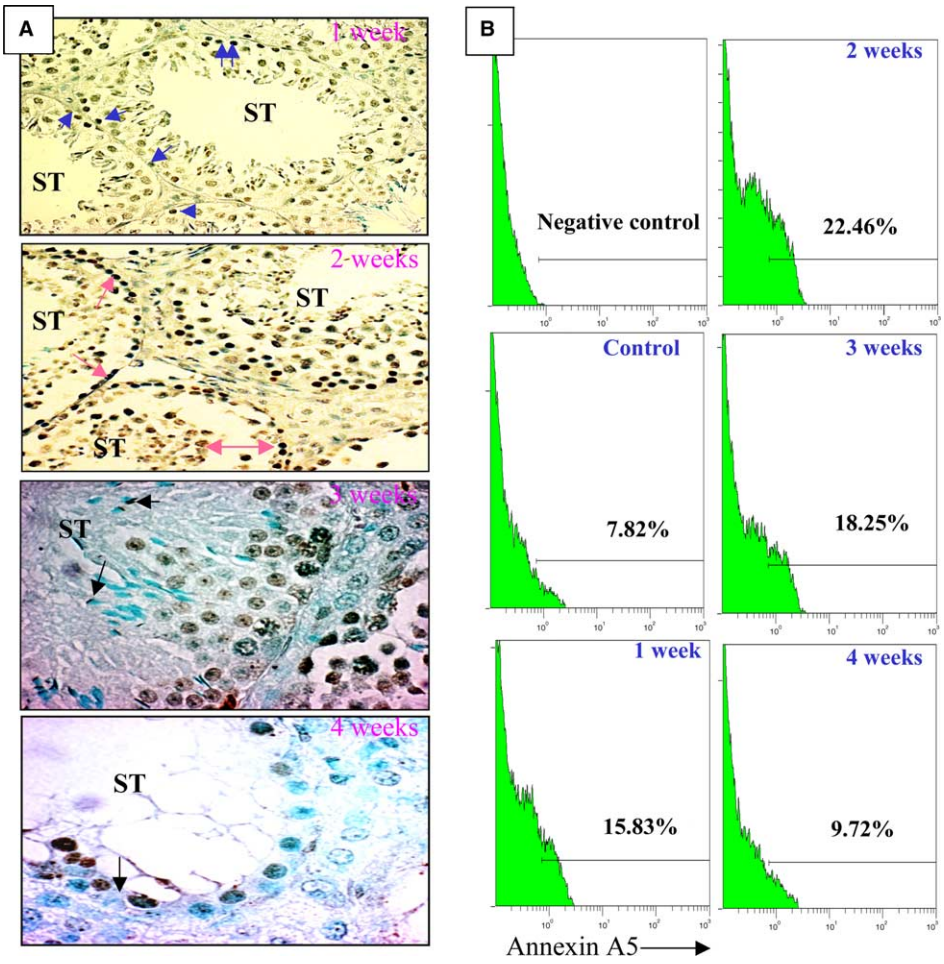

Fig. 2. Monitoring of male germ cell apoptosis following busulfan treatment by the TUNEL and annexin V assays. For assay details, see Section 2. (A) TUNEL analysis of sections prepared from one to four weeks after busulfan treatment. Male germ cell apoptosis is indicated by brown coloration. One week after treatment, apoptotic cells are limited to spermatogonia, but at two to four weeks, they include meiotic to postmeiotic germ cells. Arrows on the 1, 2, and 3 weeks panels indicate apoptotic male germ cells; arrow on the 4 weeks panel indicates a surviving spermatogonial cell. ST, seminiferous tubule. (B) A representative analysis of apoptotic cells in busulfan-treated testes as detected by Annexin V staining. Male germ cells were selected on a laminin-coated dish. and then fractionated into Annexin V-positive (apoptotic) subpopulations. The distributions of apoptotic subpopulations are shown for cells assayed with the secondary IgG alone (negative control), control cells, and cells 1, 2, 3, and 4 weeks after busulfan treatment. The frequency of Annexin 5-positive cells increases one week after busulfan treatment, peaks at week 3, and drops rapidly to control levels at four weeks.

four weeks, these cells were undetectable (Fig. 4A, panel 4 week).

Immunohistochemical analysis of normal adult (Fig. 6A, panel b) and postnatal day 5 (Fig. 6A, panel a) testes revealed extensive PCNA-positive staining in all seminiferous tubules. However, 66% (97/147) of the seminiferous tubules in busulfan-treated mice were negative for PCNA, with only a few tubules weakly staining (Fig. 6A, panel c). To determine the mechanism by which busulfan restricts spermatogonial proliferation, we profiled pRB, a phosphoprotein that controls the expression of genes necessary for progression through the cell cycle. Testes of busulfan-treated mice showed a higher level of pRB expression (Fig. 6A, panel f), whereas control adult testis showed very low levels of expression (Fig. 6A, panel e), and postnatal day 5 testes exhibited intermediate levels of pRb (Fig. 6A, panel d). Consistent with this view, Rb phosphorylation (Fig. 6C, busulfan) and PCNA expression (Fig. 6B) were inhibited in the bulsufan-treated testis, compared to that of adults or neonates (Fig. 6C and B). The hypophosphorylated form of p110Rb has been found to complex with the cellular transcription factor E2F. Thus, busulfan-induced hypophosphorylation causes p110Rb to remain bound to E2F, thereby maintaining E2F in an inactive form that cannot promote transcription of the PCNA gene (Fig. 8). Therefore, we examined the inhibitory effects of busulfan on cdk2, cdk4, cyclin D1, and cyclin E expression using RT-PCR (Fig. 7). Busulfan inhibited cdk2 and 4 as well as cyclin E, but not cyclin D transcripts, indicating that it is a specific inhibitor of cdk/cyclin complexes. Through this pathway, busulfan may prevent apoptosis in spermatogonial stem cells by inhibiting the expression of proteins critical for G1 progression, such as PCNA.

4. Discussion

This study revealed that busulfan treatment results in germ cell damage and that germ cell apoptosis mediated by loss of the c-kit/SCF pathway is independent of p53 or Fas/FasL. In experimentally induced cryptorchid animals, pre-meiotic

leptotene, zygotene, pachytene spermatocytes and first-stage spermatids are more sensitive to testicular heat stress [19,24]. Round spermatids are exquisitely sensitive to alkylating agents such as cyclophosphamide [23,25]. Since the precise molecular target of alkylating agents differs according to their chemical structure, it is possible that the biological effects and target(s) of busulfan are different from those of cyclophosphamide. Previous studies [16–19,25,37] suggested that different spermatogenic cell types exhibit remarkable variation in their susceptibility to stressful stimuli and that several apoptotic pathways are activated during male germ cell differentiation in response to different stress conditions.

We analyzed the mRNA levels of the pre-meiotic markers c-kit, Stra8, Gli-1, and Gli-3 [26,27], the meiotic markers prohibitin, PCNA, SRF, Mak, and Rad51 [28–30] and the post-meiotic markers FasL, TGF betaRI, and Iba [31,32]. In the adult testis, the c-kit receptor is expressed in differentiating spermatogonia but not in spermatogonial stem cells. However, SCF is expressed in Sertoli cells [33]. Stra8 is a retinoic acid-inducible gene which is expressed in all spermatogonia and it is present at the highest levels in preleptotene spermatocytes [26]. Therefore, c-kit, Gli-1, and Stra8 mRNA were examined to determine the relationship between susceptibility to busulfan-induced apoptosis and spermatogonial cell types (Fig. 3). At week 1 post-treatment, c-kit expression was reduced to undetectable levels. Notably, this decrease in c-kit expression occurred earlier than the increase in TUNEL-positive cells. The expression of other spermatogonial markers also decreased, albeit with different kinetic patterns as compared to c-kit. The decrease in Stra8 mRNA levels in the busulfan-treated testis was accompanied by an increase in TUNEL-positive cells. Thus, downregulation of c-kit mRNA levels appears to reflect a cellular response to busulfan treatment, whereas reduced Stra8 gene expression is closely associated with an enhancement of busulfan-induced death in differentiating spermatogonial cells and preleptotene spermatocytes. During this time, however, p53, Bax and Bcl-2 mRNA expression was not reduced. Therefore, the premature termination of spermatogenesis was accompanied by increased apoptosis, which must be due to loss of a survival factor such as c-kit. Although differentiating spermatogonia from busulfan-treated testes were repopulated from the surviving spermatogonial stem cells, Stra8 gene expression was consistently downregulated until six weeks after busulfan treatment, indicating that the Stra8 function is not important for spermatogonial differentiation but that it is essential for preleptotene spermatocytes.

In the mouse testis, TNFα R55 is expressed in Leydig cells and Sertoli cells, but SCF is exclusively produced in Sertoli cells [33]. A slight, albeit significant, decrease in TNFα R55 and SCF mRNA levels was observed one to three weeks after busulfan treatment, but the expression of both increased from week 4 onward and reached maximal levels at four to six weeks (Fig. 3). This observation indicates that somatic cells were not damaged by busulfan treatment and that busulfan is not toxic for Sertoli cells. Given that TNFα R55 is a receptor that regulates differentiated functions of testicular cells and that SCF is a spermatogonial survival factor [33], their increased expression four weeks after busulfan treatment suggests that these factors might induce DNA repair or the resumption of spermatogonial stem cell proliferation after chemotherapy. Also, four weeks after busulfan treatment, c-kit expression was detectable, and it was restored to control levels at five weeks. This result suggests that mitotic proliferation resumed and that proliferating type A spermatogonia reappeared in some seminiferous tubules four weeks after busulfan treatment. It also indicates that the c-kit/SCF pathway is critical for male germ cell survival and that busulfan treatment results in transient, rather than permanent, infertility due to the downregulation of c-kit expression.

The second wave of apoptosis induced by busulfan treatment affected meiotic spermatocytes. It is well known that pachytene spermatocytes express high levels of RAD51 and that transcript decreases to nearly undetectable levels in elongating spermatids, whereas PCNA is expressed at various stages of spermatocyte development from pachytene spermatocytes to round spermatids [24]. It is also postulated that

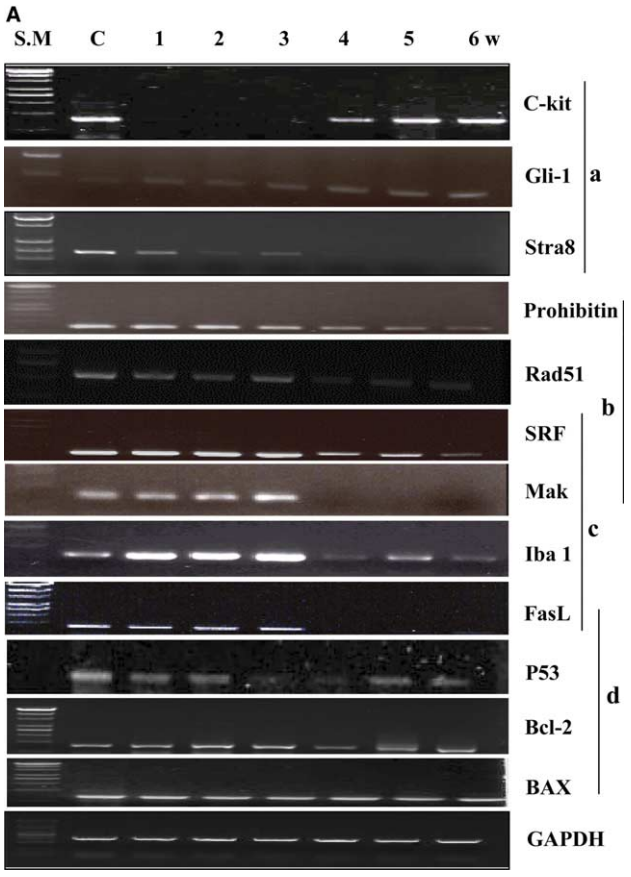

Fig. 3. Male germ cell-specific gene expression levels in busulfan-treated adult testis. (A) RT-PCR analysis. (a) A representative analysis of spermatogonia-specific markers. c-kit is expressed mainly from type A to early meiotic stages, and Stra 8 is expressed mainly in type A spermatogonia. Both OCD and Gli-1 mRNA are expressed in type B and early spermatocytes, including type B spermatogonia. (b) Analysis of meiotic germ cell-specific markers. Rad51is expressed mainly in pachytene stage spermatocytes, but prohibitin and SRF are expressed mainly in leptotene spermatocytes. (c) Analysis of haploid-specific markers. FasL and Iba I are expressed mainly in round and elongated spermatids. (d) Analysis of apoptosis related markers. Apoptosis in male germ cells is mediated by a P53 and/or FasL/Fas-independent pathway. (B) Densitometric analysis of the RT-PCR results shown in (A). Values are shown relative to the highest value, which was normalized to 100% ($P < 0.05$). The time points are indicated at bottom. c-kit and Stra8 mRNA are lost one week following busulfan treatment, whereas Rad51 mRNA is lost at week 2 and prohibitin and SRF are reduced from 3 weeks onward. FasL and Iba I are lost at week 4, but Fas mRNA and Bax are constantly expressed.

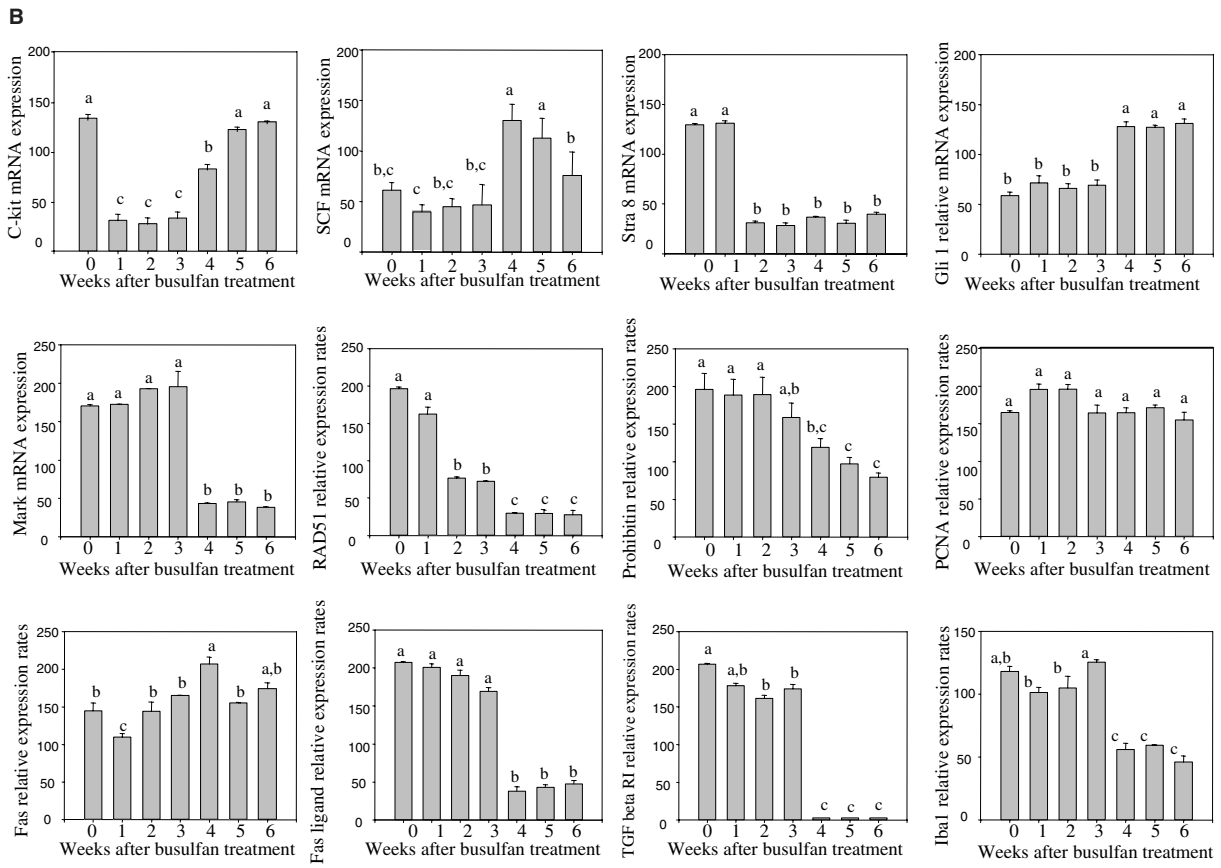

Fig. 3 (*continued*)

PCNA and RAD51 may be involved in excision repair up to mid-spermatogenesis. Mak mRNA intensely localizes in late pachytene (stage X) and diplotene (stage XI) spermatocytes, and immunostains weakly in dividing spermatocytes (stage XII) and early round spermatids (stages I–II) [29]. However, a recent study showed that $Mak^{-/-}$ mice develop normally and without gross abnormalities [34], indicating that Mak function is not essential for spermatogenesis and male fertility. Although PCNA mRNA remained constant after busulfan treatment, the busulfan-treated testis exhibited decreased expression of prohibitin, Stra8, Mak mRNAs, and RAD51, which are involved in DNA damage repair. Therefore, it is plausible that preleptotene spermatocytes and pachytene spermatocytes are more sensitive to stress stimuli, such as busulfan treatment, relative to mid-spermatocytes and that some busulfan-induced damage in these cells might be caused by the loss of mediators of DNA repair such as RAD51.

Alessio et al. [31] demonstrated that FasL mRNA is predominantly expressed in round spermatids and to a lesser extent in pachytene spermatocytes in mouse and rat testis, whereas only the protein is expressed in mature spermatozoa. Iba I mRNA appeared to be expressed in spermatids at steps 1–12 and Iba I protein were detectable at steps 10–19 [32]. However, FasL, Iba I, and TGFβ RI mRNA in busulfan-treated mice, as compared to control adult mouse testis, was constantly expressed for three weeks and then rapidly reduced from four weeks (Fig. 3). Although Iba I expression, compared to FasL and TGFβ RI, was reduced during this time, Iba I was expressed at somewhat higher levels, indicating that a third wave of apoptosis induced by busulfan treatment affects round spermatids rather than elongated spermatids. Taken together, our observations support the report by D'Alessio et al. [31] that FasL is expressed in male germ cells but not in Sertoli cells. However, Fas mRNA was constantly expressed even after germ cell loss. Therefore, the long-term adverse biological effects of Fas mRNA expression after busulfan treatment must be further investigated.

Jordan et al. [35] previously demonstrated that spermatogenesis in W^v/W^v (c-kit receptor deficient) mice can be rescued by loss of the p53 gene. However, a more recent study has shown that the loss of the p53 function does not promote or rescue spermatogenesis in Sl/Sl^d mutant testes, indicating that germ cell deficiency in the c-kit/SCF mutant is not dependent on p53-mediated apoptosis [36]. We observed TUNEL-positive germ cells in differentiated spermatogonia and meiotic male germ cells of the busulfan-treated testis (Fig. 2A). RT-PCR analysis showed that the busulfan-treated testis lacked male germ cells, as evidenced by c-kit expression (Fig. 3). In addition, it exhibited a low but constant level of p53 mRNA (Figs. 3 and 5). This result, coupled with a previous observation [37], suggests that DNA-damaging agents do not result in increased levels of p53 mRNA and that the half-life of the p53 protein is prolonged in the testis of the busulfan-treated mouse. Therefore, the fact that p53 protein expression did not correlate with mRNA levels might reflect the autoregulatory control of p53 expression, because the protein binds to and inhibits the translation of its own mRNA [37]. In this study, it is notable that the apoptotic mechanisms in male germ cells

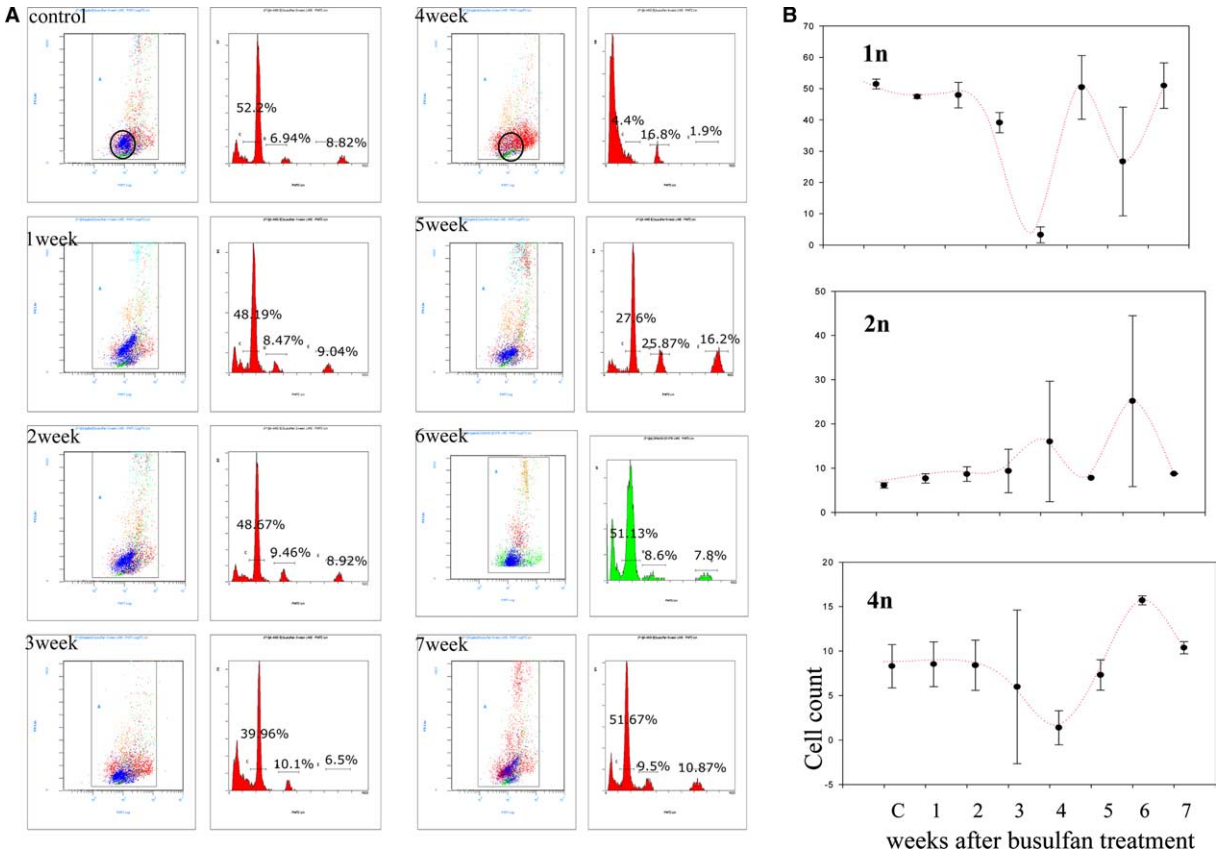

Fig. 4. A representative flow cytometric analysis of surviving testicular cells after injection of a single dose of busulfan. (A) Time course analysis reveals that the percentages of both haploid and G2/M cells, which consist of primary spermatocytes, decrease over time as compared to control testicular cells, and reaches a minimum four weeks after busulfan treatment. No further decrease occurs and the cell cycle resumes at five weeks. (B) This observation was reanalyzed with respect to germ cell stages. The percentages of 1*n* (postmeiotic) and 4*n* (meiotic) germ cells are significantly lower in busulfan-treated testes than in control testes at all timepoints, whereas 2*n* (somatic and premeiotic germ) cells slightly increase in frequency, peaking at four weeks, compared to control testes, indicating that spermatogonia and somatic cells are not damaged by busulfan treatment.

exposed to busulfan are quite distinct from those triggered by X ray exposure or seen in the cryptorchid-induced testis and c-kit receptor-deficient mice. Our results suggest that the loss of c-kit/SCF signaling results in busulfan-induced male germ cell death.

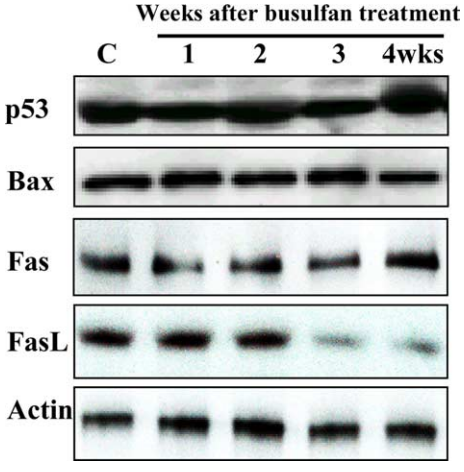

Fig. 5. Western blot analysis of the FasL/Fas- and p53-independent pathways. P53, Bax, Fas, and FasL protein expression are unchanged one to three weeks after busulfan treatment. At four weeks, p53 and Fas expression slightly increases, but Bax and FasL expression is reduced, indicating that male germ cell apoptosis after busulfan treatment does not occur via the p53 or Fas/FasL pathways.

In vivo spermatogonial growth is regulated by the degree to which stem cells in basal seminiferous tubules remain in the quiescent (G0) or proliferative (S/G2/M) phases of the cell cycle. This process is aberrant in the testis of the busulfan-treated mouse. Spermatogonial hyperproliferation in the busulfan-treated mouse testis is characterized by a markedly increased percentage of normally quiescent spermatogonial stem cells in the proliferative phases of the cell cycle (Fig. 4A). The data presented here indicate that busulfan does not affect stem cells. As shown in Fig. 8, busulfan acts after cyclin E induction, but prior to PCNA expression and DNA synthesis, in developing male germ cells. Although PCNA expression was markedly reduced, it was not completely abolished by busulfan treatment (Fig. 6). To determine if the degree of PCNA reduction was associated with a failure to progress into DNA synthesis per se (S/G2/M), mice were treated with busulfan and their testicular cells were analyzed by flow cytometry. This experiment indicated that the G1 block results from the busulfan-induced inhibition of PCNA synthesis. However, PCNA expression was higher in control adult and postnatal day 5 testes than in the busulfan-treated testis.

Rb is present in an unphosphorylated form, which hinders the transition from G1 to S phase, and in a phosphorylated form, which induces cell-cycle progression [38]. The active

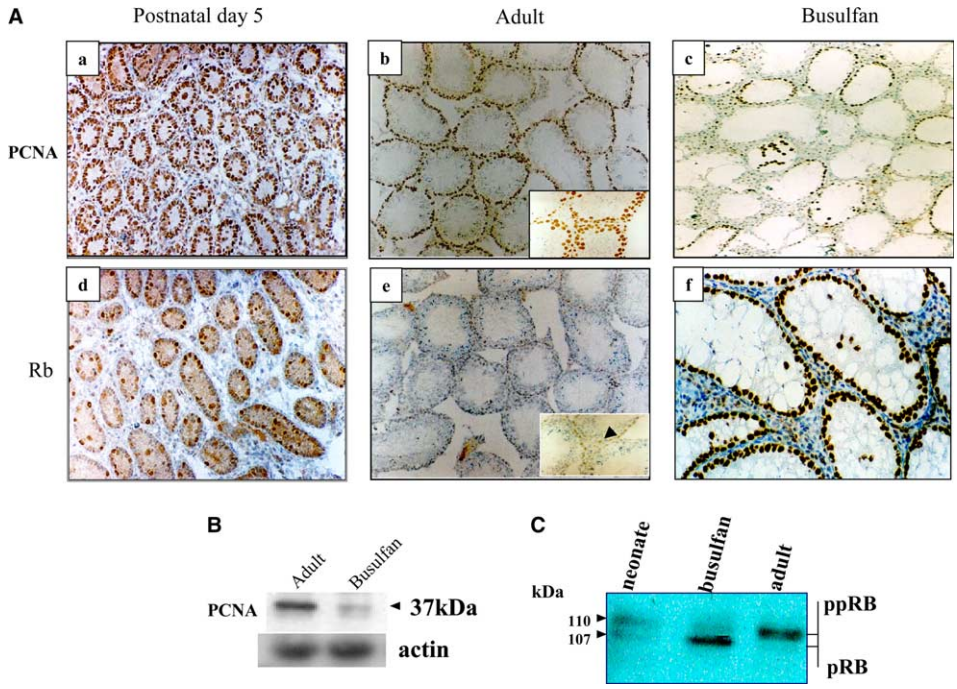

Fig. 6. Localization of Rb and PCNA expression in control adult, postnatal day 5, and busulfan-treated testis. (A) PCNA is very strongly expressed in control and postnatal day 5 testes but is expressed at reduced levels in busulfan-treated testis. Although Rb expression is almost undetectable in the control adult testis, it increases after busulfan treatment and peaks at four weeks. However, postnatal day 5 testis shows a moderate level of Rb expression. (B and C) Western blot analysis of PCNA and pRB/ppRB expression, respectively. pRB and ppRB are expressed at equal levels in postnatal day 5 testis (neonate in C). Busulfan-treated testis exhibits strong pRB (busulfan in C) and weak PCNA signals (busulfan in B), but control adult testis exhibits strong ppRB (adult in C) and PCNA signals (adult in B). pRB, the hypophosphorylated form of Rb; ppRB, the hyper-phosphorylated form.

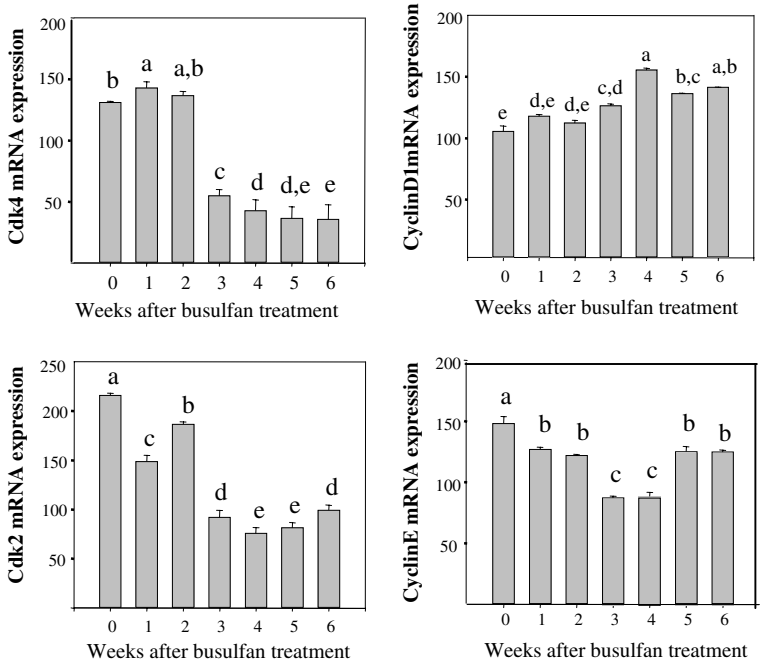

Fig. 7. RT-PCR analysis of cell cycle-specific gene expression levels in busulfan-treated adult testis from week 0 to week 6. Timepoints are indicated at bottom. Temporal changes in expression levels were plotted based on densitometric analysis of RT-PCR. Values are shown relative to the highest value, which was normalized to 100 percent ($P < 0.05$). (A, B, C and D) show RT-PCR analyses of CDK4, cyclin D1, CDK2, and cyclin E, respectively. The expression of cdk4, cdk2 and cyclin E mRNA is slowly reduced, whereas cyclin D1 is not affected.

form (pRB) is primarily found in resting cells, whereas the hyperphosphorylated form (ppRB) is primarily found in proliferating cells. Previous studies of Rb in mammalian testes did not distinguish between the two forms [39,40]. In this study, we used the G3-245 antibody to distinguish between the two forms. In the control adult testes, pRB staining was negative in most cells, including germ cells and somatic cells, and a few spermatogonia located along the basement membrane were stained (e of Fig. 6A), whereas moderate levels of expression could be detected in spermatogonia of postnatal day 5 testes (d of Fig. 6A). However, testes of busulfan-treated mice stained more intensely for pRB (Fig. 6A, panel f). This observation agrees with the results of Western blot analysis (Fig. 6C). Therefore, the upregulation of Rb expression in the busulfan-treated testis is likely to be induced by its effects on spermatogonial germ cells. According to this concept, we hypothesize that RB phosphorylation is normally repressed in resting spematogonial stem cells after busulfan treatment and that it is de-repressed for normal spermatogenesis after loss of busulfan activity (Fig. 8). Given the its established role in protection against cell death [41], pRB reduces but does not eliminate apoptosis in actively developing male germ cells. However, moderate RB expression and high PCNA expression in the postnatal day 5 testis indicate that it contains actively dividing spermatogonia rather than resting spermatogonial stem cells, explaining why neonatal spermatogonia are less able to colonize a recipient testis than are adult stem cells [42]. To our knowledge, this is the first report showing evidence for the survival of spermatogonial stem cells after busulfan treatment, which can be directly related to protection of male germ cells from cell death.

In view of the increased usage of high dose chemotherapy with autologous or allogeneic stem cell transplantation and a gradually increased cure rate of patients that have undergone such therapy, the prospect of young patients maturing and establishing families is increasing. A large fraction of such patients are, however, infertile because of the previous high dose therapy [21]. The basis for understanding how to address this problem starts with understanding the molecular mechanisms underlying the infertility. In this study, these questions were addressed in an experimental setting in the present studies. In conclusion, we observe that (1) the loss of spermatogonia through apoptosis is mediated by c-kit, and (2) this loss is not associated with changes in the expression of P53, Bax or Bcl-2 protein, and (3) the survival of spermatogonia stem cells is mediated by the cell cycle.

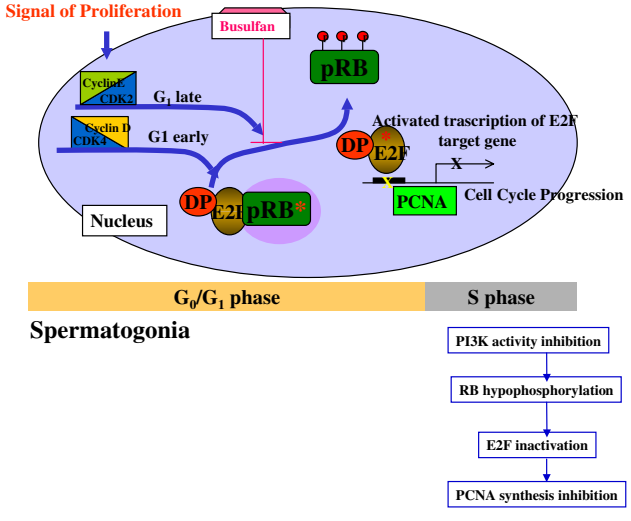

Fig. 8. Schematic depicting the survival of spermatogonial stem cells following busulfan treatment. The active form of Rb (pRB) is primarily found in resting cells, whereas the hyperphosphorylated form (ppRB) is primarily found in proliferating cells. Testes of busulfan-treated mice show a higher level of pRB expression, whereas control adult testis shows a very high level of ppRB expression. Therefore, bulsufan treatment may inhibit Rb phosphorylation in the testis via the inhibition of cyclin E. In its hypophosphorylated form, p110Rb remains bound to E2F, which in the complexed form is inactive and cannot transcribe the PCNA gene. Therefore, spermatogonial stem cells may be protected from cell death by cell cycle arrest at G0/G1.

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