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GENOTOXICITY

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Doxorubicin induces male germ cell apoptosis in rats

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Abstract To clarify whether apoptosis is involved in doxorubicin (DXR)-induced testicular toxicity and to identify the target germ cell type, adult Sprague-Dawley rats were treated with a single intravenous dose of DXR (8 or 12 mg/kg) and euthanized at 3, 6, 12, 24, and 48 h subsequently. Histologically, germ cell degeneration was first found 6 h after dosing in meiotically dividing spermatocytes and early round spermatids of seminiferous tubules at stage I, and subsequently observed in spermatogonia at stages I–VI showing ultrastructural characteristics of apoptosis. Coincident with the appearance of morphological changes, degenerating germ cells were shown to be undergoing apoptosis as revealed by in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The frequency of TUNEL-labeled germ cells increased in a stage- and cell type-specific manner, the peak of frequency gradually progressing from stage I of seminiferous tubules to later stages with time after dosing, suggesting that the damaged germ cells, especially spermatogonia, gradually underwent the processes leading to apoptosis. DNA laddering on gel electrophoresis was apparent 24 and 48 h after dosing. The results demonstrate that apoptosis plays an important role in the induction of testicular toxicity caused by DXR with meiotically dividing spermatocytes and type A and intermediate spermatogonia as highly vulnerable target cells.

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Introduction

Doxorubicin (DXR), one of the most widely used and effective anthracycline anticancer agents, has been found to exert multiple effects in target cells, including intercalation with DNA, induction of DNA breaks, inhibition of topoisomerase II, and formation of toxic free radicals (Cummings et al. 1991; Powis 1991). DXR has been shown to cause significant DNA damage in rodent spermatogenic cells as measured by chromosome aberration (Au and Hsu 1980), sister-chromatid exchange (Abraham and Fränz 1983), as well as meiotic micronucleus assays in vivo and in vitro (Lähdetie et al. 1983; Toppari et al. 1986). Inhibition of stage-specific DNA synthesis in rat testes has been described (Parvinen and Parvinen 1978) and furthermore, a number of reports have indicated that DXR alters fertility in men (Shamberger et al. 1981) and animals (Meistrich et al. 1985; Imahie et al. 1995).

Although many investigations of DXR-induced testicular toxicity in rodent models have been reported (Parvinen and Parvinen 1978; Lui et al. 1986; Russell and Russell 1991; Matsui et al. 1993; Lähdetie 1994), little is known about the processes leading to germ cell death. Recent studies have shown that acute exposure to DXR causes apoptosis in several cell lines in vitro (Ling et al. 1993; Skladanowski and Konopa 1993) or in murine transplantable tumors in vivo (Meyn et al. 1995). It has also been demonstrated that DXR induces apoptosis in small intestinal epithelial cells (Anilkumar et al. 1992), renal tubular epithelial cells (Zhang et al. 1996), hair follicle cells (Cece et al. 1996), and female germ cells or oocytes in vivo (Perez et al. 1997).

In the testis, it is known that significant germ cell degeneration occurs during normal spermatogenesis, and this has recently been found to involve apoptosis (Brinkworth et al. 1995; Blanco-Rodríquez and

Martínez-García 1996). In addition, there is increasing evidence that germ cell degeneration caused by testicular toxicants including chemotherapeutic compounds, such as cyclophosphamide (Cai et al. 1997) and mitomycin C (Nakagawa et al. 1997), is linked to apoptosis.

We wished to clarify whether apoptosis is involved in DXR-induced testicular toxicity, and if this is the case, to identify the target germ cell type. For this purpose, the extent and nature of nuclear DNA fragmentation in the testes of rats given a single treatment of DXR were evaluated using both in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and DNA gel electrophoresis, together with conventional histological and electron microscopic approaches.

Materials and methods

Animals and treatments

Adult male Sprague-Dawley rats at 10 weeks of age obtained from Charles River Breeding Laboratories (Shiga, Japan) were housed in polycarbonate cages with a 12:12 h light/dark cycle, at a temperature of 23 ± 2 °C and humidity of 55 ± 5%, and given MF diet (Oriental Yeast Co. Ltd., Tokyo) and water ad libitum. The animals received single intravenous doses of 8 or 12 mg/kg of doxorubicin hydrochloride (Wako Chemical Co., Tokyo) in saline at a concentration of 0.2% (w/v) and were euthanized under ether anesthesia after 3, 6, 12, 24, and 48 h ($n = 3$–4, at each time point). The doses were chosen on the basis of the literature (Bertazzoli et al. 1985; Lui et al. 1986) and data from our own preliminary experiments. Control rats ($n = 3$) received 6 ml/kg of saline vehicle and were euthanized 24 h subsequently. Immediately after killing, both testes from each animal were removed, one being immersed in Bouin's fixative for histological and TUNEL evaluations. The contralateral testes were frozen in liquid nitrogen and stored at −70 °C for subsequent detection of DNA fragmentation by gel electrophoresis, or punctured through the tunica albuginea with a 26-gauge needle followed by infusion with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) for electron microscopic evaluation. In addition to the above mentioned principal experiments, a supplementary study was conducted only for gel electrophoresis, in which animals ($n = 2$) were treated with 12 mg/kg of DOX and euthanized 72 h after dosing.

Histological and electron microscopic evaluation

For light microscopy, testes fixed in Bouin's fixative were embedded in paraffin followed by sectioning and staining with hematoxylin-eosin (HE). For electron microscopy, cubes of 3 to 5 mm^3 from infused testes were immersed in 2.5% glutaraldehyde, postfixed in 1.0% osmium tetroxide, dehydrated through a graded series of ethanols, and embedded in Epon 812 after several changes of propylene oxide. Ultrathin sections stained with uranyl acetate and lead citrate were examined under a JEOL 1200EX transmission electron microscope.

TUNEL

Histological detection of DNA fragmentation in testes was performed by the TUNEL method using an ApoTag-peroxidase kit (Oncor, Gaitherburg, Md., USA). Briefly, sections of 5 μm in thickness from testes fixed in Bouin's fixative and embedded in paraffin were mounted on glass slides, deparaffinized by clearing with xylene, and hydrated through a graded series of ethanols to deionized water. Sections were treated with proteinase K (20 μg/ml) for 10 min for digestion of nuclear proteins and hydrogen peroxide (2%) for 5 min for inactivating endogenous peroxidase. Subsequently, sections were incubated in a solution of terminal deoxynucleotidyl transferase (TdT) and digoxigenin-dUTP in a humidified chamber at 37 °C for 1 h and then treated with anti-digoxigenin-peroxidase for 30 min at room temperature. Digoxigenin-dUTP end-labeled DNA was visualized by peroxidase detection with diaminobenzidine (0.05%) and hydrogen peroxide (0.02%) for 5 min. Sections were counterstained with hematoxylin. For the quantification of TUNEL-labeled germ cells, the seminiferous tubules were divided into seven groups, i.e., stages I, II–IV, V–VI, VII–VIII, IX–XI, XII–XIII, and XIV, based on the cell types of spermatogonia and spermatocytes according to the description by Hess (1990). The TUNEL-labeled germ cells were identified as spermatogonia, spermatocytes, and spermatids by their morphological features and location within the seminiferous epithelium.

DNA gel electrophoresis

The detection of low molecular weight DNA ladders was performed as described by Strauss (1994) with modifications. A whole frozen testis from each animal was minced on dry ice and digested with proteinase K (100 μg/ml; Sigma) for 90 min. After addition of an equal volume of phenol, the preparations were placed on a roller apparatus for 15 min followed by centrifugation at 27 000 *g* for 15 min. The aqueous phase was extracted with an equal volume of phenol/chloroform (1:1) for 15 min and subjected to RNase A (10 mg/ml; Sigma) treatment for 60 min followed by repeated phenol/chloroform extraction. The resultant solution was ethanol-precipitated overnight, pelleted, dried, and resuspended in TE buffer [10 mM TRIS-HCl and 1 mM ethylenediaminetetraacetic acid (EDTA)]. Following spectrophotometric estimation of the DNA concentration, aliquots of 3 to 5 ng of DNA were loaded onto 1.8% agarose gels and separated by electrophoresis (100 V, 1 h). DNA was stained with ethidium bromide and visualized with an ultraviolet transilluminator (NLMS-20 E; UVP, Upland, Calif., USA) at 302 nm. The sizes of resulting DNA bands were estimated by comparison with standard molecular markers (pHY markers; Takara Co. Ltd., Tokyo).

Statistical analysis

The quantification of TUNEL-labeled germ cells was assessed on >2000 cross-sectioned seminiferous tubules from each rat ($n = 3$) and expressed as numbers of TUNEL-labeled germ cells per 100 Sertoli cells in each group of stages. Statistical analyses were performed with one-way analysis of variance and Dunnett' s multiple comparisons. All results were presented as mean ± SE and the level of significance taken as $P < 0.05$, compared with the respective control.

Results

Morphological evaluation

No remarkable changes were apparent in testes from rats euthanized 3 h after dosing with either 8 or 12 mg/kg. Germ cell degeneration was first found 6 h after dosing at 12 mg/kg, in meiotically dividing spermatocytes and early round spermatids with increased cytoplasmic eosinophilia of seminiferous tubules at stage I (Fig. 1C). Such degenerating cells were rare in testes from the control (Fig. 1A) or the 8 mg/kg groups. At 12 h after dosing, degenerating meiotically dividing spermatocytes of seminiferous tubules at stage I were

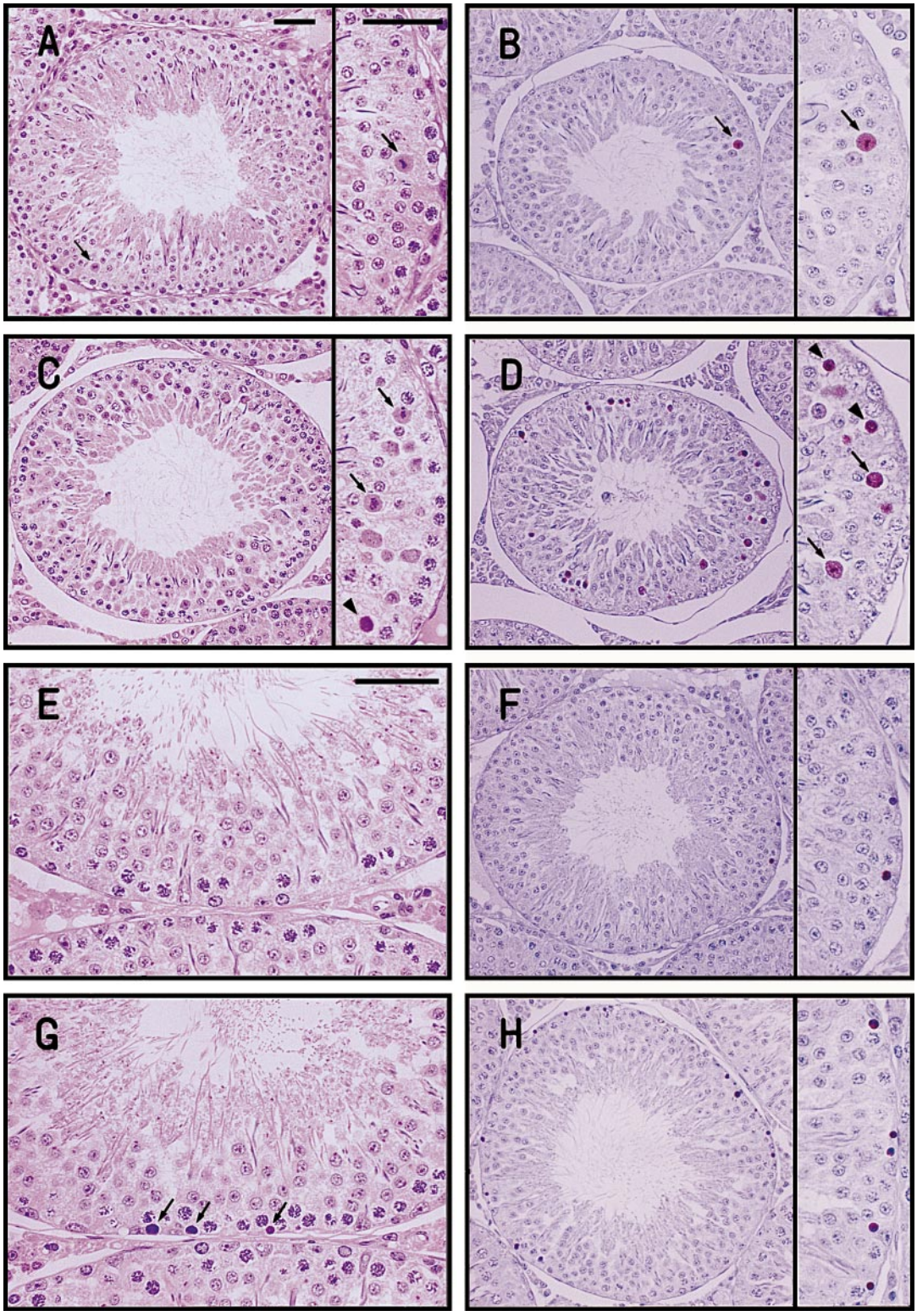

Fig. 1A–H Light micrographs of seminiferous tubules from control and doxorubicin (DXR)-treated rats. **A**, **B** Stage I tubule from a control rat. Degenerating meiotically dividing spermatocytes (*arrow*) with increased eosinophilia and TUNEL-labeling are rare in the control. **C**, **D**. Stage I tubule from a DXR (12 mg/kg)-treated rat at 6 h after dosing. Note the increase in numbers of degenerating meiotically dividing spermatocytes (*arrows*) and round spermatids (*arrowheads*). **E**, **F** Stage III tubule from a control rat. Note the small number of spermatogonia with TUNEL-labelling. **G**, **H** Stage III tubule from a DXR (12 mg/kg)-treated rat 24 h after dosing. Note the degenerating spermatogonia with pyknotic nuclei (*arrows*) and TUNEL-labelling. **A**, **C**, **E**, **G** Hematoxylin-eosin; **B**, **D**, **F**, **H** TUNEL staining. *Bar*, 50 μm (*TUNEL* Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling)

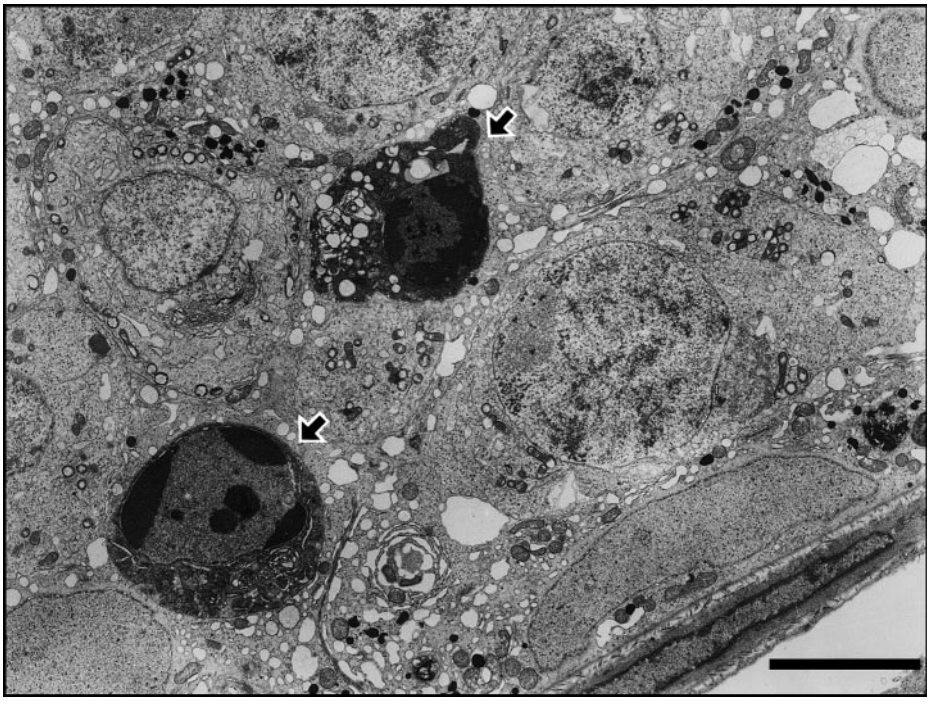

Fig. 2 Electron micrograph of a seminiferous tubule from a DXR (12 mg/kg)-treated rat 24 h after dosing. Note the degenerating spermatogonia (*arrows*) with marked nuclear chromatin condensation at the nuclear periphery and crowding of cytoplasmic constituents. *Bar*, 2 μm

present, and spermatogonia with pyknotic nuclei slightly increased in number at stages I and II–IV of seminiferous tubules from the 12 mg/kg group. Such degenerating spermatogonia were frequently noted in seminiferous tubules at stages II–IV and V–VI in both 8 and 12 mg/kg groups by 48 h after dosing (Fig. 1G). Electron micrographs of degenerating spermatogonia showed marked nuclear chromatin condensation at the nuclear periphery and crowding of cytoplasmic constituents, which are characteristic of apoptosis (Fig. 2).

In situ TUNEL evaluation

Quantitative date for TUNEL-labeled germ cells are shown in Figs. 3 and 4. In testes from the control rats, a few TUNEL-labeled cells in seminiferous tubules were found at stages I, II–IV, XII–XIII, and XIV, but were rare at stages V–VI, VII–VIII, and IX–XI (Fig. 3, control). These spontaneously labeled cells predominantly corresponded to spermatogonia (Fig. 1F). In comparison, the frequency of TUNEL-labeled germ cells after a single dose of DXR increased in a stage-specific and dose-dependent manner; such changes were observed in seminiferous tubules at stage I at 6 h, stages I and II–IV at 12 h, stages II–IV at 24 h, and stages V–VI at 48 h after dosing (Fig. 3). Thus the peak of frequency gradually progressed from stage I to later stages with time after dosing.

Examination of the TUNEL-labeled germ cells showed that spermatogonia, meiotically dividing spermatocytes, and early round spermatids were preferentially labeled, depending on the stages of the seminiferous tubules and the time after dosing: i.e., meiotically dividing spermatocytes and early round spermatids at 6 h, and meiotically dividing spermatocytes and spermatogonia (type A) at 12 h in stage I tubules (Fig. 4A); spermatogonia (type A and/or intermediate) at 12 h and 24 h in stage II–IV tubules (Fig. 4B); spermatogonia (type A and/or B) at 48 h in stage V–VI tubules (Fig. 4C). At all other stages, there were no cell type-specific increases in the frequency of TUNEL-labeled germ cells (data not shown). TUNEL-labeling demonstrated almost the same distribution pattern as that of degenerating germ cells observed in HE-stained preparations. Leydig cells and Sertoli cells failed to show TUNEL-labeling throughout this study.

DNA gel electrophoresis evaluation

In order to confirm the occurrence of fragmented DNA assessed using in situ TUNEL, we examined the DNA fragmentation pattern in testes of the control and DXR-treated rats (Fig. 5). DNA ladders with an ordered pattern of multiple bands on agarose gels were apparent for testes from rats euthanized 24 and 48 h after dosing with either 8 or 12 mg/kg, becoming less detectable 72 h after dosing.

Discussion

Apoptosis is an active cellular process of gene-directed self-destruction, in which cells die in a controlled fashion either spontaneously or in response to changes in the levels of specific physiological stimuli. It is thought to be the predominant mechanism for elimination of cells that

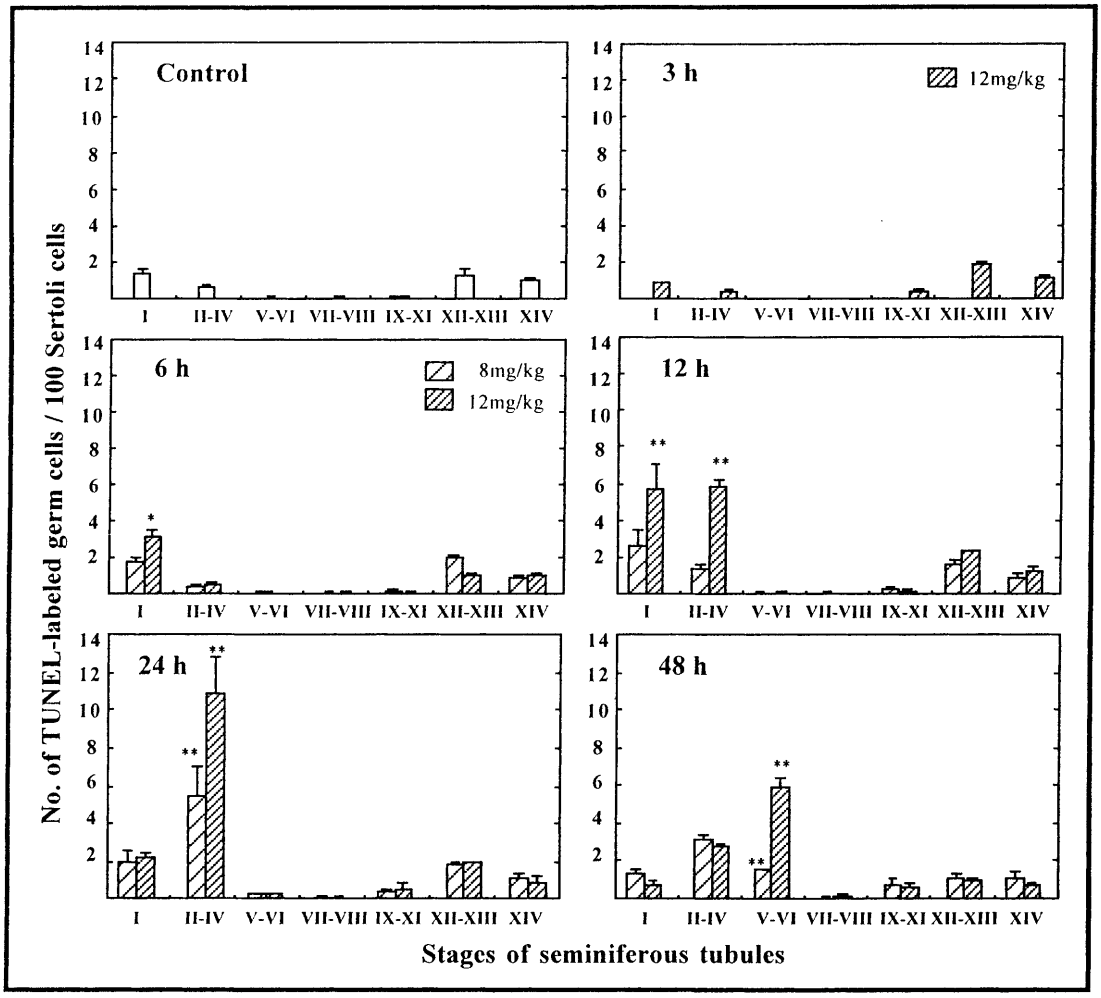

Fig. 3 Stage-specific quantification of TUNEL-labeled germ cells in control and DXR-treated rats. Data are means ± SE ($n=3$). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with values for the respective stages of seminiferous tubules in controls

have been produced in excess, have developed improperly, or have sustained genetic damage (Schwartzman and Cidlowski 1993; Thompson 1995). In the present study, morphological evaluation, in situ analysis of DNA fragmentation (TUNEL), and examination of DNA ladder patterns on gel electrophoresis all demonstrated increase in features of apoptosis after DXR treatment. The electron microscopic examination revealed degenerating spermatogonia with marked nuclear chromatin condensation at the nuclear periphery and crowding of cytoplasmic constituents, which are characteristic of apoptosis. These results strongly indicate that apoptosis is a result of the testicular toxicity caused by DXR.

Although TUNEL staining is routinely used to detect apoptotic cells in various tissues, including the testis, it has been demonstrated that nuclei of necrotic cells can also be labeled under certain conditions (Ansari et al. 1993; Grasl-Kraupp et al. 1995). In the present study, therefore, apoptosis was confirmed with a variety of methods, including electron microscopic analysis and examination of DNA ladder formation on gel electrophoresis. Our results for the stage-specific distribution pattern and frequency of TUNEL-labeled cells in the control testes are generally in agreement with previous reports (Brinkworth et al. 1995; Cai et al. 1997; Sinha Hikim et al. 1997). In response to DXR treatment, the frequency of TUNEL-labeled cells increased in a stage-specific and dose-dependent manner. The peak of frequency gradually progressed from stage I seminiferous tubules to the later ones with time after dosing. This finding suggested that damaged germ cells, especially spermatogonia, gradually undergo the process leading to apoptosis. DXR exerts multiple effects on DNA, with DNA cross-linking thought to be mainly responsible for its cytotoxic activity, causing G_2 arrest of the cell cycle (Konopa 1983, 1988). We therefore consider that the DNA repair might occur in arrested cells, but when damage is beyond a certain threshold , apoptosis is initiated.

The presence of DNA ladders on gel electrophoresis, in general, is regarded as a hallmark of apoptosis, although cells exhibiting necrotic death, in rare cases, also show similar patterns (Collins et al. 1992). In response to DXR treatment, DNA laddering was apparent at the 24 and 48 h time points, but not after 6 and 12 h when

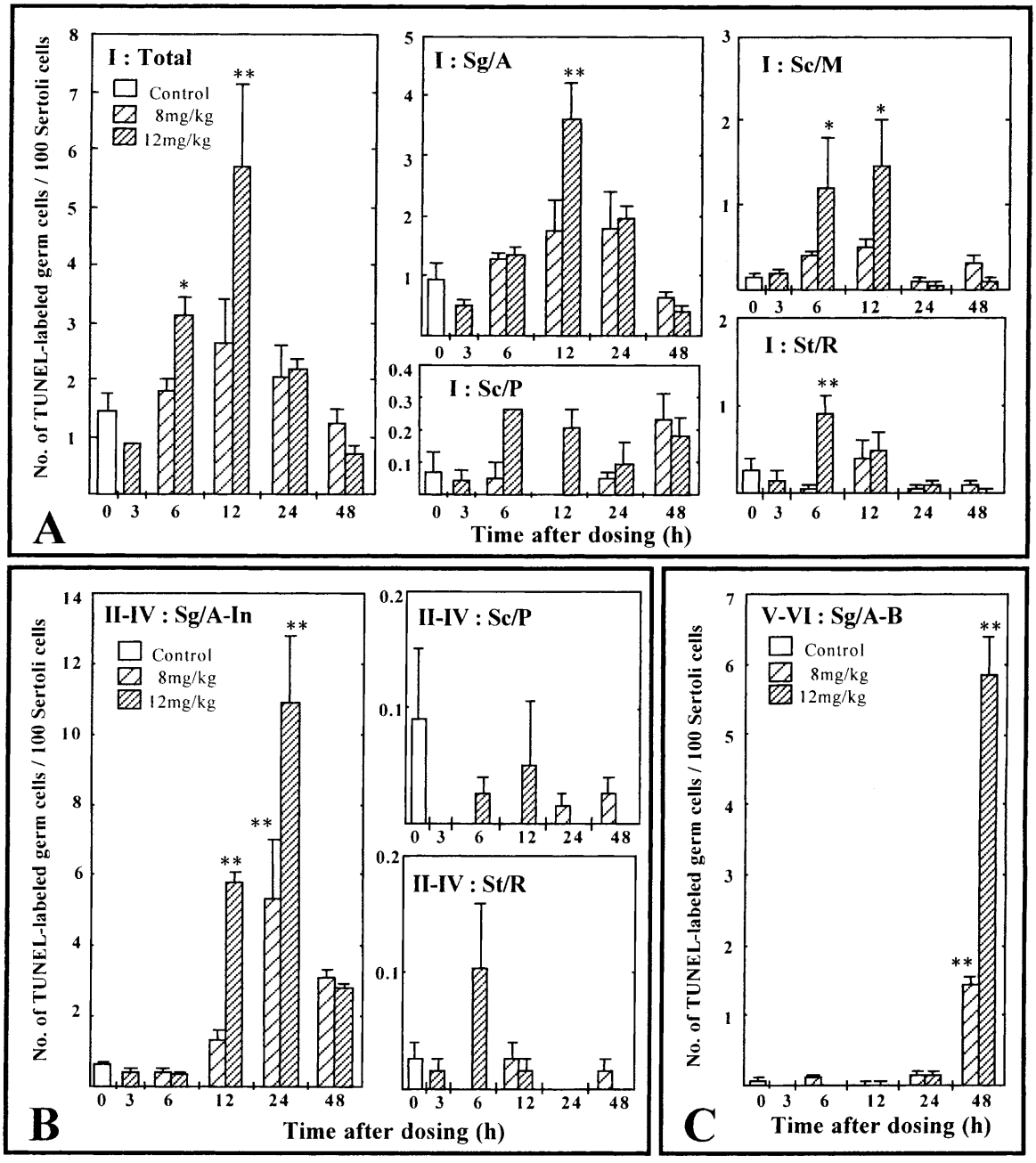

Fig. 4A–C Cell-type specific quantification of TUNEL-labeled germ cells in control and DXR-treated rats. Data are means ± SE ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control values (0 h). The cell types are as follows: Sg/A, Sg/I, and Sg/B are type A, intermediate, and type B spermatogonia, respectively; Sc/P, pachytene spermatocytes; Sc/M, meiotically dividing spermatocytes; St/R, round spermatids. **A–C** The frequencies of TUNEL-labeled cells in seminiferous tubules at **A** stage I, **B** stages II–IV, and **C** stages V–VI. At stages V–VI, numbers of the germ cells with TUNEL-labeling other than spermatogonia were extremely small. The total frequencies at stages II–IV and V–VI were nearly equal to those evaluated for spermatogonia

TUNEL-labeling showed occurrence of apoptosis. However, DNA fragmentation was measured for the whole testis and thus reflected the average for all 14 stages of the seminiferous epithelial cycle. Therefore, the relative numbers of apoptotic germ cells, depending on the ratios of the seminiferous tubules at the target stages, could be responsible for this discrepancy.

Although DXR is well known to induce testicular toxicity characterized by the depletion of germ cells followed by infertility in rats, there are some discussions with regard to the target germ cell. Histologically, Parvinen and Parvinen (1978) showed that preleptotene, mid-pachytene, and meiotically dividing spermatocytes were the cell types most sensitive to DXR. Russell and Russell (1991), however, performed a detailed examination and demonstrated that type A, intermediate, and type B spermatogonia were highly vulnerable. Matsui et al. (1993) also suggested that DXR is effective at killing type A spermatogonia especially in seminiferous

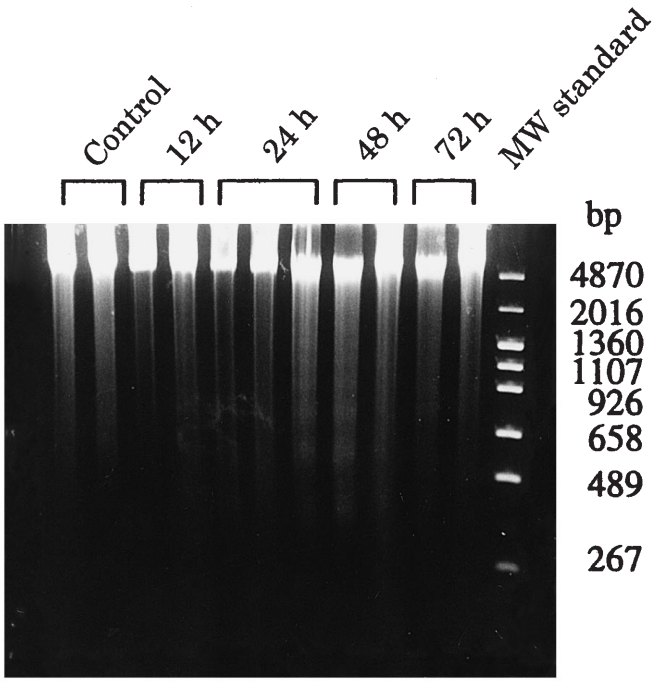

Fig. 5 Gel electrophoresis of testicular low-molecular-weight DNA isolated from control and DXR-treated rats. Each lane demonstrates the pattern for an individual rat. The *numbers* at the *right* indicate molecular weights (MW) of the size standard (in bp)

tubules at stages XI–XVI and I. Furthermore, DNA flow cytometric analysis revealed a high sensitivity of type A spermatogonia to DXR (Lähdetie et al. 1994).

In the present study germ cell degenaration, indicative of apoptosis, was first found in meiotically dividing spermatocytes and early round spermatids in stage I tubules at 6 h after dosing. During normal spermatogenesis in rats, meiotically dividing spermatocytes are generally not observed at stage I but at stage XVI. Our observations suggest that meiotically dividing spermatocytes damaged by DXR at stage XVI fail to complete the meiotic division followed by degeneration, or formation of degenerating round spermatids at stage I of seminiferous tubules. A pharmacokinetic study showed that when DXR was administered as a single intravenous injection, the plasma concentration decayed as a double exponential function of time over the first 48 h, and the plasma T½ α was estimated to be 20 min in rats (Wilkinson and Mawer 1974). Thus DXR might exert its cytotoxicity on target germ cells within only a short period immediately after dosing.

On TUNEL evaluation, significant increase of TUNEL-labeled spermatogonia was first observed in stages I and II–IV tubules at 12 h, but not in stage V–VI tubules up to 24h after dosing. The duration of each step of rat spermatogenesis has been reported (Clemont and Harvey 1965). Taking into consideration the time periods before sacrifice, our results suggest that type A and intermediate spermatogonia of seminiferous tubules at stages XVI and I–III are highly vulnerable to the cytotoxic effects of DXR, and undergo apoptosis later. One explanation for discrepancies regarding the target germ cells between the present and previous investigations may lie in differences in the dosage, the administration route, and the time points of sacrifice after dosing: 0.25 and 0.125 mg/rat, intratesticular, at 12 and 24 h by Parvinen and Parvinen (1978); 7 mg/kg, intravenous, at 7.5 days by Russell and Russel (1991); 8 mg/kg, intravenous, at 3 days by Matsui et al. (1993); 10 mg/kg, intraperitoneal, at 18 days by Lähdetie et al. (1994). We consider that a shorter duration before sacrifice might be more suitable for identifying the target cell type, and propose that TUNEL may be most appropriate for detection of germ cells degeneration caused by DXR.

Much attention has been recently focused on signal transduction events, which plays a critical role in regulating the process of apoptosis. A number of investigations have indicated involvement of p53 (Lowe et al. 1993), Fas/Fas-ligand (Friesen et al. 1996), and bcl-2/bax (Ohmori et al. 1993; Perez et al. 1997) in DXR-induced apoptosis in vitro. In the testis, p53 (Stephan et al. 1996), bcl-2/bcl-xL/bax (Rodriguez et al. 1997), and Fas/Fas-ligand (Suda et al. 1993) have been shown to be present, and the Fas system has been identified as a key regulator of Sertoli cell-directed germ cell apoptosis (Lee et al. 1997). Further studies are now necessary to elucidate the underlying transduction events for DXR-induced male germ cell apoptosis.

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