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Expression of $P16^{INK4a}$ in testis of rhesus monkey during heat stress and testosterone undecanoate induced azoospermia or oligozoospermia☆

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Abstract

Previous studies on azoospermia or oligozoospermia induced by heat stress or high doses of testosterone mainly focused on germ cell apoptosis; no data regarding their possible effect on spermatogonia mitosis are available. We have established unilateral cryptorchid and testosterone undecanoate (TU)-treated monkey models and examined expression of $P16^{INK4a}$ in the testis to look at its possible role in azoospermia or oligozoospermia induced by the heat stress or the TU treatment. The results showed that both heat stress and TU were capable of inducing expression of $P16^{INK4a}$ mainly in spermatogonia and other types of germ cells as well as Sertoli cells at the later stage of germ cell apoptosis, namely on Day 10 after operation or on Day 60 after TU injection. It is, therefore, suggested for the first time that $P16^{INK4a}$ protein may inhibit the spermatogonia mitosis in the testis at the later stage of the germ cell apoptosis, resulting in arrest of spermatogenesis.

Keywords: $P16^{INK4a}$; Heat stress; Testosterone undecanoate; Azoospermia; Oligozoospermia; Mitosis

1. Introduction

Our previous studies showed that both heat stress and high doses of testosterone undecanoate (TU) are capable of inducing animal azoospermia or oligozoospermia, and the spermatocytes and spermids undergo apoptosis [1,2]. Germ cell apoptosis may play an important role in azoospermia or oligozoospermia induced by heat stress or high doses of TU treatment. However, if spermatogonia proliferation in the testis was not influenced by the treatment, spermatogonia should develop into spermatocytes and spermids and replace the reduced apoptotic germ cells. In fact, morphologically, only spermatogonia and Sertoli cells were observed; nearly all the spermatocytes and spermids underwent apoptosis in the seminiferous tubules at the later stage of germ cell apoptosis [2]. We, therefore, speculate that inhibition of spermatogonia mitosis may occur during azoospermia or oligozoospermia induced by heat stress or high doses of TU.

The enforced expression of INK4 proteins of cyclin-dependent kinase inhibitors in mammalian cells inhibits the activity of all G1-phase cyclin-dependent kinases (CDKs), which positively regulates cell proliferation and induces growth arrest by preventing entry into S phase of cell cycle [3–6]. $P16^{INK4a}$ is one member of the INK4 family [7].

In this study, we examined the expression of $P16^{INK4a}$ to look at the possible effect on mitosis of spermatogonia during azoospermia or oligozoospermia induced by heat stress or high doses of TU treatment by immunohistochemistry and Western blot.

2. Materials and methods

2.1. Animals and tissue preparations

Thirty male adult rhesus monkeys (including three normal monkeys as a control group) at ages between 5–7 years were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences (CAS), and approved for this study by the Academic Committees of both the Institute of Zoology and the Kunming Institute Primate Research Center, CAS.

To induce unilateral cryptorchidism, 15 animals were anesthetized by injection of pentobarbital sodium and a small incision was made in the abdomen. The gubernacu-

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lum was cut on the right side to displace the testis into the abdomen. Testis decent was prevented by closure of the inguinal canal on the right side by suturing. Another 12 monkeys were treated either weekly by intramuscular injection of TU 20 mg/kg, or twice weekly injection of TU 10 mg/kg, on Days 1 and 4, respectively. The testes were removed on various days and decapsulated and divided into quarters. One quarter was fixed in Bouin's solution and embedded in paraffin prior to sectioning (6 μm) for immunohistochemistry. The other quarters were snap-frozen in liquid nitrogen and stored at −70°C for Western analysis.

2.2. Materials and reagents

Rabbit anti-$P16^{INK4a}$ protein monoclonal antibody was purchased from Santa Cruz, CA, US. Alkaline phosphatase conjugated anti-DIG IgG and CDP-Star chemiluminescence reagent were purchased from Boehringer Mannheim GmbH Biochemical (Mannheim, Germany). Substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium were obtained from Promega Corporation (Madison, WI, US). Levamisole and paraformaldehyde were from Sigma Chemical Company (St Louis, MO, US). Zeta-Probe nylon membrane and Gene Linker ultraviolet light chamber were purchased from Bio-Rad Laboratories (Richmond, CA, US). Formamide and 3-aminopropyl-trithoxysilane were obtained from Beijing Zhongshan Biotechnology, Inc.

2.3. Immunohistochemistry

Immunohistochemistry was carried out with a Vectastain ABC Kit, as recommended by the manufacturer. Deparaffinized sections were incubated with 10% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 30 min. The primary antibody (rabbit anti-$P16^{INK4a}$ monoclonal antibody) was diluted in PBS containing 10% NGS and incubated with the sections for 1 h (the control groups were incubated with 10% NGS in PBS instead of primary antibodies). Then the sections were washed in PBS for 3 × 5 min, incubated with biotinylated second antibody for 1 h and washed in PBS for 3 × 5 min. After incubation with avidin-biotin-peroxidase complex in PBS for 1 h and washing in PBS for 3 ×5 min, the sections were incubated in diaminobenzidine tetrahydrochloride in 0.05 mol/L Tris-HCl (pH 7.2) with 0.01% H2O2 for 2–7 min. The sections were dehydrated through a graded series of ethanol, cleared in xylene, and then mounted.

2.4. Immunoblot analysis

Testes were homogenized in lysis buffer (5 mmol/L phosphate buffer, pH 7.2, containing 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride, 1 mg/L leupeptin, and 1 mg/L chymostatin). The protein content of the preparation was determined by spectrophotometer, using bovine serum albumin as a standard. Sample lysates were mixed with the loading buffer (final concentration, 62.5 mmol/L 1, 4-dithiothreitol, 5% sodium dodecyl sulfate (SDS), and 10% glycerol), boiled for 8 min, separated by SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred on to polyvinylidene difluoride membranes. The membranes were blocked for 1 h in Tris buffered saline (pH 7.6) with 5% skim milk, and incubated for 2 h with 1:500 rabbit anti-$P16^{INK4a}$ protein sera, and then for 1 h Alkaline phosphatase-conjugated goat anti-rabbit IgG as second antibody. Bands were visualized by CDP-Star. The values of fold-increases were determined by densitometric analysis of the stained bands from three independent experiments.

2.5. Data analysis

Testes from three individual monkeys for each treatment group were analyzed. Experiments were repeated at least three times, and one representative from at least three similar results is presented. The quantitative data represent the mean ± SEM of three results.

3. Results

3.1. Immunohistochemical localization of $P16^{INK4a}$ protein in the cryptorchid testis

As shown in Fig. 1, the expression level of $P16^{INK4a}$ protein was low, not obviously changed in the cryptorchid testis on Day 5 after operation compared with the control testis. However, the expression level of $P16^{INK4a}$ increased dramatically in spermatogonia and Sertoli cells in the cryptorchid testis on Day 10; morphologically almost all spermatocytes and spermatids were lost. The $P16^{INK4a}$ level in the spermatogonia remained high thereafter.

3.2. Immunohistochemical localization of $P16^{INK4a}$ protein in the testis after TU injection

The expression of $P16^{INK4a}$ protein was almost unchanged and was very low in the TU-treated testes on Days 14 and 30 after injection compared with the normal testis (Fig. 2). However, its expression level increased dramatically in spermatogonia, the other germ cells, as well as Sertoli cells on Day 60 after treatment and then remained at its high level.

3.3. Western blot analysis of $P16^{INK4a}$ protein in the cryptorchid and TU-treated monkey testes

The content of $P16^{INK4a}$ protein in the cryptorchid and the TU-treated monkey testes was analyzed by Western blotting with rabbit anti-$P16^{INK4a}$ monoclonal antibody. Fig. 3 revealed that, compared to its expression in normal testis, $P16^{INK4a}$ protein content in the cryptorchid testis on Day 5 after operation was the same as the control. A rapid

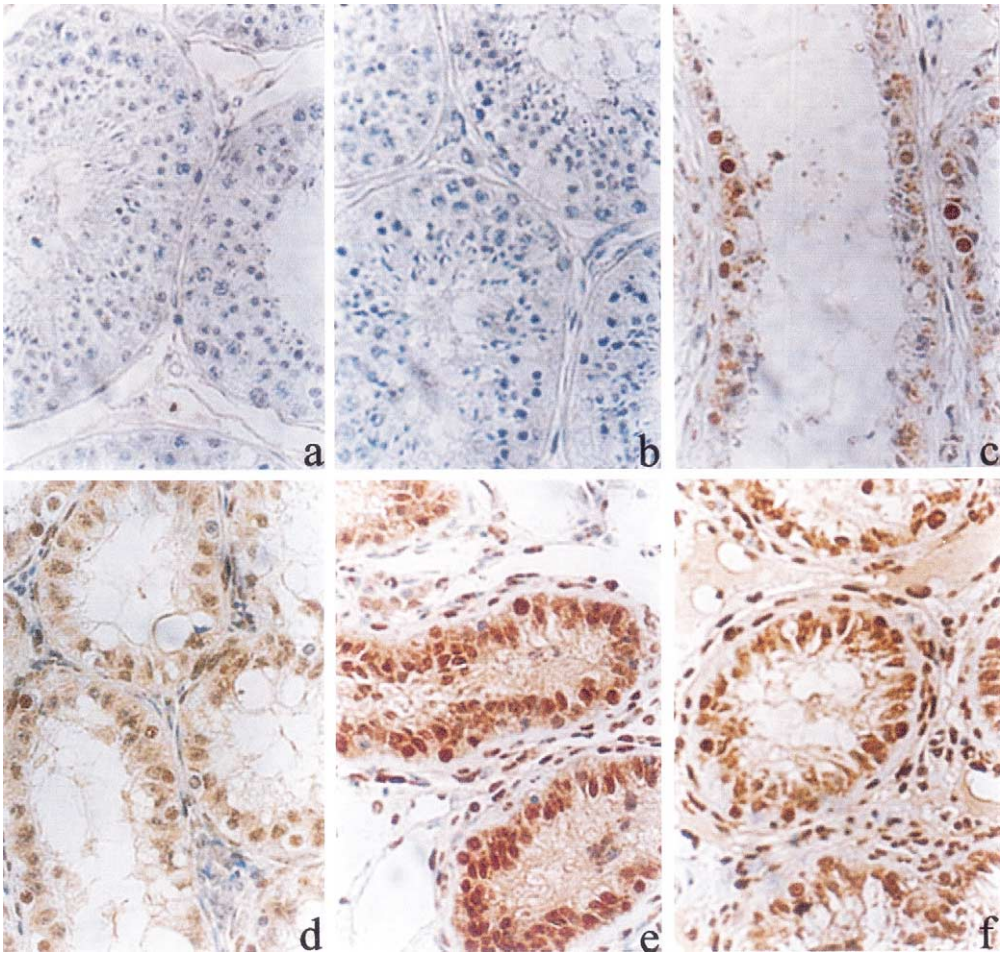

Fig. 1. Immunohistochemical localization of $P16^{INK4a}$ protein in the cryptorchid testes. (a) normal testis. (b-f) the cryptorchid testis on Days 5 (b), 10 (c), 15 (d), 30 (e), and 60 (f), respectively; magnification ×400.

increase of $P16^{INK4a}$ protein content at about one-fold on Days 10 and 15 and about two-fold on Day 30 were observed, respectively. Fig. 4 shows the changes of $P16^{INK4a}$ protein content of the TU-treated testes. The protein level was almost unchanged on Day 30 after the injection and followed by rapid increase to about one-fold on Day 60, and then it remained at the high level.

4. Discussion

Azoospermia or oligozoospermia can be induced by heat stress [2,8] or high doses of testosterone [1,9,10]. In our previous studies, we examined the role of TR2 [2,11] and Hsp70–2/HSF2 [12] in germ cell apoptosis induced by cryptorchid and high doses of TU treatment. In the present study, we further demonstrated for the first time that $P16^{INK4a}$ may inhibit mitosis of spermatogonia at the later stage of germ cell apoptosis induced by the heat stress and high doses of TU treatment.

Cell proliferation is positively regulated by CDKs. In normal cells, progression through G1-phase of cell cycle depends on the activities of cyclin D-dependent CDK4 or CDK6, and later, on cyclin E- and A-dependent CDK2 [13]. However, the activities of G1 CDKs can be blocked by CDK inhibitors that, in mammalian cells, fall into one of two distinct families [7,14]. The INK4 class (inhibitors of CDK4) consists of four members ($P16^{INK4a}$, $P15^{INK4b}$, $P18^{INK4c}$, and $P19^{INK4d}$) that exclusively bind to and inhibit the cyclin D-dependent catalytic subunits CDK4 and CDK6. By contrast, the Cip/Kip family includes three members ($P21^{CIP1}$, $P27^{KIP1}$, and $P57^{KIP2}$) that bind to both cyclins and CDKs to preferentially inhibit cyclin E- and A-dependent CDK2. The induced expression of INK4 family proteins will inhibit cell proliferation [4].

Our previous studies demonstrated that the strongest apoptotic signal was observed in the testis on Day 5 after cryptorchid operation and on Day 30 after injection of TU [15,16]. Here the immunohistochemical results further showed that the expression level of $P16^{INK4a}$ protein was low, and no change in the testis at the early stage of germ cell apoptosis induced by heat stress or high doses of TU was observed compared with the normal testes. However, at the later stage of germ cell apoptosis, the expression of

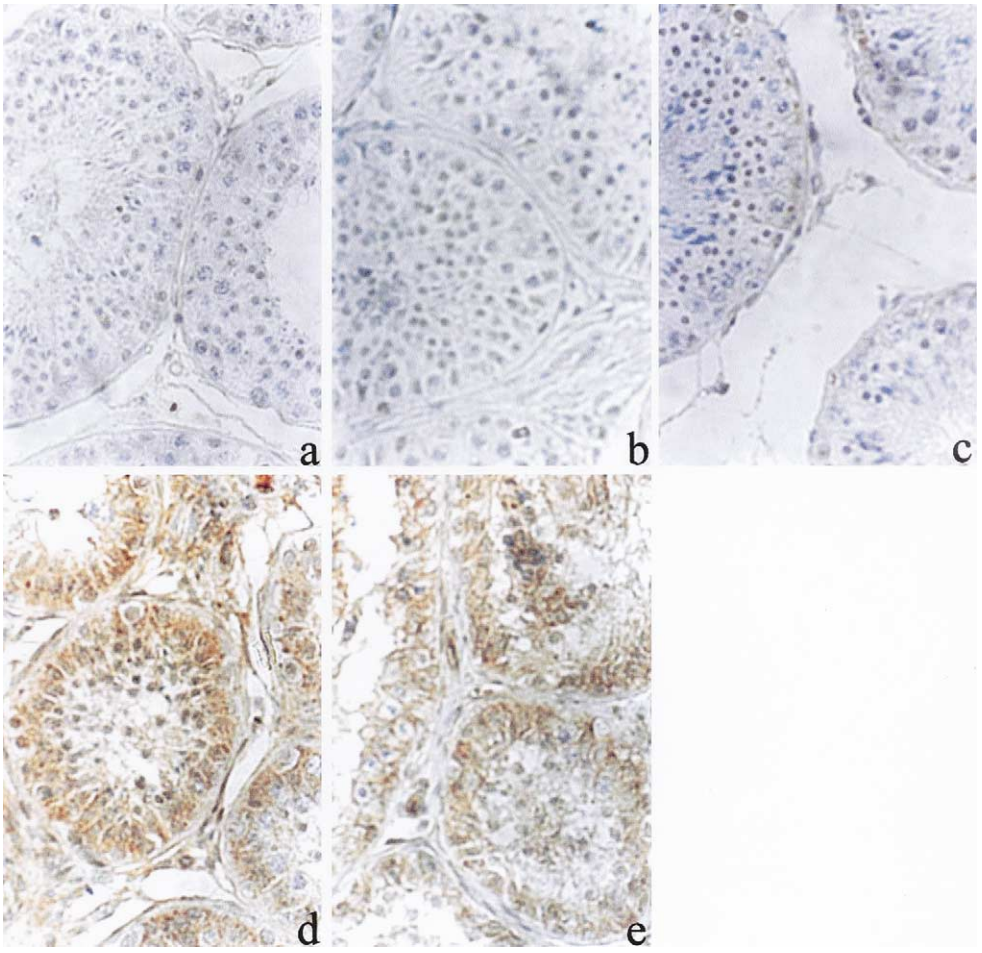

Fig. 2. Immunohistochemical localization of $P16^{INK4a}$ protein in the TU-treated monkey testes. (a) normal testis. (b-e) TU-treated monkey testes on Days 14 (b), 30 (c), 60 (d), and 90 (e), respectively; magnification ×400.

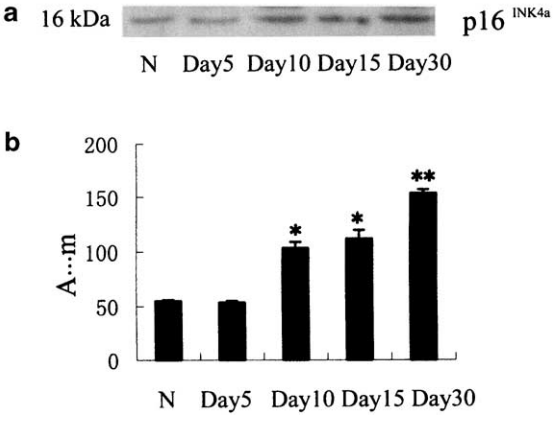

Fig. 3. Western blot analysis of $P16^{INK4a}$ protein in the cryptorchid testes. (a) Fifty micrograms of protein from testicular cell lysates were electrophoresed, transferred to a membrane, and hybridized as described in Materials and Methods. N: normal testes; D5, D10, D15, and D30 represent the Days 5, 10, 15, and 30 after operation, respectively. (b) Quantitative analysis of $P16^{INK4a}$ protein content in the testes. Each data point represents the mean ± SEM (n = 3). A. . .m is optical density. (* $p < 0.05$, ** $p < 0.01$ vs. control group).

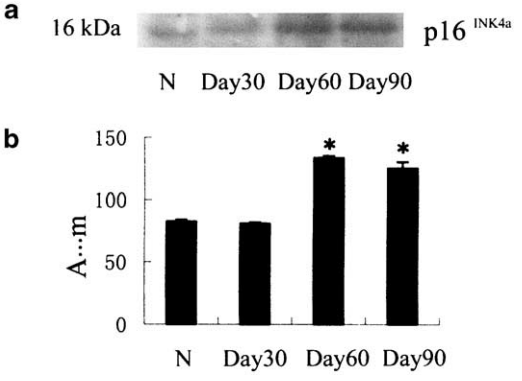

Fig. 4. Western blot analysis of $P16^{INK4a}$ protein in the TU-treated monkey testes. (a) Fifty micrograms of protein from testicular cell lysates were electrophoresed, transferred to a membrane, and hybridized as described in Materials and Methods. **N**: normal testes; D30, D60, and D90 represent the Days 30, 60, and 90 after TU treatment, respectively. (b) Quantitative analysis of $P16^{INK4a}$ content in the testes. Each data point represents the mean ± SEM (n = 3). A. . .m is optical density. (* $p < 0.05$ vs. control group).

$P16^{INK4a}$ protein was induced dramatically and then remained at its high level in spermatogonia. These changes were also confirmed by Western blot analysis. It is, therefore, suggested that $P16^{INK4a}$ may inhibit spermatogonia mitosis at the later stage of germ cell apoptosis.

In summary, both heat stress and TU treatment are capable of inducing germ cell apoptosis at early stage on one hand, and inhibiting spermatogonia mitosis at the later stage in the testis on the other hand, which may result in azoospermia or oligozoospermia.

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