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Role of *Fas/FasL* genes in azoospermia or oligozoospermia induced by testosterone undecanoate in rhesus monkey[1]

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KEY WORDS *Fas/FasL* genes; testosterone; apoptosis; macaca mulatta

ABSTRACT

AIM: To investigate role of apoptosis related genes *Fas/FasL* in azoospermia or oligozoospermia induced by testosterone undecanoate. **METHODS**: TUNEL was used to detect the apoptotic signal of testicular cells. Immunohistochemistry and Western blot were used to quantitatively or qualitatively analyze the expression of these apoptosis-related proteins. **RESULTS**: After injection of testosterone undecanoate, both the apoptotic signal in germ cells and the expression of Fas/FasL in testis increased correlatively in a time-dependent manner, reaching a maximum level on day 30. **CONCLUSION**: Fas system may initiate and regulate the germ cell apoptosis induced by testosterone undecanoate.

INTRODUCTION

It has been reported that a high dose of testosterone is capable of functioning as a male contraceptive. Testosterone has been demonstrated to suppress spermatogenesis through inhibition of pituitary gonadotropin secretion[1]. Studies by landmark WHO contraceptive efficacy have established that testosterone enanthate-induced azoospermia[2] and severe oligozoospermia[3] provided highly effective and reversible contraception with minimal short-term side effects. Despite its role in contraception was definite, the contraceptive molecular mechanism of testosterone was still unclear. The androgen withdrawal as a result of hypophysectomy[4], or by injection of GnRH antagonist[5] or ethane-1, 2-dimethane sulphonate (EDS)[6], increased degeneration of pachytene spermatocytes and spermids by cell apoptosis. These data suggest that the degenerated germ cells by withdrawal of the endogenous testosterone after administration of testosterone undecanoate (TU) may undergo apoptosis.

A histochemical approach such as terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) has turned out to be a convenient way to detect apoptotic nuclei with double-stranded DNA breaks[7]. However, the most fascinating aspect of apoptosis may be the requirement for activation of certain genes[8].

Fas (APO-1, CD95), a transmembrane receptor glycoprotein, belongs to the tumor necrosis factor/nerve growth factor receptor family[9]. It contains a "death domain" and is capable of initiating apoptosis when stimulated by its ligand, FasL[9]. It is generally accepted that both Fas and FasL are expressed in the seminiferous epithelium of the testis. However, there is a discrepancy in the precise cellular localization of Fas and FasL in the seminiferous tubules in the literature. Several evidences showed that Fas was expressed in germ cells, including spermatocytes and spermatids[10], but also detected in Sertoli cells[6,11]. Some reports showed FasL mainly localized in Sertoli cells[10,12], while others detected FasL in the germ cells[6,11]. Although the Fas system has been thought to be involved in the testicular cell apoptosis induced by environmental toxicants[10] or testosterone withdrawal after EDS injection[6], a relationship between the Fas pathway and apoptosis induced by a high dose of testosterone is still unknown.

To investigate the molecular mechanism related to testosterone-suppressed spermatogenesis and germ cell apoptosis, we have established a TU-treated monkey model which is similar to the human case[13]. The experiments were designed to examine the testicular cell apoptosis in relation to the expression of Fas/FasL during

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the TU treatment.

MATERIALS AND METHODS

Animals and tissue preparations Male adult rhesus monkeys (7 – 8 year old) were obtained from the Primate Research Centre, Kunming Institute of Zoology, Chinese Academy of Sciences. The animals were treated either weekly by im injection of TU 20 mg/kg, or twice weekly injection of TU 10 mg/kg on day 1 and 4 (D_7 group). Their testes were removed at 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 TUNEL, *in situ* hybridization or immunohistochemistry. The other quarters were snap-frozen in liquid nitrogen and stored at – 70 ℃ for mRNA analysis.

Materials and reagents Rabbit anti-Fas and anti-FasL antibodies were from Santa Cruz Co. RNA labeling mixture, *in situ* cell death detection kit (AP), alkaline phosphatase conjugated anti-DIG IgG and CDP-Star™ chemiluminescence reagent were purchased from Boehringer Mannheim GmbH Biochemical (Mannheim, Germany). T3 and T7 RNA polymerase, *Eco*R Ⅰ and *Hind* Ⅲ and substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) were obtained from Promega Corporation (Madison, WI, USA). Levamisole and paraformaldehyde were from Sigma Chemical Company (St Louis, MO). Zeta-Probe nylon membrane and Gene Linker UV chamber were purchased from Bio-Rad laboratories (Richmond, CA). Acid guanidine thiocyanate and agarose gel were from Gibco BRL (Life Technologies Inc, Gaithersbury, MD). Formamide and 3-aminopropyl-triethoxysilane (APES) were obtained from Beijing Zhongshan Biotechnology Inc.

***In situ* analysis of testis cell DNA fragmentation** DNA fragmentation in histological sections was done using non-radioactive detection method. Namely, sections (6 μm) were mounted on coated slides, deparaffinized, and hydrated. The slides were incubated with proteinase (20 mg/L) for 15 min at 37 ℃ and washed in PBS. Then DNA 3'-end labeling with digoxigenin-dideoxyuridine triphosphate (ddUTP) was preformed after incubation of the slides for 10 min in terminal transferase buffer (potassium cacodylate 200 mmol/L, Tris 25 mmol/L, BSA 0.25 g/L, and $CoCl_2$ 5 mmol/L, pH 6.6) at room temperature. Terminal transferase (1×10^6 U/L), digoxigenin-ddUTP (1 μmol/L), and dideoxy ATP (ddATP, 49 μmol/L) were added in fresh buffer and incubated at 37 ℃ in humidified chamber for 1 h. After three times washes in Tris buffer, the slides were incubated with a blocking buffer [Tris 100 mmol/L, NaCl 150 mmol/L, pH 7.5, and 2 % (w/v) blocking reagent] for 30 min at room temperature before addition of antidigoxigenin antibody conjugated to alkaline phosphatase. After incubation with the antibody [1∶10 000 in 2 % (w/v) blocking reagent, Tris 100 mmol/L, and NaCl 150 mmol/L, pH 7.5] at room temperature for 2 h in a humidified chamber, the slides were washed three times in Tris buffer and finally equilibrated in alkaline phosphatase buffer (Tris 100 mmol/L, NaCl 100 mmol/L, and $MgCl_2$ 50 mmol/L, pH 9.5) before addition of enzyme substrates (nitro-blue tetrazolium 337.5 mg/L and BCIP 175 mg/L) for alkaline phosphatase. After 60 min, the color reaction was terminated with Tris 10 mmol/L, edetic acid 1 mmol/L, pH 8, and counter-stained with eosin. Blue staining represented positive reaction.

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 PBS for 30 min. The primary antibody (rabbit anti-Fas or anti-FasL 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 % H_2O_2 for 2 – 7 min. The sections were dehydrated through a graded series of ethanol, cleared in xylene, and then mounted.

Western blot analysis Testes were homogenized in lysis buffer (phosphate buffer 5 mmol/L, pH 7.2, containing 0.1 % Triton X-100, phenylmethylsulfonylfluoride 1 mmol/L, leupeptin 1 mg/L, and chymostatin 1 mg/L). The protein content of the preparation was determined by spectrophotometer, using BSA as a standard. Sample lysates were mixed with the loading buffer (final concentration, 1,4-dithiothreitol 62.5 mmol/L, 5 % SDS, and 10 % glycerol), boiled for 8

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min, separated by SDS-PAGE, 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-Fas and anti-FasL sera, and then incubated for 1 h AP-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.

Data Analysis Testes from at least 2 individual monkeys for each treatment group were analyzed. Experiments were repeated at least three times, and one of representatives from at least three similar results is presented. The quantitative data represent $\bar{x} \pm s$ of three results at least.

RESULTS

***In situ* analysis of apoptotic DNA fragmentation in germ cells of TU-treated monkey testes** The apoptotic signals appeared, but were very weak in germ cells on day 7 after injection of TU (Fig 1b); they became stronger on day 14 (Fig 1c); the strongest signals were observed on day 30 (Fig 1d). However, the signals decreased dramatically on day 60 (Fig 1e). Moreover, these labeled cells were identified as primary spermatocytes and round spermatids (Fig 1f). In contrast, in the control testes, only a few spermatogenic cells showed DNA fragmentation (Fig 1a).

Immunohistochemical localization of Fas and FasL protein in testes after TU injection To identify positive cells for Fas and FasL, we localized Fas and FasL immunohistochemically in the paraffin sections at various intervals after TU injection. As shown in Fig 2, staining for Fas was in a broad range of germ cells, mainly including spermatocytes and spermatids, and also in Sertoli cells. The intensity of positive staining cells initially decreased (Fig 2b), but then increased time-dependently, reaching a maximum level at day 30 after TU injection. On the other hand, FasL positive staining cells were also detected in Sertoli cells and germ cells. The patterner of FasL expression is consistent with that of Fas expression as shown in Fig 3. In the sections incubated with normal rabbit serum as negative control, no staining was found (data not shown).

Western blot analysis of Fas and FasL We examined the content of Fas and FasL proteins by Western blotting with rabbit anti-Fas and anti-FasL antibodies in the testes after injection of TU. Fig 4 reveals that as compared with their expression in normal testis, both Fas and FasL initially slightly dropped on day 7 and followed by increase in a time-dependent manner, reaching maximum level on day 30. Both 1.5-fold in Fas and 2-fold in FasL content in the testis were observed on that day after TU-treatment, respectively. After that time, their expression declined again.

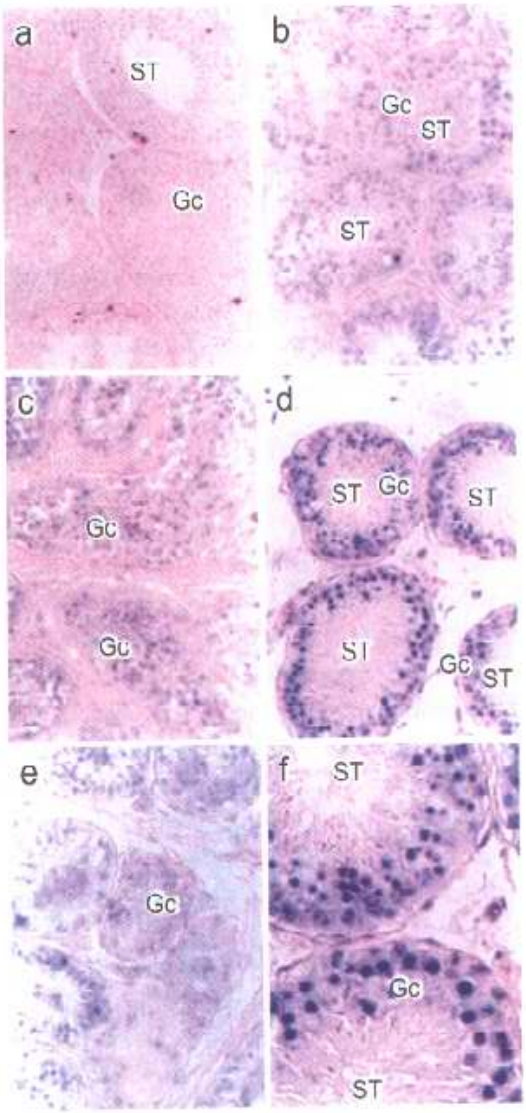

Fig 1. *In situ* 3'-end labeling of DNA fragmentation on testis sections from TU-treated monkeys. a: normal testis. b – e: TU-treated monkey testes on day 7 (b), 14 (c), 30 (d), and 60 (e), respectively. f: the higher magnification of d. ST: seminiferous tubule; Gc: germ cell. Magnification × 200 for a – e and × 400 for f.

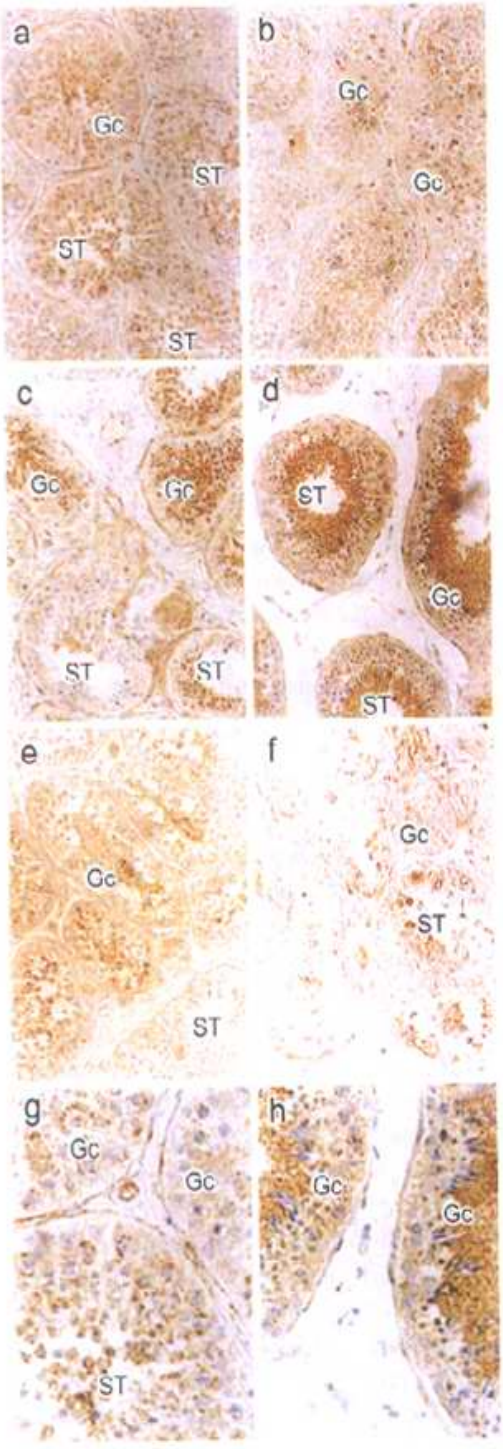

Fig 2. Immunohistochemical localization of Fas protein in monkey testes. a: normal testis; b – f: TU-treated monkey testes on day 7(b), 14(c), 30(d), 60(e), and 90(f), respectively. g and h: the higher magnification of a and d, respectively. ST: seminiferous tubule; Gc: germ cell. Magnification × 200 for a – f and × 400 for g and h.

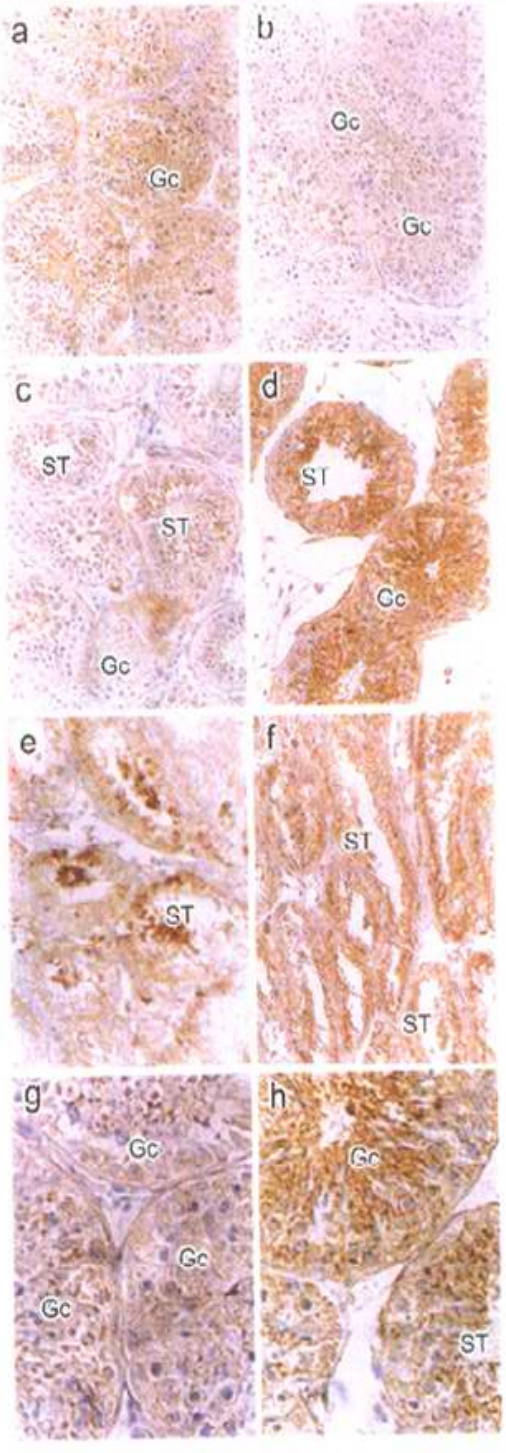

Fig 3. Immunohistochemical localization of FasL protein in the testes. a: normal testis; b – f: TU-treated monkey testes on day 7(b), 14(c), 30(d), 60(e), and 90(f), respectively. g and h: the higher magnification of a and d, respectively. ST: seminiferous tubule; Gc: germ cell. Magnification × 200 for a – f and × 400 for g and h.

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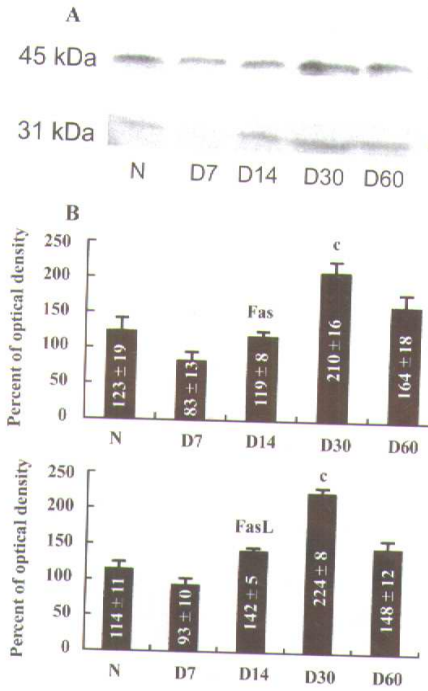

Fig 4. Western blot analysis of the expression of Fas and Fas L protein in monkey testes. A: Western blot analysis using specific polyclonal antibodies against Fas and Fas L. Fifty micrograms of protein from testicular cell lysates were electrophoresed, transferred to a membrane, and hybridized as described in materials and methods. N: normal testes; D7, D14, D30, D60 represent the day after TU treatment. B: Quantitative analysis of Fas and FasL content in the testes. $n = 3$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control group.

DISCUSSION

Testosterone functions as a contraceptive[1-3] by suppressing secretion of piptuitary gonadotropic luteinizing hormone and follicle stimulating hormone. Low levels of these hormones decrease endogenous testosterone secretion from the testes and deprive developing sperm of the signals required for normal maturation, which causes a decline in sperm production and can result in reversible infertility in men[14]. However, the exactly molecular mechanism of testosterone as a contraceptive is unclear.

It is well known that EDS can induce germ cell apoptosis. EDS is a unique testicular toxin with cytotoxic action confined almost exclusively to the Leydig cells[15]. EDS selectively eliminates both basal and LH-stimulated testosterone production, resulting in complete androgen ablation within the testes. After administration of EDS, the germ cells undergo apoptosis as a result of endogenous androgen withdrawal induced by this drug[6], and the germ cell apoptosis may be mediated by Fas pathway[6]. These data tempted us to raise the possibility that induction of germ cell death as result of endogenous testosterone withdrawal after injection of TU might also take the form of apoptosis and be regulated by Fas pathway.

We have previously reported that administration of a high dose of TU induced azoospermia or oligozoospermia in rhesus monkeys in a time-dependent manner, and followed by increase of plasminogen activator activity in semen[13]. To assess the involvement of testicular cell death in azoospermia or oligozoospermia induced by a high dose of testosterone, we first examined the presence of apoptotic cells by means of biochemical methods in monkey testes after TU injection. Our TUNEL result revealed that the apoptotic signal gradually increased in germ cells, reaching a maximum level on day 30 after TU injection. As a next step, we examined whether Fas and FasL expression may be associated with the germ cell apoptosis. Western blot analysis showed that injection of TU time-dependently enhanced the expression of both Fas and FasL in the testes after the initial slight drop on day 7; both Fas and FasL reached a maximum level on day 30 after TU injection. Positive staining for Fas protein was detected in germ cells and also in Sertoli cells, and the staining intensity in germ cells, especially in spermatocytes and spermids, increased in a time-dependent manner after TU treatment, reaching a maximum level on day 30. In parallel with the result of Western blotting, the staining intensity for FasL protein was increased, the positive staining was detected not only in Sertoli cells, but also in germ cells. It is interesting to note co-localization and co-increase of Fas and FasL in spermaocytes and spermatids which were undergoing apoptosis. Fas/FasL system has been widely accepted as a major molecular mechanism to induce apoptosis on the Fas-bearing cells whenever FasL binds it (FasL receptor)[16]. This finding therefore suggests that the Fas system may take part in initiating and regulating the germ cell apoptosis induced by TU.

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Fas/FasL 基因在 11 酸睾酮诱导恒河猴无精子症或少精子症中的作用[1]

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关键词　*Fas/FasL* 基因；睾酮；细胞凋亡；猕猴

目的：研究凋亡相关基因 *Fas/FasL* 在 11 酸睾酮诱导无精子症或少精子症的作用．**方法**：TUNEL 用于检测睾丸细胞的凋亡信号．免疫组化和 Western blot 用于对这些凋亡相关基因所表达的蛋白进行定量或定性分析．**结果**：11 酸睾酮处理后，生精细胞的凋亡信号和 Fas/FasL 蛋白的表达呈现时间依赖性方式增强，至第 30 天达到最大值．**结论**：Fas 系统可能参与启动和调节 11 酸睾酮诱导的生精细胞的凋亡．

（责任编辑　吴民淑）