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Differential Expression of Transforming Growth Factor-β in the Interstitial Tissue of Testis During Aging

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Abstract Transforming growth factor-βs (TGF-βs) have significant effects on testis development. The pattern of TGF-β expression in aging testis has not been established to date. We examined age-related changes in the expression of TGF-β and its receptors in the testis using Western blot analysis. TGF-β1 expression increased continuously in aging rat testis, whereas no age-associated changes were observed for TGF-β3. Strong expression of TGF-β2, as well as type I and II receptors was observed in 12-month-old testis, but following this time, expression decreased dramatically. Interestingly, TGF-β2 and -β3 displayed strong and similar expression patterns in liver, regardless of age, suggesting that the down-regulation of TGF-β2 is testis-specific. We observed significant induction of p53 and $p21^{WAF1}$ in 18-month-old testis that appeared to correspond with aging. Moreover, caloric restriction (CR) prevented age-related decrease in TGF-β2 expression. Using immunohistochemistry, we showed that all TGF-β1, -β2, and -β3 proteins are expressed primarily in interstitial cells, which are located in the space between adjoining seminiferous tubules. Our data collectively indicate that aging in the testis is regulated by differential expression of TGF-β proteins, and decreased levels of TGF-β2 contribute to the aging process. J. Cell. Biochem. 92: 92–98, 2004.

Key words: senescence; TGF-β; testis

Both the development and aging of cells involve altered patterns of gene expression. Aging involves progressive loss of physiological capacity at both the cellular and tissue levels [Helenius et al., 1996]. Numerous in vitro and in vivo studies suggest that transforming growth factor-β (TGF-βs) play an important role in the early stages of testis development.

TGF-β is a multi-functional cytokine involved in the regulation of the cell cycle, differentiation, apoptosis, tissue repair, angiogenesis, and extracellular matrix accumulation. Since TGF-β has potent biological effects, it is important that activating the latent TGF-β complex tightly controls its activity. TGF-β1, -β2, and -β3 are differentially expressed in mammalian tissues. However, the isoform-specific roles of each TGF-β are not well-defined, and each has slightly different biological effects [Mullaney and Skinner, 1993]. Each TGF-β binds with different affinities to TGF-β receptors. Consequently, the Smads protein is phosphorylated, activated, and translocates to the nucleus where it cooperates with other transcription factors to coordinate the expression of target genes.

Abbreviations used: LC, Leydig cells; TGF-β, transforming growth factor-β; TGF-β-R, transforming growth factor-β receptor.

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The testis has two interrelated functions, specifically, the production of gametes and steroids. TGF-βs and their receptors are involved in testis development [Watrin et al., 1991; Mullaney and Skinner, 1993; Le Magueresse-Battistoni et al., 1995; Ingman and Robertson, 2002]. Both somite and germ cells express TGF-β1, while only somite cells express TGF-β2 and -β3. TGF-β receptor I (TGF-β-RI) and II (TGF-β-RII) are expressed predominantly in the immature testis, suggesting that the cytokine plays a key role in early testicular development. Typically, hormones control and regulate the expression of TGF-β in testicular target cells. Steroid hormones, primarily testosterone, function in regulating the development of spermatozoa as well as the growth, development, and maintenance of accessory reproductive glands.

The population of interstitial cells, which mainly comprises Leydig cells (LC), is the chief source of testosterone in the male. LC are classified as fetal or adult, according to whether they differentiate prenatally or postnatally around the second postnatal week [Lording and De Kretser, 1972; Mendis-Handagama and Ariyaratne, 2001]. LC secretes testosterone during the fetal period, which promotes the development of the male reproductive tract. The testis becomes essentially devoid of recognizable LC soon after birth. At puberty, in response to gonadotropic stimulation, adult LC re-develop and begin synthesizing and secreting primarily testosterone. Thereafter, the number of LC remains constant, and does not decline in elderly individuals. A number of researchers suggest that luteinizing hormone (LH) stimulates LC proliferation, dependent on the relative amounts of TGF-α, TGF-β, insulin-like growth factor-1, and interleukin-1β [Teerds and Dorrington, 1993].

Several lines of evidence show that aging is associated with increased levels of TGF-β. In this investigation, we demonstrate differential expression and localization of TGF-β isoforms to determine their possible roles in the aging process in testis. Here, we report that aging is associated with strongly decreased expression of TGF-β2, but not TGF-β1 and -β3, which remain up-regulated or unaffected in the testis of aging rats. The data imply that different forms of TGF-β proteins specifically expressed by interstitial tissue regulate aging. Moreover, the aging process may repress the function of the TGF-β2 signaling system in vivo.

MATERIALS AND METHODS

Animals and Materials

We used testes from young adult (6-month-old), middle-aged (12- and 18-month-old) and half-old (2-year-old) male Sprague–Dawley rats in the study. Animals were housed in the animal facility under pathogen-free conditions and fed standard rat chow. Animals in the 'caloric restriction (CR)' group were fed 60% of the food intake of ad libutum-fed littermates, beginning at 6 weeks of age. Animals were killed using carbon dioxide. Testes of three rats from each group were removed, washed in ice-cold phosphate buffered saline (PBS), frozen in liquid nitrogen, and stored at −80°C for Western blot analysis or embedded into OCT compound (Western Sakura Finetech, Torrance, CA) for immunohistochemistry.

Western Blot Assay

Frozen whole testes were homogenized with a sonicator in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM NaCl, 5 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and various protease inhibitors). Homogenates were incubated for 30 min and centrifuged at 25,000*g* for 15 min at 4°C. Soluble proteins were recovered by centrifugation at 25,000*g* for 15 min at 4°C. The concentration of total protein in samples was measured using the Bradford protein dye reagent (Bio-Rad, Hercules, CA). TGF-β proteins were assayed using primary antibodies (Santa Cruz, Santa Cruz, CA) against TGF-β1, -β2, and -β3. Each protein sample (25 μg) was separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (using a 10% gel) under reducing conditions. Proteins were transferred to nitrocellulose membranes, blocked with 10% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature, and incubated with the primary antibodies for 1 h at room temperature. Next, membranes were incubated in secondary goat anti-rabbit IgG conjugated with horseradish peroxidase for 1 h at room temperature. Proteins were detected on autoradiography film using an enhanced chemiluminescence detection kit (Amersham Biosciences, Seoul, Korea).

Immunohistochemistry

Cryostat sections (8 μm) were cut and placed on poly-L-lysine coated slides. Slides were dipp-

ed into methanol:3% hydrogen peroxide (4:1) for 15 min to remove endogenous peroxidase enzyme activity, rinsed with PBS, and blocked with normal goat serum for 1 h. Sections were incubated in a humidified chamber at room temperature for 30 min with anti-TGF-β1, -β2, and -β3 antibodies (Santa Cruz). After washing, sections were incubated for 30 min with diluted biotinylated secondary antibody. Tissues were washed twice in PBS for 5 min each and incubated for 30 min with vectastain ABC reagent (Vecta Laboratories, Burlingame, CA). After washing in PBS for 15 min, sections were treated with peroxidase substrate enzyme solution (3,3′-diaminobenzidine) until the desired stain intensity was obtained. Tissues were washed in PBS, mounted with Permount (Fisher Scientific, Fair Lawn, NJ), and photographed using a Nikon microscope equipped with a digital camera.

RESULTS

Differential Expression Patterns of TGF-β Proteins in Aged Testis

Levels of TGF-β proteins in the testes of aging rats were analyzed by Western blotting. We mainly detected bands corresponding to the latent forms of TGF-β1, -β2, and -β3 at 50 kDa (Fig. 1). However, the activated form of TGF-β was not observed. Expression of the latent form of TGF-β1 increased continuously from 6 months up to 24 months of age. However, in the aging testis, TGF-β3 protein levels were not affected. TGF-β3 displayed similar profiles of constant expression at all the examined periods. Surprisingly, the amounts of the TGF-β2 and type I and type II TGF-β receptor decreased significantly by 18 months. This decreased protein level persisted until 2 years of age (Fig. 1A).

Accumulating evidence suggests that cytotoxic drugs, particularly at subcytotoxic concentrations, induce terminal growth arrest with features of senescence. p53 and the cyclin-dependent kinase inhibitor, $p21^{WAF1}$, are major players in this process [Chang et al., 1999; Han et al., 2002]. As expected, levels of p53 and p21 were dramatically increased by 18 months of age, which continued up to 24 months (Fig. 1A). This finding suggests that testis aging may begin at 18 months.

We examined whether TGF-β2 down-regulation is tissue-specific in aged tissues by comparing expression in liver (Fig. 1B). Quantitative analyses revealed no differences in TGF-β2 and -β3 expression in liver aged from 6 to 24 months (Fig. 1B). This finding suggests that the down-regulation of age-related expression of TGF-β2 occurs in a tissue-specific fashion. CR enhances longevity in many different animal species, and

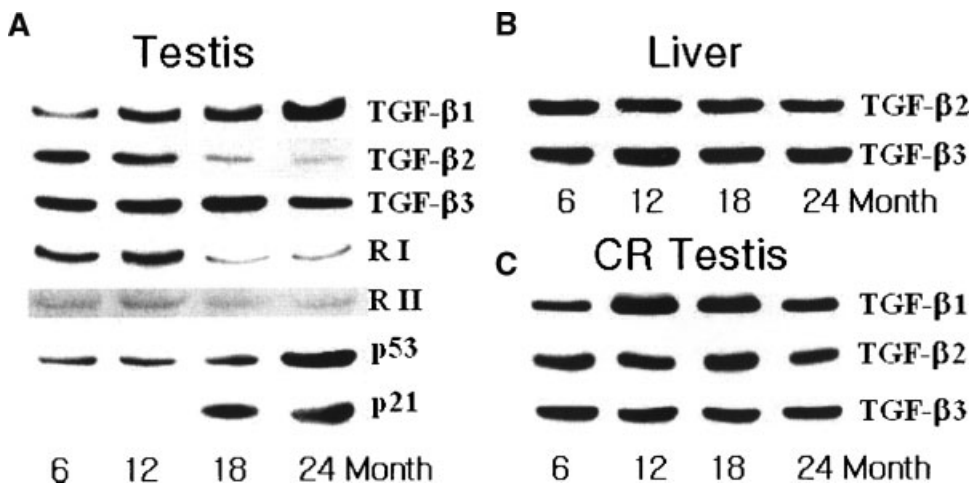

Fig. 1. Expression levels of transforming growth factor-β (TGF-β) proteins and receptors, as well as p53 and p21, in aging rat testis. **A**: Equal amounts of testis lysates (25 μg) from 6-month-, 12-month-, 18-month-, and 24-month-old rats were separated on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel, transferred to nitrocellulose membrane, and subjected to immunoblot analysis with anti-TGF-β1, -β2, -β3, type I (RI) and type II (RII) TGF-β receptor, p53, and p21 antibodies. **B**, **C**: Equal amounts of liver and caloric restriction (CR) testis lysates (25 μg) from 6-month-, 12-month-, 18-month-, and 24-month-old rats were separated on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose membranes, and subjected to immunoblot analysis with anti-TGF-β1, -β2, and -β3 antibodies. Notably, specific down-regulation of TGF-β2 was detected in aged testis, but not aged liver and caloric restricted testis. Data are presented as results of a typical experiment performed four times, with reproducible patterns.

prevents oxidative damage in mammalian cells [Sohal and Weindruch, 1996]. Accordingly, we investigated whether CR prevents down-regulation of age-related expression of TGF-β2. As expected, there was little change in TGF-β2 expression in the CR group, even in senescent 24-month-old rat testis (Fig. 1C). Our data clearly show that the aging process of rat testis is accompanied by decreased expression of TGF-β2. Moreover, CR is responsible for its powerful anti-aging action.

Localization of TGF-βs in Aged Testis

To evaluate the localization of TGF-β expression, we analyzed frozen sections of testis from both 6-month (Fig. 2A) and 24-month-old rats (Fig. 2B–F). Representative photomicrographs depict the localization of TGF-β immunoreactivity and relative intensity of staining (Fig. 2). All examined TGF-β1 (Fig. 2A–C), -β2 (Fig. 2D), and -β3 (Fig. 2E) isoforms were predominantly detected in the cytoplasm of interstitial cells between the seminiferous tubules, presumably in LC. No significant age-related differences in localization were observed. As with the adult testis, no major or systematic variation in immunostaining was detected for any of the TGF-β isoforms examined in the testes of aged rats. Additionally, immunostaining was not observed in the control sample in which testis from a 24-month-old rat was incubated without the primary antibody (Fig. 2F).

DISCUSSION

TGF-βs are directly secreted from LC [Robinson et al., 2001]. These cytokines influence numerous cellular functions, and their aberrant expression is implicated in a number of disease processes. TGF-β proteins play a variety of important roles in testicular development and function. However, to date, no data have been published on the expression levels of TGF-β in testis with aging. Accordingly, in this study, we investigate the expression patterns of TGF-β1, -β2, and -β3, as well as the type I receptor in the aging rat testis. In adult testis, expression of these cytokines was predominantly observed in interstitial tissue, whereas no staining was detected in Sertoli cells and germ cells in seminiferous tubules, consistent with previous data. Immunolocalization experiments cannot be used to identify the cellular site of production of these TGF-β isoforms as they are secreted.

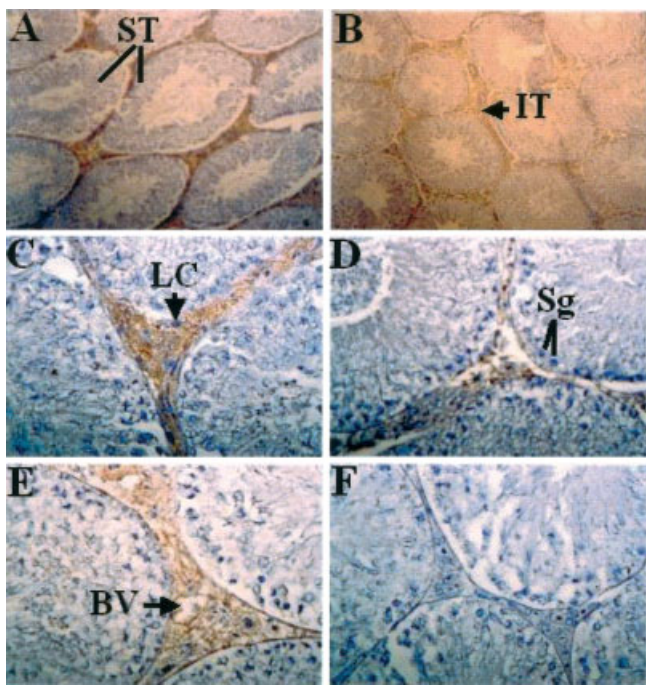

Fig. 2. Immunohistochemical localization of TGF-βs in aging rat testes. Sections of testis tissue from a 6-month-old (**A**) and 24-month-old rat (**B**–**F**) were detected using anti-TGF-β1 (A–C), -β2 (D), and -β3 antibodies (E). For the negative control, incubation was performed without primary antibody (F). LC, Leydig cell; IT, interstitial tissue; Sg, spermatogonia; ST, seminiferous tubule; BV, blood vessel. Magnifications: ×10 (A, B); ×40 (C–F).

The proteins may bind to the extracellular matrix or directly to cells. Thus, it is likely that TGF-βs are directly secreted from LC to mediate specific functions.

LC differentiate and secrete testosterone during the fetal period, which promotes the development of the male reproductive tract. However, the testis becomes essentially devoid of recognizable LC soon after birth. At puberty, LC re-develop in response to LH [Huhtaniemi et al., 1981; Benton et al., 1995], and begin synthesizing and secreting primarily testosterone. Differential levels of TGF-β isoforms are expressed in interstitial tissue. Thus, it is possible that the combinatorial action of TGF-βs in the aging testis is essential for testosterone synthesis. Specifically, this action of TGF-βs may be necessary for the proliferation and differentiation of spermatogenic cells within the seminiferous epithelium.

Temporal/spatial expression of three TGF-β isoforms and their receptors is necessary for both embryonic and adult testis [Watrin et al., 1991; Mullaney and Skinner, 1993; Le Magueresse-Battistoni et al., 1995]. In rat testis, TGF-β1 and -β2 displayed temporal-spatial expression between spermatocytes and spermatids especially during spermatogenesis [Teerds and Dorrington, 1993]. In postnatal testis, TGF- β1, but not TGF-β2, was consistently observed in LC, and neither protein was expressed in interstitial tissue at day 35 [Teerds and Dorrington, 1993]. TGF-β3 inhibits the expression of tight junctions between cultured Sertoli cells [Lui et al., 2001]. In cultured LC, TGF-β1 reduces responsiveness to LH and inhibits steroidogenesis [Avellet et al., 1987]. The significantly higher level of TGF-β1 in 24-month-old testis (Fig. 1A) may affect the rate of testosterone synthesis in LC and inhibit spermatogenesis. Follicle stimulating hormone (FSH) decreases TGF-β2 synthesis in pubertal Sertoli cells [Mullaney and Skinner, 1993]. In contrast, co-treatment with LH stimulates TGF-β1 secretion in fetal testis [Gautier et al., 1997]. In this case, we postulate that FSH also negatively regulates the synthesis of TGF-β2 in LC between 18 and 24 months in vivo. Moreover, low levels of TGF-β2 and high levels of LH, in turn, enhance TGF-β1 expression. Both TGF-β-RI and TGF-β-RII are strongly expressed in the immature testis, and possibly play key roles in early testicular development [Le Magueresse-Battistoni et al., 1995].

Down-regulation of TGF-β-RI in the aged testis (between 18 and 24 months) (Fig. 1A) may also be implicated in the process of aging, but its relative importance for downstream signaling remains to be investigated. Our present data indicate that differentially co-expressed amounts of TGF-β proteins and their receptors in interstitial tissue control steroidogenic activity in concert with various cytokines and hormones. In a pericellular environment of adult testis, it is possible that LH resulting from the cooperation with FSH and TGF-β isoforms as well as their receptors stimulates spermatogenesis. In view of earlier findings, it appears that TGF-βs are directly involved in the process of testis aging.

A number of studies show that TGF-β is involved in cell death in the testis. Previously, researchers associated the activity of differential TGF-β isoforms in the postnatal development of testis with germ cell apoptosis [Olaso et al., 1998]. Cryptorchidism leads to infertility due to reduction in germ cells through apoptosis that correlates with the induction of TGF-βs, tumor necrosis factor-α, Fas, Fas ligand, and decreased levels of testosterone [Ohta et al., 1996]. Recent studies on Smad4 transgenic mice show that over-expression of these cytokine isoforms causes apoptosis of germ cells and LC hyperplasia. It is therefore concluded that TGF-β signaling pathways are carefully orchestrated [Narula et al., 2002]. Interestingly, p53 and p21 are implicated in the process of apoptosis [Embree-Ku et al., 2002; Lin et al., 2002; Yin et al., 2002; Ohta et al., 2003]. The following results were obtained with testis: (i) expression of p53 and p21 was induced by 18 months of age, and (ii) maximum levels of expression were observed in 24-month-old testis (Fig. 1A). Our data, along with previous studies, suggest that cooperative interactions between TGF-βs and their receptors, hormones, and other cytokines contribute to testis apoptosis through p53 downstream signaling. Since p21 is induced by p53-dependent mechanisms, it is possible that the p21 oncogene is partly responsible for aging phenotypes in rat testis.

In our system, all TGF-β isoforms appear as the predominantly latent form. The latent form of TGF-β may bind to extracellular matrix, prevent diffusion and confer high degree of local control of activity. Latent TGF-β1, -β2, and -β3 are differentially expressed in mammalian tissues. Each isoform binds with varying affinities to TGF-β receptors, and has slightly

different biological effects [Mullaney and Skinner, 1993]. Moreover, higher homology exists between the mature regions compared to precursor regions, suggesting distinct functions of the precursor region. In general, TGF-β1 is produced and secreted as a latent, high molecular weight complex consisting of mature TGF-β1, latency-associated peptide (LAP), and latent TGF-β1 binding protein (LTBP-1). The precursor or LAP has an important function in the latency of TGF-β complex. A number of researchers have demonstrated that LTBP facilitates the secretion of latent TGF-β and may modulate activation of latent TGF-β [Miyazono et al., 1991; Flaumenhaft et al., 1993]. Depending on cell type, immunoprecipitation experiments led to the identification of several forms of TGF-β protein and revealed that the small latent complex of TGF-β (50 kDa) was biologically active [Baillie et al., 1996]. Importantly, both TGF-β2 and -β3 displayed a constant expression pattern in liver tissue as well as caloric restricted testis, regardless of aging (Fig. 1B,C). This finding suggests that the TGF-β isoform-mediated aging process is tissue-specific. It remains to be determined whether the forms of TGF-β (50 kDa) targeted and stored in interstitial tissue are biologically active in the testis.

In conclusion, we have demonstrated that TGF-β isoforms are differentially expressed in aged rat testis and co-localized in interstitial tissues. Our results suggest that these proteins are involved in steroidogenesis at each time-period. These cytokines may additionally function in the paracrine regulation of spermatogenesis.

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