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Telomerase activity in the testis of infertile patients with selected causes

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In human testes, stem cells such as spermatogonia need to produce progeny cells continually. Telomere length is maintained throughout spermatogenesis, i.e. from spermatogonia to spermatozoon, and telomerase is reported to be present in the testes. In this study, we measured the activity of telomerase in the human testes of 16 cases of idiopathic azoospermia, 10 of obstructive azoospermia, and 17 of oligozoospermia in order to understand the role of telomerase in spermatogenesis. Telomerase activity in the testes with Sertoli cell-only and in testes with maturation arrest were 0.08 ± 0.05 optical density (OD) (mean ± SD) and 1.96 ± 0.98 OD, respectively ($P < 0.05$). Classifying those testes with maturation arrest into two groups, the telomerase activity of those with early maturation arrest (arrest at spermatocyte) and of those with late maturation arrest (arrest at round spermatid) was 1.82 ± 0.82 OD and 2.10 ± 1.14 OD respectively. There was no significant difference between the two groups. The telomerase activity in the testes showing hypospermatogenesis in obstructive azoospermia and in those of oligozoospermia with hypospermatogenesis was 1.89 ± 1.06 OD and 1.92 ± 1.02 OD respectively. No difference in telomerase activity existed between the testes with maturation arrest and those with hypospermatogenesis in obstructive azoospermia or oligozoospermia. Sertoli cell-only testes without germ cells showed no telomerase activity. The source of the telomerase activity was likely to be germ cells. The telomerase activity in the testes ($n = 63$) was related to the histology of the testes. The activity of telomerase showed no significant correlation with the sperm concentration in each patient. Only serum oestradiol level significantly correlated with telomerase activity ($P < 0.05$). The concentrations of follicle stimulating hormone, luteinizing hormone, or testosterone had no significant relationship with the telomerase activity. Therefore similar levels of telomerase activity were detected in the testes of infertile men with azoospermia and oligozoospermia and in testes showing maturation arrest.

Key words: human testes/hypospermatogenesis/maturation arrest/Sertoli cell-only/telomerase activity

Introduction

Spermatogenesis is a complex process, including mitosis, meiosis and spermiogenesis. In human testes, four spermatogonial cell types have been identified, descriptively termed Along, Adark, Apale, and B. Type B spermatogonia are the differentiated precursors to preleptotene spermatocytes (Clermont, 1963). It remains unclear as to which spermatogonia are the stem cells (Clermont, 1966; Chowdhury *et al.*, 1975). Stem cells may actively divide into progeny cells, or remain in a quiescent or non-dividing state for much of their life span. In order to constantly divide to produce progeny, stem cells in the testes need to replicate their telomeres. Telomeres are heterochromatic structures at the ends of eukaryotic chromosomes and consist of simple, highly conserved, repeated DNA sequences (TTAGGG) (Counter, 1996). Their function is to protect and stabilize the chromosome ends. Chromosomes normally shorten with mitosis because of the inherent inability of DNA polymerases to replicate telomeres (Harley *et al.*, 1990). Ageing and replication of normal cells are accompanied by decreases in telomere lengths. The progressive reduction of telomere lengths to critically short sizes has been correlated with the cessation of cell division and the onset of senescence.

Telomerase is a ribonucleoprotein that extends telomeres by the addition of six base pair direct repeats onto chromosome ends (Morin, 1989; Blackburn, 1991; Counter, 1996). All tissues do not necessarily show evidence of active telomerase, even after screening with highly sensitive, polymerase chain reaction (PCR)-mediated telomeric repeat amplification protocol (TRAP) assay (Kim *et al.*, 1994). Telomerase has not been detected in most normal human somatic cells *in vivo* or *in vitro* (Counter *et al.*, 1992; Yasumoto *et al.*, 1996), nor has it been identified in epithelial tissues which contain reproductive somatic cells (Harley *et al.*, 1992, Wright *et al.*, 1996). In contrast, telomerase activity has been observed only in germ cells, along with some activity in normal bone marrow and peripheral blood leukocytes (Kim *et al.*, 1994; Harley *et al.*, 1995; Prowse *et al.*, 1995; Burger *et al.*, 1997). In this report, we investigated telomerase activity in the testes with regard to the various dysfunctions of spermatogenesis, using a TRAP assay associated with an enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Patients

A total of 43 patients were involved in this study: 17 oligozoospermic men, 10 obstructive azoospermic men, and 16 idiopathic azoospermic men whose condition was due to germinal cell aplasia ($n = 5$) or maturation arrest ($n = 11$). Semen was analysed using a Makler

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Table I. Semen analysis and concentrations of serum hormones

Clinical diagnosis	Semen analysis		Pathology	Serum hormone concentrations				
	Concentration (10^6/ml)	Motility (%)		FSH (mlU/ml)	LH (mlU/ml)	Testosterone (ng/ml)	Prolactin (ng/ml)	Oestradiol (pg/ml)
Idiopathic azoospermia	0		MA	5.3 ± 4.5	4.5 ± 2.2	4.0 ± 1.9	10.3 ± 8.5	23.1 ± 7.8
			SO	15.6 ± 4.7*	6.4 ± 2.4	3.7 ± 1.4	10.7 ± 3.4	29.0 ± 11.2
Obstructive azoospermia	0		HYPO	5.6 ± 6.3	3.2 ± 2.0	4.8 ± 1.9	6.7 ± 3.3	24.9 ± 8.1
Oligozoospermia	9.49 ± 8.39	40.2 ± 22.1	HYPO	7.7 ± 5.8	4.4 ± 2.6	4.2 ± 1.5	8.8 ± 5.4	23.8 ± 10.2

*Statistically significant difference $P < 0.05$.
MA = maturation arrest; HYPO = hypospermatogenesis; SO = Sertoli cell-only; FSH = follicle stimulating hormone; LH = luteinizing hormone.

chamber at least three times on three separate occasions after 5-day periods of abstinence, according to the World Health Organization (1987) standards of classification. Serum concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, prolactin, and oestradiol were measured by radioimmunoassay in the morning of the study. Testicular biopsies were performed in all patients. A total of 72 testicular specimens was obtained, including 25 specimens from the 16 men with idiopathic azoospermia, 15 specimens from the 10 men with obstructive azoospermia and 32 specimens from the 17 men with oligozoospermia. For the histopathological examination, the testicular specimens were fixed in Bouin's solution (Wako, Japan). A 5 µm section of each specimen was stained with haematoxylin and eosin, and spermatogenic function was then evaluated according to Johnsen's mean score (JS) (Johnsen, 1970). The rest of each specimen was snap-frozen in liquid nitrogen and stored at –70°C until assayed for telomerase activity. Each subject gave his informed consent for participation.

Telomerase assay

Telomerase assay was performed using the telomerase PCR-ELISA kit (Boehringer Mannheim, Tokyo, Japan), based on the TRAP assay. Briefly, the frozen specimen was homogenized individually in a glass homogenizer and centrifuged at 13 000 *g* for 20 min at 4°C. The supernatant was collected and the protein concentration was determined by Bradford (Bio-Rad, Tokyo, Japan). The supernatant containing 6 µg protein was used for PCR. The thermal cycler was set as follows: primer elongation, 25°C at 30 min; telomerase inactivation, 94°C at 5 min; denaturation, 30 s at 94°C; annealing, 30 s at 50°C; polymerization, 90 s at 72°C. The telomerase activity was detected by the colour change of 3,3′,5,5′-tetramethyl benzidine with peroxidase and was expressed as optical density (OD).

Statistical analysis

The Pearson's correlation coefficient was calculated, and the statistical difference of the activity among patient groups was evaluated by analysis of variance (ANOVA). The statistical significance was defined as $P < 0.05$.

Results

Clinical features of patients tested

The results of semen analysis and the levels of FSH, LH, testosterone, prolactin and oestradiol in each group are shown in Table I. There were no significant differences in the levels of LH, testosterone, prolactin and oestradiol among each group. The concentration of FSH in azoospermic patients with Sertoli cell-only was significantly higher than in the other three groups ($P < 0.05$) (Table I). The mean sperm count and motility in

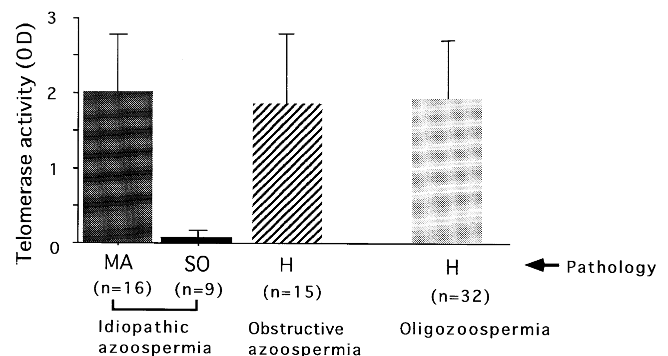

Figure 1. Telomerase activity in the human testes. MA = maturation arrest; SO = Sertoli cell-only; H = hypospermatogenesis; OD = optical density.

cases of oligozoospermia was 9.49 ± 8.39×10^6/ml and 40.2 ± 22.1%, respectively.

Pathological examination of testicular biopsy

Out of 25 specimens from men with idiopathic azoospermia (n = 16), 16 showed maturation arrest. The remaining nine specimens demonstrated Sertoli cell-only. Fifteen specimens from cases of obstructive azoospermia (n = 10) and 32 specimens from cases of oligozoospermia (n = 17) showed hypospermatogenesis. The JS of the testes showing maturation arrest and of those with Sertoli cell-only was 5.5 ± 1.6 (mean ± SD) and 1.97 ± 0.04, respectively. The JS of those showing obstructive azoospermia was 8.6 ± 0.3. In contrast, the JS of those with oligozoospermia was 7.3 ± 2.3.

Telomerase activity in the human testes

Telomerase activity in the testes with Sertoli cell-only and in those with maturation arrest was 0.08 ± 0.05 OD (mean ± SD) and 1.96 ± 0.98 OD, respectively (Figure 1). There was a significant difference in telomerase activity between the two groups above ($P < 0.05$). Within the group with maturation arrest, telomerase activity of the testes showing early maturation arrest (arrest at spermatocyte) and of those with late maturation arrest (arrest at round spermatid) was 1.82 ± 0.82 OD and 2.10 ± 1.14 OD respectively. No significant difference was observed between the two groups. Dividing the 16 specimens with maturation arrest into three groups according to JS, the telomerase activity was 1.12 ± 0.48 OD in the case of specimens with JS <4 (n = 3), which was much lower

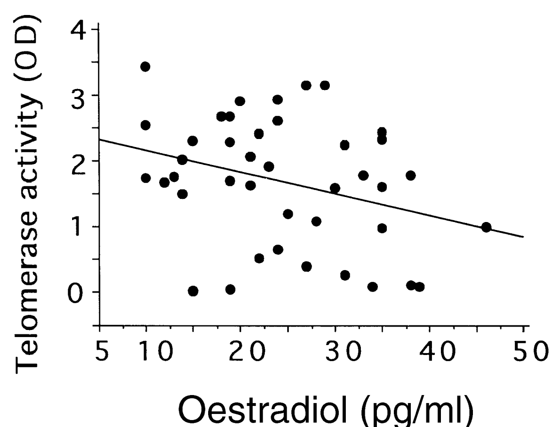

Figure 2. Telomerase activity and oestradiol level. $n = 41$; $R = 0.313$; $P < 0.05$. OD = optical density.

than that of the specimens with 4 ⩽ JS <6 (2.23 ± 0.70 OD, $n = 5$) or JS ⩾6 (2.10 ± 1.15 OD, $n = 8$). These results may suggest that the presence of spermatocytes or spermatids contributed to a higher level of telomerase activity. The activity in the testes with hypospermatogenesis in obstructive azoospermia and in testes with oligozoospermia was 1.89 ± 1.06 OD and 1.92 ± 1.02 OD, respectively. Telomerase activity did not differ between the testes with maturation arrest and testes with hypospermatogenesis in obstructive azoospermia, or those with oligozoospermia. Sertoli cell-only testes without germ cells showed no telomerase activity. The source of the telomerase activity is likely therefore to be germ cells.

Testicular volume and telomerase activity

The testes were divided into four groups, according to the testicular volume: A <14 ml; 14 ml ⩽ B <18 ml; 18 ml ⩽ C <22 ml; 22 ml ⩽ D. The activity of group A (1.16 ± 1.20 OD) was significantly lower than those of group B (1.85 ± 1.15 OD), C (1.77 ± 1.08 OD), or D (1.98 ± 0.87 OD), ($P < 0.05$).

Telomerase activity and Johnsen's mean score

Eliminating the cases showing Sertoli cell-only testes, we examined the correlation of telomerase activity and JS in 63 specimens. The telomerase activities in the testes did not correlate with their corresponding JS.

Telomerase activity and sperm concentration

We selected 21 oligozoospermic patients and examined the correlation of the level of telomerase activity and the sperm count. There was no significant correlation between telomerase activity and sperm concentration.

Telomerase activity and the concentrations of serum hormones

The correlation of the telomerase activity with the levels of FSH, LH, testosterone, prolactin, or oestradiol was examined. Only the oestradiol level correlated significantly with telomerase activity ($P < 0.05$; Figure 2). The oestradiol:testosterone ratio also bore a significant relationship with the telomerase activity ($P < 0.05$).

Discussion

Spermatogenesis is very complicated, consisting of such processes as mitosis, meiosis, and spermiogenesis. Stem cells actively divide into progeny cells to produce spermatocytes, spermatids and spermatozoa. In these processes, it is necessary that telomeric DNA is consistently maintained. We have reported previously that the significantly lower activities of DNA polymerase α, β, and γ were the cause of hypospermatogenesis in the testes of oligozoospermic men with varicocele (Fujisawa *et al.*, 1988). These enzymes are involved in DNA synthesis and play an important role in germ cell differentiation. However, telomeric DNA is not completely synthesized by conventional DNA polymerases (Harley *et al.*, 1990; Blackburn *et al.*, 1991). It is telomerase that is involved in the elongation of telomere, and thus enables germ cells to divide continuously (Counter, 1996). It has been reported that telomerase activity is present in the pachytene spermatocyte and round spermatid of rat, but not in spermatozoa from either the caput or the cauda epididymis, and that the telomerase activity in spermatids is higher than in pachytene spermatocytes (Eisenhauer *et al.*, 1997). Since it is necessary for these germ cells in the human testes to divide for much of the individual's life, telomerase that has been reported to be present in human testes (Kim *et al.*, 1994; Burger *et al.*, 1997) is essential.

In this study, we measured the telomerase activity in the human testes with selected diseases. Sertoli cell-only testes showed almost no activity whereas the activity of telomerase in the testes with maturation arrest, or in those with hypospermatogenesis showed similar activity. Therefore, according to our data, the telomerase activity seemed to be present in germ cells, but not in Sertoli cells or Leydig cells. Even if the pathohistological examination showed maturation arrest, telomerase activity in the testes with maturation arrest was the same as that in the testes of oligozoospermic men showing hypospermatogenesis with some spermatozoa. It has been reported that the level of telomerase activity from human cells does not vary significantly during the cell cycle but is repressed as cells exit the cell cycle (G0) via either differentiation or reversible quiescence (Holt *et al.*, 1996). If most of the germ cells in the testes with maturation arrest exist in the G0 phase of the cell cycle, no telomerase activity or a lower level of telomerase activity in the testes with maturation arrest is expected to be detected in comparison with telomerase activity in those with hypospermatogenesis. In contrast, our data show similar activity was detected in the testes with maturation arrest and hypospermatogenesis, suggesting that most of the germ cells in maturation arrest are not present in G0 phase. Bestilny *et al.* (1996) demonstrated that terminal differentiation of HL-60 human promyelocytic leukaemia cells to monocytic and granulocytic lineages is accompanied by a loss of telomerase activity. Therefore, it is assumed that the germ cells in maturation arrest may actively divide into progeny and may undergo programmed cell death due to loss of telomerase only at the terminal stages of the differentiation, such as spermatocyte or spermatid. However, we did not examine the distribution or show the loss of telomerase in specific germ cells at the terminal stage in this study.

In humans, there are four spermatogonial cell types, descriptively termed Along, Adark, Apale, and B (Clermont, 1963). It remains unclear as to which spermatogonia are the stem cells (Clermont, 1966; Chowdhury *et al.*, 1975). Spermatogonia may remain in a quiescent or non-dividing state for much of their life span or may actively divide into progeny cells. Several studies have reported that primitive non-dividing stem cells may not exhibit telomerase activity (Hiyama *et al.*, 1995; Chiu *et al.*, 1996). CD34CD38-positive haematopoietic stem cells have very low levels of telomerase activity, while their dividing CD34CD38-positive progeny have increased concentrations of telomerase activity (Blackburn, 1991; Chiu, 1996). Thus, it is possible that non-dividing spermatogonia have no need to replicate their telomeres, and thus may be capable of down-regulating telomerase, and that actively dividing spermatogonia need telomerase to produce differentiated precursors to spermatocytes. Dividing the specimens into three groups according to JS, the telomerase activity was 1.12 ± 0.48 OD in the specimens with JS <4 that had maturation arrest at spermatogonia, which was much lower than that of the specimens with $4 \leqslant$ JS <6 (2.23 ± 0.70 OD) or JS $\leqslant 6$ (2.10 ± 1.15 OD). The fact that the specimens with maturation arrest at spermatogonia (JS <4) had lower levels of telomerase activity might suggest that telomerase activity is likely to be higher in spermatocytes and spermatids than in spermatogonia. These data also suggest that some spermatogonia may be present in G0 phase, while spermatocyte and spermatids appear to divide more actively and most of them are not likely to exist in G0 phase.

In the human testes, differentiated spermatozoa do not contain telomerase activity. Telomerase is expected to remain active in germline cells to ensure the transmission of full-length chromosomes to progeny and may also be important during meiosis in the testes (Wright, 1996; Eisenhauer *et al.*, 1997). The regulating mechanism for the loss of telomerase activity in some differentiated cell types such as spermatozoa is not clearly understood. Moreover, it is still unclear whether telomerase plays a critical role in maintaining full-length chromosomes during meiotic progression and gamete differentiation in germ cells.

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