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Detection of a Short CCR5 Messenger RNA Isoform in Human Spermatozoa

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ABSTRACT: It has recently been reported that the Regulated upon Activation of Normal T-cells Expressed and Secreted (RANTES) chemokine may exhibit a chemotactic effect on sperm. The RANTES chemokine acts on target cells by binding to the CCR5 receptor, which is present on the surface of various cells. Spermatozoa contain a complex repertoire of messenger RNAs (mRNAs) that may provide an insight into past events of spermatogenesis. The type and amount of CCR5 chemokine receptor transcript were investigated in spermatozoa that were isolated by the swim-up method from semen samples of men with normozoospermia. Using reverse transcription and real-time quantitative polymerase chain reaction (RQ-PCR) analysis, we found that the CCR5 mRNA isoform in human spermatozoa consists of exons 3 and 4, and is shorter than the transcript in leukocytes. This CCR5 transcript may represent a more stable mRNA isoform; one that is used to biosynthesize the CCR5 receptor in spermatogenesis or the early stages of embryonic development.

Key words: Sperm, mRNA, CCR5, RQ-PCR.

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The presence of messenger RNA (mRNA) in human spermatozoa has been documented as comprising a wide range of somatic and germ cell–specific transcripts. These identified spermatozoal mRNAs include the transcripts of members of the β1 integrin family, c-myc, estrogen receptor, protamine Prm2, and cyclic nucleotide phosphodiesterase (Kumar et al, 1993; Durkee et al, 1998; Miller et al, 1999; Richter et al, 1999). These mRNAs in mature spermatozoa may represent remnants of untranslated stores (ie, reflections of a historic record of spermatogenesis) (Ostermeier et al, 2002).

Chemokines belong to the cytokine superfamily; they represent a group of small proteins that stimulate the attraction of leukocytes and mediate inflammation (Murdoch and Finn, 2000). The two main subfamilies of chemokines include the α-chemokines (CXCs) and the β-chemokines (CCs) (Murdoch and Finn, 2000; Kaplansky and Bongrand, 2001).

Recent findings suggest that certain chemokines may be involved in human reproduction and may play profound roles in ovulation, menstruation, implantation, cervical ripening, preterm labor, and sperm chemotaxis (Garcia-Velasco and Arici, 1999).

Follicular fluid causes chemotaxis of sperm to the fertilization site in the female genital tract, but the chemoattractant in follicular fluid has not yet been identified in humans (Eisenbach, 1999; Garcia-Velasco and Arici, 1999).

It has been shown that chemokines such as Regulated upon Activation of Normal T-cells Expressed and Secreted (RANTES) exist in the genital tract fluids (Rohwedder et al, 1996; Hornung et al, 2001). This chemokine affects various cells via binding to CC chemokine receptor 5 (CCR5), which is present on the surface of various cell types (Alkhatib et al, 1996; Filippatos et al, 2003). RANTES exhibits a chemotactic effect on human sperm, and this observation may suggest that the CCR5 receptor transcript can be used during spermatogenesis to biosynthesize this receptor (Isobe et al, 2002).

The CCR5 gene is organized into 4 exons and 2 introns; exons 2 and 3 are not interrupted by an intron (Figure 1A). The presence of CCR5 transcript isoforms has been studied only in leukocytes. The CCR5 mRNA isolated from leukocytes exists mainly as 2 isoforms, which consist of exons 1, 2, 3, and 4, or a second isoform without exon 2 (Figure 1B) (Mummidi et al, 1997).

Using reverse transcription and real-time quantitative polymerase chain reaction (RQ-PCR) analysis, we evaluated the CCR5 mRNA isoform profiles in spermatozoa that were isolated from semen samples of healthy, fertile volunteers.

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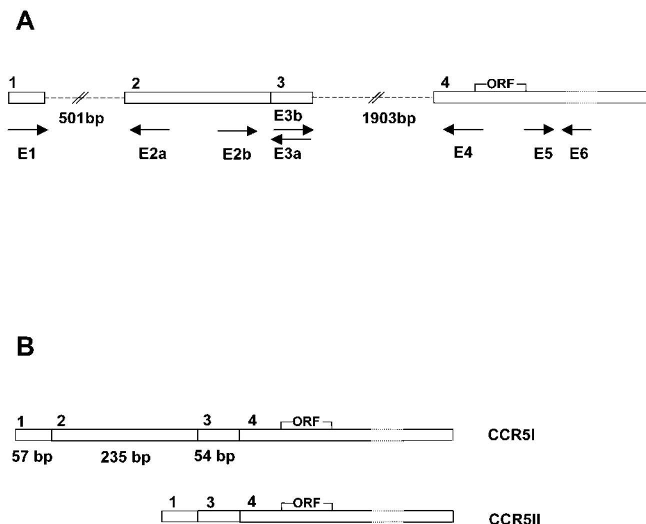

Figure 1. Diagram of CCR5 receptor gene map and composition of transcript isoforms. **(A)** The CCR5 receptor gene map. Open boxes and dashed lines represent exons and introns, respectively. Exons are numbered at the upper left of the corresponding box. Exon 4 contains an open reading frame. The arrows represent positions of primer pairs: E1,E2a; E1,E3a; E2b,E3a; E3b,E4; E5,E6, which were used to determine the presence of, respectively, exons 1,2; 1,3; 2,3; 3,4; and 4 in CCR5 transcript isoforms (Table). **(B)** The composition of 2 main CCR5 transcript isoforms present in human leukocytes. The exons are represented as numbered open boxes. The CCR5I and CCR5II transcript isoforms differ in only in the presence or absence of exon 2.

Materials and Methods

Spermatozoa Purification

Human ejaculates were obtained from 13 healthy volunteers of proven fertility, and normal semen quality as assessed by World Health Organization (1999) criteria (Dickey et al, 1999). These ejaculates were obtained from the Department of Gynecology and Obstetrics, University of Medical Sciences, Poznań, Poland. Spermatozoa were purified by centrifugation through discontinuous Percoll (Amersham Bioscences, United Kingdom) density gradient (80:40, vol/vol) and the swim-up technique. Liquefied semen samples were diluted with an equal volume of minimal essential medium (MEM) and layered on top of a 2-mL Percoll solution. After centrifugation for 20 minutes at 800 × *g*, Percoll pellets were washed twice (300 × *g* for 10 minutes) with 10 mL MEM containing 1 mg/mL bovine serum albumin and 50 mM benzamidine chloride. The pellets were gently overlaid with 1.2 mL human tubal fluid medium (HTFM) modified by the addition of 20 mM HEPES (Sigma, Deisenhofer, Germany) and 25 μg/mL streptomycin, 15 μg/mL penicillin, and 10 mg/mL human serum albumin. The tubes were placed in an incubator at 37°C for 60 minutes. The top 1.0 mL of the HTFM was removed and the purity of the dissolved spermatozoa was examined using an optical microscope equipped with a 100× oil objective. These separated spermatozoa were used immediately to isolate RNA, which were reverse transcribed and investigated by RQ-PCR analysis.

Isolation of Peripheral Blood Mononuclear Cells

Blood samples were obtained from the same individuals whose semen samples were used to purify spermatozoa. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll-Hypaque (density 1.077 g/cm^3) and were used immediately to isolate the RNA, which was reverse transcribed and investigated by RQ-PCR analysis for the presence of CCR5 mRNA isoforms.

RQ-PCR Analysis of CCR5 mRNA Isoforms in Spermatozoa and PBMCs

Total RNA was isolated from spermatozoa or PBMCs according to the methods described by Chomczynski and Sacchi (1985). RNA was treated with DNase I (Promega, Madison, Wis) and reverse-transcribed into complementary DNA (cDNA) using random hexamer priming and Maloney murine leukemia virus (MMLV) reverse transcriptase (RT-Kit; Sigma Chemical Company, St Louis, Mo). Quantitative analysis of CCR5 cDNA was performed by RQ-PCR SYBR Green I analysis (Light Cycler, Roche Diagnostics GmbH, Mannheim, Germany). The CCR5 cDNA was amplified using pairs of primers as shown in the Table.

For amplification, 2 μL of cDNA was added to 18 μL of PCR mix containing HotStart*Taq* DNA polymerase, reaction buffer, deoxynucleotide triphosphate mix, SYBR Green I dye, 2.5 mM $MgCl_2$, and primers (QIAGEN Inc, Valencia, Calif). Quantification of copy number was derived from a standard curve of known amount of synthetic DNA template. One RNA sample of each preparation was processed without MMLV RT (RT reaction) to provide a negative control in subsequent PCR reactions. To normalize for the quantity of cellular CCR5 transcripts in each sample, copy numbers were corrected to the amount of cellular glyceraldehyde-3-phosphate dehydrogenase (Table). To ensure that granulocytes did not contaminate the spermatozoa, we excluded them by RT-PCR analysis of myeloperoxidase transcript, which was restricted to granulocytes.

The amount of CCR5 mRNA was expressed in the number of CCR5 transcript copies shared per 10^6 heterogeneous population of spermatozoa (Table).

Results and Discussion

Using RQ-PCR analysis we have shown that spermatozoa obtained by swim-up preparation from semen samples of 13 fertile volunteers contain the CCR5 mRNA isoform, which comprises exons 3 and 4 (Figure 2; Table). Because of the heterogeneity of the spermatozoal population, we were able to determine the number of CCR5 transcripts that were shared per 10^6 cells. Employing RQ-PCR analysis we indicated that the average number of CCR5 transcript was 20 320 ± 11 990 copies per 10^6 heterogeneous population of spermatozoa (Table).

This spermatozoal CCR5 mRNA isoform was shorter than the CCR5 transcript found in human leukocytes (Figure 2; Table). These immune cells contain two major CCR5 mRNA isoforms that comprise exons 1, 3, and 4,

*Detection of exons in CCR5 mRNA isoforms present in spermatozoa and PBMCs.**

Primer	Sequence (5′-3′ direction)	Position	GenBank Accession Number	Exons	Product Size	CCR5 Transcript Copies Shared per 10^6 Heterogeneous Spermatozoa Population	Number of Positive Individuals: Sperm-atozoa	Number of Positive Individuals: PBMCs
E1	5′-ATATCTGGAGTGAAGGATCCT-3′	14–34	AF031237	1,2	140 bp	0	0	13
E2a	5′-GGTCTCTAGATAAGAGATCGA-3′	634–654						
E1	5′-ATATCTGGAGTGAAGGATCCT-3′	14–34	AF031237	1,3	313 and 78 bp	0	0	13
E3a	5′-GGAACGGATGTCTCAGCTC-3′	809–827						
E2b	5′-TTTAACTCCACCCTCCTTCAA-3′	722–742	AF031237		106 bp	0	0	13
E3b	5′-GGAACGGATGTCTCAGCTC-3′	809–827						
E3b	5′-GAGCTGAGACATCCGTTCC-3′	809–827	AF031237	2,3	139 bp	20 320 ± 11 990	13	13
E4	5′-GCTGCGATTTGCTTCACATTG-3′	2830–2850		3,4				
E5	5′-GGCAAAGACAGAAGCCTCAC-3′	4452–4471	AF031237	4	101 bp	18 920 ± 9780	13	13
E6	5′-CTAGCATTGACCTTCCTCCC-3′	4533–4552						
MYP	5′-CCTTGCTGGGCTGGGGTCTCAC-3′	226–248	NM 000250	1,3	394 bp	0	0	13
	5′-GGGCGGGCGTCAGCACATCAG-3′	559–619						
GPDH	5′-GTCTGTCGTGGACCTGACCT-3′	1002–1021	BC036373	9,10	143 bp	45 678 ± 5796	13	13*
	5′-CGGGAAGTCCGTAGAGACG-3′	1125–1144						

* Low level of GPDH isoenzyme transcript in PBMCs.

and they differ in the presence of exon 2 (Figure 1B) (Mummidi et al, 1997). The differences between the composition of CCR5 transcripts, which were detected in these two types of cells, may result from different requirements for CCR5 mRNA stability in spermatozoa and leukocytes.

It seems that the leukocyte CCR5 mRNAs are use immediately for translation to biosynthesize the mature molecule of the receptor, or they can be degraded by ribonucleases. This relatively short life span of leukocyte CCR5 mRNA allows control of the rate of protein biosynthesis at the level of transcript stability (Schwartzbauer and Menon, 1998). The different composition of CCR5 mRNA in spermatozoa may be attributed to a higher stability of this transcript in the postmeiotic stages of spermatogenesis. This short CCR5 transcript isoform can be used to biosynthesize the CCR5 receptor either during spermatogenesis or during the early stages of embryonic development.

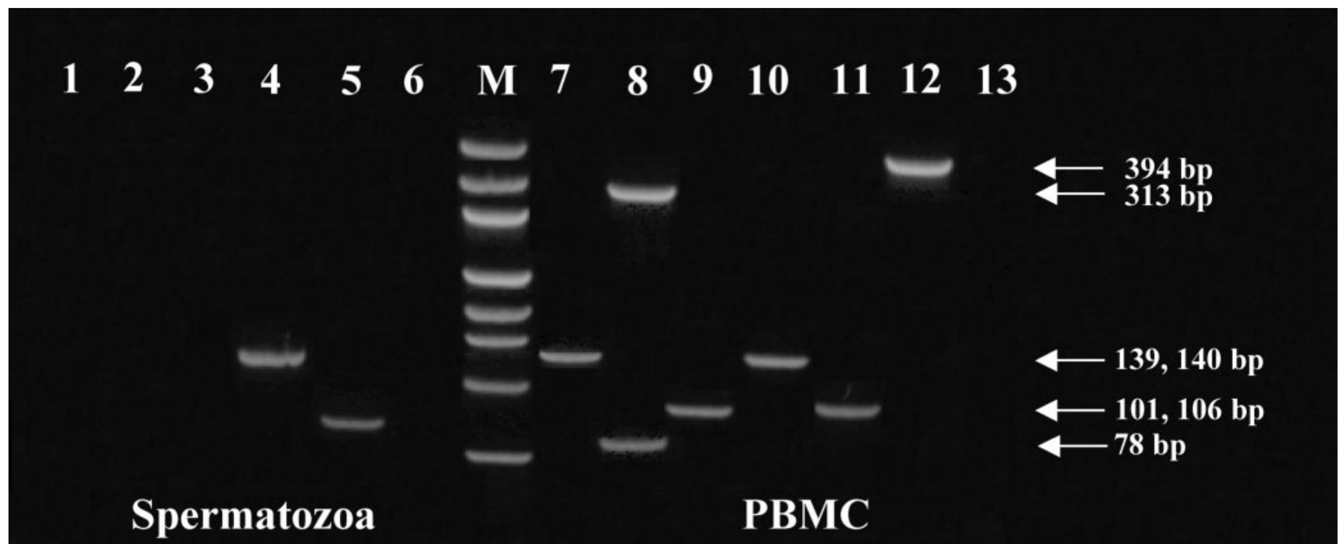

Figure 2. Representative picture of agarose gel electrophoresis of RQ-PCR analysis used to identify exons in spermatozoal and PBMC CCR5 transcripts. Swim-up–separated spermatozoa and PBMCs isolated from whole blood of the same individuals were used immediately for total RNA isolation, which was reverse-transcribed into cDNA. The RQ-PCR amplification products of spermatozoal (lanes 1–6) and PBMC (lanes 7–12) cDNA were analyzed on 2.5% agarose gels, stained with ethidium bromide, visualized under UV light, and photographed. The presented amplification products were obtained using primer pairs E1,E2a (140 bp); E1,E3a (78 and 313 bp); E2b,E3a (106 bp); E3b,E4 (139 bp); and E5,E6 (101 bp), which were complementary to, respectively, exons 1,2 (lanes 1 and 7), exons 1,3 (lanes 2 and 8), exons 2,3 (lanes 3 and 9), exons 3,4 (lanes 4 and 10), and exon 4 (lanes 5 and 11) (Table; Figure 1). Amplification products of myeloperoxidase transcript are shown in lanes 6 and 12. Lanes M and 13 represent molecular weight marker and negative control of amplification, respectively.

Transcription of many genes in various stages of spermatogenesis may result in biosynthesis of alternatively spliced variants of somatically expressed genes (Hecht, 1990). In general, transcription takes place throughout spermatogenesis from the spermatogonial stage to the haploid, round-elongating spermatid stage. Investigations of the packaging of DNA in human sperm chromatin revealed that in late spermatogenesis, 85% of the DNA is packaged into nucleoprotamine, and 15% into nucleohistone (Gatewood et al, 1990). The biological significance of the two chromatin fractions suggests that they may exhibit different roles. The nucleoprotamine complex is highly condensed and is transcriptionally inactive, whereas the nucleohistone complex could be involved in chromatin decondensation and transcription of genes, which is necessary in the last step of spermatogenesis (Gatewood et al, 1990).

It has been reported that transcription of many genes occurs in the postmeiotic stages of spermiogenesis. These large numbers of mRNAs can be stored for long periods in the nonpolysomal messenger ribonucleoprotein particles prior to their translation in round spermatids (Hecht, 1990). Many transcripts survive the condensation of the spermatid nucleus and are present in human ejaculated spermatozoa (Miller et al, 1994; Wykes et al, 1997). Further in situ investigations are needed to determine the transcriptional activity of CCR5 gene during the different stages of spermatogenesis.

Chemotaxis may serve as a principal process in bringing together human gametes by the transfer of sperm during the fertilization process (Eisenbach and Tur-Kaspa, 1999). The induction of chemotaxis in human spermatozoa by follicular fluid in vitro has been well documented, whereas the chemoattractant in follicular fluid remains unidentified. Follicular fluid contains several types of chemokines, including RANTES, which exhibits a chemotactic effect on human sperm (Machelon et al, 2000). The RANTES effect on target cells is mediated by the binding of this ligand to the CCR5 chemokine receptor. This finding may suggest that the CCR5 mRNA in spermatozoa could be used to biosynthesize CCR5 receptor during spermatogenesis. Further studies should endeavor to find the intracellular localization of CCR5 receptor in sperm and the relationship between the spermatozoal CCR5 transcript and sperm function.

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