Meiotic defects in a man with non-obstructive azoospermia: Case report

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Infertile men have an increased frequency of aneuploid sperm. We have determined that decreased recombination is associated with the production of aneuploid sperm in humans. The aim of this study was to determine whether some cases of infertility are associated with decreased meiotic recombination. Analysis of the early stages of meiosis was performed in a 33-year-old man with non-obstructive azoospermia. Newly developed immunocytogenetic techniques were used to identify the synaptonemal complex (SC) in various stages of prophase. Antibodies to meiotic proteins identified the SC (SYN1/SCP3), the centromere (CREST) and recombination sites (MLH1). Only 36 meiotic spreads were recovered from the infertile man, compared with hundreds available from controls. One-third of the cells were in zygotene compared with 4% in controls, demonstrating an inability of bivalents to synapse and progress to pachytene. The infertile man had a greatly reduced frequency of recombination, with a mean of only 32.7 MLH1 foci/cell (range 1–60) compared with 46.0 (range 21–62) in control donors. A high proportion of cells (73%) contained at least one autosomal bivalent with zero MLH1 foci, compared with only 4.5% in control donors. Discontinuities in the SC were also more prevalent (68% of cells versus 26% in controls). This is the first demonstration of dramatic pachytene-stage abnormalities in an infertile man using these powerful new immunocytogenetic techniques.

Key words: male infertility/meiotic recombination/non-obstructive azoospermia/pachytene spermatocytes/synaptonemal complex analysis

Introduction

The association of chromosome abnormalities with male infertility has been recognized for decades. The most common types of karyotypic abnormalities detected among infertile populations include sex chromosomal abnormalities and translocations (Johnson, 1998). With the introduction of fluorescence in-situ hybridization (FISH) analysis of human sperm, it has become evident that infertile men with a normal somatic karyotype also have an increased frequency of chromosome abnormalities in their sperm (reviewed in Shi and Martin, 2001). This has been reflected in an increased frequency of chromosome abnormalities observed in children conceived with the use of ICSI for male factor infertility (Aboulghar et al., 2001; Van Steirteghem et al., 2002). Our group and others have suggested that the increased frequency of sperm chromosome abnormalities in infertile men could be associated with abnormalities of chromosome pairing during meiosis (Egozcue et al., 1983; Martin, 1996). Since anomalies of meiotic chromosome pairing and recombination are associated with increased frequencies of aneuploidy in lower organisms (Bascom-Slack et al., 1997), it is possible that pairing abnormalities in infertile men could lead to meiotic arrest in some cells, causing oligozoospermia, and aneuploidy in other cells capable of completing spermatogenesis. We have shown by single sperm typing that aneuploid XY sperm have a decreased frequency of recombination in the pseudoautosomal region (Shi et al., 2001). Thus the link between abnormalities of meiotic chromosome pairing, recombination, aneuploidy and male infertility appears plausible.

Recent developments in the identification of proteins important in meiotic chromosome pairing and recombination have provided a means of studying the progress of early meiosis in humans. Various important meiotic structures can be identified by the use of immunofluorescence. Antibodies against elements of the synaptonemal complex (SC) can be
used to monitor prophase of meiosis I, and the centromere can be localized with CREST antisera. Most excitingly, sites of meiotic exchange on the SC can be identified with antibodies to the mismatch repair protein MLH1. Studies in both mouse (Baker et al., 1996; Anderson et al., 1999) and human (Barlow and Hultén, 1998; Lynn et al., 2002) spermatocytes have demonstrated that the number and location of the MLH1 foci conform to that expected of a molecule that marks the site of recombination, and, recently, Marcon and Moens (2003) have demonstrated that MLH1 localizes to chiasmata.

We have used this immunocytogetic approach to study early meiosis in men with non-obstructive azoospermia to determine whether errors in chromosome pairing and recombination are prevalent in this population. In this report, we present analysis of meiotic prophase in a man with severely compromised progression of meiosis I.

Case report

The patient with non-obstructive azoospermia was 33 years of age at the time of the analysis. He had been trying to conceive for more than 2 years without success. Physical examination revealed normal external secondary sex characteristics, normal testes, epididymides, and both vasa present and normal. Hormone evaluation revealed normal levels of testosterone, prolactin, LH and FSH, with FSH at the upper limit of normal (8 IU/l). Two semen analyses indicated azoospermia. Cystic fibrosis testing demonstrated that he was a carrier for the delta F508 allele. Testicular sperm aspiration was attempted but there was no evidence of spermatozoa in three aspirations. The patient had three brothers and two sisters ranging in age from 27 to 38 years. None of these siblings had reproduced, but only one sister was known to be infertile. The patient had non-consanguineous parents, with no evidence of infertility in their extended families (further investigation of siblings was declined).

Materials and methods

The patient gave informed consent and the research was approved by our Institutional Review Board. The testicular tissue was processed using a modification of the technique of Barlow and Hultén (1998). Testicular biopsy tissue was shredded with two pairs of forceps, and the released pachytene cells were spread evenly over microscope slides layered with paraformaldehyde solution at pH 9.2 (PFA; Fisher Scientific, Edmonton, Alberta, Canada)/Triton-X (Sigma, Oakville, Ontario, Canada). Slides were dried for 10 min, and soaked for 30 min in antibody dilution buffer (ADB) at room temperature in a humid chamber, then drying was completed by air 24 h at 37°C in a humid chamber for 90 min, were washed three times in phosphate-buffered saline solution for 10, 20 and 30 min (agitating every 5 min), and then a glass cover slip was applied and sealed with rubber cement.

Slides were scanned with a Zeiss Axioshot microscope, locations of the spreads were determined using a gridded finder slide, and images of the SC, MLH1 sites and CREST locations were captured using an Applied Imaging Cytovision 2.81 work station. Prints of the captured images were analyzed to determine number of MLH1 foci on each individual SC and in the whole cell.

Since the meiotic phenotype of our patient resembled that of a mouse mutation for Dnmt3L (Bourc’his et al., 2001), we sequenced the human Dnmt3L gene.

Results

The stages of meiotic prophase can be distinguished by the appearance and chronology of the proteins when examined by immunofluorescence. Cells at leptotene had multiple short SCP3-positive segments (the start of formation of lateral elements of the SC, binding sister chromatids); at a zygotene-like stage, 46 complete but unpaired SCP3-positive elements with 46 CREST signals (centromeres) were observed; at pachytene, synopsis of homologues was complete with 23 SCs, 23 CREST signals and the appearance of MLH1 foci (marking recombination sites).

Since pachytene is the longest stage of prophase I, most cells are observed in this stage. In six control donors (mainly cancer patients), 88% of cells were in pachytene, with only 8% in leptotene and 4% in zygote (Sun and Martin, unpublished data). However, in our infertile male many cells seemed to be blocked in a zygote-like stage, with 33% at this stage, and 64% in pachytene. This zygote-like stage, SCs were more condensed than regular zygote SCs, and there were 46 SCs with little or no evidence of pairing between homologous chromosomes (Figure 1A). Another striking feature of meiosis in our infertile male was that there were very few meiotic cells. In control donors, hundreds of meiotic cells are observed from a small testicular sample, whereas the patient had a total of only 36 cells.

The 22 cells in pachytene exhibited many abnormalities in chromosome pairing and recombination. Normally in pachytene, all chromosome regions are paired; in analysis of 600 cells from control donors only 2.3% cells with unpaired chromosome regions were found (Sun and Martin, unpublished data). In our infertile male, 64% of cells had unpaired regions (Figure 1B). Discontinuities (gaps) in the SC are relatively frequent in control donors (26% of cells), but these were much more prevalent in the infertile male (68% of cells) (Figure 1B). Recombination was greatly reduced, with a mean of 32.7 MLH1 foci/cell (range 1–60) compared with 46.0 MLH1 foci/cell (range 21–62) in control donors. In addition, a high proportion of cells (73%) contained at least
one bivalent with zero MLH1 foci, compared with only 4.5% in control donors.

There were no mutations discovered in the sequence of the human Dnmt3L gene.

Discussion

This is the first report of dramatic meiotic abnormalities in a man with non-obstructive azoospermia observed during the pachytene stage using these powerful new immunocytogenetic techniques. It is not surprising that no sperm could be recovered from three testicular biopsy samples, as very few meiotic cells were found. Several abnormalities indicated defects in chromosome pairing: a much higher than normal proportion of cells in a zygotene-like stage demonstrated a block in pairing of homologous chromosomes, and pachytene-like cells had a much higher percentage of unpaired chromosome regions and more discontinuities in the SCs than controls. Recombination was also affected, with a reduced mean frequency of MLH1 foci/cell and the majority of cells containing at least one bivalent with no recombination sites. Bivalents with no recombination foci (chiasmata) are unable to orient properly on the metaphase plate or to segregate chromosomes to daughter cells. A number of studies have demonstrated that meiotic chromosomes with unpaired regions lead to meiotic arrest of spermatogenesis (Hultén et al., 1970; Egoscue et al., 1983). Thus the lack of sperm in this male could have been caused by abnormalities

Figure 1. (A) Example of a human spermatocyte in a zygotene-like stage with lateral elements of the SC (red) but no synopsis of homologous chromosomes. Centromeric regions are shown in blue. (B) Example of a human spermatocyte in pachytene stage. Chromosome bivalents are paired with 22 mature autosome SCs (red) and sex chromosomes (marked X and Y) synapsed at the pseudoautosomal region. Centromeric regions are shown in blue. There are 28 MLH1 foci (yellow) indicating recombination sites. One SC has a discontinuous region or gap (g); other SCs have unpaired regions (*).
in the pairing of homologous chromosomes, as demonstrated by both the block in zygotene in some cells and the unpaired regions observed in pachytene cells.

The precise underlying cause of the meiotic defects in this infertile man is unknown. Many specific meiotic genes have been described and mammalian mutants of these genes have been reported (Hunt and Hassold, 2002). One possible candidate gene could be \textit{Dmnt3L}, a DNA methyltransferase that regulates imprint establishment. The \textit{Dmnt3L} gene is expressed during gametogenesis, and targeted disruption of the gene causes azoospermia in mice (Bourc'his \textit{et al.}, 2001). The prophase phenotype of this mouse is similar to our case in that there are very few meiotic cells, in some cells the chromosomes remain unpaired but progress to a pachytene-like state of contraction, and in other cells the majority of chromosomes pair and progress to pachytene with pairing abnormalities. We sequenced the \textit{Dmnt3L} gene in our infertile male and did not discover any abnormalities in this gene. Other candidate genes could be involved with bivalent pairing, such as \textit{SCP1}. These investigations are currently in progress.

The study of this one infertile man demonstrates the power of new immunocytogenetic techniques in the investigation of meiotic abnormalities in male infertility. Information can be derived on the abundance of meiotic cells, the progress through meiotic stages including the pairing of chromosomes, and recombination in individual bivalents.

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