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Sperm ubiquitination in patients with dysplasia of the fibrous sheath

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BACKGROUND: Human sperm with structural abnormalities display an increased content of the cellular proteolytic marker peptide, ubiquitin. We investigated whether dysplasia of the fibrous sheath (DFS), a severe structural anomaly found in the sperm of some asthenozoospermic patients, is accompanied by (i) increased ubiquitination of the sperm surface and (ii) by increased ubiquitination of the sperm mitochondria. METHODS AND RESULTS: Five DFS patients and eight fertile donors were studied by immunocytochemistry with anti-ubiquitin antibodies. Increased cross-reactivity of the ubiquitinated mitochondrial epitopes was seen in 32–50% of DFS sperm, but only 2–4.1% of sperm from fertile donors. Sperm surface ubiquitination assessed by sperm-ubiquitin tag immunoassay (SUTI) and immunofluorescence demonstrated an increased sperm ubiquitination in all DFS patients. The average median value of ubiquitin-induced fluorescence in DFS patients was 25.8 counts (range 19.8–37.9), as opposed to 13.4 counts range (9.3–16.6) in fertile men. Sperm with 'stump tails', coiled tails, twin and triplet sperm, and clusters of immature spermatogenic cells were common. CONCLUSIONS: DFS sperm have increased cross-reactivity to anti-ubiquitin antibodies, a finding consistent with the ubiquitination of defective sperm shown in animal models. These results justify the use of ubiquitin-based assays for objective semen analysis in infertile men with heritable defects.

Key words: asthenozoospermia/dysplasia of the fibrous sheath/male infertility/sperm mitochondria/ubiquitin

Introduction

Dysplasia of the fibrous sheath (DFS), or stump tail syndrome, is a form of flagellar pathology, which causes extreme asthenozoospermia or sperm immotility. Marked hyperplasia and disorganization of the fibrous sheath (FS) and other axonemal structures, as well as severe mitochondrial sheath alterations or absence of FS are common characteristics (Chemes *et al.*, 1987, 1991, 1998; Torikata *et al.*, 1991; Rawe *et al.*, 2001). DFS is sometimes associated with the absence of dynein arms in sperm flagella and respiratory cilia (Chemes *et al.*, 1990, which makes it a variant of the immotile or dyskinetic cilia syndrome (Eliasson *et al.*, 1997; Kastury *et al.*, 1997). Transmission electron microscopy was particularly useful for understanding severe axonemal and periaxonemal alterations present in DFS sperm. Sterile males showing similar defective sperm tails have been described by other authors in humans and in several mammalian species (Antonelli *et al.*, 1981; Maqsood, 1951; Coubrough and Barker, 1964; Blom, 1976; Bisson *et al.*, 1979; Barth and Oko, 1989; Baccetti *et al.*, 1993;).

Ubiquitin is a small, 8.5 kDa proteolytic polypeptide (Goldstein *et al.*, 1975; Ciechanover *et al.*, 1984), involved in the regulation of proteolysis in a diverse array of cellular events. These include recycling of the outlived cytoplasmic proteins, endocytosis of membrane receptors and cell cycle control through the destruction of the cyclin component of maturation promoting factor (MPF) at the metaphase/anaphase transition (Hershko, 1998; for review), but also the formation of Alzheimer's plaques (Perry *et al.*, 1987) and HIV viral infection (Strack *et al.*, 2000). Ubiquitination of multiple substrates occurs during the final stages of spermatogenesis (Bebington *et al.*, 2001; Sutovsky, 2002) and mitochondria appear to be one of the primary targets of ubiquitination in normal sperm. During epididymal passage, the ubiquitinated substrates in the sperm mitochondria are sterically hidden from antibody detection by the formation of disulphide bonds in the mitochondrial membranes. The ubiquitination of sperm mitochondrial membrane proteins may be necessary for the recognition and proteolysis of sperm mitochondria inside the fertilized oocyte, thus promoting the maternal mode of mitochondrial DNA inheritance in mammals (Sutovsky *et al.*,

1999, 2000). As it was reported (Sutovsky *et al.*, 2001a,b), some epididymal and ejaculated sperm, particularly those with structural abnormalities, can display partial to entire cell ubiquitination. These sperm are found predominantly in the supernatants from Percoll separation gradients, which contain immotile, dead and defective sperm (Sutovsky *et al.*, 2001a). Failed disulphide bond stabilization and/or apoptosis-related alterations of the sperm plasma membrane could be a possible cause of the binding of epididymis-secreted ubiquitin-cross-reactive proteins to the surface of defective sperm (Sutovsky *et al.*, 2001a). Consequently, ubiquitin-cross-reactive substrates can be detected on the cell surface and in the mitochondria of the aldehyde fixed, or even unfixed, defective sperm without permeabilization, which is a necessary step for the detection of ubiquitinated mitochondrial substrates in normal, motile sperm. Based on these findings, a novel, ubiquitin-based, flow cytometric assay of semen quality has been developed—sperm-ubiquitin-tag immunoassay (SUTI) (Sutovsky *et al.*, 2001b), with possible applications in the diagnostics of male infertility. The surprising phenomenon of extracellular sperm ubiquitination may be more widespread than originally thought, as the new evidence validating such hypothesis comes from the studies of ascidian fertilization (Sawada *et al.*, 2002).

In the present work, we report the presence and incidence of sperm-surface ubiquitination in sperm from patients with DFS. While the increased sperm ubiquitination was previously associated with male infertility (Sutovsky *et al.*, 2001a), this is the first report showing this phenomenon in a group of patients suffering from a well defined, presumably heritable sperm anomaly.

Materials and methods

Patients and andrological evaluation

Clinical and laboratory evaluations were carried out in five men with complete (nos. 1–3) and incomplete forms (nos. 4, 5) of DFS diagnosed with asthenozoospermia. Physical examination of testes and scrotum was normal in all cases and no further indication of clinical problems was found. The patients' hormonal profiles were normal, and the immunological and bacteriological studies of their semen did not show any abnormalities. DFS was diagnosed using transmission and scanning electron microscopy. For this, the fresh semen samples were processed within 30 min of ejaculation, according to previously described methods (Chemes *et al.*, 1998). To identify the complete and incomplete forms of DFS, quantification of axonemal anomalies and analysis by transmission electron microscopy was carried out in at least 100 flagella per patient. The samples were further probed by hypo-osmotic swelling test (HOST) (Jeyendran *et al.*, 1984). Briefly, semen samples were incubated for 60 min at 37°C in a 1:10 dilution of hypo-osmotic solution and then the viability was assessed. A small aliquot of fresh semen was studied under phase contrast microscopy, and motility, viability and morphology were studied according to standard methods (World Health Organization, 1987). Sperm from anonymous, fertile donors were purchased from Fairfax Cryobank, Fairfax, VA, USA.

Clinical evaluation of sperm ubiquitination by immunofluorescence with anti-ubiquitin antibodies

Sperm were attached to microscopy coverslips coated with 1% Poly-L-lysine and fixed in 2% formaldehyde in 0.1 mol/l phosphate buffered saline (PBS) (Sigma St. Louis, MO, USA) (Sutovsky *et al.*, 2001a,b). To detect the ubiquitin signal in sperm mitochondria, sperm were permeabilized for 40 min in PBS with 0.1% Triton X-100 (TX-100), and incubated overnight at 4°C with mouse monoclonal immunoglobulin M (IgM) antibody KM 691 (Kamiya Biomedical Comp., Seattle, WA, USA), raised against human recombinant ubiquitin. KM 691 was diluted 1:100 in PBS containing 0.1% bovine serum albumin (BSA), 0.02% sodium azide and 0.1% Triton X-100. Samples were washed in PBS, and further incubated in TRITC-conjugated goat anti-mouse IgM (diluted 1:40; Zymed Inc., San Francisco, CA, USA) for 40 min at room temperature. For negative control staining, PBS + BSA alone replaced the first antibody solution. A parallel positive control with sperm from three fertile donors (nos. 6–8) was performed for comparison. Coverslips with sperm were washed in PBS and counterstained with Hoechst 33258 (1 mg/ml) for 5 min at room temperature, washed again in PBS, mounted on slides, and sealed with a clear nail polish. Samples for clinical evaluation (patient nos. 1–5 and fertile donor nos. 6–8) were examined using an Olympus BX-40 epifluorescence microscope and photographed on Kodak Ektachrome 1600 film. Images were scanned and processed using Adobe Photoshop 5.0 software (Adobe System Inc., Mountain View, CA, USA). To ascertain that this labelling was indeed confined to the sperm mitochondria, unfixed sperm were labelled with a vital mitochondrial probe, MitoTracker Green FM (Molecular Probes Inc., Eugene, OR, USA) as described previously (Sutovsky *et al.*, 2000).

Flow cytometric SUTI assay

SUTI was performed as described previously (Sutovsky *et al.*, 2001b). Sperm samples (250 µl) were pelleted by a 5 min centrifugation at 500 *g* in TALP-HEPES medium and fixed for 40 min in 2% formaldehyde in PBS. No permeabilization was performed. Samples were blocked for 25 min in 5% normal goat serum (NGS) in PBS and processed with anti-ubiquitin antibody KM691 (1/100) and FITC-conjugated goat anti-mouse IgM (1/80). All washings were performed by resuspension/centrifugation in PBS with 1% of NGS. At the end, the samples were resuspended in 500 µl of pure PBS without serum. Blank, control samples were prepared by omitting the primary, anti-ubiquitin antibody.

Ubiquitin median values (channel number at which half the cells are dimmer and half the cells are brighter), the histograms of relative fluorescence and the diagrams of the visible light scatter, were generated by FACS Calibur Analyzer (Becton Dickinson, San Diego, CA, USA) at 488 nm wavelength. Relative levels of fluorescence in 5000 individual cells per sample were recorded in each of the three repeats. A blank sample from the corresponding donor/patient, labelled with secondary antibody alone, was measured prior to each anti-ubiquitin-labelled sample. Samples from five DFS patients (nos. 1–5) and five fertile donors (nos. 9–13) were measured and compared.

Sperm samples already processed for flow cytometry were also screened by high resolution epifluorescence microscopy. Representative images are shown in Figure 3. Briefly, 2–3 µl of the processed sperm suspension in PBS were pipetted onto a microscopy slide in a 10 µl of VectaShield (Vector Labs, Burlingame, CA, USA) mounting medium containing 5 µg/ml of DNA stain, Hoechst 33258. Drops of sperm were covered with a coverslip and sealed with a clear nail polish. Samples were examined and photographed with a Nikon Eclipse 800 biological research microscope equipped with infinity-corrected, planar, apochromatic lenses (CFI60 Plan Apo Series, Nikon), and a CoolSnap HQ CCD camera (Roper Scientific, Tucson, AZ, USA), operated by MetaMorph 4.6.5 software (Universal Imaging Corp., Downington, PA, USA). Images were edited using Adobe Photoshop 6.0 software. Most reagents were purchased from Sigma and secondary antibodies were obtained from Zymed.

Table IA. Clinical parameters of five examined DFS sperm samples and three fertile donors used for epifluorescence microscopic evaluation

Patient no.	1	2	3	4	5	Mean ± SD (1–5)	6	7	8	Mean ± SD (6-8)
Volume (ml)	4	1.3	3.4	4.3	3.5	3.3 ± 1.2	4.5	3.4	2.0	3.3 ± 1.3
Sperm conc.($\times 10^6$/ml)	5	18	54	44	23	28.8 ± 19.9	90	38	115	81.0 ± 39.3
Total motility (%)	0.4	0.16	0.92	20.4	4.34	5.2 ± 8.6	65	50	63	59.3 ± 8.1
Progressive motil. (%)	0	0	0	0	0	N/A	25	22	23	23.3 ± 1.5
Viability (%)	27	21	20	48	58	34.8 ± 17.2	69	71	67	69.0 ± 2.0
DFS form (C/I)*	C	C	C	I	I	N/A	N/A	N/A	N/A	N/A
Morphology (%)	4	3	2	13	17	7.8 ± 6.8	14	17	14	15.0 ± 1.7

*C = complete form; I = incomplete form of DFS.

Table IB. Incidence of sperm-mitochondrial ubiquitination in five DFS semen samples (nos. 1–5) and three fertile donors (nos. 6–8), evaluated by epifluorescence microscopy*

Patient no.	1	2	3	4	5	6	7	8	$P<$
High signal (%)	320 (32)	401(40.1)	445 (44.5)	503 (50.3)	409 (40.9)	20 (2)	41 (4.1)	29 (2.9)	0.0001
Low signal	680	599	555	497	591	980	959	971	0.0001
Total (100%)	1000	1000	1000	1000	1000	1000	1000	1000	0.0001

*Values represent number of sperm with or without signal. Values in parenthesis are percentages. Samples 1, 2 and 3 are DFS semen samples with complete form of the pathology. Samples 4 and 5 represent the incomplete form of DFS. Samples 6–8 are control samples from fertile donors.

Table IC. Available clinical parameters of semen samples from five fertile donors used as control, standard samples in the flow-cytometric SUTI assay

Donor no.	9	10	11	12	13	Mean ± SD
Sperm conc. ($\times 10^6$/ml)	167	68	227	155	106	144.6 ± 60.7
Total motility (%)	70	60	55	60	65	62.0 ± 5.7
WHO abnormal (%)	27	27	19	20	24	23.4 ± 3.8
Age	29	24	30	20	32	27 ± 4.9

Statistical analysis

The χ^2 test has been used to compare the frequency of ubiquitin signal in the sperm mitochondria between DFS semen samples and three normal semen samples. DFS semen samples from five men (patient nos. 1–5) and three normal samples (donor nos. 6–8) were compared. This was also used to test the significance of differences between ubiquitin medians of five DFS samples and one standard, control sample in SUTI assay.

Results

Light and electron microscopic evaluation of DFS semen samples

Electron microscopic analysis first revealed the presence of a complete form of DFS in patient nos. 1–3 (more than 95% affected sperm) and an incomplete form in patient nos. 4 and 5 (70–75% pathological sperm; Table IA). Several consecutive semen analyses consistently showed 0% progressive motility and low total motility in all patients with complete and incomplete forms of DFS (Table IA). Variable sperm concentrations were found (Table IA). Upon light microscopic examination, sperm flagella were short, rigid, and unusually thick (hence the synonym 'stump tail syndrome'). Average semen parameters in three examined semen samples from fertile donors were within the normal range (Table IA; donor nos. 6–8).

Further electron microscopic analysis confirmed severe flagellar alterations with thickening of the fibrous sheath and short tails of irregular contours. Numerous sperm and immature spermatids had coiled or folded tails, as well as large cytoplasmic droplets at the connecting piece. Even though none of these cells had the typical appearance of sperm, their nature was ascertained by the identification of heads with acrosomal caps and tail structures (Figure 1 A–C). Their largest diameter ranged from 9–15 μm which far exceeds that of normal sperm heads.

Clinical evaluation of the ubiquitination of sperm mitochondria

A total of 1000 sperm with DFS were analysed for each of five patients in order to detect the presumably defective mitochondria with unmasked, ubiquitinated substrates, in clinical settings and with clinical equipment. The purpose of this evaluation was to determine whether there is a difference in mitochondrial ubiquitination between sperm from fertile donors and DFS patients. This ubiquitin-based evaluation was performed using a clinical grade microscope, without an expensive top-of-the-line microscope and camera. TX-100

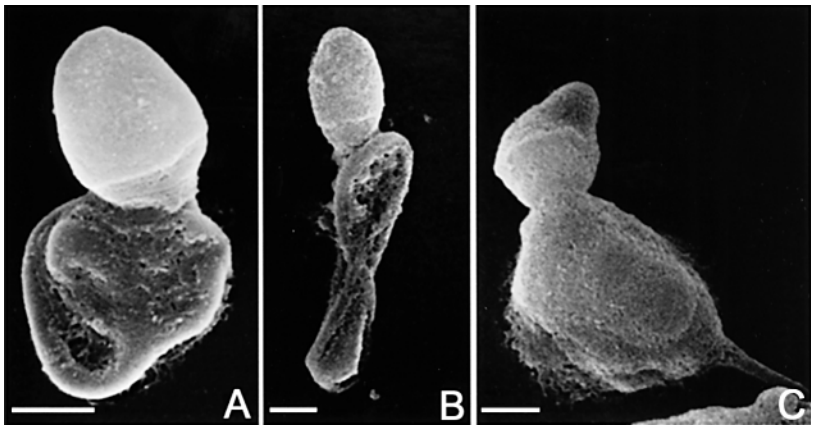

Figure 1. Scanning electron micrographs of DFS spermatozoa with bent or coiled tails (**A** and **B**), and irregular cytoplasmic remnants at the sperm head-tail connecting piece (A and C). All sperm display heads with acrosomes. Bars represent 2 μm.

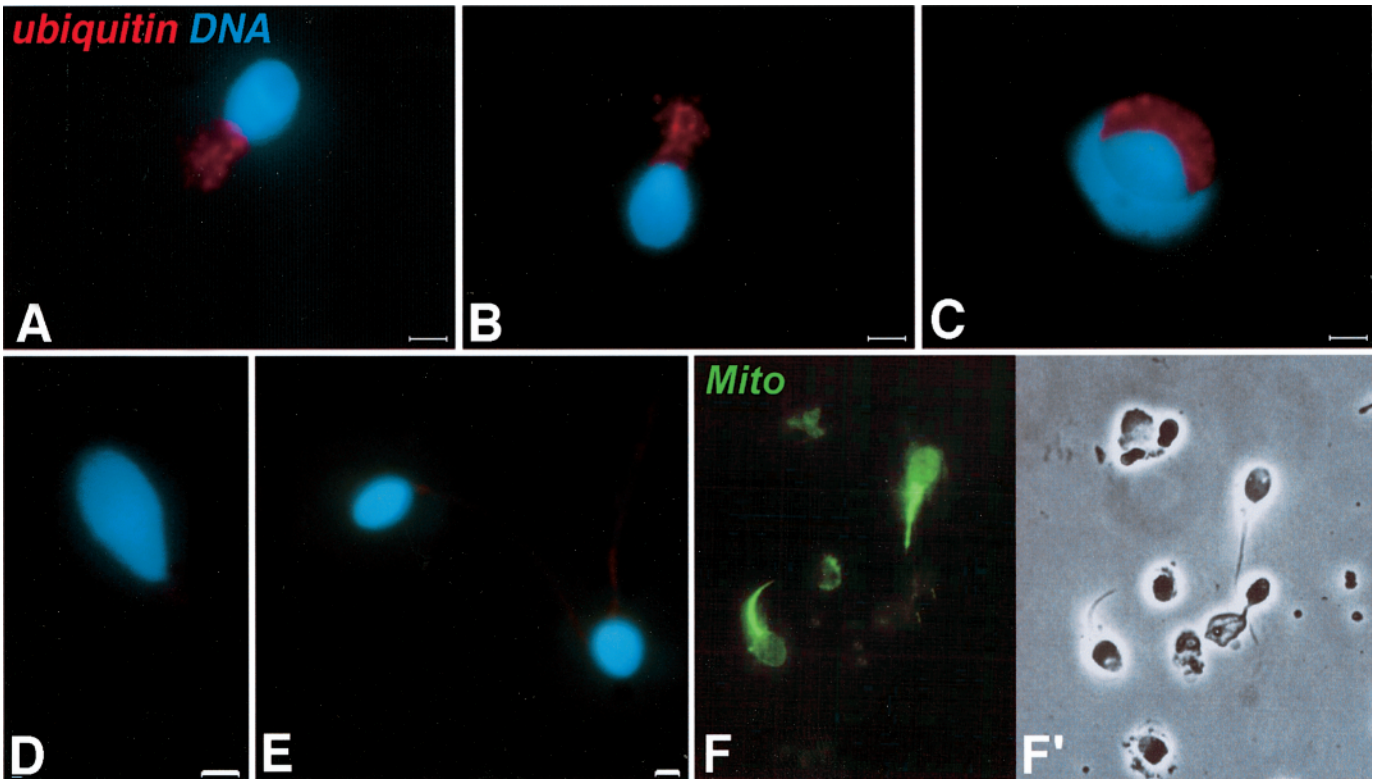

Figure 2. Clinical evaluation of sperm ubiquitination in defective (**A–D**) and normal (**E**), permeabilized human spermatozoa. (**A,B**) Examples of cells with strong cross-reactivity in the mitochondrial sheath/sperm tail mid piece are shown. (**C**) Round spermatid showing an intense ubiquitin signal. (**D**) Negative control shows the absence of ubiquitin signal. (**E**) Standard sperm sample from a fertile donor shows low ubiquitin signal. Ubiquitin was detected by a monoclonal antibody KM 691 raised against human recombinant ubiquitin, and a secondary, TRITC-conjugated antibody (red). Sperm DNA was counterstained using Hoechst 33258 (blue). (**F**) Labelling of sperm mitochondria with a vital mitochondrial probe MitoTracker Green FM ascertains the co-localization of ubiquitin-signal with the sperm mitochondrial sheath. (**F′**) A phase-contrast double of (**F**). Scale bars = 2 μm.

permeabilization was used to increase the labelling of sperm tail mid-pieces with anti-ubiquitin antibodies. Overall, the strongest signal is present in the sperm tail mid-piece corresponding to the sperm mitochondrial sheath and it is the most easy to detect signal when clinical epifluorescence microscopes are used. In the clinical evaluation, described here, the prevalent fluorescence was indeed detected mainly in this compartment of DFS sperm. In DFS patients, 32–50.3% of the studied sperm (Table IB) showed high levels of anti-ubiquitin labelling in the mitochondrial sheath region (Figure 2A,B). At the same time, it was possible to detect many round spermatid-like cells with a high ubiquitin signal (Figure 2C). Negative controls (primary antibody omission) showed weak or no signals (Figure 2D). For comparison, semen samples from three fertile donors (nos. 6–8) were analysed, showing very low levels of ubiquitin in the mitochondria of 96–98% of sperm (Figure 2E; Table IB). Ubiquitin-signal in the sperm midpiece coincided with the sperm mitochondria, as ascertained in some sperm samples by labelling with a vital mitochondrial probe MitoTracker Green FM (Figure 2F, F′). χ^2 analysis showed a highly significant

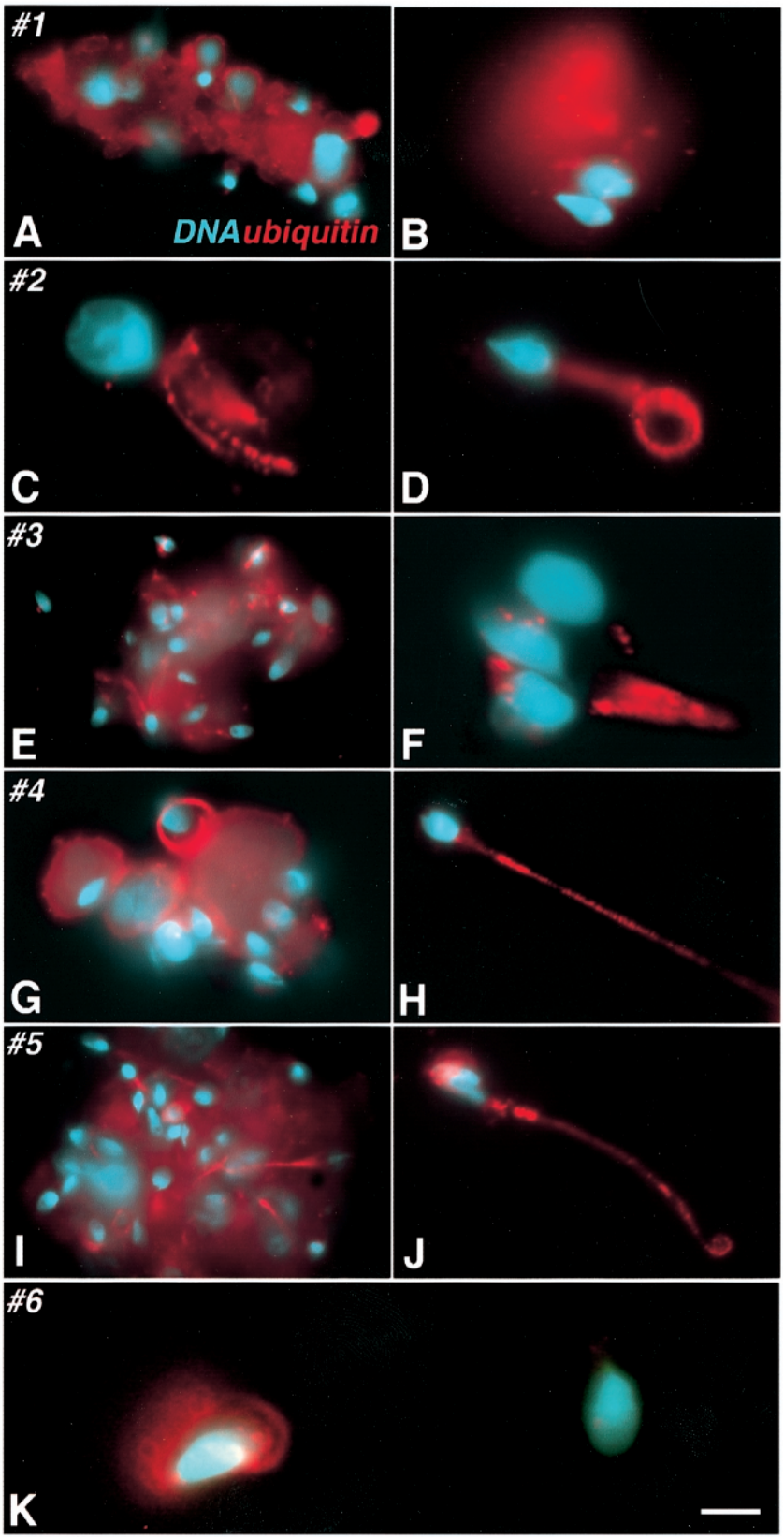

Figure 3. Representative sperm samples from DFS patients (**A–J**; patient nos.1–5) and a standard sample (**K**; no. 6), stained for ubiquitin (red) and DNA (blue) in the preparations for flow cytometry/SUTI. Samples were processed without permeabilization and examined by a high resolution, research epifluorescence microscope. Note the presence of clumped testicular/epididymal cells (**A, E, G, I**), twin (**B**) and triplet (**F**) sperm, round headed sperm (**C**), and sperm with abnormal tails (**C, D, J**, and most sperm in the other images). The ubiquitinated spermatozoon in (**H**) does not display major morphological defects and could be scored as normal in conventional semen analysis by light microscopy. The normal spermatozoon in the standard sample (**K**, right) shows a low level of ubiquitination, as opposed to the high ubiquitination of a spermatozoon with a coiled tail on the left. Scale bar = 10 μm.

difference ($P < 0.001$) in the presence of mitochondrial ubiquitin signal between the sperm samples from fertile men and DFS patients.

Patterns of sperm surface ubiquitination in DFS sperm

Representative samples were taken from each aliquot of sperm processed for flow cytometry/SUTI without permeabilization (see Materials and methods) and evaluated by a high resolution, research immunofluorescence microscope. The common denominator of all DFS samples (nos. 1–5) was the presence of strong surface labelling in ubiquitinated sperm (Figure 3B–D,F,H,J). Large clumps of sperm mixed with (presumably) immature spermatogenic cells were often seen (Figure 3A,E,G,I). Another possible source of such contamination is the white blood cells present in the epididymis. While these cells were ubiquitinated on their surface, the overall intensity of their fluorescence was lower than that of the ubiquitinated sperm (Figure 3 B–D,F,H,J). Large clumps of ubiquitinated, defective sperm (Figure 3E,I), and the twin and triplet sperm (Figure 3B,F) were frequently seen. The control samples from fertile donors (nos. 9–13; Table IC) contained abundant defective sperm with strong labelling (Figure 3K, left), but also numerous morphologically normal sperm with low levels of ubiquitin labelling (Figure 3K, right).

Flow cytometric SUTI assay

Sperm samples from all five DFS patients (nos. 1–5) and five fertile donors (nos. 9–13) were analysed using the flow cytometric SUTI (Sutovsky *et al.*, 2001b). Five thousand sperm per sample were screened in each of three consecutive repeats with almost identical results.

The analysis of DFS semen samples by SUTI posed a unique challenge due to the presence of an unusually large number of cells other than normal sperm. Initially, the median values were obtained for the whole samples (R0: Table IIA, Figure 4A) and then the cells within the screened pool were divided into two groups, based on the arbitrary subdivision of scatter diagrams of the cell size measured in the visible light spectrum (Figure 4B). (i) Cells of prevailing size, considered to be normal size sperm (R2), which formed a tight focus in the centre of the scatter diagrams (Figure 4B); and (ii) cells of either small or large size (R1; Figure 4B), scattered outside the R2 region. High median values for all cells measured (R0) were detected in all five patients (19.8–37.9; Table IIA). The fertile samples displayed significantly lower overall median values (9.3–16.6; mean 13.4), as well as lower fluorescence of normal size-cells (R2; 8.7–15.4; as opposed to 16.6–27.4 in DFS samples) and somewhat lower fluorescence of the large cells (R1, 16.6–52.3 in the fertile samples; 22.9–67.3 in DFS samples). Overlapping of the flow cytometric histograms of overall fluorescence (R0) of all five patients with that of the fertile donor with highest ubiquitin median (donor no. 7) revealed a shift of the curve towards the highly fluorescent cells in all patients (examples shown in Figure 4A).

All DFS samples displayed an increased percentage of small/large cells (R1), ranging from 31.1% in patient no. 3 to 61.0% in patient no. 1 (Table IIB). In contrast, the standard samples contained only 15.3–21.5% of abnormally sized cells. The standard samples therefore had the highest proportion of prevailing, presumably normal sized cells (R2; 79.1–84.8% as opposed to 39.0–68.9% in DFS patients).

In summary, the results of the SUTI assay showed that the semen samples of all five DFS patients displayed the following common attributes: (i) high overall median fluorescence of ubiquitin; (ii) higher median fluorescence of the prevailing size cells; and (iii) higher percentage of small and large cells.

Discussion

In addition to causing the abnormal configuration of the sperm tail fibrous sheath, DFS affects various cytoskeletal components including axonemal microtubule doublets, outer dense fibres and the mitochondrial sheath (Chemes *et al.*, 1987). According to the prevalence of sperm tail abnormalities, two groups of patients have been described in previous studies. In some patients, all sperm were affected, while in others, the percentage of abnormal tails was ~70–80%, with 20–30% in the normal configuration (Chemes *et al.*, 1998). These two groups have distinct characteristics and correspond to the complete and incomplete form of DFS. While these defects are presumed to be heritable, no major mutations have been reported in DFS patients in the genes encoding major FS proteins AKAP 3 and AKAP 4 (Turner *et al.*, 2001). In mice, a globozoospermia-like fertility defect, associated with the absence of perinuclear theca and acrosome, was linked to a mutation in the catalytic subunit of casein kinase II alpha (Xu *et al.*, 1999), which seems to be an upstream signalling element that acts during spermatogenesis. The sperm tail abnormalities associated with DFS could be caused by a mutation of such signalling elements or by a mutation in the yet to be identified structural proteins of the FS.

With regard to the ubiquitination of the sperm tail structures in these DFS and other patients, it is important to distinguish between two different steps of ubiquitination that occur during spermatogenesis in the testis and during sperm maturation/storage in the epididymis respectively. At the round spermatid stage of spermiogenesis, the mitochondria in both normal and defective sperm cells acquire ubiquitin cross-reactivity. This ubiquitin tag is probably masked by disulphide bond formation in the caput epididymis (Sutovsky *et al.*, 2000), and later may serve as a signal for the recognition and elimination of the sperm mitochondria at fertilization (Sutovsky *et al.*, 1999, 2000). By immunofluorescence, the mitochondrial sheath is the only sperm structure displaying a detectable ubiquitin cross-reactivity in human and animal testicular sperm prior to epididymal passage (Sutovsky *et al.*, 2000, 2001a). In the defective sperm, such a ubiquitination pattern may diverge. The failure of disulphide bond cross-linking and/or the irreversible changes of the sperm plasma membrane may render the defective sperm prone to further ubiquitination by the ubiquitin cross-reactive proteins inside the epididymal lumen (Sutovsky *et al.*, 2001a). Thus the cross-reactivity of the ubiquitinated substrates in the mitochondria of DFS sperm may be the results of failed cross-linking of the mitochondrial membranes, and/or de-novo ubiquitination of the mitochondrial membranes during epididymal passage. The ubiquitin cross-reactive proteins, detected in DFS samples on the surface of the sperm tail

Table IIA. Median values of the ubiquitin-induced fluorescence in sperm samples from five DFS patients (nos. 1–5) and five fertile men (nos. 9-13), screened by flow cytometric SUTI assay[a]

Patient no.	1	2	3	4	5	9	10	11	12	13	Mean 1–5	Mean 9–13
R0	37.86[c]	19.81	22.07	29.43	19.81	15.40	16.55	9.31[b]	12.41	13.34	25.796	13.402
R1	67.32[c]	22.88	58.29	62.64	43.71	24.58	38.54	16.55[b]	52.33	40.68	50.968	34.536
R2	27.38[c]	22.88	17.15	16.55	16.55	14.86	15.40	8.66	10.75[b]	11.55	20.102	12.224

[a]Values represent the median, ubiquitin-activated fluorescence of the whole sperm samples (R0), the cells of prevailing size (R2) and the atypical, small or large cells present in the semen (R1; see Figure 4B for explanation). Values are the averages from three repeats. Samples 1–3 are DFS semen samples with the complete form of the pathology. Samples 4 and 5 represent the incomplete form of DFS. Samples 9–13 are the control standard semen samples from fertile donors.
[b]Denotes the lowest value in the row.
[c]Denotes the highest value in the row.

Table IIB. Relative rates of prevailing size-cells (R2) and small/large cells (R1) in semen samples from 5 DFS patients (#1-5) and 5 fertile men (#9-13), measured by visible light-scatter in flow cytometer[a]

Patient no.	1	2	3	4	5	9	10	11	12	13	Mean 1–5	Mean 9–13
R0	100	100	100	100	100	100	100	100	100	100	100	100
R1	61.02[c]	54.56	31.14	51.60	47.72	20.88	15.84	15.33[b]	20.79	21.46	49.208	18.74
R2	38.98[b]	45.44	68.86	48.40	52.28	79.12	84.56	84.77[c]	79.21	78.64	50.792	81.26

[a]Values represent % of sperm/cells within the appropriate segment of the visible light-scatter diagrams (see Figure 4B), representing normal, prevailing cell size (R2), or small and large cells (R1); 5000 sperm were measured by flow cytometry for each sample. Samples 1, 2 and 3 are DFS semen samples with complete form of the pathology. Samples 4 and 5 represent the incomplete form of DFS. Samples 9–13 are control, standard sample from fertile donors.
[b]Denotes the lowest value in the row.
[c]Denotes the highest value in the row.

principal-piece and on the sperm head, most likely originate from the epididymal secretion (Fraile *et al.*, 1996; Sutovsky *et al.*, 2001a). The microscopic assessment of ubiquitin-cross-reactivity in sperm tail mitochondria could therefore be a good marker of infertility wherever flow cytometry is not available. More sensitive tests could be performed using flow cytometric SUTI assay, as described here and previously (Sutovsky *et al.*, 2001b), or by other immunological methods (Western blotting, ELISA, proteomics).

Sperm evaluation by SUTI poses a unique challenge in DFS patients because of the high number of cells other than mature sperm present in their semen samples. On the one hand, these cells may affect the overall reading of ubiquitin medians as their median fluorescence may be lower than that of the defective sperm (see Figure 3). On the other hand, due to their large size and dense cytoplasm, these cells may cause a higher autofluorescence in blank, negative control samples than the normal and defective sperm. It was therefore useful to subdivide the scatter diagrams generated by flow cytometer into areas representing normal/prevailing size sperm cells (R2 in Figure 4A) and small/large cells (R1 in Figure 4A). The standard, fertile sperm samples showed the lowest overall ubiquitin medians, as well as the lowest ubiquitin medians of prevailing-size cells. While the median values for the small and large, R1 cells were lower in the fertile samples than in the DFS semen, the fluorescence levels were comparable. It has been shown previously that the leukocytes, residual bodies and cellular debris in human semen contain both intracellular and cell surface-bound ubiquitin-cross-reactive proteins (Sutovsky *et al.*, 2001b).

Bongso *et al.* (1989) demonstrated that human oocytes can be fertilized successfully with immotile spermatozoa by micro-injection. The use of intracytoplasmic sperm injection (ICSI) has resulted in pregnancies in 60% of patients with DFS (Stalf *et al.*, 1995; Terriou *et al.*, 1995; Chemes *et al.*, 1998; Brugo Olmedo *et al.*, 1997; 2000). Although having an elevated rate of epifluoresecence-assessed sperm mitochondrial ubiquitination (~10× higher than standard sperm) and higher sperm surface ubiquitination (~2× higher than standard samples), treatment by ICSI in patient no. 1 led to a triplet pregnancy, and birth of one boy and two girls (unpublished results). This implies that DFS sperm could be used for ICSI, though all necessary precautions should be taken with regard to possible embryonic abnormalities. It is yet to be determined what happens with the ubiquitinated epitopes on the sperm surface once they are injected into the oocyte cytoplasm. Since the sperm plasma membrane, mitochondria, axoneme and perinuclear skeleton are disposed of after natural fertilization (Sutovsky and Schatten, 2000; review), it is possible that their ubiquitination prior to sperm injection into the oocyte may not interfere with normal zygotic and embryonic development. However, there could be some cases in which the sperm centriole, an organelle required for successful pronuclear apposition in humans, could also be compromised (V.Rawe *et al.*, unpublished observations).

In summary, the present study demonstrates that the anti-ubiquitin antibodies recognize defective sperm in a presumably heritable, male infertility syndrome, DFS. Ubiquitin thus may be an efficient marker of defective sperm in humans regardless of the cause of infertility. Ubiquitin-based screening may not be necessary for the diagnosis of DFS, as the gross morphological

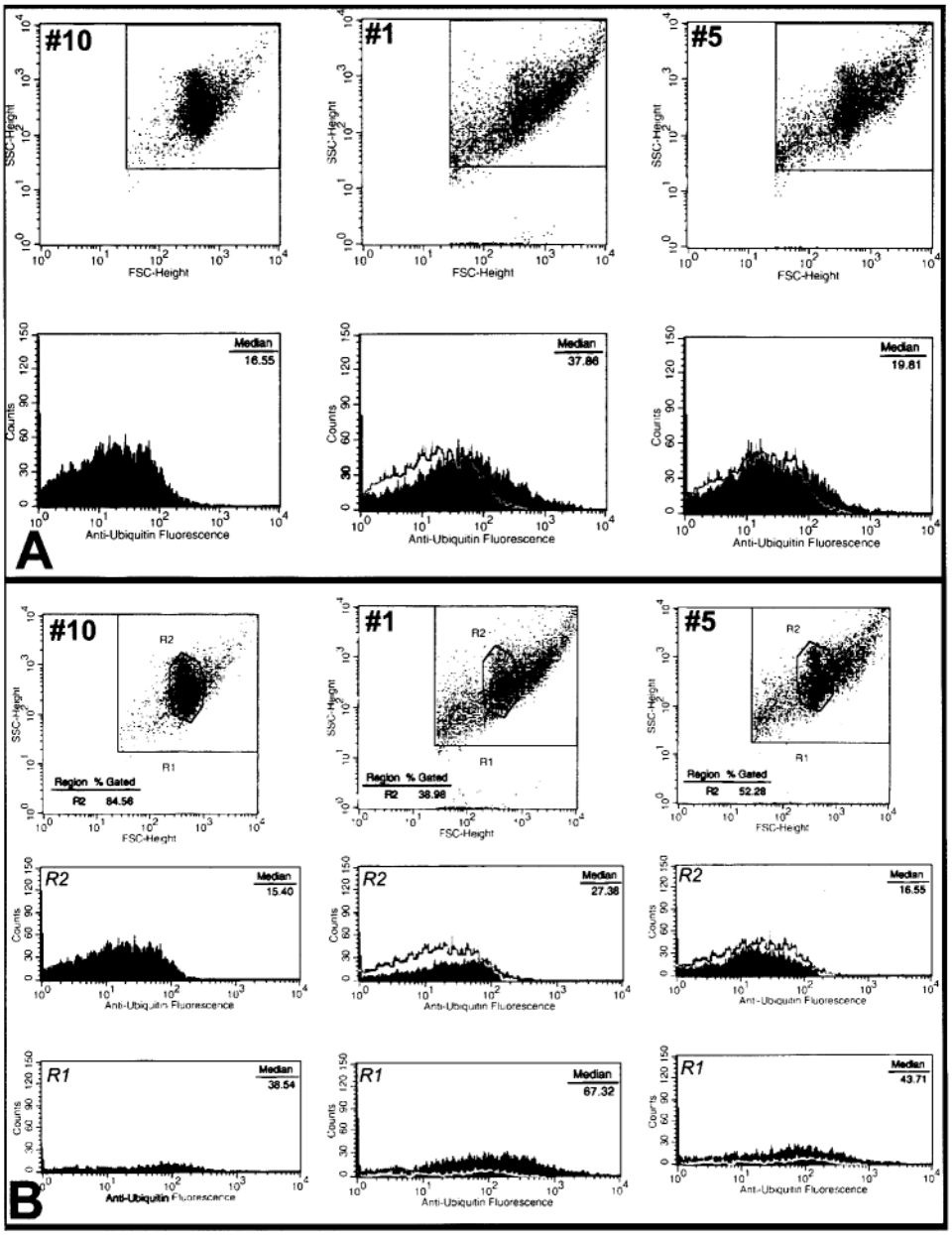

Figure 4. (**A**) Representative, light scatter diagrams (upper row) and flow cytometric histograms (lower row) of ubiquitin-induced fluorescence in samples from a fertile donor (no. 10), a patient suffering from the complete form of DFS (no. 1), and a patient with incomplete form of DFS (no. 5). The fluorescence histogram of fertile donor (empty curve) is superimposed onto the histograms of both patients, showing a shift towards highly fluorescent cells in both DFS cases (lower row). Numbering of donors/patients correspond to Results and Tables I, II. (B) Arbitrary subdivision of scatter diagrams of the cell size measured in visible light spectrum. Cells of prevailing size (R2) are considered to be normal sized sperm. Cells of either small or large size (R1, outside of the R2 region) are most likely defective sperm and other contaminants of cellular origin. Corresponding histograms of ubiquitin-induced fluorescence for R2 and R1 are shown in the second and third row respectively. The same donors as shown in Figure 3 are illustrated. Overlapping of the flow cytometric histograms in R2 and R1 reveals an increase in the number of small and large cells (R1) and a proportional decrease in the number of prevailing-size cells (R2) in both DFS patients (nos. 1 and 5).

defects in this particular type of infertility are obvious from routine microscopic sperm evaluation. However, this is the first study to show increased sperm ubiquitination in any type of presumably inherited fertility disorder, and the SUTI assay could be useful for revealing other heritable infertility cases, should these be associated with less conspicuous sperm abnormalities.

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