A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis

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SUMMARY

The normal structure and function of sperm are prerequisites for successful fertilization and embryonic development, but little is known about how defective sperm are eliminated during mammalian spermatogenesis. Here, we describe a ubiquitin-dependent, sperm quality control mechanism that resides in the mammalian epididymis, the site of sperm maturation and storage. We used immunofluorescence, electron microscopy, western blotting and pulse-chase experiments to show that ubiquitin is secreted by the epididymal epithelium and binds to the surface of defective sperm. Most of the ubiquitinated sperm are subsequently phagocytosed by the epididymal epithelial cells. A portion of defective sperm escapes phagocytosis and can be found in the ejaculate. Cultured epididymal cells maintain their ability to produce ubiquitin and phagocytose the defective sperm, as well as the ubiquitin-coated microspheres, in vitro. The surprising phenomenon of cell-surface ubiquitination in defective sperm provides a possible mechanism for sperm quality control in mammals and a new marker of semen abnormalities in men and animals.

Key words: Spermatogenesis, Ubiquitin, Epididymis

INTRODUCTION

Mammalian fertilization depends upon the normal structure and functioning of the participating gametes. This is assured at multiple levels, including selection of the fittest sperm at the egg vitellus during fertilization, as well as pre-selection of both sperm and eggs prior to their release from the gonads. The female gametes, the oocytes, undergo a dramatic reduction in their number, during which only a few oocytes from the initial pool are allowed to grow to full size and become ovulated. Meanwhile, the vast majority of oocytes, along with their somatic entourage of ovarian follicular cells, undergo an apoptotic process known as atresia (reviewed by Morita and Tilly, 1999). In contrast to our knowledge of oocyte selection, there are few reports implicating apoptosis in the quality control of the male gametes, the sperm or spermatozoa. Components of an active apoptotic pathway are present in the spermatogenic cell lineage (reviewed by Sinha Hikim and Swerdloff, 1999) and in the mature sperm of mice (Weil et al., 1998; Yin et al., 1999) and men (Sakkas et al., 1999). However, a definitive mechanism for sperm quality control is yet to be established.

After exiting the testis via the testicular rete, mammalian sperm are collected and stored in the epididymis, where they undergo final maturation. The mammalian epididymis is composed of three distinct compartments: caput, corpus and cauda. Each of these has a specific role in sperm maturation, sustenance, transport and storage (reviewed by Bedford, 1979; Cooper, 1998). Numerous proteins are secreted in apocrine fashion by the epididymal epithelium. The proteome of the epididymal fluid is composed of more than 200 major proteins (Fouchécort et al., 2000) implicated in sperm immobilization, stabilization of the sperm plasma membrane and the acquisition of fertility (reviewed by Kirchhoff, 1998). Maturation and hardening protects the sperm from oxidative damage during storage and after their discharge into the female genital tract. The droplets of a residual cytoplasm carried over from the testis (Bloom and Nicander, 1961; Hermo et al., 1988) and the abnormal sperm (Ramamohana-Rao et al., 1980; Roussel et al., 1967) are phagocytosed during sperm descent down the epididymis. Two major components of the ubiquitin-dependent proteolytic pathway, the 8.5 kDa-ubiquitin and the ubiquitin-recycling C-terminal hydrolase, gene product 9.5, are expressed in the epididymal tissue (Fraile et al., 1996; Santamaria et al., 1993). Furthermore, the ubiquitin-conjugating enzyme E2, is present in mammalian sperm and prohibitin, the major constituent of sperm mitochondrial membranes, seems to be constitutively ubiquitinated during spermatogenesis (Sutovsky et al., 2000).

Here, we show that abnormal sperm found in the fertile

males of several mammalian species, including bovines and humans, are marked with ubiquitin during epididymal passage. Ubiquitin crossreactivity can be detected in both live and fixed sperm isolated from the individual epididymal compartments. A certain portion of the ubiquitinated, defective sperm is phagocytosed prior to reaching the storage site in the cauda epididymis. The remaining defective sperm are ejaculated and can be isolated from the immotile sperm fraction. Sperm ubiquitination in the epididymis, representing a new role for ubiquitin in mammalian reproduction, may therefore mediate epididymal sperm quality control and regulate male fertility.

MATERIALS AND METHODS

Antibodies and probes

Data from bovine species, including domestic bull, gaur and buffalo, were obtained using mouse monoclonal antibody MK-12-3 (MBL, Nagoya, Japan), raised against purified bovine erythrocyte ubiquitin. An unrelated antibody, Ab 1690, against bovine erythrocyte ubiquitin (Chemicon International Inc., Temecula, CA, USA), was used for staining shown in Fig. 2H. Human and rhesus data were obtained using mouse monoclonal antibody KM 691, raised against recombinant human ubiquitin (Kamyia Biomedical Comp., Seattle, WA, USA). The ubiquitin-conjugating enzyme E2, was detected using rabbit polyclonal antibody UG 9520 (Affinity Research Products Ltd., Mamhead, Exeter, UK). Secondary antibodies were purchased from Zymed Labs (South San Francisco, CA, USA). Rhodamine-phalloidin (actin stain) and DAPI (DNA stain) were purchased from Molecular Probes Inc. (Eugene, OR, USA).

Sperm

Straws of frozen bull semen were purchased from ABS Global (De Forest, WI, USA) and, where stated, were separated on a two-layer Percoll gradient (Parrish et al., 1986). Some experiments were performed using fresh bull sperm collected from the ampula of the caput epididymis by dissection and release into culture medium. Frozen gaur and buffalo semen samples were kindly donated by Dr Naida Loskutoff (Henry Doorly Zoo, Omaha, NE, USA). Frozen human sperm from fertile donors were purchased from Follas Laboratories (Indianapolis, IN, USA). Fresh rhesus semen was obtained by penile electro-ejaculation. Fresh epididymal and testicular sperm were obtained by mincing of the appropriate bovine tissues purchased from a local slaughterhouse. Cell suspensions were washed in Sperm-TL medium (modified Tyrode-lactate-Hepes medium; Parrish et al., 1986) and used as described below.

Tissue isolation and epididymal cell culture

Pieces of the caput, corpus and cauda epididymal tissue (5×5×5 mm) were transferred into TL-Hepes medium (Parrish et al., 1986) and digested using adaptations of published techniques (Moore et al., 1992; Cooper et al., 1989). The first digestion (30 minutes at 37°C, shaking) was done in TL-Hepes containing 2 mg/ml collagenase II (Sigma, St Louis, MO, USA), 3 mg/ml BSA-fraction-V, 0.2 mM pyruvate and 0.5 μl/ml gentamicin. The second digestion (20 minutes at 37°C, shaking) was done in TL-Hepes with the above supplements, 2 mg/ml hyaluronidase and 0.33 mg/ml elastase (all from Sigma). Isolated cells and tissue fragments were collected by centrifugation, washed in TL-Hepes and plated onto 6-well culture clusters (Costar, Corning, NY, USA) in DMEM medium (Gibco) supplemented with 10% fetal calf serum, 50 i.u./ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate, 0.1 μM water soluble testosterone and 1 μM dihydrotestosterone (all from Sigma). Cultures were maintained for up to 15 days with a medium change every 2 days. Ubiquitin-coated Dynabeads (ubi-beads, see below) were added to cultures in a final concentration of 10,000 beads/ml (50,000 per well) or as specified in the results section.

Immunofluorescence

Sperm were attached to poly-L-lysine-coated microscope coverslips by a 5-minute incubation in a 500 μl drop of 37°C KMT medium (Sutovsky et al., 2000) on a warm plate. Epididymal epithelial cells (EEC) were fixed in suspension (prior to in vitro culture) or in a culture dish (after in vitro culture). Both sperm and EEC were fixed for 40 minutes in 2% formaldehyde in PBS. EEC were permeabilized for 40 minutes in 1% Triton TX-100. Permeabilization was not used for the evaluation of sperm surface ubiquitination in order to avoid the contribution of constitutively ubiquitinated, intrinsic sperm substrates to the signal. The samples were blocked for 25 minutes in 5% normal goat serum (NGS) in PBS and incubated for 40 minutes with monoclonal antibody MK-12-3 (mouse IgG; diluted 1/100 for bovine sperm) or KM 691 (mouse IgM; diluted 1/100 for primate sperm). Washing and dilution of the primary and secondary antibodies were done in PBS with 1% NGS and 0.1% Triton X-100 (TX-100 was not used for sperm). After washing, the samples were incubated for 40 minutes with the TRITC-conjugated goat anti-mouse IgG/IgM (Zymed; diluted 1/80) and DNA-stain DAPI (Molecular Probes, Eugene, OR, USA; added at 5 μg/ml 10 minutes before the end of incubation), and mounted on microscopy slides in Vectashield medium (Vector Labs, Burlingame, CA, USA). In some EEC experiments, actin microfilaments were counterstained with rhodamine-phalloidin (Sigma; St Louis, MO, USA) and added to a second antibody solution at 1/40 dilution. The ubiquitin-conjugating enzyme E2, was detected in sperm as described above, using antibody UG 9520 (diluted 1/100), followed by goat anti-rabbit IgG-FITC (1/80; Zymed), with/without permeabilization. Ubi-beads were labeled with MK-12-3 as described for sperm (no permeabilization), except that a Dynal MPC device was used for washing and labeling, instead of attaching the ubi-beads to the coverslips. Paraffin sections of formaldehyde-fixed, epididymal and testicular tissues were placed on microscopy slides, dewaxed by xylene, and processed for immunofluorescence with MK-12-3/FITC and DAPI (0.1% Triton X-100 added to all processing solutions) as described for sperm and EEC. Images were captured on a Zeiss Axiophot microscope by a Princeton RTE/CCD 1217 digital camera using MetaMorph software, edited by Adobe Photoshop 4.0, and printed on a Sony UP-D 8800 dye sublimation printer.

Electron microscopy and ultrastructural immunocytochemistry

Bull sperm, EEC and ubi-beads were fixed in formaldehyde and processed with antibody MK-12-3, with permeabilization in Triton TX-100, as described for immunofluorescence. Cultured EEC were lifted from the bottom of the culture dishes using a 15 minute digestion with a trypsin-containing DMEM medium. The fluorescently conjugated secondary antibodies were replaced by a 10 nm gold-conjugated goat antimouse IgG (Jackson Immunochemicals, West Grove, PA, USA). Labeled cells, as well as fresh cells and tissue fragments for ultrastructural studies, were then collected by centrifugation, fixed in glutaraldehyde/paraformaldehyde fixative, post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon 812, as described previously (Sutovsky et al., 2000). Ultrathin sections were cut on a Sorvall MT-5000 ultramicrotome (Ivan Sorvall, Inc. Norwalk, CT, USA), stained by uranyl acetate and lead citrate, and photographed in a Phillips EM 300 electron microscope. Negatives were scanned by a Umax Powerlook 3000 flat bed scanner (Umax Technologies Inc., Fremont, CA, USA) and printed on a Sony UP-D 8800 dye sublimation printer using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA, USA).

Western blotting and pulse-chase

Following Percoll separation, fractions of motile and immotile

ejaculated sperm and isolated testicular sperm were lysed in 0.5 ml of a sample buffer containing 1 M NaCl, 20 mM imidazole, 1 mM EDTA, 5 mM benzamidine HCl, 5 mg/ml leupeptin, 1 mg/ml pepstatin A and 1% Triton-X-100 (pH 6.0). 20 μg of proteins were loaded onto each lane. Proteins were separated by SDS-PAGE, transferred to 0.2 μm nitrocellulose membranes (Sigma), blocked with non-fat dried milk, incubated overnight at 4°C with MK-12-3 antibody (3 μg/ml), then washed and incubated with goat anti-mouse IgG/horseradish peroxidase (Sigma; diluted 1/2000). Bands were revealed with chemiluminescence (Amersham Life Sci., Arlington, IL, USA) and a chromogen substrate. Negative controls were performed by the omission of the first antibody and pre-absorption of antibody MK-12-3 with purified bovine erythrocyte ubiquitin (Sigma). For pulse-chase experiments, the epididymal tubules were isolated as described above and cultured for 48 hours in DMEM medium containing 3.5 mCi of [^{35}S]methionine. At the end of culture, the tubules were washed thoroughly by repeated medium exchange and placed back in the culture in DMEM medium without radioactive precursor. Medium was collected by centrifugation after 72 hours of culture and the cells were stored separately. Both the culture medium and the cells were subjected to immunoprecipitation with antibody MK-12-3 (5 μg/ml) and the precipitated protein was electrophoresed on an SDS-gradient gel (4%-20%). The gels were exposed for 3 days and bands developed using a phosphoimager.

Ubi-beads experiments

Uncoated (4.5 μm diameter) and tosyl-activated (2.8 μm) magnetic beads were purchased from Dynal (Lake Success, NY, USA) and coated using a Dynal MPC device, according to the manufacturer's recommendations, with the purified bovine erythrocyte ubiquitin (Sigma) in PBS (pH 7.3) or bovine serum albumin, fraction-V (BSA-V; Sigma), both at a concentration of 150 μg/ml. Beads were incubated with ubiquitin or BSA overnight at 37°C in a shaking water bath, washed and stored at 4°C until used (typically on the same day). Uncoated beads were processed using the same procedure except that no protein was added before the overnight incubation. The initial concentration of beads/ml was calculated using dilution factors from the known concentration data provided by the manufacturer and the final concentrations after coating and washing were determined separately for each group using a hemocytometer. Beads were added to wells with cultured EEC on day 3 of culture at a final concentration of 150,000 and cultured for 24 hours. After 24 hours, 50 plaques were selected randomly from four wells in each group (Ubi-beads, BSA-beads, uncoated beads), and the beads bound to each individual plaque were counted using a Nikon Eclipse 300 inverted microscope under the Hoffman modulation contrast optics at a primary magnification of 20×. During each counting, the stage was shaken repeatedly and vigorously, and only those beads that did not move were counted as attached to a plaque. The experiment was repeated 3× with similar results (representative data from one experiment are shown), the standard deviations were calculated for each group and the averages were compared by Student's *t*-test. Numerical data shown in Figs 2 and 6 were analyzed using single factor Analysis of Variance (ANOVA). Standard deviations were computed as described (Snedecor and Cochran, 1973).

Labeling of live sperm with anti-ubiquitin antibodies

Fresh and frozen-thawed sperm were resuspended in 500 μl of KMT medium on the surface of a poly-L-lysine-coated coverslip, placed on the cross of a four well, 100×15 mm Petri dish, and incubated for 5 minutes on a slide warmer (37°C). The attached sperm were incubated sequentially with antibody MK-12-3 (1/100) and a goat-anti-mouse IgG-FITC (1/80), 40 minutes each, both diluted in warm KMT medium. Washings and incubations were done in warm KMT in a Petri dish placed inside a bench top incubator

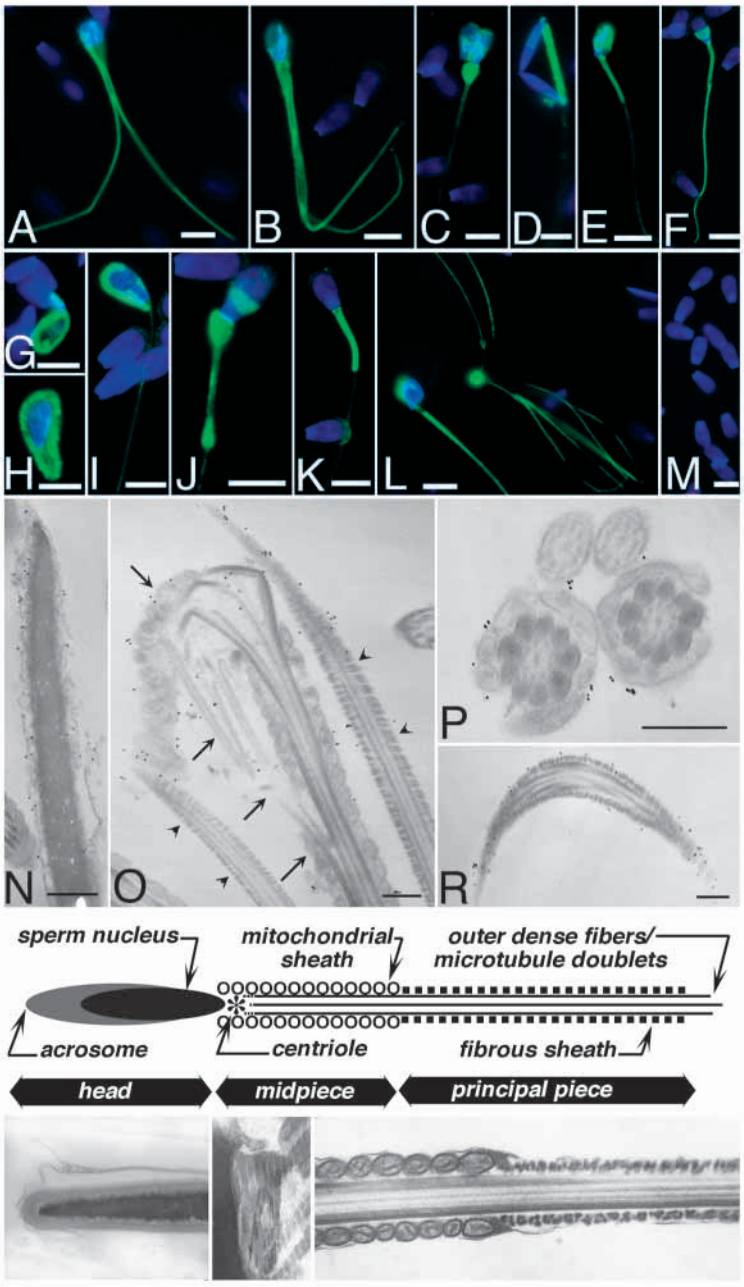

Fig. 1. Ubiquitinated, abnormal spermatozoa in the ejaculate of a domestic bull. Abnormalities shown include twin spermatozoa (A), twin tail (B), twin head (C,D), misshaped sperm heads (E), ubiquitinated post-acrosomal sheath (F), coiled (G) and lasso tails (H,I), abnormal mitochondrial sheath (J), ubiquitinated mitochondrial sheath (K), and the clustering of ubiquitinated sperm (L). Control sperm without surface ubiquitination are shown in (M). Colloidal gold labeling of formaldehyde-fixed and permeabilized sperm (N-R) shows ubiquitination of the sperm head (N), mid piece (arrows) and principal piece (arrowheads) of the sperm tail (O), mitochondrial sheath (P) and fibrous sheath (R). The diagram and the representative EM figures below it show the major structural components of mammalian spermatozoa, as referred to in this paper. Green in A-M, anti-ubiquitin MK-12-3/FITC (see Materials and Methods); blue, DNA stain DAPI. Scale bars, 10 μm (A-M), 500 nm (N-R).

(37°C). The coverslips with the labeled sperm were mounted on the microscopy slides in a 10 μl drop of warm KMT, sealed with clear nail polish and imaged immediately by epifluorescence microscopy. Many sperm retained their motility after this labelling procedure, as evidenced by the beating of the loose sperm tails.

Isolation and fractionation of epididymal secretory vesicles

The epididymal fluid and sperm were released from the dissected pieces of caput epididymal tissue into Sperm TL medium in a 100 mm Petri dish, and the cellular debris and large cells were removed by filtration through 53 μm pore size nylon mesh (Spectrum Lab Products, Houston, TX, USA). Collected medium was then filtered through a 20 μm mesh into 15 ml centrifugation tubes (Falcon) and pelleted by a 10 minute centrifugation at 2100 rpm (400 ***g***) in a clinical centrifuge. The pellet (<20 μm pellet fraction) was saved and supernatant was recentrifuged for 10 minutes at 3300 rpm. The resulting pellet (<20 μm supernatant fraction) was saved and supernatant was filtered through a 0.45 μm syringe filter (Corning-Costar), then repelleted for 10 minutes at 3300 rpm (1000 ***g***; 0.45 μm fraction). All fractions were resuspended in KMT on the surface of lysine-coated coverslips, as described for sperm, fixed in 2% formaldehyde and stored in PBS without Triton-X-100. Where permeabilization is indicated in the Results section, coverslips were incubated for 40 minutes with 0.1% Triton-X-100 prior to their labeling with MK-12-3. Leftover pellets were processed for electron microscopy, as described for sperm.

RESULTS

The presence of the ubiquitinated spermatozoa in the ejaculate of different mammalian species

Ubiquitinated sperm were initially detected in both the fresh and frozen-thawed, formaldehyde-fixed semen from domestic bulls *(Bos taurus)*, by immunofluorescence labeling with the anti-bovine ubiquitin antibody MK-12-3. Invariably, ubiquitinated sperm cells displayed visible defects of the sperm head and/or axoneme. Ubiquitinated twin sperm and sperm with two tails/heads were frequently seen (Fig. 1A-M). Ultrastructural analysis of the permeabilized cells showed that the defective sperm were ubiquitinated mainly on their surface (Fig. 1N-R), a presumption confirmed by the fact that immunofluorescence labeling of sperm was obtained in the absence of permeabilization or even in live, unfixed sperm (see Fig. 9A). In addition to domestic bull sperm, ubiquitin was detected by MK-12-3 in the frozen-thawed sperm of Asian wild cattle, gaur *(Bos gaurus)* (Fig. 2A), and American buffalo *(Bos bison)* (Fig. 2B). An unrelated antibody KM-691, raised against recombinant human ubiquitin, revealed ubiquitination of defective sperm in fresh semen samples from rhesus monkeys *(Macaca mullata)* (Fig. 2C), and in frozen-thawed sperm donated by fertile men (Fig. 2D). In all examined species, ubiquitinated sperm cells were apparently abnormal.

To investigate the distribution of the ubiquitinated substrates in the sperm, we performed western blot analysis of motile and immotile sperm fractions obtained by Percoll separation. There appeared to be an increase in the number and density of ubiquitin-crossreactive bands in the immotile sperm fraction, as compared with the motile sperm, or the isolated testicular sperm (Fig. 2E). The representative images of ubiquitinated sperm in these sperm fractions, stained with MK-12-3, are shown in Fig. 2F,G. An unrelated antibody against bovine erythrocyte ubiquitin, Ab1690, also yielded similar staining of abnormal sperm (Fig. 2H).

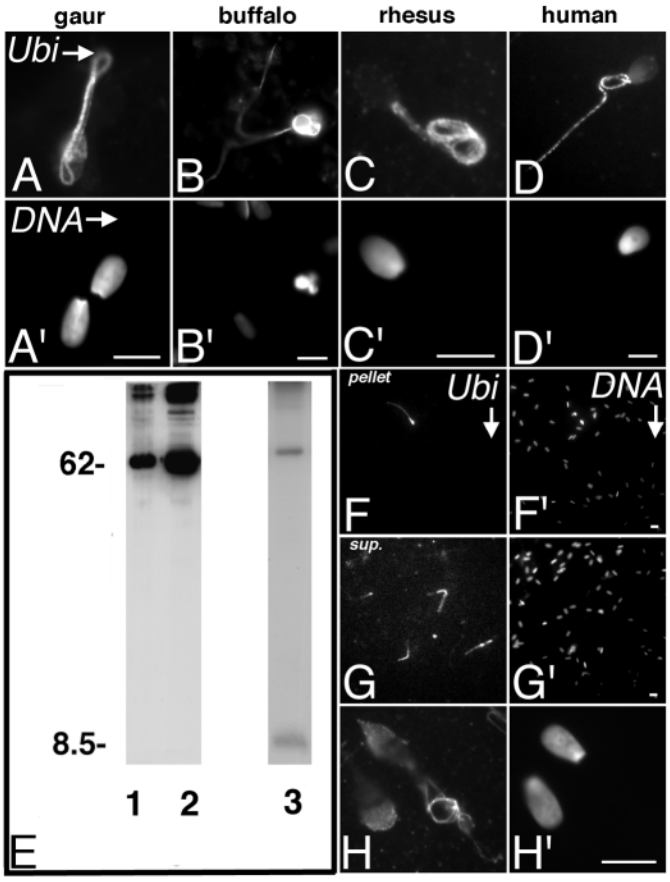

Fig. 2. Ubiquitinated sperm found in mammalian ejaculates and afferent ductuli. Defective, ubiquitinated sperm of gaur (A), buffalo (B), rhesus monkey (C) and man (D). Fresh (C) or frozen/thawed (A,B,D) sperm were fixed in formaldehyde and processed without permeabilization. (E) Western blot of the ejaculated, domestic bull sperm, separated by a Percoll gradient into motile (lane 1) and immotile (lane 2) sperm fractions. Isolated testicular sperm that did not enter the epididymis are shown in lane 3. (F,G) Ubiquitinated sperm in the motile (F, Percoll pellet) and immotile (G, Percoll supernatant) sperm fractions. (H). Detection of abnormal sperm with an unrelated anti-ubiquitin antibody, Ab1690. Scale bars, 10 μm.

Ubiquitination and phagocytosis of the defective sperm in the epididymis

The site of the ubiquitination of defective sperm was revealed by comparing the percentages of ubiquitinated sperm in the individual compartments of the genital tract of two different bulls (Fig. 3A-D). 1000 sperm were screened randomly for each bull after immunostaining with MK 12-3 in two replicates (total of 2000 sperm/bull). Although only 0-0.4% of sperm were ubiquitinated in the testicular rete (Fig. 3A,E), the rate of the ubiquitinated sperm rose to 5.3% in bull #1 and 5.2% in bull #2, and then decreased to 0.8% and 0.9%, respectively in cauda epididymis (Fig. 3E). Values in the corpus-epididymal samples reached approximately 55-70% of those in the caput. The types of defects and ubiquitination patterns found in epididymal sperm (Fig. 3A-D) were essentially the same as those seen in the ejaculated sperm (Fig. 1A-M).

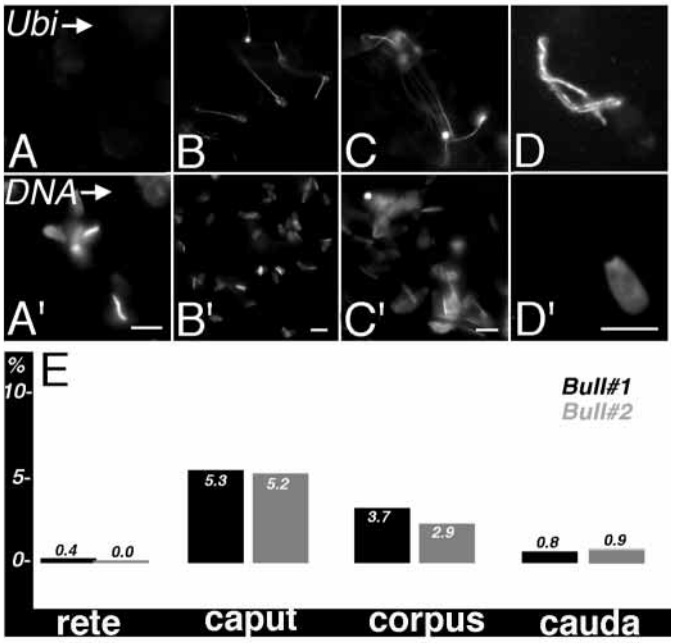

Fig. 3. Ubiquitination of defective sperm begins in the proximal epididymal compartment, caput epididymis. Representative samples of fresh (no freeze/thaw) sperm from the rete testis (A), and the caput (B), corpus (C) and cauda (D) epididymis. Samples were labeled with MK-12-3/FITC (A-D) and DAPI (for DNA; A′-D′). (E) Relative abundance of ubiquitinated sperm in the rete testis and the individual epididymal compartments. ANOVA: F=105.2; $F_{critical}$=6.59; P<0.01; Scale bars, 10 μm.

Immunostaining of the tissue sections revealed the localization of ubiquitin-crossreactive substrates in the caput epididymis (Fig. 4A). The microvillar accumulation of ubiquitin in EEC (Fig. 4B) may reflect the apocrine secretion of ubiquitin into the lumen of the epididymal ductuli, consistent with previous descriptions of this secretory pathway (Agrawal et al., 1988; Fouchécort et al., 2000; Manin et al., 1995). Sperm cells with coiled, ubiquitinated tails were occasionally detected in the sections (Fig. 4C). Most sperm cells in the lumen of the caput epididymal ductuli also carried ubiquitinated, proximal cytoplasmic droplets (Fig. 4D). The distribution of ubiquitin in the corpus and cauda epididymis (Fig. 4E) was similar to that of the caput, whereas the epithelial cells were shorter, the wall of the epididymal tubules was thinner, and the apical ubiquitin staining was less intense. Sperm in the rete testis were not ubiquitinated and no distinct ubiquitination of abnormal sperm was found in the seminiferous tubules adjacent to the rete (Fig. 4F). The binding of the second antibody was not detected after the omission of the first antibody in the negative controls of the epididymal tissue sections (not shown).

Ultrastructural analysis revealed the presence of disintegrating sperm tails and heads deep in the cytoplasm of the EEC (Fig. 5A). Numerous sperm with abnormal configurations of the perinuclear skeleton and axoneme (Fig. 5B,C) were found attached to, or internalized by the apical cytoplasm of the EEC in the caput epididymis. Cytoplasmic droplets filled with membrane vesicles were frequently found on the mid-piece of the caput epididymal sperm, or shed into the lumen of the epididymal ductuli (Fig. 5D). Colloidal gold labeling of permeabilized cells with antibody MK 12-3 revealed strong ubiquitination of the cytoplasmic droplets (Fig. 5D), as well as the presence of ubiquitin in the secretory bodies (Fig. 5E) and vesicles (Fig. 5F). Such ubiquitin-positive structures were often found attached to the sperm heads (Fig. 5G) and/or tails (Fig. 5H; see also Fig. 9).

Reconstitution of the ubiquitin-dependent sperm phagocytosis in vitro

An in vitro system to study epididymal sperm ubiquitination was developed using EEC isolated by enzymatic digestion of epididymal tissue. Individual cells (Fig. 6A) and the aggregates of elongated EEC (Fig. 6B) were obtained and plated in 6-well culture dishes containing medium with serum and testosterone (see Materials and Methods). Concomitantly, the magnetic spheres were coated with purified bovine erythrocyte ubiquitin (ubi-beads; Fig. 6C,D). Both the isolated cells and the ubi-beads crossreacted with MK-12-3 (Fig. 6A-C), whereas no crossreactivity was seen with the control, uncoated beads (Fig. 6D). The isolated cell suspensions also contained sperm killed by enzymatic digestion. Those sperm did not crossreact with MK-12-3 (not shown). On day 2 of culture, the cells adhered to the bottom of the culture dishes and coated ubi-beads were added to the culture at a final concentration of 150,000 beads/well. On day 3-4, the sperm introduced into culture with isolated EEC became strongly ubiquitinated (Fig. 6E). The EEC formed large epithelial plaques (Fig. 6F-H), often containing attached beads. Some of the plaques already contained the internalized ubi-beads and sperm at this point. The assembly of microfilaments around the internalized beads was visualized by rhodamine-phalloidin. (Fig. 6F,G). On day 10 of culture, the epithelial plaques engulfed most of the beads, often forming large clusters of beads next to internalized sperm (Fig. 6H). Such plaques still produced ubiquitin and actively assembled actin microfilaments, as documented by double labelling with MK-12-3 and rhodamine-phalloidin (Fig. 6H). A quantitative study (Fig. 7) demonstrated that ubiquitin-coated beads had a higher affinity to the cultured EEC than beads coated with a control protein (BSA-V) or uncoated beads.

Ultrastructural studies confirmed that cells actively phagocytosing sperm also internalized the ubi-beads (Fig. 8A). Sperm at various stages of disintegration were found next to the ubi-beads in the cytoplasm of such cells (Fig. 8B). Large lysosomal vesicles were regularly found next to internalized ubi-beads and sperm (Fig. 8B). We also observed the internalization of mouse and rhesus monkey sperm added to EEC cultures (not shown). Although the ubi-beads displayed strong labeling when processed with MK-12-3/gold prior to coculture with EEC (Fig. 8C), this was diminished in the internalized beads by day 3 (Fig. 8D), and almost completely disappeared from the internalized beads by day 10 (Fig. 8E). The cells with internalized beads often displayed strong cortical and surface labeling (Fig. 8E). Similar to ubi-beads, internalized sperm did not display detectable ubiquitin labeling by day 10, even though ubiquitin was detected on the adjacent lysosomes and in the surrounding cytoplasm (Fig. 8F). In contrast, most EEC displayed strong surface labeling (Fig. 8G), and secretory bodies similar to those found in situ (see Fig. 5E,F) were often detected (Fig. 8H).

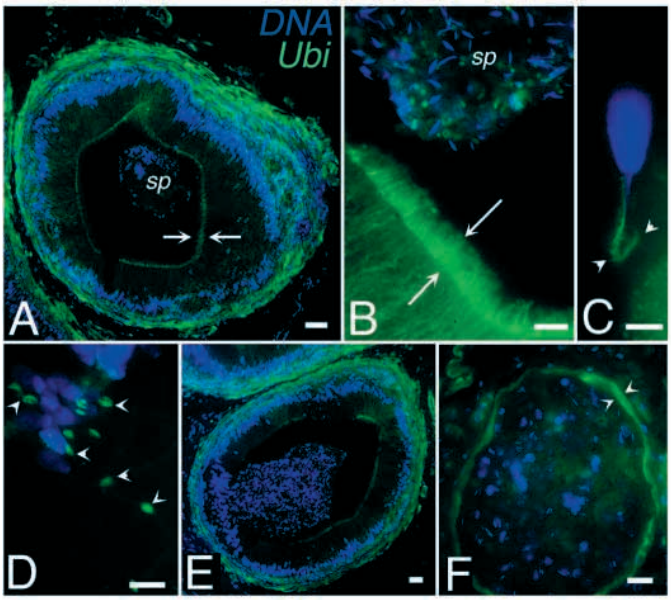

Fig. 4. Immunofluorescence detection of ubiquitin (green) in the paraffin sections of the epididymal tissue of a domestic bull. (A-D) Epididymal ductuli in the caput epididymis. Note the ubiquitin-crossreactive layer lining the lumen (arrows in A,B), corresponding with the apical microvilli. (B) Detail of the microvillar layer (arrows) and a clump of sperm with ubiquitinated residual droplets in the lumen of an epididymal ductus. (C) A sperm with a coiled, ubiquitinated tail (arrowhead) near the epithelial surface. (D) Ubiquitinated residual cytoplasmic droplets (arrowheads). (E) A cross-section of a caput-epididymal ductus. (F) The absence of the ubiquitinated sperm from the rete testis. Note the crossreactivity in adjacent epithelium (arrowheads). Ubiquitin was visualized by MK-12-3/FITC, DNA (blue) by DAPI. Scale bars, 50 μm (A,E,F); 20 μm (B); 5 μm (C); 10 μm (D).

Further evidence for surface ubiquitination of defective sperm

Our data demonstrate that the surface of defective mammalian sperm becomes crossreactive to anti-ubiquitin antibodies during epididymal passage. Surface ubiquitination is a novel and surprising phenomenon that had to be validated by further experiments. Thus, live sperm were labeled with MK-12-3 at the physiological temperature. The morphologically normal sperm, often retaining their motility after processing at 37°C, displayed a low level of labeling. In contrast, the patterns of ubiquitin fluorescence in defective, unfixed sperm (Fig. 9A) were identical to those seen in formaldehyde-fixed sperm (Fig. 1).

Ubiquitin-conjugating enzyme E2, a crucial component of the ubiquitin-conjugation machinery, was detected in all sperm after permeabilization (Fig. 9B), but only in ubiquitinated, defective sperm when the permeabilization was omitted (Fig. 9C), suggesting that the plasma membrane is altered in these cells during epididymal passage. Ubiquitin on the surface of secretory vesicles and bodies, which can be isolated from the epididymal fluid (Fig. 9D-K), may thus interact directly with ligases on the surface of defective sperm.

A pulse-chase experiment, coupled with the immunoprecipitation of ubiquitin from culture medium, demonstrated that isolated EEC secreted ubiquitin-crossreactive proteins into the culture medium (Fig. 9L). Two

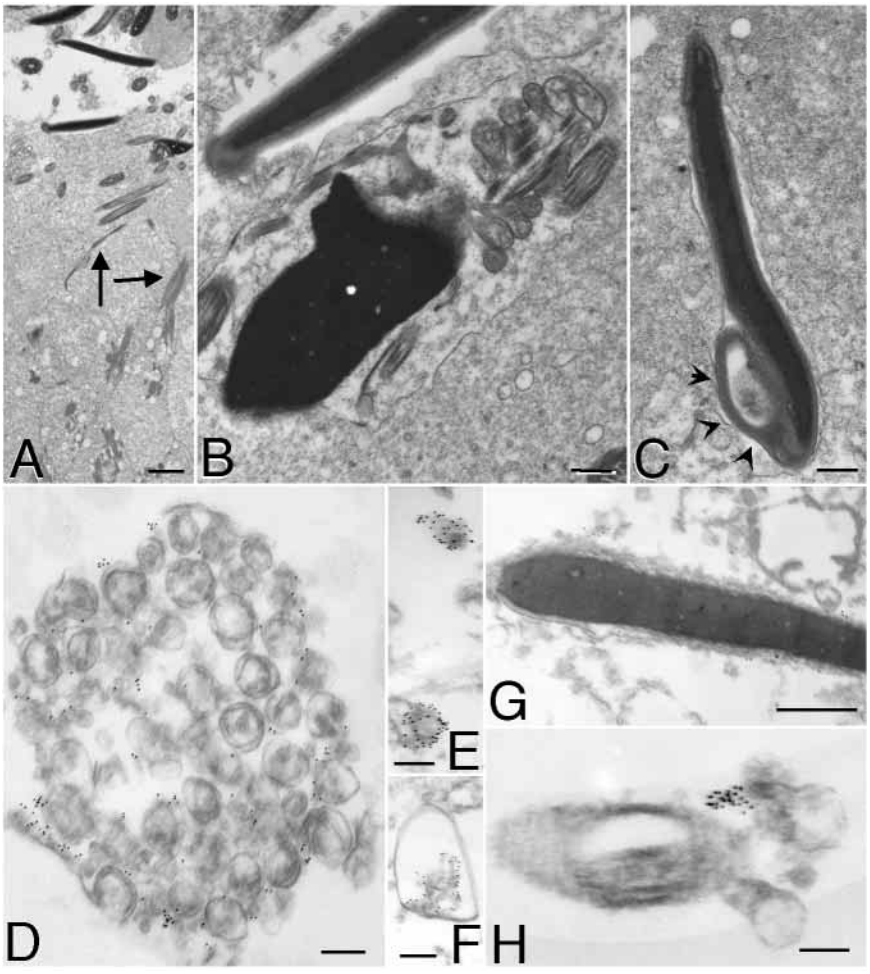

Fig. 5. Ultrastructure of sperm ubiquitination and phagocytosis in a bovine epididymis. (A) Apical surface of the caput epididymal epithelium with the sperm attached to it. Note the disintegrating sperm axonemes deep in the epithelial cell cytoplasm (arrows). (B) A spermatozoon with a misshaped nucleus and a defective axonemal midpiece, surrounded by the cytoplasm of the caput epididymal epithelium. (C) Phagocytosed sperm head with the abnormal perinuclear theca (arrowheads). (D) Colloidal gold labelling of ubiquitin in a residual cytoplasmic droplet, composed of a number of small membrane vesicles. (E,F) Ubiquitin-containing secretory bodies (E) and membrane vesicles (F) in the caput epididymis. (G,H) Binding of ubiquitin (gold particles) to a sperm head (G) and a sperm axoneme (H; cross-section of the principal piece) in the caput epididymis. Ubiquitin was detected by MK-12-3/gold (see Materials and Methods) in the formaldehyde-fixed, permeabilized cells. Scale bars, 2 μm (A); 250 nm (B); 500 nm (C,G); 200 nm (D-F).

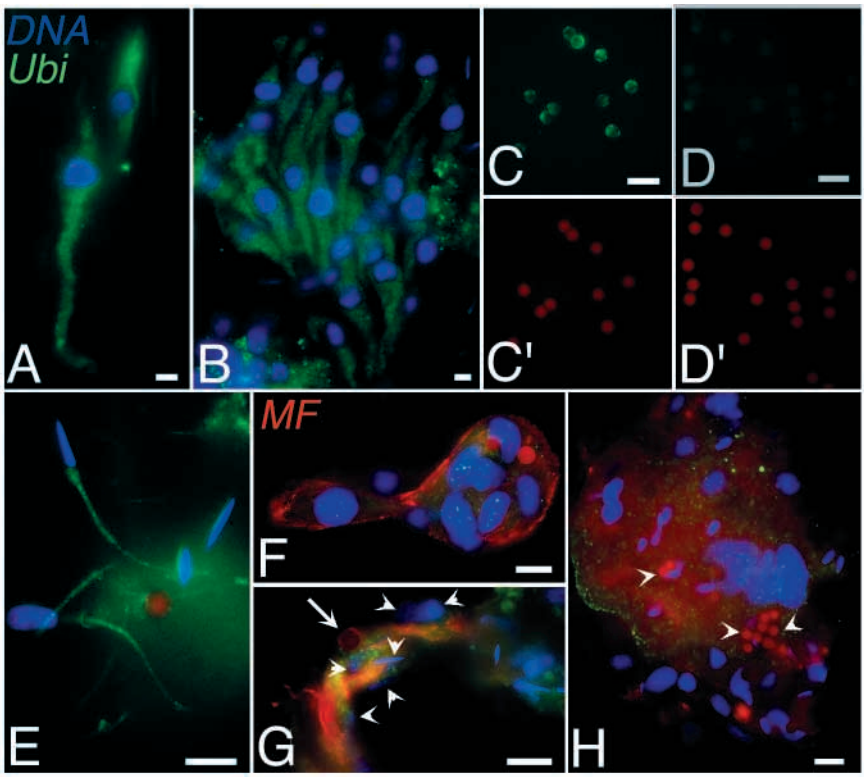

Fig. 6. In vitro reconstitution of ubiquitin-based sperm phagocytosis in cultured epididymal cells. (A,B) Ubiquitin (green) in freshly isolated epididymal epithelial cells (A) and epithelial plaques (B). (C) Ubiquitin-coated magnetic beads, used for the resorption experiments (C, green = MK 12-3/FITC; C′, red autofluorescence at 580 nm peak emission wave length). (D,D′) Autofluorescence (D, green at 488 nm; D′, red at 580 nm emission wavelength; contrast-enhanced for better visibility) of the uncoated control beads probed with MK-12-3/FITC. (E) Surface ubiquitination of dead sperm introduced into culture with the enzymatically digested tissue. (F) An epithelial plaque with phagocytosed ubi-beads at day 3 of culture, 2 days after bead addition. (G) A detail of the microfilament bundles (red), assembled around phagocytosed ubi-beads (arrow) and sperm (arrowheads). (H) An epithelial plaque at day 10 of coculture displays a well-defined microfilament cytoskeleton (red) and ubiquitin accumulation at the leading edge (green). Note the clusters of the phagocytosed ubi-beads (arrowheads). Scale bars, 10 μm. Ubi, ubiquitin; MF, microfilaments.

major bands were obtained after immunoprecipitation of the ubiquitin-crossreactive substrates from the EEC-conditioned medium. One of those bands had a molecular mass consistent with that of unconjugated ubiquitin (8.5 kDa). This observation supports the hypothesis that surface ubiquitination of defective sperm in EEC cultures is due to the binding of newly secreted ubiquitin or ubiquitin-protein conjugates, rather than to the unmasking of substrates ubiquitinated during spermatogenesis in the testis.

DISCUSSION

Ubiquitin was previously detected in human epididymal cells (Fraile et al., 1996; Santamaria et al., 1993) and seminal plasma (Lippert et al., 1993), though no connection was made to sperm quality control or fertility. The presence of ubiquitinated epitopes in motile sperm can be explained by the presence of intrinsic, ubiquitinated substrates inside sperm organelles that are carried over from the final steps of spermatogenesis (Rajapurohitam et al., 1999). Sperm mitochondrial membrane proteins such as prohibitin become ubiquitinated during spermiogenesis, and are masked by disulfide bond crosslinking during the epididymal passage (Sutovsky et al., 1999; Sutovsky et al., 2000). Similarly, nuclear histones (Baarends et al., 1999a; Baarends et al., 1999b; Chen et al., 1998), and possibly other sperm substrates, are subject to ubiquitination during spermatogenesis. The epididymal sperm undergo extensive membrane remodeling by the insertion of new, epididymis-secreted proteins (Cooper,

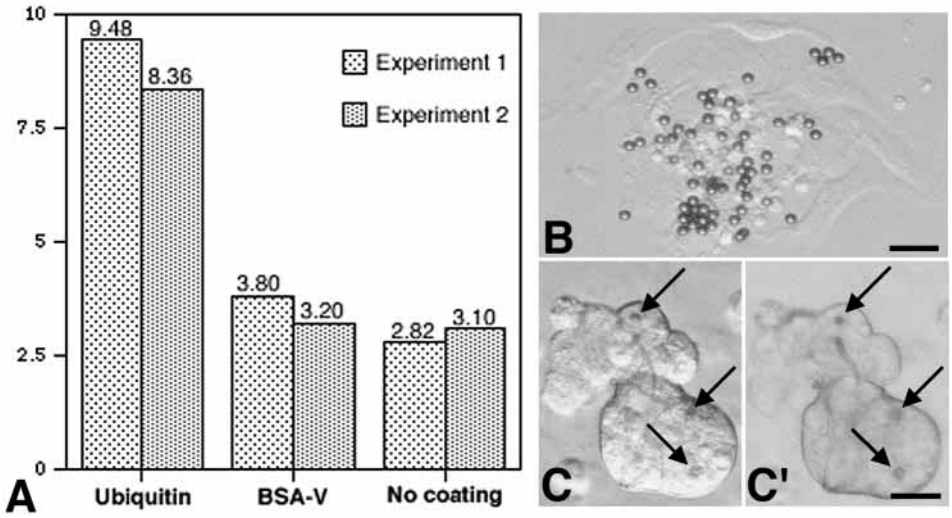

Fig. 7. Binding of ubi-beads and control, uncoated or BSA-coated beads to cultured epididymal epithelial cells. Beads were introduced to the EEC culture at day 2, 48 hours after EEC isolation. (A) The average number of bound beads per plaque; results are from two experiments, as scored 24 hours after bead introduction into EEC culture (150,000 beads/ml). (B) Ubiquitin-coated beads bound to the surface of an EEC-plaque after 24 hours of coculture, as shown by Hoffman modulation contrast microscopy. (C,C′) Hoffman modulation contrast (C) and phase contrast (C′) images of partially internalized, ubiquitin-coated beads (arrows) at day 6 of coculture. Scale bars, 20 μm.

1998), phospholipid substitution (Parks and Hammerstedt, 1985), and disulfide bond-crosslinking (Bedford, 1979). Therefore, both normal and defective sperm may carry intrinsic, constitutively ubiquitinated substrates, while only the defective sperm become surface-ubiquitinated during epididymal passage.

The present study demonstrates that defective mammalian sperm acquire ubiquitinated, surface-exposed epitopes during epididymal passage. Although it is likely that defective sperm are ubiquitinated because of structural damage, it is not clear how such sperm are recognized by the ubiquitination machinery, and how they are disposed of after phagocytosis. One possible explanation is that epididymal ubiquitination is at the intersection, or perhaps at the common end-point of several apoptotic mechanisms operating in the testis (Sinha Hikim and Swerdloff, 1999). Such mechanisms may recognize structural damage of sperm DNA (Sakkas et al., 1999a) and/or damaged sperm accessory structures. Apoptotic antigens, such as Fas-ligand, were found in defective human sperm (Sakkas et al., 1999b). An alternative apoptotic signal leading to the ubiquitination of defective sperm could be the release of cytochrome-*c* from the mitochondria. Bursting of the outer mitochondrial membrane during apoptosis (Martinou et al., 1999) may also expose proteins of the inner mitochondrial membrane. A unique, putatively ubiquitinated isoform of prohibitin, a highly conserved, 30 kDa protein of the inner mitochondrial membrane, is present in the sperm mitochondria (Sutovsky et al., 2000). Surface-ubiquitination of the mitochondrial sheath alone in some sperm with no apparent structural abnormalities was often observed in this study (see Fig. 1K). Misfolding or denaturation of sperm-surface antigens could also lead to their ubiquitination. The amino acid sequence of the N-terminal domain determines the half-life of most proteins and is subject to ubiquitination after its unfolding (N-end rule pathway; Varshavsky, 1997). Other motifs and signals, including hydrophobic protein surface domains, phosphorylation and destruction box sequences, are implicated in substrate targeting by ubiquitin (reviewed by Laney and Hochstrasser, 1999).

Ubiquitination is also involved in the endocytosis of membrane receptors and plasma membrane-anchored transporters (reviewed by Hershko and Ciechanover, 1998; Strous and Govers, 1999). Our study suggests that a whole [sperm] cell can become ubiquitinated on its surface and eventually be phagocytosed. Similar to the proteolysis of endocytosed receptors (Strous and Govers, 1999), the destruction of the phagocytosed sperm seems to occur by means of lysosomal/vacuolar proteolysis (see Fig. 8B). The proteins of the SNARE hypothesis, such as VAMP and syntaxin, are present on the sperm surface (Ramalho-Santos et al., 2000) and could mediate the fusion of the epididymal secretory vesicles with the plasma membrane of defective sperm. Such suggestions are consistent with our ultrastructural observations of membrane ruffling and binding of the secretory vesicles to the surface of defective epididymal sperm (Fig. 9J,K). Ubiquitin could be removed from phagocytosed sperm and recycled in a manner similar to the recycling of the epididymal SGP-2 antigen (Adonian and Hermo, 1999; Igdoura et al., 1994) and immobilin (Hermo et al., 1992). Ubiquitin crossreactivity was lost from the surface of phagocytosed ubi-

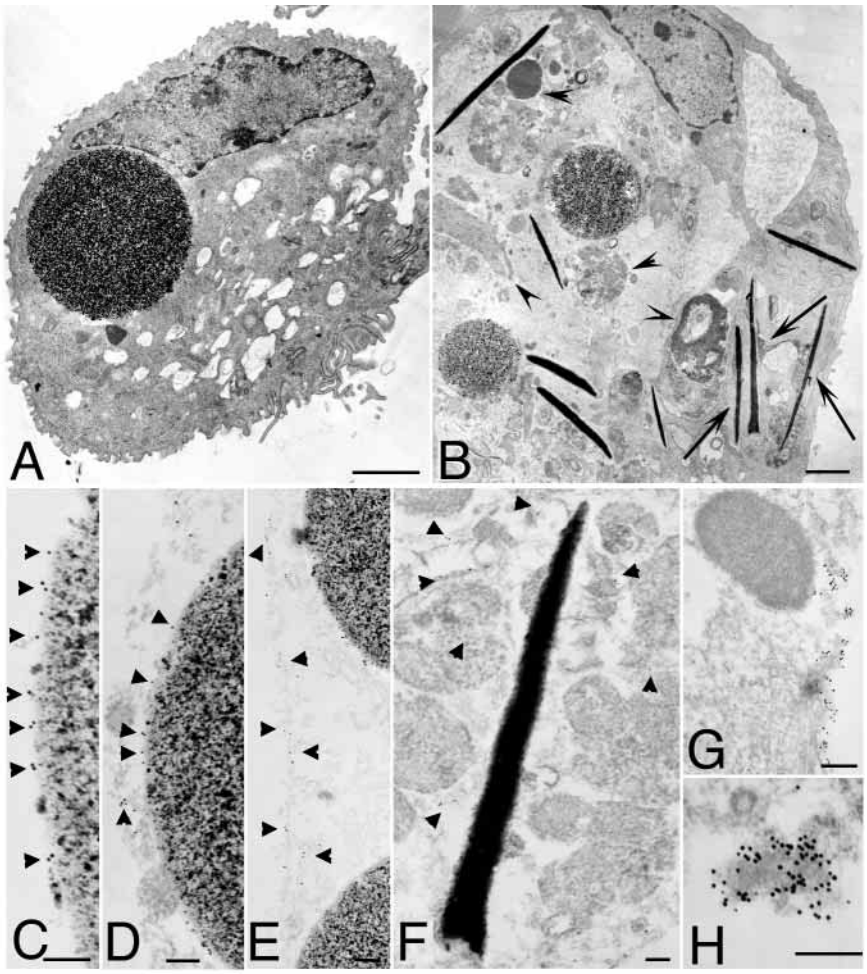

Fig. 8. Ultrastructure of epididymal epithelial cells, cocultured for 9 days with ubi-beads (4.5 μm). (A) A cultured epididymal cell with a phagocytosed ubi-bead. (B) Cells with phagocytosed ubi-beads also contained numerous sperm. Note the disintegration of the phagocytosed sperm (arrows) and the clustering of lysosomes (arrowheads) around the sperm and ubi-beads. (C) Ubiquitin-gold (arrowheads) is detectable on the surface of ubi-beads prior to co-culture. The crossreactivity (gold particles, arrowheads) seems to be reduced shortly after the bead phagocytosis by the cultured cells (D), and is almost completely absent from beads exposed to the cytoplasm of cultured epithelial cells for 9 days (E). Note the presence of ubiquitin in the cell cortex in E (arrowheads). (F) An internalized sperm head, surrounded by lysosomes and ubiquitin-bound colloidal gold particles (arrowheads). (G) Ubiquitin labeling in the cortex and on the surface of a cultured epididymal cell. (H) Ubiquitin-containing secretory bodies in the cultured EEC were similar to those produced by the epididymal cells in situ. Scale bars, 2 μm (A,B), 500 nm (C-G); 200 nm (H).

beads in our studies (see Fig. 8E). Rapid turnover of ubiquitin in EEC is consistent with the abundance of the ubiquitin-recycling enzyme, ubiquitin-C-terminal hydrolase/gene product 9.5, in the epididymis (Fraile et al., 1996; Santamaria et al., 1993).

The prevailing defect in the ubiquitinated sperm of bulls, men and rhesus monkeys was the coiled tails. This so-called 'Dag defect' was described in the sperm of subfertile bulls (Barth and Oko, 1989; Blom, 1966) and *c-ros*-tyrosine kinase-mutant mice (Yeung et al., 1998; Yeung et al., 1999). Such observations warrant further inquiry into possible relationships between sperm ubiquitination and fertility. In farm animals, quantification of ubiquitinated sperm in ejaculates, as well as patterns of sperm ubiquitination, could provide useful information for the selection of the best breeders at an early stage of their reproductive lives. In humans, ubiquitin-based assays could be used to diagnose male infertility and in clinical toxicologic studies (Sutovsky et al., 2001). Epididymal markers for human infertility have long been sought after (Cooper et al., 1988; de Kretser et al., 1998). Ubiquitinated epitopes on the sperm surface could be a good target for an immunocontraceptive interfering with the fertilization process. In humans, even normal sperm contain a certain amount of ubiquitin (Sutovsky et al., 2001), often most prominent on the surface of the sperm head's equatorial segment, a region to which the oocyte microvilli bind first during fertilization (Yanagimachi, 1994). Epididymal contraception can be achieved through chemical interference with the sperm metabolic pathways in the epididymis (Jones, 1998) and immunological targeting of epididymal sperm antigens (Beagley et al., 1998). The alteration of the sperm tail structure (i.e. the

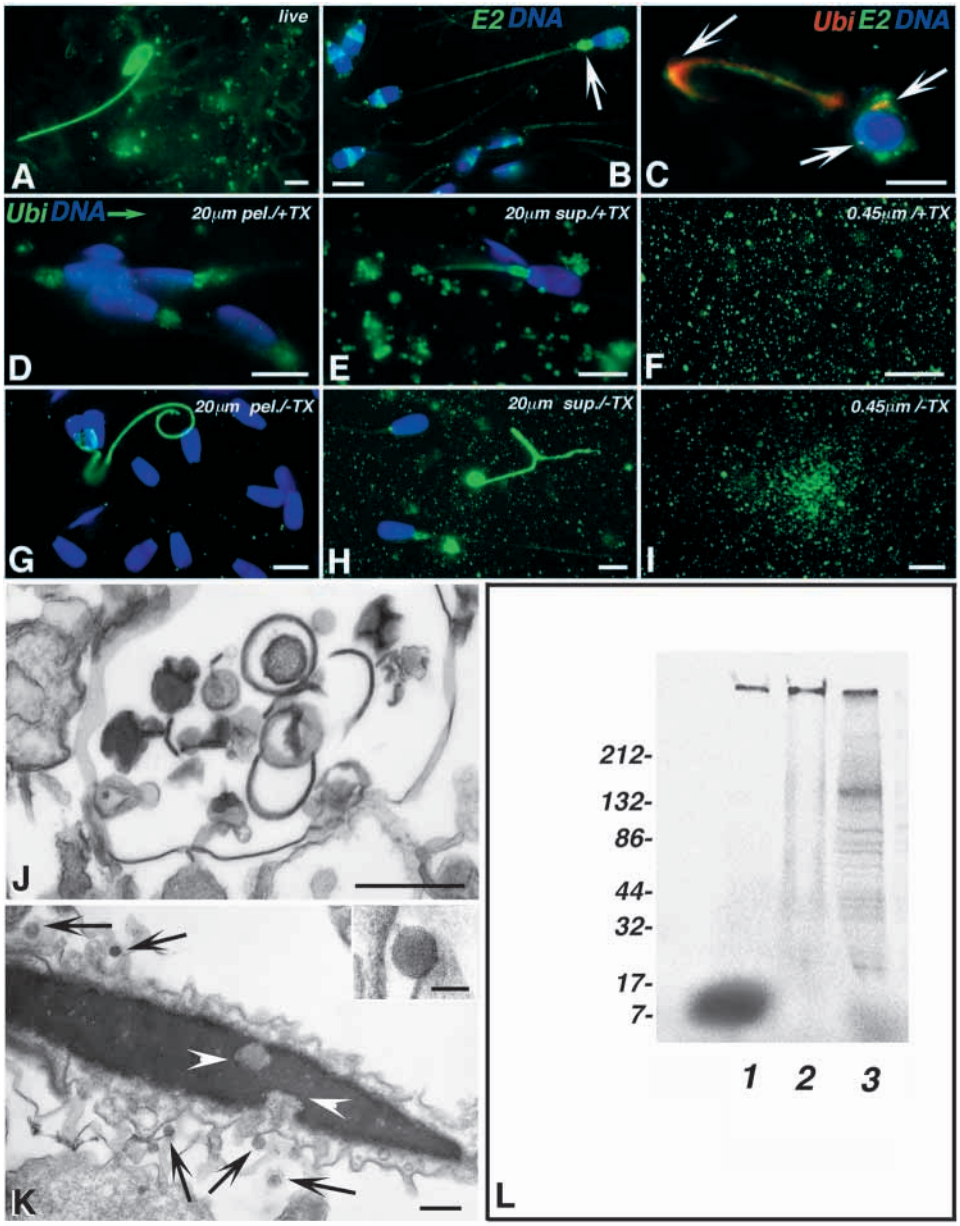

Fig. 9. The observations supporting the occurrence of ubiquitination on the surface of defective epididymal sperm. (A) Direct labeling of live, unfixed sperm with anti-ubiquitin antibodies (green). Note a ubiquitinated, defective sperm in the center. (B,C) Ubiquitin-conjugating enzyme E2 is revealed in all formaldehyde-fixed epididymal sperm after permeabilization with Triton X-100 (B, arrow), but only in defective ones (C, arrows; a round-headed sperm is shown) when the permeabilization is omitted. (D-I) Ubiquitin is present in the secretory vesicles and bodies isolated from the caput-epididymal fluid. Vesicles and sperm isolated from <20 μm fraction pelleted by centrifugation (D), from the remaining supernatant, re-pelleted at high speed (E), and from the <0.45 μm fraction (F), then fixed in formaldehyde and permeabilized with Triton X-100. Identical fractions were labeled without permeabilization and are shown in (G; <20 μm pellet), (H; <20 μm supernatant) and (I; <0.45 μm fraction). Cytoplasmic droplets (D) are labeled in the permeabilized sperm only. (J-K) Electron micrographs of the isolated 0.45 μm (J) and 20 μm supernatant (K) fractions. Note the binding of secretory vesicles (K; arrows and insert) to the ruffled membranes of a spermatozoon with a visible chromatin defect, known as nuclear vacuoles or 'crater defect' (arrowheads; Barth and Oko, 1989). (L) Incorporation of [^{35}S]methionine in newly synthesized proteins after pulse-chase and immunoprecipitation of ubiquitin from an EEC-conditioned culture medium (lane 1) and cultured EEC (lane 2). Lane 3 shows the total cell extract without immunoprecipitation of ubiquitin, showing all proteins that incorporated the isotope-labeled precursor. Note the band with molecular mass consistent with that of unconjugated ubiquitin (8.5 kDa), present in lane 1 (culture medium after pulse-chase), but not in other lanes. Scale bars, 10 μm (A-I); 250 nm (J,K); 100 nm (K, insert).

induction of the sperm tail coiling) in the epididymis has been suggested as a promising route to reversible epididymal sterility (Cooper and Yeung, 1999). It is possible that ubiquitin, which is associated with the coiled sperm tails, may contribute to this effect. Ubiquitin may also be involved in the resorption of sperm and spermatic granula (Flickinger, 1982) after vasectomy.

In summary, our data suggest a new role for ubiquitin in mammalian spermatogenesis and sperm quality control. Such a finding may affect the fields of andrology, infertility treatment and biotechnology by providing the means for a more accurate assessment of male fertility, diagnostics of infertility, male fertility drug testing and reproductive toxicology, as well as new targets for male contraceptives.

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