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Deterioration of Plasma Membrane Is Associated With Activated Caspases in Human Spermatozoa

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ABSTRACT: Spermatozoa with deteriorated plasma membranes can be separated by magnetic-activated cell sorting (MACS) after binding superparamagnetic annexin V-conjugated microbeads (ANMBs) to membrane phosphatidylserine (PS). Semen samples from 15 donors and 25 infertile patients were divided into 2 spermatozoal fractions by annexin V-MACS. Activated caspases (aCPs), which mediate degradations of cell quality, were determined by CaspaTag in the 2 subpopulations. Spermatozoa from donors showed lower levels of bound annexin V (3.6% ± 0.5% vs 11.9% ± 1.1%; $P < .01$) and aCPs (21.8% ± 2.6% vs 43.2% ± 2.1%; $P < .01$) than did spermatozoa from infertile patients. MACS resulted in a decrease of spermatozoa with aCPs from 21.8% ± 2.6% (before separation) to 9.2% ± 1.4% (in the ANMB-negative fraction) in donors and from 43.2% ± 2.1% to 18.8% ± 2.6% in infertile patients (mean ± SEM; $P < .01$). Separation effects of the MACS technique were confirmed with flow cytometry using anti-annexin V antibodies and with electron microscopy. ANMB-MACS removes spermatozoa with PS-bound annexin V and produces a higher quality spermatozoal fraction. Spermatozoa with a deteriorated membrane are characterized by an increase in aCPs. A higher percentage of spermatozoa with ANMBs bound to PS and with aCPs were found in infertile patients.

Key words: Annexin V, immunomagnetic cell depletion, human spermatozoa, caspases, electron microscopy.

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Successful fertilization requires a sperm plasma membrane with normal integrity and function (Flesch and Gadella, 2000). A spermatozoal membrane with impaired integrity is known to occur more frequently in infertile men and contributes to infertility despite normal sperm parameters (Sugkraroek et al, 1991; Glander and Schaller, 1999; Duru et al, 2001). In vital cells with an intact plasma membrane, the phospholipid phosphatidylserine (PS), is located on the inner leaflet of the plasma membrane only (Vermes et al, 1995). PS has a high and selective affinity for annexin V, a 35–36 kd phospholipid-binding protein (van Heerde et al, 1995), but annexin V cannot pass through an intact plasma membrane. Therefore, finding that annexin V has bound to a spermatozoon indicates a cellular membrane that is impaired. The binding process can be the result of translocation of PS from the inner to the outer leaflet of the plasma membrane resulting in PS exposure on the external surface (Vermes et al, 1995). This translocation of PS is one of the earliest detectable features of cells undergoing the terminal steps of apoptosis, or so-called programmed cell death. However, PS can also bind annexin V in apoptotic and dead spermatozoa. Assays to detect membrane function by annexin V–binding have demonstrated spermatozoal deterioration at a subcellular level (Glander and Schaller, 1999). Superparamagnetic microbeads (about 50 nm in diameter) conjugated with annexin V (called ANMBs) allow these low-quality spermatozoa to be eliminated via magnetic-activated cell sorting (MACS) (Meng et al, 1996; von Schönfeldt et al, 1999). Spermatozoa with bound beads are greatly enriched within a column containing iron balls when the spermatozoa are placed within a very strong magnetic field. Unlabeled cells pass through the column, whereas labeled cells are retained. The retained cells are easily eluted when the column is removed from the field (Margolis et al, 1983). The procedure delivers either ANMB-positive ($ANMB^+$) or ANMB-negative ($ANMB^-$) sperm fractions.

In somatic cells, the deterioration of membrane integrity, which is detectable by annexin V and PS binding, is associated with an activation of caspases, the cytosolic cysteine-containing aspartate-specific proteases (Vermes et al, 2000). These enzymes, called activated caspases (aCPs), play an important role in cell quality degradation (Enari et al, 1998; Thornberry and Lazebnik, 1998; Earnshaw et al, 1999; Wolf and Green, 1999) and eventually induce DNA fragmentation within the apoptosis signaling cascade (Cohen, 1997). Cells with fragmented DNA result in lower rates of conception via in vitro fertilization (Sun et al, 1997) and intracytoplasmic sperm injection (Lopes et al, 1998). Therefore, we examined the associ-

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Table 1. *Semen parameters and basal hormone concentrations†*

Characteristic	Donors (mean ± SEM)	Patients (mean ± SEM)
Abstinence (days)	2.9 ± 0.1	6.0 ± 0.7*
Volume (mL)	2.8 ± 0.3	4.0 ± 0.4*
Sperm concentration (million/mL^{-1})	99.9 ± 8.0	45.2 ± 7.9*
Total sperm count (million/ejaculate)	269.5 ± 33.8	153.5 ± 28.5*
Normal morphology (%)	22.7 ± 1.2	7.9 ± 1.0*
Total sperm with normal morphology (million/ejaculate)	63.5 ± 10.2	15.4 ± 5.6*
Progressively motile spermatozoa‡	65.0 ± 1.2	57.6 ± 2.2
VSL (μm/s)	23.2 ± 2.5	20.9 ± 1.4
VAP (μm/s)	35.6 ± 4.0	30.4 ± 1.9
VCL (μm/s)	56.1 ± 7.4	47.3 ± 3.4
FSH (U/L)	3.4 ± 0.4	5.7 ± 0.8*
FH (U/L)	3.6 ± 0.3	5.2 ± 0.9*
Testosterone (nmol/L)	22.5 ± 1.0	15.4 ± 1.7*

†VSL indicates velocity straight line; VAP, velocity average path; and VCL, velocity curve linear.

‡Motility figured using World Health Organization formula a + b, where a and b indicate rapid and slow progressively motile spermatzoa, respectively.

*$P < .05$, Mann-Whitney *U* test.

ation between plasma membrane disintegration and caspase activation in human spermatozoa. To this end, cells with an intact membrane were separated from those in which the membrane had deteriorated via the MACS technique using ANMBs. Afterward, the aCPs were detected with a fluorescein caspase activity kit (CaspaTag; Intergen Company, Oxford, United Kingdom) to evaluate the coincidence of deteriorated membranes and the activation status of caspases. The separation effect of MACS was evaluated by electron microscopy and flow cytometric analysis using fluorescein isothiocyanate (FITC)-conjugated anti-annexin V antibodies.

Materials and Methods

Semen Sample Selection Criteria and Sperm Preparation

Sixty-eight semen samples were provided by 15 selected donors and 25 patients who attended the Department of Andrology for infertility problems. In the experiment in which caspase activation was measured, we used only 1 semen sample from each individual. All experiments and procedures were conducted in accordance with standard ethical guidelines, and written informed consent was received from all participants.

Semen samples were collected by masturbation into sterile plastic Petri dishes after 3 days of sexual abstinence. The classical semen parameters of spermatozoal concentration, motility, and morphology were examined according to World Health Organization (1999) criteria. The ejaculates from donors fulfilled the following criteria: ratio of spermatozoa to leukocytes >100:1; spermatozoal concentration >20 million/mL, >15% spermatozoa with normal morphology (strict criteria) and >50% progressively motile (WHO a + b, where a and b indicate rapid and slow progressively motile spermatazoa, respectively; Table 1). Highly viscous ejaculates and semen samples with a positive mixed antiglobulin reaction test (ie, >10% spermatozoa with adherent particles) were excluded. The semen samples were filtered through glass wool to remove the gelatinous masses, diluted in human tubal fluid (HTF; Quinn et al, 1985), washed twice with HTF, and centrifuged at 400 × *g* for 5 minutes. The pellets were suspended in a calcium-containing annexin-binding buffer (Dead Cell Removal Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) for further experiments without capacitation.

Depletion of Spermatozoa With Deteriorated Membrane by Magnetic Cell Separation

The sperm suspensions were divided into 2 sperm fractions by passage through a magnetic field (MiniMACS, Miltenyi Biotec) as a function of the binding of ANMBs to PS. The power of the magnetic field was measured as 0.5 tesla between the poles of the magnet and up to 1.5 tesla within the iron globes of the column. Briefly, the washed spermatozoa were incubated with 100 μL of ANMBs at room temperature for 15 minutes, and placed atop the column. The ANMB-labeled spermatozoa were retained in the separation column, which was placed in the magnet, whereas spermatozoa with intact membranes passed through. After removing the column from the magnetic field, the retained fraction was eluted using an annexin-binding buffer (Margolis et al, 1983). The procedure delivers 2 sperm fractions: ANMB^{+} and ANMB^{-} spermatozoa.

Evaluation of ANMBs on Spermatozoa

Flow cytometric analysis of the samples using anti-annexin V–FITC antibodies (BMS147FI, Bender MedSystems, Austria) evaluated the MACS system for sorting ANMB^{x} and ANMB^{-} spermatozoa. The separated spermatozoal populations were adjusted to a concentration of 2.0×10^{6} spermatozoa/mL^{-1} HTF, incubated with FITC-conjugated anti-annexin V antibodies for 60 minutes at 4°C, and centrifuged at 400 × *g* for 5 minutes. The fluorescence signals of labeled spermatozoa were analyzed with the FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) flow cytometer. A minimum of 10 000 spermatozoa were examined for each assay at a flow rate of <100 cells/s. The spermatozoal population was gated using 90-degree forward-angle light scatter to exclude debris and aggregates. The excitation wave length of 488 nm was supplied by an argon laser at 250 mW. Green (FITC-derived) fluorescence was measured using a 530 ± 30 nm band-pass filter. The percentage of positive cells and the mean fluorescence were calculated with CellQuest (Becton Dickinson) software on a 1023 channel scale. The superparamagnetic microbeads, which are about 50 nm in diameter, are too small to be detected by optical methods or by changing the scatter properties of spermatozoa in the flow cytometer (Miltenyi et al, 1990). As a positive control, the binding of FITC-conjugated *Pisum sativum* agglutinin to the acrosome was employed according to the method described by Miyazaki et al (1990) for setting the bitmap on a dot plot. The negative controls

Table 2. *Fresh spermatozoa with PS-ANMB⁺ binding*

	N	ANMB⁺ (%)*
Donors	15	3.6 ± 0.5
Patients	20	11.9 ± 1.1

* $P < .001$.

represented spermatozoa that were treated with buffer containing immunoglobulin G_1 only (Glander and Schaller, 1999).

Electron Microscopy

Spermatozoa from 3 donors were washed twice in Hepes buffer, fixed in 2% glutaraldehyde (pH 7.3 in 0.1 M cacodylate buffer) for 2 hours, and centrifuged for 10 minutes at 800 × *g* rpm. The pellets were placed in gelatin and postfixed in ice-cold 1% OsO_4 for 2 hours. Samples were dehydrated through a graded series of acetone, en bloc contrasted with uranyl acetate and phosphotungstic acid, dehydrated through a graded series of acetone, and embedded in Durcupan (Fluka AG, Buchs, Switzerland). Semithin sections were stained with toluidine blue to determine orientation, and ultrathin sections were cut (Ultracut, Reichert-Jung), mounted on uncoated copper grids, and counterstained with Reynolds lead citrate. Observations of ultrathin sections were made with a Zeiss (Oberkochen, Germany) EM 900 electron microscope.

Detection of Cytosolic Cysteine-Containing Aspartate-Specific Proteases in Vital Spermatozoa

Active caspases 1–9 were detected in living spermatozoa by the carboxy-fluorescein labeled caspase inhibitor FAM-VAD-FMK (carboxyfluorescein, FAM, a derivative of benzyloxycarbonyl valylalanyl aspartic acid fluoromethyl ketone, z-VAD-FMK). This cell-permeable and noncytotoxic caspase inhibitor binds covalently to active caspases 1–9 (Ekert et al, 1999). The fluorogenic substrate becomes fluorescent when it is cleaved by the caspases (Vaux and Korsmeyer, 1999). The detection of aCPs by the inhibitor was performed with controls according to the instruction manual provided by the manufacturer of the fluorescein caspase (VAD) activity kit (CaspaTag, S7300, Intergen).

Statistical Analysis

Evaluation of differences and data correlations were performed by nonparametric tests (Mann-Whitney *U* test, Wilcoxon sum-rank test, Spearman test) as appropriate for data type and distribution (investigated with the Shapiro-Wilk test). Calculations were performed with the Statistica version 6.0 statistical computer program (StatSoft Inc, Tulsa, Okla). *P* values $< .05$ were considered statistically significant. All values provided are means ± SEM.

Table 3. *Spermatozoa exhibiting activated caspases before and after MACS*

	Activated caspases		
	In unseparated sperm (%)	In ANMB⁻ (%)	In ANMB⁺ (%)
Donors	21.8 ± 2.6[ad]	9.2 ± 1.4[ab]	97.7 ± 1.0
Patients	43.2 ± 2.1[cd]	18.2 ± 2.6[bc]	99.2 ± 0.4

* Values with identical superscripts are significantly different ($P < .05$).

Results

Spermiogram parameters and hormone concentrations were significantly different between donors and patients ($P < .05$), with the exception of velocities (straight line velocity, VSL; average path velocity, VAP; and curvilinear velocity, VCL), as determined via computer-aided semen analysis (CASA) (Table 1). The semen samples from donors contained significantly fewer spermatozoa with bound ANMBs than did samples from patients (3.6% ± 0.5% vs 11.9% ± 1.1%; $P < .01$; Table 2).

Fluorescence staining of aCPs localized the enzymes mainly to the postacrosomal region in sperm from donors. This pattern differed slightly from that of patients, in whom additional cytoplasmic residues were found to be highly positive (Fig. 1). Semen samples from donors showed lower percentages of spermatozoa with aCPs than did samples from patients before MACS (aCPs, 21.8% ± 2.6% vs 43.2% ± 2.1%; $P < .01$; Table 3).

The fractions of ANMB⁻ spermatozoa from patients and donors alike showed significantly smaller amounts of aCPs compared with unseparated spermatozoa samples and ANMB⁺ fractions ($P < .01$; Table 3). In donors, aCPs

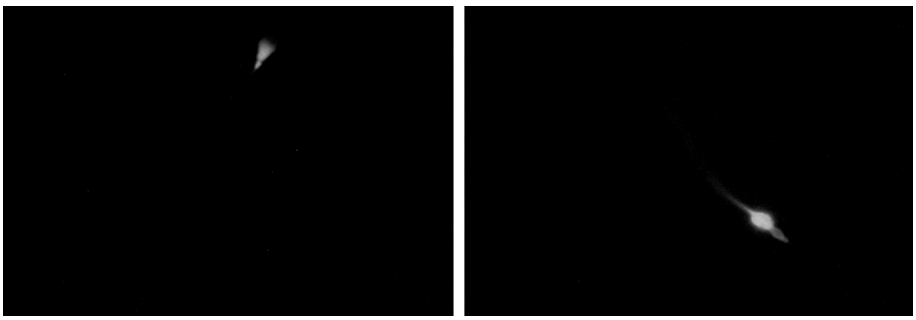

Figure 1. Fluorescence staining pattern of activated caspases (aCPs) in donors (left, postacrosomal region) and patients (right, cytoplasmic residues).

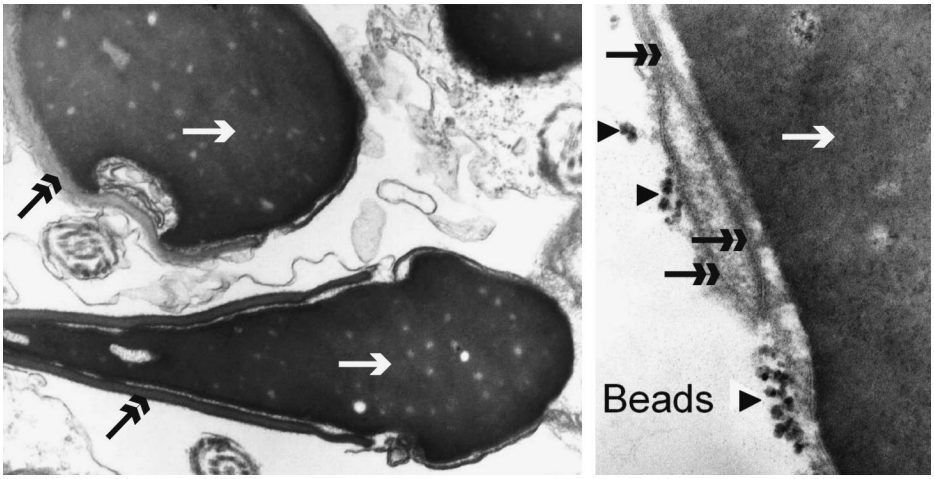

Figure 2. Electron microscopy of ANMB⁻ sperm of donors without bound beads (magnification 20 000×, left) and of ANMB⁺ sperm of donors with tightly bound beads (magnification 85 000×, right). Short black arrow indicates beads; long black double arrow, membrane; long white arrow, sperm nucleus.

decreased from 21.8% ± 2.6% to 9.2% ± 1.4% ($P < .01$), and in patients it dropped from 43.2% ± 2.1% to 18.2% ± 2.6%. Spermatozoa with aCPs were found mainly within the ANMB⁺ fractions ($P < .01$; Table 3). A significant correlation between the overall percentage of spermatozoa with aCPs and those that were able to bind annexin V microbeads was calculated in donors as well as in patients ($r = .97$ and $r = .72$; $P < .01$).

Electron microscopy revealed microbead binding on membranes at the acrosomal and postacrosomal regions in the ANMB⁺ fraction only (Fig. 2). Signs of nuclear degradation were also visible in the ANMB⁺ fraction as well.

Flow cytometric analyses using FITC-conjugated anti-annexin V antibodies confirmed the separation effect of MACS in donor sperm. In the ANMB⁻ fraction, 5.2% ± 1.0% of spermatozoa showed fluorescent signals, however, 72.2% ± 2.7% of labeled spermatozoa were found in the ANMB⁺ fraction ($P < .01$, Table 4). The specificity and sensitivity of the system were determined to be 94.8% and 72.6%, respectively. Because this method of evaluation using monoclonal antibodies bears its own limitations, differences in overall sensitivities and specificities from their actual values must be considered. The presence of beads in up to 73% of ANMB⁺ cells is rather low. It could be that after separation, not all cells could be detected by the antibodies because of ongoing membrane degradation. Although 5% of ANMB⁻ sperm showed binding of the anti-annexin V antibody via FACS analysis, no beads were found in that group by electron microscopy. Unspecific binding probably contributes to the 5% figure.

Table 4. *Percentage of spermatozoa demonstrating FITC-conjugated anti-annexin V antibodies*

	ANMB⁻	ANMB⁺
Spermatozoa binding FITC-conjugated anti annexin V antibodies	5.2% ± 1.0%*	72.2% ± 2.7%*

* $P < .001$.

Passage through the column led to a spermatozoal loss of 1.2% ± 1.1%. The separation column and the magnetic field had no significant influence on progressively motile spermatozoa. Passage through the column changed the percentage of progressively motile spermatozoa from 59.6% ± 3.7% to 47.8% ± 7.1% in donors (n = 15) and from 44.2% ± 5.9% to 33.6% ± 5.2% in patients (n = 10; $P > .05$). A significant alteration in motility quality by MACS was detectable in the ANMB⁻ fraction, whereas it was not detectable in untreated spermatozoa (Table 5; $P < .05$). The slight decrease in motility may be caused by the preparation and centrifugation of samples for MACS and not by the MACS process itself. All velocities of progressively motile spermatozoa (VSL, VCL, VAP), as measured by CASA in ANMB⁻ aliquots were found to be significantly greater than those of the ANMB⁺ aliquots (Table 5). VSL declined in patients to a greater degree than it did in donors (from 25.8 ± 3.0 μm/s to 15.4 ± 2.0 μm/s vs from 29.4 ± 2.1 μm/s to 21.1 ± 3.6 μm/s; $P < .05$). VAP was slower in donors ($P < .05$).

Discussion

After spermatozoal suspensions had been divided into 2 sperm fractions by MACS with the use of ANMBs, their aCPs were examined by fluorescence microscopy. The spermatozoa with a deteriorated plasma membrane

Table 5. *Velocity parameters**

	Progressive motile sperm (%)	VSL (μm/s)	VAP (μm/s)	VCL (μm/s)
Donors				
Fresh spermatozoa	59.6 ± 3.7	29.4 ± 2.1[a]	39.5 ± 2.5[b]	59.3 ± 4.2
AMNB⁻	47.8 ± 7.1	21.1 ± 3.6[ad]	32.7 ± 3.1[be]	48.5 ± 3.8[f]
ANMB⁺	0.7 ± 0.7	5.9 ± 2.7[d]	8.3 ± 3.4[e]	13.0 ± 4.9[f]
Patients				
Fresh spermatozoa	44.2 ± 5.9	25.8 ± 3.0[c]	34.8 ± 3.5	56.0 ± 5.2
ANMB⁻	33.6 ± 5.2	15.4 ± 2.0[c]	29.4 ± 2.1	46.2 ± 4.4
ANMB⁺	0.0	0.0	0.0	0.0

* Wilcoxon test values with identical superscripts are significantly different ($P < .05$).

showed a significantly greater degree of aCPs than did spermatozoa with an intact membrane.

ANMBs are a useful, specific, and sensitive method for eliminating spermatozoa at early stages of membrane deterioration, including apoptosis. Liposomes that bear antifibronectin antibodies being associated with ferromagnetic particles were first described in 1983 as a means of cell sorting, and was named MACS (Margolis et al, 1983). The method was first introduced to deplete T cells from bone marrow (Kogler et al, 1990; Kato and Radbruch, 1993) and is now used as part a broad spectrum of scientific, diagnostic, and even therapeutic strategies (Despres et al, 2000), including detection and enrichment of carcinoma cells in semen (Meng et al, 1996).

Annexin V binding can be the result of a disturbed membrane integrity and an externalization of PS on the sperm surface, which is recognized by annexin V. Externalization of PS is an early detectable event on membranes during apoptosis (Martin et al, 1995; Vermes et al, 1995). Recent models of apoptosis include receptor-mediated pathways and intrinsic apoptosis triggered by caspases (Faleiro and Lazebnik, 2000; Vermes et al, 2000).

We examined spermatozoal fractions with different membrane structures with regard to aCPs. Intact chicken sperm cultured with a global inhibitor of the caspase enzyme family showed no enhancement of overall sperm survival (Weil et al, 1998), although aCPs were found in germ cells at late stages (Tesarik et al, 1998). Therefore, the question was whether caspases are fully removed or inactive in spermatozoa from donors and patients. Furthermore, it was of interest to know whether abnormal sperm morphology or membrane defects are associated with aCPs as a possible cytoplasmic sign of ongoing degradation or apoptosis.

In donors as well as in patients, the spermatozoa that were able to bind ANMBs (ie, with deteriorated plasma membranes), also showed aCPs, which mediate apoptosis (Vermes et al, 2000). Fluorescence microscopy detected pronounced aCPs in the postacrosomal region and in cytoplasmic residues of abnormally shaped spermatozoa (Fig. 1). Subsequently, the prominent presence of those proteins in abnormally shaped ejaculated spermatozoa may be linked to defects or incomplete cytoplasmic remodeling during the later stages of spermatogenesis (Blanco-Rodriguez and Martinez-Garcia, 1999; Sakkas et al, 2002). Despite this, a group of cells (9%–18%, depending on the group) exist with an intact membrane (ie, ANMB⁻) and which contain aCPs, whereas a significant difference between donors and patients was detectable (Table 3).

This finding might reflect that caspases are residual elements from spermatogenesis and are prone to become activated due to membrane damage even in morphologically intact spermatozoa. Membrane damage, however, might be a possible trigger because almost all spermatozoa with PS bound to annexin V are found to have aCPs, possibly indicating that prolonged rather than immediate nuclear changes appear (Bratton et al, 1997; Duru et al, 2001; Schuffner et al, 2001). Further studies are required to differentiate the degree to which DNA fragmentation is linked to problems of the nuclear remodeling process during spermatogenesis or to the caspase-driven activation of DNA-degrading enzymes. Regardless of its origin, DNA fragmentation results in reduced male fertility (Zini et al, 2001). On the other hand, caspases may contribute to immotility by blocking mitochondrial functions.

Caspases are a family of highly specific proteases that contain the amino acid cysteine in their active sites. After proteolytic activation in a cascade (Wolf and Green, 1999), caspases cleave their targets (Thornberry and Lazebnik, 1998) at amino acid aspartate (Alnemri, 1997; Wolf and Green, 1999). From a functional point of view, caspases involved in apoptosis act either as initiators (caspases 8, 9, 10) or as effectors (caspases 3, 6, and 7) of apoptosis (Earnshaw et al, 1999; Bratton et al, 2000). CP3, the most important among them, executes the final disassembly of the cell by cleaving a variety of cell structure proteins and generating DNA strand breaks (Enari et al, 1998). In our experiments, signs of nuclear degradation were visible in ANMB⁺ spermatozoa, corresponding to the results reported by Blanc-Layrac et al (2000). Modifications of membrane functions have also been linked

to caspase 3, which cleaves protein kinase, thereby facilitating bidirectional migration across the bilayer of all phospholipid classes by retaining scramblase active (Frasch et al, 2000). Collapse of lipid asymmetry, manifested by externalization of PS on the cell surface, subsequently results (Bevers et al, 1999). The ability of annexin V to detect PS in spermatozoal suspensions depends on the primary spermatozoal membrane structure, cytological stress treatments (Glander and Schaller, 2001), or the quality of the membrane bilayer (Schiller et al, 2000). The specificity of annexin V binding to PS allows a magnetic depletion of dead and apoptotic cells (Martin et al, 1995) (ie, low quality spermatozoa from donors and patients).

The specificity and sensitivity of the magnetic separation technique using ANMBs was proved by ultrastructural analysis with electron microscopy and by flow cytometric analysis of spermatozoa exposed to FITC-conjugated anti-annexin V antibodies. The small size of microbeads, about 50 nm in diameter, is advantageous in flow cytometry because ANMBs are unable to change the scatter properties of spermatozoa (Miltenyi et al, 1990), but they can prevent identification by light microscopy. Therefore, the localization of binding demands examination by electron microscopy. As a side effect, the nuclear degradation in $ANMB^+$ spermatozoa was visible in ultrastructural investigations. Nevertheless, the $ANMB^-$ fractions may contain spermatozoa that can bind only a few beads, which is an insufficient for their retention in the column. However, no beads were found within $ANMB^-$ fractions of samples investigated with electron microscopy, which almost rules out this possibility.

The separation columns and their magnetic field did not exert detectable adverse effect on the motility of spermatozoa in our experiments, which satisfies the prerequisite for harmless application in the clinical laboratory. Magnetic tagging and separation with improved beads has been shown not to affect cell viability or proliferation of various cell types and is already used to extract active germ cells from animal testis (von Schönfeldt et al, 1999) as well as from human testicular cancer (Meng et al, 1996). The established feasibility and safety of the MACS enrichment procedure in patients with autologous transplantation of peripheral blood stem cells (Despres et al, 2000) were confirmed in our study.

In summary, the annexin V MACS separation technique removed spermatozoa with deteriorated membrane structures, and it may show better results in attempts to evaluate the quality of semen samples.

Fluorescence staining of active caspases showed their presence in subpopulations of mature sperm and even more intense staining was found in sperm from infertile patients. This finding contributes new details to current concepts of cytoplasmic remodeling defects during spermatogenesis. However, caspases are not completely removed during undisturbed spermatogenesis and may therefore be contributors of inhibition of normal sperm function.

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