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Apoptosis in human sperm: its correlation with semen quality and the presence of leukocytes

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BACKGROUND: Apoptosis plays an important role in regulating spermatogenesis. However, the biological significance of apoptosis in ejaculated sperm is not yet clear. This study set out to investigate how apoptosis correlates with semen quality and the presence of seminal leukocytes. METHODS: Fifty-seven semen samples from the male partners of infertile couples were classified as normal or abnormal according to World Health Organization guidelines. Flow cytometry was used to evaluate sperm populations and seminal leukocytes. Preliminary flow cytometry analysis using 6-carboxyfluoresceindiacetate (6-CFDA), which identifies live cells, and propidium iodide (PI), which stains only dead cells, was performed in order to pinpoint the sperm region accurately. Having thus gated the sperm population, bivariate Annexin V/PI analysis was then carried out in order to measure the percentage of apoptotic and necrotic sperm and the apoptotic index (the ratio between apoptotic:live sperm). Leukocytes were counted by the standard peroxidase test and by flow cytometry using monoclonal antibody (mAb) anti-CD45 or anti-CD53. RESULTS: No significant differences in the apoptotic index and the percentage of live and apoptotic sperm were detected between the subjects with normal and abnormal semen. A significant inverse correlation between sperm concentration and the apoptotic index was observed only in the normal sperm group. There was no correlation between the concentration of leukocytes, detected either by peroxidase or by mAb anti-CD45 or anti-CD53, either with the percentage of apoptotic sperm or with the apoptotic index. In contrast, the ratio between CD45 positive leukocytes and sperm showed a significant correlation with the apoptotic index. A weaker correlation was found when leukocytes were counted by peroxidase, while no correlation was observed using mAb anti-CD53. CONCLUSIONS: Sperm apoptosis did not seem to be correlated with semen quality. In the absence of genito-urinary infection, one of the main functions of seminal leukocytes is probably to provide for the removal of apoptotic sperm.

Key words: apoptosis/flow cytometry/membrane phosphatidylserine/seminal leukocytes/sperm

Introduction

Recent studies have demonstrated that apoptosis is the underlying mechanism of germ cell death during normal spermatogenesis and that it is a major mechanism in regulating spermatogenesis of various mammalian species, including man (Bartke, 1995; Sinha Hikim *et al*., 1998; Print and Loveland, 2000). Apoptosis has been extensively investigated in spermatogonia, spermatocytes and spermatids in the testis, and many apoptotic factors have been identified (Tapanainen *et al*., 1993; Callard *et al*., 1995; Billig *et al*., 1996; Hadziselimovic *et al*., 1997; Rodriguez *et al*., 1997; Fan and Robaine, 1998; Sinha Hikim and Swerdloff, 1999; Jurisicova *et al*., 1999; Pentikainen *et al*., 2000), while conflicting data have been reported on apoptosis in ejaculated sperm. Gorczyca *et al*. first demonstrated, in abnormal human sperm cells, the presence of DNA strand breaks and an increased sensitivity of DNA in-situ denaturation, as characteristic of apoptosis of somatic cells (Gorczyca *et al*., 1993). These authors speculated that the activation of endogenous endonuclease, which causes extensive DNA damage in somatic cells, might be responsible for functional elimination of defective germ cells from the reproductive pool.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) investigation detected higher DNA fragmentation in ejaculated sperm from infertile men (Baccetti *et al*., 1996; Gandini *et al*., 2000). The percentage of sperm containing fragmented DNA correlates negatively with

fertilization rates in IVF (Sun *et al*., 1997), ICSI (Lopes *et al*., 1998) and the percentage of ejaculated sperm with endogenous DNA nicks has been correlated with reduced fertility (Manicardi *et al*., 1995; Bianchi *et al*., 1996; Sakkas *et al*., 1996; Donnelly *et al*., 2000).

However, although many studies have demonstrated that apoptotic DNA fragmentation of somatic cells can be reliably detected using TUNEL, it has not been proven that this method is suitable to evaluate apoptosis of mature ejaculated sperm (Manicardi *et al*., 1998; Barroso *et al*., 2000). Furthermore, the authors who first demonstrated a classical ultrastructural apoptotic pattern in ejaculated sperm (Baccetti *et al*., 1996), in recent studies using TUNEL and electron microscopy have shown that DNA fragmentation was not associated with an apoptosis-like phenomenon in ejaculated sperm, and have concluded that DNA fragmentation should be considered a sign of defective sperm maturation probably dating back to the time of DNA packing (Muratori *et al*., 2000).

It has been shown that the loss of phospholipid asymmetry, leading to exposure of phosphatidylserine on the outside of the plasma membrane, is an early event of apoptosis of all human cells tested. The anticoagulant Annexin V preferentially binds to negatively charged phospholipids such as phosphatidylserine (Koopman *et al*., 1994; Martin *et al*., 1995; van Heerde *et al*., 1995). By conjugating fluorescein to Annexin V it has been possible to use the marker to identify apoptotic cells by flow cytometry. During apoptosis the cells bind Annexin V prior to the loss of the plasma membrane's ability to exclude propidium iodide (PI). Therefore, by staining cells with a combination of Annexin V and PI it is possible to simultaneously distinguish live, apoptotic and necrotic sperm populations. This method does not involve enzyme activity and does not require cells to be previously fixed. Accordingly, unlike the TUNEL assay, this assay enables living sperm to be evaluated. The method has been used by two authors to investigate sperm apoptosis, but conflicting results have been obtained (Glander and Schaller, 1999; Oosterhuis *et al*., 2000). In the first study the percentage of apoptotic sperm in the ejaculate positively correlated with motility, while in the second study a negative correlation was observed between apoptotic cells and sperm motility and concentration. This surprising difference could be due to the different method used and/or to the different patient population studied, or more probably to the fact that in one study the analysis was carried out on semen (Oosterhuis *et al*., 2000) and in the other study it was carried out on sperm separated from seminal plasma by Percoll density gradient centrifugation (Glander and Schaller, 1999).

Many studies have demonstrated that there is enormous potential for interactions between leukocytes and the male reproductive system (Rossi and Aitken, 1997 for review). However, the precise significance of leukocytes in the semen is not fully understood. To date, no data have been published on the relationship between seminal leukocytes and apoptosis in sperm.

The objectives of this study were: (i) to study apoptosis in human sperm using a modified flow cytometric method; (ii) to evaluate the relationship between apoptosis and seminal leukocytes using flow cytometry.

Materials and methods

Patients

Semen samples were obtained from 57 patients (mean age 35.7 ± 6.0 years) attending the Department of Reproductive and Developmental Sciences of the University of Trieste. All subjects were Caucasians and were the partners of women who had failed to conceive after 24 months of unprotected intercourse. All subjects were asymptomatic for genito-urinary infections. Informed consent for participation in the study was obtained from all patients.

Semen analysis

Samples were collected by masturbation into sterile containers after 3–4 days of sexual abstinence and were delivered to the laboratory within 45 min after ejaculation. Routine analysis was performed following published guidelines (World Health Organization, 1999). The variables taken into account were: volume of ejaculate (ml), round cell concentration ($\times 10^6$/ml), sperm concentration ($\times 10^6$/ml), forward motility (%), morphology (% of normal forms). A leukocyte count was carried out using peroxidase test and flow cytometry combined with monoclonal antibody anti-CD45 and anti-CD53 as previously described (Ricci *et al*., 2000). Briefly, 20 µl of fluorescein isothiocyanate (FITC)-conjugated anti-CD45 and 20 µl of PE-conjugated anti-CD53 (Pharmingen; Becton Dickinson, San José, CA, USA) were added to 100 µl of the semen sample and incubated at room temperature for 20 min in the dark. Samples then were prepared for flow cytometric analysis using a Coulter Immunoprep (Coulter Beckman, Fullerton, CA, USA).

Fluorescent staining of sperm

Staining with Annexin V/PI was performed using a commercial kit from Bender Diagnostics GmbH (MedSystems, Vienna, Austria). An aliquot of semen specimen (50 µl of samples containing 0.5×10^6/ml sperm) was added to 145 µl of binding buffer prepared according to the manufacturer's instructions, plus 5 µl of Annexin V–FITC, and incubated at room temperature for 10 min. Samples were then washed once with binding buffer, centrifuged at 200 *g* for 10 min, and, finally, the pellets were resuspended in 200 µl of binding buffer containing 1 µg/ml of PI (Molecular Probes, Eugene, OR, USA).

In order to assess the sperm region correctly during flow cytometric analysis, sperm viability was checked by staining with 6-carboxy-fluoresceindiacetate (6-CFDA; Molecular Probes) as follows. Aliquots of 100 µl of sperm suspension were mixed into a solution of 6-CFDA 0.015 µg/ml in phosphate-buffered saline (PBS). Following 30 min incubation at 37°C in CO_2 atmosphere, cells were washed with PBS and resuspended in 200 µl of PBS with 1 µg/ml final concentration of PI.

Flow cytometric analysis

Samples were acquired using a FACScalibur cytometer (Becton Dickinson, San José, CA, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser. FL1 (FITC) signals were detected through a 530/30 nm band pass filter, FL2 (PI) signals were detected through a 585/42 nm band pass filter. 20 000 events were recorded in list mode and analysed using the Cell Quest Pro software (Becton Dickinson). The sperm population was gated on the basis of the linear forward (FSC) and side-scatter (SSC) properties. In order to correctly set the region on the sperm and avoid debris or fragments of cells, we used a combination of the two fluorescent dyes, 6-CFDA to identify live sperm and PI to identify dead cells or cells with

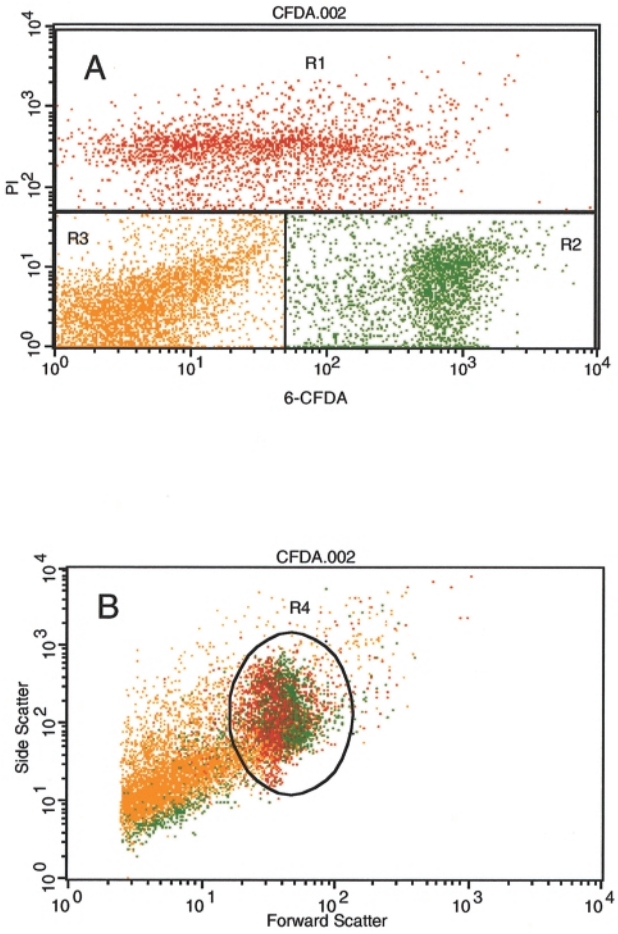

Figure 1. Sample cytogram of a sperm sample stained with propidium iodide (PI) and 6-carboxyfluoresceindiacetate (6-CFDA) indicating the gate strategy used to correctly identify the sperm population. In (**A**) (PI fluorescence versus 6-CFDA fluorescence), region R1 represents PI positive (red-stained) dead sperm, region R2 6-CFDA positive (green-stained) live sperm, and region R3 a third population which does not absorb any dye and is constituted by cellular debris or fragments of cells (orange-stained). In (**B**), a region R4, which identifies only the sperm cell population, was set on side-scatter versus forward-scatter setting.

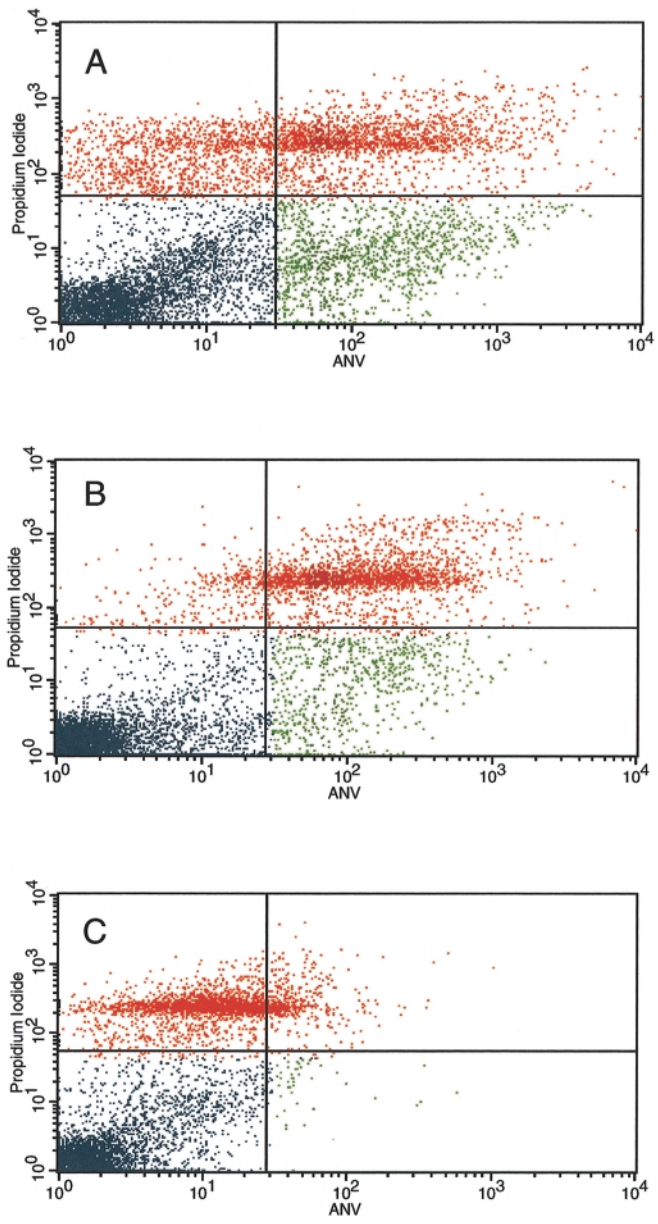

Figure 2. Annexin V/propidium iodide (PI) bivariate analysis used to identify the different cell populations. The lower left quadrant of each graph contains Annexin V negative/PI negative (blue-stained) viable, non-apoptotic sperm. The lower right quadrant shows Annexin V positive/PI negative (green-stained) early apoptotic sperm. The upper right quadrant represents Annexin V positive/PI positive (red-stained) apoptotic sperm. The upper left quadrant contains Annexin V negative/PI positive sperm (red-stained) necrotic sperm. (**A**) A seminal sample with high apoptotic index. (**C**) A seminal sample with low apoptotic index. In this case, most cells are either viable (lower left quadrant) or necrotic (upper left quadrant), and the low rate of the apoptotic index calculated does not exclude any other type of cell damage. (**B**) A seminal sample with intermediate characteristics.

damaged membranes. The fraction of particle that is negative for both dyes corresponds to debris identified on the basis of light scatter properties and can be excluded from the analysis (Figure 1).

The different labelling patterns in the bivariate PI/Annexin V analysis identify the different cell populations (Figure 2). We classified sperm as normal (negative Annexin V and PI), early apoptotic (positive Annexin V and negative PI), apoptotic (positive Annexin V and positive PI) and necrotic (negative Annexin V and positive PI). We defined as the apoptotic index the ratio between the positive Annexin V negative PI sperm and the total living sperm (PI negative).

Statistical analysis

Comparison between normal and abnormal semen groups was performed using the Mann–Whitney *U*-test. Correlations between the age of patients, routine semen analysis parameters and the results obtained from flow cytometry assays were computed using the Spearman rank correlation coefficient. The Pearson correlation coefficient was used for all other correlation studies. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant.

Results

Table I reports the percentage of the cell populations identified using flow cytometry. When the patient populations were divided into normal and abnormal subjects according to the standard criteria (World Health Organization, 1999), no significant differences were found between the two groups in

Table I. Staining of sperm by Annexin V and propidium iodide (PI)

Sperm	Total (n = 57)	Normal semen subjects (n = 28)	Abnormal semen subjects (n = 29)	P
Annexin V^-/PI^- (%)	60.8 ± 14.8	59.9 ± 14.7	61.7 ± 15.0	NS
Annexin V^+/PI^- (%)	4.5 ± 3.7	4.9 ± 3.3	4.1 ± 4.1	NS
Annexin V^+/PI^+ (%)	15.7 ± 9.4	17.8 ± 8.9	13.7 ± 9.5	NS
Annexin V^-/PI^+ (%)	19.0 ± 14.3	17.5 ± 14.6	20.5 ± 14.2	NS
Apoptotic index	7.1 ± 5.9	7.6 ± 5.4	6.6 ± 6.4	NS

Values are mean ± SD.
NS = not significant.

Table II. Correlation between semen parameters and results of flow cytometry assay

	AN^-/PI^-		AN^+/PI^-		AN^+/PI^+		AN^-/PI^+		Apoptotic index	
	r	P	r	P	r	P	r	P	r	P
Semen volume	0.004	NS	−0.016	NS	−0.039	NS	−0.074	NS	−0.047	NS
Round cells	−0.042	NS	−0.022	NS	−0.208	NS	0.182	NS	−0.025	NS
Sperm concentration	0.028	NS	−0.080	NS	0.037	NS	0.058	NS	−0.098	NS
Progressive motility	0.059	NS	0.020	NS	0.044	NS	−0.097	NS	−0.007	NS
% of normal forms	−0.008	NS	−0.007	NS	0.061	NS	0.022	NS	−0.009	NS

AV = Annexin V; PI = propidium iodide.
NS = not significant.

Table III. Correlation between semen parameters and apoptotic index in total samples and in normal and abnormal semen subgroups

	Apoptotic index					
	Total (n = 57)		Normal semen (n = 28)		Abnormal semen (n = 29)	
	r	P	r	P	r	P
Semen volume	−0.047	NS	−0.099	NS	−0.053	NS
Round cells	−0.025	NS	−0.269	NS	0.199	NS
Sperm concentration	−0.098	NS	−0.536	< 0.01	−0.022	NS
Progressive motility	−0.007	NS	−0.171	NS	−0.069	NS
% of normal forms	−0.009	NS	−0.414	< 0.05	0.373	< 0.05

NS = not significant.

terms of the apoptotic index and the percentage of live and apoptotic sperm.

The apoptotic index was significantly correlated with Annexin positive/PI positive sperm (r = 0.788, $P < 0.0001$) and inversely correlated with Annexin negative/PI positive sperm (r = –0.358, $P < 0.01$). Patients' ages positively correlated with the percentage of apoptotic sperm (r = 0.377, $P < 0.01$) and negatively correlated with the percentage of normal Annexin and PI negative cells (r = –0.366, $P < 0.01$). No correlation was seen between the conventional semen parameters and the percentage of apoptotic sperm when considering all the samples together (Table II). A significant inverse correlation between sperm concentration and the apoptotic index was observed only in the normal semen group (Table III). A significant positive correlation was seen between percentage of normal forms and apoptotic index in the abnormal semen group.

No correlation was observed between the percentage of sperm midpiece defects and apoptotic index (r = –0.137, P = 0.306).

The concentration of the seminal leukocytes was higher in the normal semen group compared with the abnormal semen group. However, the difference between the two groups was significant only when leukocytes were counted using flow cytometry. The median values (thousands per ml of seminal fluid) in normal and abnormal semen groups were respectively 40 (range 0–7200) and 30 (range 0–6000) using peroxidase test (P = 0.081), 690 (range 10–11 800) and 380 (range 10–6900) using monoclonal antibody anti-CD45 ($P < 0.01$) and 500 (range 10–4300) and 260 (range 10–6900) using monoclonal antibody anti-CD53 ($P < 0.05$).

The concentration of leukocytes per ml of seminal fluid did not correlate with the percentage of apoptotic sperm or with the apoptotic index. In contrast, considering the ratio

Table IV. Correlation of leukocyte/sperm (L:S) ratio evaluated counting leukocytes with monoclonal antibody (mAb) anti-CD45, mAb anti-CD53 and peroxidase test with apoptotic index in total samples and in normal and abnormal semen subgroups

	Apoptotic index					
	Total ($n = 57$)		Normal semen ($n = 28$)		Abnormal semen ($n = 29$)	
	r	P	r	P	r	P
L:S ratio (mAb anti-CD45)	0.610	< 0.0001	0.501	< 0.05	0.663	< 0.001
L:S ratio (mAb anti-CD53)	0.318	NS	0.338	NS	0.323	NS
L:S ratio peroxidase test	0.526	< 0.0001	0.236	NS	0.721	< 0.001

NS = not significant.

between CD45 positive leucocytes and sperm, a significant correlation was found between this ratio and the apoptotic index (Table IV). A slightly weaker correlation was found using the peroxidase test. No correlation was found using monoclonal antibody anti-CD53. The same significant correlation between the CD45 positive leukocyte:sperm ratio and the apoptotic index was present both in abnormal and in normal semen groups. However, using peroxidase, a significant correlation was only seen in the abnormal group.

The mean value of the calculated leukocyte:sperm ratio in our patient population was respectively 0.0057 ± 0.0158 using peroxidase to detect leukocytes, 0.0550 ± 0.0650 using monoclonal antibody anti-CD45, and 0.0515 ± 0.0577 using monoclonal antibody anti-CD53. The mean value of the leukocyte:sperm ratio in the abnormal semen group was not significantly different compared with the normal semen group.

Discussion

Flow cytometry has become the method of choice for analysis of apoptosis in a variety of cell systems, providing fast, highly accurate, automatic, multiparameter analysis of thousands of cells (Telford *et al*., 1994; Darzynkiewicz *et al*., 1997). However, when applying flow cytometry to the study of sperm populations, measurement problems due to the asymmetric shape of sperm should be taken into account.

We found a lower percentage of sperm in early apoptosis than those reported in previous studies (Glander and Schaller, 1999; Oosterhuis *et al*., 2000). Indeed, apoptosis can take place in a few hours or even minutes (Wyllie *et al*., 1980; Majno and Joris, 1995; Uchiyama, 1995; Vaux and Korsmeyer, 1999), and since it progresses rapidly, it is difficult to observe cells in the earliest stages of apoptosis (Schwartzman and Cidlowski, 1993). In an experimental study it has been shown that Annexin V binding of sperm increases shortly after addition of H_2O_2 followed immediately afterwards by an increase in PI positive cells and that after just 60 min incubation of sperm with H_2O_2 almost all cells were in apoptotic necrosis, being stained by Annexin V and PI (Ramos and Wetzels, 2001). Hence we were not surprised to observe few cells in early apoptosis. Furthermore, to obtain a more reliable evaluation of apoptotic cells in the ejaculate, we used a modified method. In the previous studies (Glander and Schaller, 1999; Oosterhuis *et al*., 2000) the sperm population was gated using forward-angle light scatter while side-angle light scatter was used to exclude debris and aggregates. However, some particles and cell debris present in the ejaculate may have frontal and side light scatter parameters similar to those of the sperm. Consequently, these particles and debris can be incorrectly quantified by the cytometer as unstained live cells. This makes it difficult to discriminate, by flow cytometry, between sperm and particles or debris, on the sole basis of light scatter parameters.

Accordingly, it would be better to be able to stain both live and dead cells, since it would enable sperm to be clearly identified, regardless of the amount of particles and cell debris present in the ejaculate.

We modified the method described by Glander and Schaller (1999), by performing a preliminary flow cytometry analysis using CFDA to identify the live sperm and using PI which only stains dead cells. Having gated the sperm population we then performed the classical bivariate Annexin V/PI analysis.

When the membrane loses its integrity, the cell becomes PI positive, indicating that the cell is necrotic. Using flow cytometry we have detected two PI positive cell fractions, one also stained with Annexin V whereas the other did not stain with Annexin V. In previous studies, PI positive/Annexin positive cells have always been classified as dead cells, while there is no agreement on how to classify PI positive/Annexin negative cells. In a study evaluating experimental models of induced apoptosis of neuroblastoma cells in culture (Van Engeland *et al*., 1998), PI positive/Annexin negative cells were classified as cells damaged during the isolation procedure. On the contrary, in accordance with another study (Glander and Schaller, 1999), we have hypothesized that this group of sperm was characterized by a very high degree of membrane disorganization, which might prevent any binding of Annexin V. In addition, we suggest that PI positive/Annexin negative cells may be sperm in the latter stage of apopotosis, and our results are consistent with this hypothesis.

In fact, we found that the apoptotic index significantly correlated with the percentage of double-stained sperm and inversely correlated with the percentage of Annexin negative/PI positive sperm. As expected, in ejaculates with active apoptosis (a high apoptotic index) double-stained apoptotic

sperm prevail over late apoptotic sperm, while in ejaculates with less active apoptosis, late apoptotic sperm prevailed over apoptotic double-stained sperm. This hypothesis, however, should be confirmed using other specific tests for late apoptosis.

In order to investigate the biological significance of apoptosis, we analysed the correlation of the results of flow cytometry with the age of the patient and semen parameters. The age of the patient positively correlated with the percentage of apoptotic sperm and negatively correlated with the percentage of normal Annexin and PI negative cells. In subjects with normal semen parameters we found a significant inverse correlation between sperm concentration and morphology and apoptotic index. These data provide further evidence that apoptosis is a physiological mechanism regulating gonadal cell proliferation. We were unable to show a similar correlation in the patient group with abnormal semen. Surprisingly, in this patient group we found a significant, positive correlation between the apoptotic index and the percentage of typical forms. In the subjects with abnormal semen it is likely that there is a disrupted regulation of apoptosis, as suggested by many studies on the testis. However, this defect probably involves the earlier stages of spermatogenesis, hence it is not detectable in the sperm population in the ejaculate. It is likely that most apoptotic cells do not appear in the ejaculate because of phagocytosis during epididymal transit. The fact that no significant differences were observed between subjects with normal and abnormal semen regarding the apoptotic index, percentage of normal, apoptotic and necrotic sperm, supports this hypothesis.

However, as AnnexinV is only a marker of early apoptosis, we cannot exclude that, in the subjects with abnormal semen, apoptotic cells may be in the later phases of apoptosis, as suggested by several previous studies using other methods (Sun *et al*., 1997; Donnelly *et al*., 2000; Gandini *et al*., 2000), or that in these patients there may also be other types of cell damage.

In order to check whether apoptosis might be an expression of a defect in sperm maturation we correlated, on the basis of previous studies (Huszar *et al*., 1998; Gergely *et al*. 1999), the percentage of midpiece defects with the apoptotic index. No significant correlation was found between midpiece defects and the apoptotic index. However, we would like to point out that we did not adopt a specific method for the visualization of the midpiece in sperm, such as one previously suggested (Gomez *et al*., 1996). Consequently we cannot rule out that different results might be obtained if such a method was followed.

No correlation was found between the concentration of leukocytes per ml of seminal fluid and the percentage of apoptotic sperm. Considering the leukocyte:sperm ratio, a highly significant correlation was found between this ratio and the percentage of apoptotic sperm. Thus, there would seem to be a very close relationship between the presence of leukocytes in the semen and the sperm apoptosis. This might be interpreted in two ways: the leukocytes might be responsible for sperm apoptosis, or, alternatively, the apoptotic sperm might induce a chemotactic response.

The first hypothesis is supported by various data. Leukocytes are considered the main source of reactive oxygen species (ROS) in sperm suspensions (Aitken *et al*., 1992; Kessopoulou *et al*., 1992; Aitken *et al*., 1994; Griveau *et al*., 1995; Ford *et al*., 1997). It has been demonstrated that exogenous ROS generation causes an increase in DNA fragmentation in human sperm and that the administration of antioxidants prevents the amount of DNA damage observed (Lopes *et al*., 1998). ROS are also a known inductor of apoptosis in somatic cells (Ratan *et al*., 1994) and in mature sperm (Ramos and Wetzels, 2001). However, a clear demonstration *in vivo* of the relationship between leukocyte contamination of semen and male infertility has not yet been provided.

The alternative hypothesis is that leukocytes are attracted by apoptotic sperm.

Apoptotic cells are believed to undergo heterophagic elimination by phagocytes without releasing pro-inflammatory mediators or ROS. It has been hypothesized that a similar mechanism probably provides for senescent or abnormal sperm removal (Rossi and Aitken, 1997 for review). It has been recently demonstrated that many spermatogenetic cells die by apoptosis and are subsequently phagocytosed by Sertoli cells and that translocation of phosphatidylserine to the outer part of the plasma membrane is the signal for phagocytosis (Shiratsuchi *et al*., 1997).

Both the results of previous studies and this study show that mature sperm also undergo apoptosis, leading to exposure of phosphatidylserine on the outside of the plasma membrane which is a chemotactic factor for leukocytes and is recognized by phosphatidylserine receptors present on the surface of the phagocyte. Thus, leukocytes could remove apoptotic sperm without causing an inflammatory reaction.

Our results seem to support this hypothesis because we found a significant correlation between the leukocyte:sperm ratio and the apoptotic index both in abnormal and normal semen. The fact that there is also a good correlation between the leukocyte:sperm ratio and the apoptotic index in subjects with normal sperm concentration, motility and morphology, suggests that seminal leukocytes have a physiological cell function. In accordance with previous observations (Tomlinson *et al*., 1992; Kiessling *et al*., 1995) we are of the opinion that seminal leukocytes might constitute a control system of senescent or abnormal sperm. Indeed their concentrations correlate with sperm concentrations, and when the rate of sperm dying by apoptosis increases, the leukocyte:sperm ratio increases proportionally. These results are also consistent with the findings of Tomlinson *et al*., who, in a prospective analysis of 229 couples, demonstrated that neither the leukocyte total, nor any of the individual leukocyte phenotypes, nor ROS levels—which correlated highly with leukocyte concentrations—were associated with reduced natural conception rates or a drop in semen quality (Tomlinson *et al*., 1993). The same authors, having observed that phagocytic cells were very strongly correlated with an increased percentage of morphologically normal sperm, hypothesized that in certain subjects leukocytes may increase semen quality by removing abnormal and degenerate forms.

We observed different results when counting seminal leukocytes using peroxidase, mAb anti-CD45 and anti-CD53. In a previous study we reported a stronger correlation between CD45 and peroxidase than CD53 and peroxidase (Ricci *et al*., 2000), and the data from the current study confirm that mAb anti-CD45 and anti-CD53 might identify a different activation or function of leukocytes.

In conclusion, flow cytometry is a useful method to study the biology of seminal cell populations, and it is the only method that enables apoptosis of live sperm to be monitored. This is the first study that has investigated, simultaneously, sperm apoptosis and seminal leukocytes using flow cytometry. In our study, membrane changes in ejaculated sperm, as can be detected by Annexin V, do not appear to correlate with semen abnormalities. It is unlikely that leukocyte contamination expresses a defective defense mechanism associated with subfertility. More probably, in the absence of genitourinary infection, one of the most important functions of seminal leukocytes is the removal of apoptotic sperm.

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