The flow cytometry study of AnnexinV binding by human spermatozoa – is it a marker of apoptosis?

MONIKA PODHORECKA[1], GRZEGORZ BAKALCZUK[1], GRZEGORZ JAKIEL[2], JACEK ROLINSKI[1]

[1]Department of Clinical Immunology, University School of Medicine in Lublin, Poland; [2]Department of Reproduction and Andrology, University School of Medicine in Lublin, Poland

Abstract

Apoptosis is a physiological process whereby cell activates an intrinsic death or suicide and kills itself in a controlled way. A sequence of morphological and biochemical changes occurs in cell during this process, one of these is the loss of plasma membrane asymmetry. In early phase of apoptosis the particles of phosphatydylserin are presented on the outer leaflet of plasma membrane, what can be detected by Annexin V binding. However, AnnexinV identifies both apoptotic and necrotic cells. To distinguish these two groups the DNA binding propidium iodide that stains permeable cells should be used. In this study to broaden the knowledge of the spermatozoa programmed cell death we analyzed Annexin V binding by these cells as early marker of apoptosis. We tested semen samples from 31 patients diagnosed because of married infertility. Annexin V binding was assessed with flow cytometry technique. We observed that human ejaculated spermatozoa do bind either AnnexinV or AnnexinV along with PI, however only very low number are double negative. Thus, it can be concluded that changes in spermatozoa membrane structure are not caused by the beginning of apoptosis, as in somatic cells. These changes may be a consequence of some other physiological processes like capacitation or spermatogenesis, what requires to be elucidated precisely yet.

Key words: *Annexin V, apoptosis, spermatozoa, flow cytometry*

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Introduction

Apoptosis is a physiological process occurring during embryonic development as well as in mature tissues to remove unwanted cells. It is called programmed cell death or cell suicide because the cell actively participates in its own destruction, in contrast to necrosis that is caused by cell injury. A sequence of morphological and biochemical changes occurs in cell during the process of apoptosis that result in efficient elimination of such a cell from tissues without eliciting an inflammatory response [1, 2]. The most characteristic change in apoptotic cells is the loss of plasma membrane asymmetry. This change occurs early during apoptosis, regardless of whether apoptosis is induced by activation the receptors on the plasma membrane or by DNA damage [3, 4]. In vital cells the particles of phosphatydylserin (PS) are located on the inner leaflet of cell membrane, in early phase of apoptosis PS residues are presented on the outer leaflet [5]. The exposure of phosphatydilserine on the outer leaflet of the plasma membrane preconditions the apoptotic bodies (remnants of apoptotic cells) to become a target for phagocytes. Phosphatidylserine can be detected by fluorochrome-labeled Annexin V, the anticoagulant that reacts with high affinity of this phospholipid [5, 6]. AnnexinV can be used to identify both apoptotic and necrotic cells. To distinguish these two groups the DNA binding propidium iodide (PI) that stains permeable cells should be used. Thus Annexin V+/PI- cells can be considered apoptotic ones, while these binding both Annexin V and PI can be considered dead ones.

Correspondence: Monika Podhorecka, MD PhD, Department of Clinical Immunology, University Medical School in Lublin, Jaczewskiego 8, 20-572 Lublin, Poland. Fax +48 81 724 47 01, e-mail: mpodhor@asklepios.am.lublin.pl

Table 1. Sperm characteristic. SD-standard deviation; Min-minimum; Max-maximum; Med-median

	Mean	SD	Min	Max	Med
Concentration (x10^6 cells/mL)	39	27	11	108	28
Cells of pathological morphology (%)	49	11	28	70	49
Motility of A category (%)	25	13	5	45	26

In recent years the role of apoptosis during normal spermatogenesis has been proved [7]. However much less is known about the role of this process in male infertility. To broaden the knowledge of the spermatozoa programmed cell death the assessment of the main steps of the process is required. In this study we focused on analysis of AnnexinV binding, which is an early marker of apoptosis.

Material and Methods

Patients

Thirty-one patients who visited Reproductive and Andrology Department because of diagnosis of married infertility were studied. All procedures were approved by Ethics Committee of University Medical School of Lublin. Semen was obtained by masturbation and allowed to liquefy at room temperature. All tests are started within 2 hours after collection. Routine screening was performed according to World Health Organization standards [8] and included such parameters as concentration, morphology and motility of spermatozoa. Motility was measured in A, B, C and D categories (propulsive, rapid or slow movements, in place moving, immotile spermatozoa, respectively). The spermatozoa characteristic is shown in table 1.

Cell preparation

AnnexinV/FITC binding procedure was performed with *Annexin V/FITC kit* (BenderMedSystems, Austria) according to manufacture instruction. About 10^6 spermatozoa per tube were washed twice in phosphate-buffered saline (PBS) and finally resuspended in 500 µl of binding buffer of 10 mM HEPES ph 7.4, 150 mM NaCl, 5 mM KCL, 1 mM $MgCL_2$, 1.8 mM $CaCl_2$. Then the cells were incubated with 5 µl of AnnexinV/FITC, followed by PI of concentration 50 µg/mL staining. After incubation at room temperature for 10 minutes the stained cells were immediately analyzed by flow cytometry technique.

Flow cytometry analysis

The FACSCalibur (Becton Dickinson, USA) flow cytometer was used The following parameters were detected for analyzed samples forward scatter (FSC), side scatter (SSC), fluorescence intensity log FL-1 (Annexin V FITC), log FL3 (PI). The FSC versus SSC dot plot was used to establish spermatozoa gate. A negative control without the presence of Annexin V was included for each test. At least 10 000 cells were examined for each sample. All measurements were done under the same instrument settings. The data analysis was performed using Cell Quest software. The flow cytometry dot plots of AnnexinV/FITC and PI labeling are shown on Fig. 1.

Statistical analysis

Statistical analysis was performed using STATISTICA 5.0 software for Windows. The data were expressed as mean

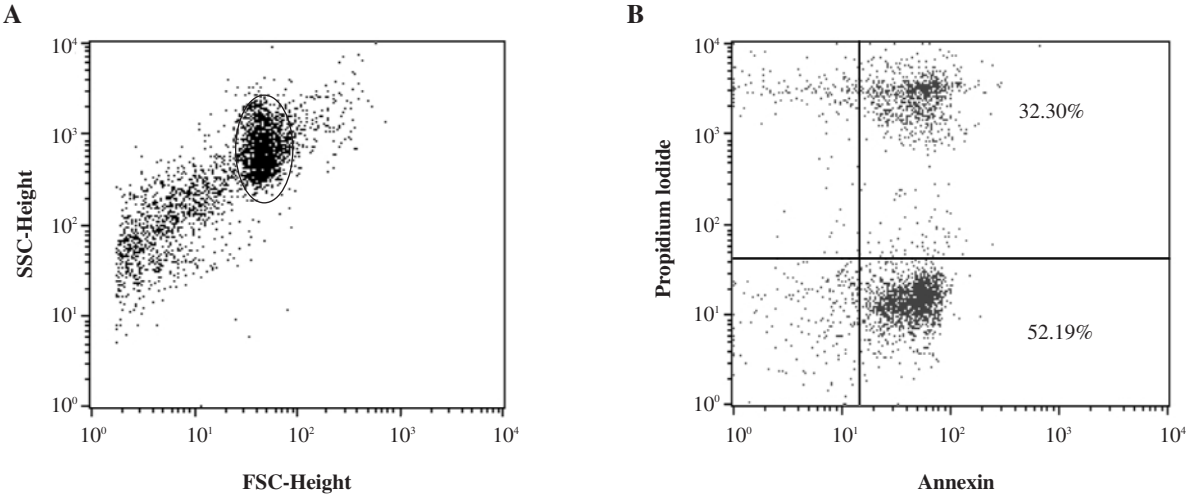

Fig. 1. The flow cytometry analysis of AnnexinV/FITC binding by spermatozoa. (A): Forward scatter/side scatter dot plot. (B): AnnexinV/FITC and propidium iodide (PI) expression

± standard deviation (mean±SD). The relationships between percentage of AnnexinV+/PI- cells or AnnexinV+/PI+ cells and such parameters as concentration, motility and morphology of spermatozoa were analyzed by Spearman rank correlation. Statistical significance was set at $p \leq 0.05$.

Results

The mean percentage of AnnexinV+/PI- cells was 45.38±16.51, while percentage of AnnexinV+/PI+ spermatozoa was 38.74±14.60. The percentage of double negative cells (living spermatozoa) was only 10.43±7.82. However, we detected a positive statistically significant correlation between the percentages of AnnexinV+/PI- with motile of A category (R=0.40, p=0.03). This correlation is shown on fig. 2. The percentage of AnnexinV+/PI+ spermatozoa positively and significantly correlated with the percentage of pathological morphology (R=0.39, p=0.04). This correlation is shown on fig. 3.

Discussion

AnnexinV binding by particles of phosphatydylserin (PS) translocated from the inner to outer leaflet of cell membrane, is one of the methods important in detection of apoptosis process. The simultaneous labeling by PI allows identifying both apoptotic and necrotic cells [6]. The labeled cells can be detected by flow cytometry technique that offers the possibility of rapid and accurate measurement of a multitude of cells attributes in large cell population [9]. In this study we analyzed Annexin V and PI binding with use of flow cytometry method to broaden the knowledge of programmed cell death in human spermatozoa.

The obtained results indicate interestingly that human ejaculated spermatozoa do bind either AnnexinV or AnnexinV along with PI, however only very low number are double negative. Although the detection of PS exposure on cell membrane and its binding by Annexin V is a well-established marker of early apoptosis, it is not known whether this can be such a marker for mature sperm. There are other studies regarding AnnexinV/FITC binding by spermatozoa, however our results do not seem to be consistent with them [10,11,12]. Oosterhuis et al. [10] detected the mean 20±10% of apoptotic cells (Annexin V+/PI-) among human spermatozoa and 19±7% Annexin+/PI+ cells. Shen et al. identified these populations with a median value of about 20% for each category as well. However, the percentage of AnnexinV+/PI- cells detected in our study positively and statistically significant correlated with percentage of spermatozoa of A motility that may be in contrast with designating them as apoptotic cells. On the other hand the percentage of AnnexinV+/PI+ positively and significantly correlated with the percentage of pathological morphology of analyzed samples, thus these cells can be assumed the dead ones. In this case plasma membrane permeability for PI rather then

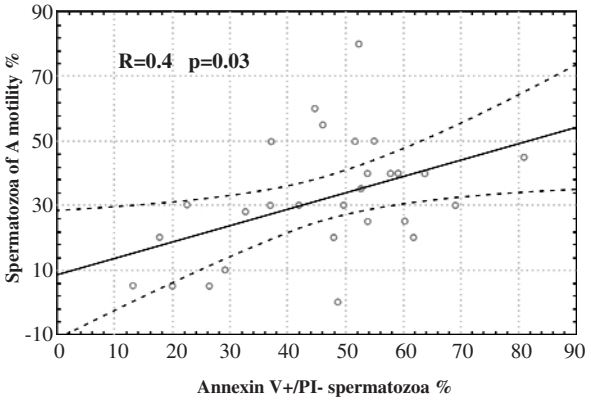

Fig. 2. Percentage of AnnexinV+/PI- spermatozoa correlates with percentage of spermatozoa of A motility

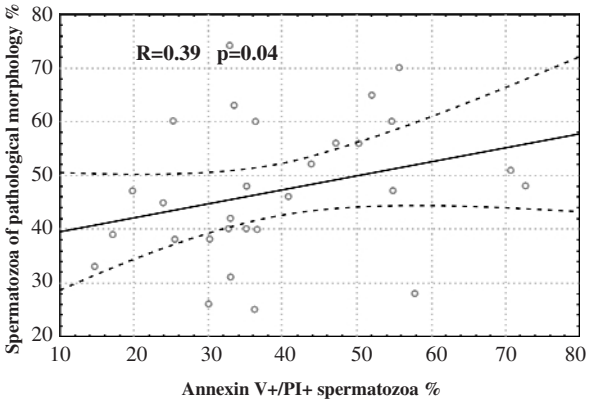

Fig. 3. Percentage of AnnexinV+/PI+ spermatozoa correlates with percentage of spermatozoa of pathological morphology

expression of PS and Annexin V binding seems to be marker of dead cells. The obtained results indicate that changes in spermatozoa membrane structure do not have to be caused by the beginning of programmed cell death, as in somatic ones, but may be a consequence of some physiological processes. It may be originated from the process of spermatogenesis or capacitation, what requires to be elucidated precisely yet.

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