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Technical Article

Isolation of apoptotic mouse fetal oocytes by AnnexinV assay

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ABSTRACT Expression of phosphotidylserine by fetal oocytes in culture renders significant numbers of such cells able to bind AnnexinV-coated microbeads and allows their separation from Annexin V-negative oocytes on a Magnetic Cell Separation (MACS) column in a magnetic field. The majority of oocytes (≥75%) which bound Annexin V-coated microbeads were viable, as indicated by their propidium iodine (PI) negativity. However, they showed apoptotic morphologies and were found to be TUNEL-positive. On the other hand, AnnexinV-negative oocytes, besides being PI negative, appeared morphologically healthy and TUNEL negative. Moreover, AnnexinV-positive oocytes showed a marked lower ratio of Bcl-xL/Bax transcripts in comparison to AnnexinV-negative oocytes. We conclude that the present method is able to separate fetal oocytes in two distinct populations: AnnexinV-positive oocytes showing features typical of apoptotic cells and AnnexinV-negative oocytes comprising for the most part viable non-apoptotic cells. This procedure should greatly facilitate studies aimed to identify the currently poorly understood molecular pathways governing apoptosis in mammalian fetal oocytes.

KEY WORDS: *apoptosis, oocytes, annexinV, meiosis*

Introduction

In mammals, extensive degeneration of female germ cells has been described during embryonic and fetal stages of oogenesis. According to several reports fetal oocyte death takes place as programmed cell death (PCD) in the form of apoptosis. However, the cause/s and the molecular mechanisms underlying oocyte PCD are little understood (for reviews, see Morita and Tilly, 1999; Tilly, 2001; Morita *et al.,* 2001; De Felici *et al.,* 2005). In general a major problem in studying the molecular mechanisms of cell apoptosis is to obtain sufficient quantities of homogeneous experimental material for biochemical analyses. *In vivo* the number of apoptotic cells present at any time inside tissues is low. In fact, in the mouse fetal ovaries only a few oocytes showing apoptotic markers such as TUNEL and/or Poly (ADP-Ribose) Polymerase (PARP) staining are identifiable (Pesce *et al.,* 1997; Reynaud and Driancurt, 1999; Pepling and Spradling, 2001; Kasai *et al.,* 2003). In previous works, we showed that isolated mouse fetal oocytes cultured *in vitro* in conditions allowing their meiotic progression, underwent cell death resembling apoptotic waves occurring in oocytes within the fetal ovaries (De Felici *et al.,* 1999; Lobascio *et al.,* 2006). In particular, we found that the number of oocytes showing TUNEL staining and AnnexinV binding increased as a function of the culture time and meiotic progression (Lobascio *et al.,* 2006 submitted). While positive TUNEL staining reveal DNA cleavage associated to relatively late stages of apoptosis (Gavrieli *et al.,* 1992), AnnexinV binding is known to result from the loss of phospholipid asymmetry and phosphotidylserine (PS) exposure on the outside surface of the plasma membrane occurring in the early stages of apoptosis (Martin *et al.,* 1995). Taking advantage of this latter process, we sought to devise a method to separate AnnexinV positive and negative oocytes in culture and to verify if distinct apoptotic and non apoptotic oocyte populations could be obtained.

Experimental Protocols

Oocyte isolation and culture

Oocytes were isolated in M_2 medium (Sigma, Milan, Italy) from 10-20 ovaries of 15.5 dpc CD-1 mouse embryos by EDTA-stab method as described in De Felici and McLaren (1982). Briefly, after collection, ovaries were transferred to 0.01% EDTA solution in PBS for 15-20 min at room temperature. The ovaries were then washed in M_2 and cells released by pricking the ovaries with fine needles. This method allows to isolate monodispersed cell population containing about 70-80% oocytes. Oocytes (about 2-4x10^4/ml) were then cultured in 5 ml Falcon tube for three days at 37°C in 5% CO2 in 0.5 ml of MEM supplemented with 5% horse serum (HS, Invitrogen, Milan, Italy) and 2.5% heat-inactivated fetal calf

Abbreviations used in this paper: dpc, days post coitum; GCNA, germ cell nuclear antigen; MACS, magnetic cell separation; PI, propidium iodide.

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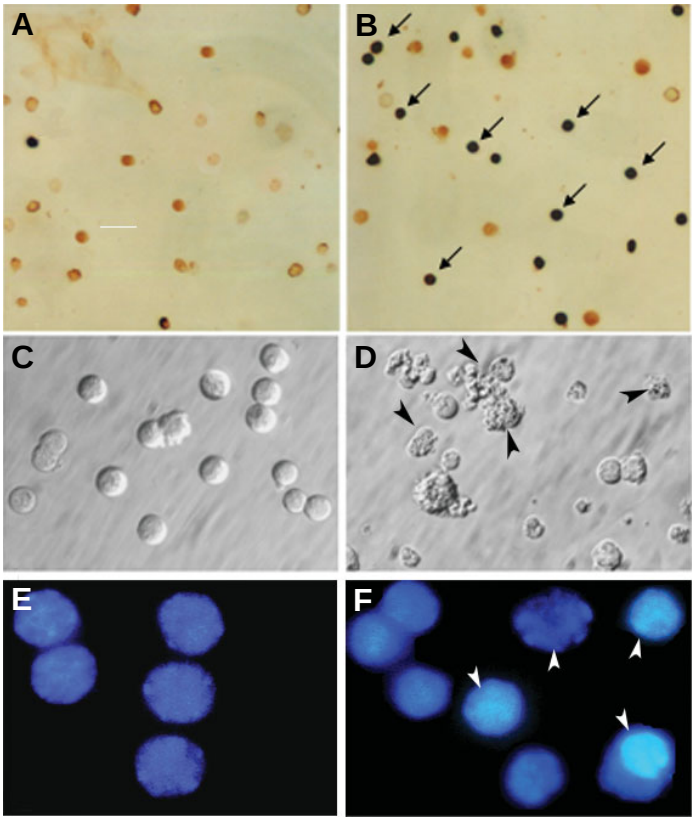

Fig. 1. Analysis of 15.5 dpc oocytes cultured for 3 days and sorted with AnnexinV-coated microbeads. *While the most part of Annexin V-negative sorted oocytes were negative to TUNEL staining* **(A)**, *the most part of the AnnexinV-positive sorted oocytes resulted TUNEL positive* **(B)** *(see arrows). Higher magnifications under light microscope of AnnexinV-negative* **(C)** *and -positive* **(D)** *sorted oocytes. Note the high frequency of poor morphologies (arrowheads) in this latter oocyte population.* **(E,F)** *Nuclear staining with Hoechst 33248 of Annexin V-negative and positive oocytes respectively. Note chromatin condensation typical of apoptotic cells (arrowheads) in* **(F)**.

serum (FCS, Invitrogen) as described in De Felici *et al.* (1999).

Annexin V binding and cell sorting

At the beginning (T0) and after 3 days of culture (T3) samples of oocytes were examined for AnnexinV binding and propidium iodure (PI) staining using the Annexin V-FITC detection kit (Calbiochem, VWR International s.r.l., Milan, Italy) following the protocol of the manufacture.

AnnexinV cell sorting was carried out using the AnnexinV Microbead kit from Miltenyi Biotechnology (Bologna, Italy). Briefly, samples of oocytes were washed in AnnexinV binding buffer by centrifugation (800g x 15 min at room temperature), resuspended in 100 μl of AnnexinV-coated microbeads and incubated for 15 min at room temperature in continuous agitation. At the end of incubation 0.9 ml of M_2 medium containing 5% BSA (Sigma) was added to the cells and the sample passed through a MS^+ column in a magnetic field (Miltenyi Biotechnology). Flushed AnnexinV-negative cells were collected in 1 ml of medium in a 1-ml Eppendorf tube. The column was then washed two times with 1 ml of M_2 in the magnetic field. To elute AnnexinV-positive cells retained by the column, this was removed from the magnetic field, placed on a 1-ml Eppendorf tube and flushed out with 1 ml of M_2 using the plunger supplied with the column. Samples of effluent and retained cells were then processed for Germ Cell Nuclear Antigen 1 (GCNA-1) staining and TUNEL assay or subjected to RT-PCR analysis.

GCNA-1 staining, TUNEL assay and Hoechst labeling

GCNA-1 immunolabelling specific for oocytes (Enders and May, 1994) and TUNEL assay for apoptosis evaluation were performed on separate cell samples of the same cell populations as described in detail in Lobascio *et al.* (2006, Submitted).

Briefly, for GCNA-1 labelling, 20 μl of the cell suspension were spotted onto L-polylysine-coated slides and fixed with 4% paraformaldehyde for 10 min, washed three times each for 10 min in PBS containing 0.5% BSA. A 15 min block in PBS containing 0.3% BSA, 0.05% X-Triton, 1% goat serum was performed. The slides were then incubated over night treated with 1:2 anti GCNA-1 antibody kindly provided by Dr. G. Enders (University of Kansas, Kansas, KS; Enders and May, 1994). The next day the slides were washed in PBS plus 0.5% BSA and incubated for 45 min with a goat anti-rat IgM fluorescein isothiocyanate (FITC) conjugated antibody (AlexaFluor, Invitrogen).

For TUNEL staining, 50 μl of the cell suspension were mixed with an equal volume of 1.5 % low melting point (37°C) agarose in PBS. Aliquots (50 μl) were smeared onto glass slides and allowed to solidify at room temperature. TUNEL-positive cells were identified according to the protocol of the *in situ* cell death detection kit, POD (Roche Diagnostics, Monza, Italy).

GCNA-1 and TUNEL-positive cells were scored in several fields for a total of at least 300 cells under a 40X objective of a Zeiss Axioplan 2 microscope.

Nuclei of cells fixed with 4% paraformaldehyde for 10 min were stained with 1 μg/ml Hoechst 33248 (Sigma), for 5-10 minutes at room temperature.

RT-PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used in order to analyse the level of Bax and Bcl-xL transcripts. Briefly, approximately 100 cells were used for each reaction and mRNA was obtained adding 1.5 μl lysis buffer (0.1M DTT, 0.48 μl Igepal, 0.6U RNase OUT). mRNA was reversed-transcribed with Super Script First Strand Synthesis System for RT-PCR (Invitrogen) according to manufacture instruction. The resulting cDNA fragments were amplified using Taq DNA polymerase (Invitrogen). Primer sequences for Bcl-*xL* were forward primer: 5'-GACCGCGTATCAGAGCTTTG-3' and reverse primer: 5'-TCACTTCCGACTGAAGAGTG-3', for Bax were forward primer: 5'-ATGCGTCCACCAAGAAGCTGAG-3' and reverse primer: 5'-CCCCAGTTGAAGTTGCCATCAG-3' and for β-actin as an internal control were forward primer: 5'-AGCCACGATTTCCCTCTCAGC-3' and reverse primer: 5'-GGCCCAGAGCAAGAGAGGTATCC-3' (Nichols *et al.,* 1998).

Results and Discussion

Co-staining with PI to determine the integrity of the plasma membrane and FITC-AnnexinV to determine the transition of PS was used to distinguish necrotic, apoptotic and viable not apop-

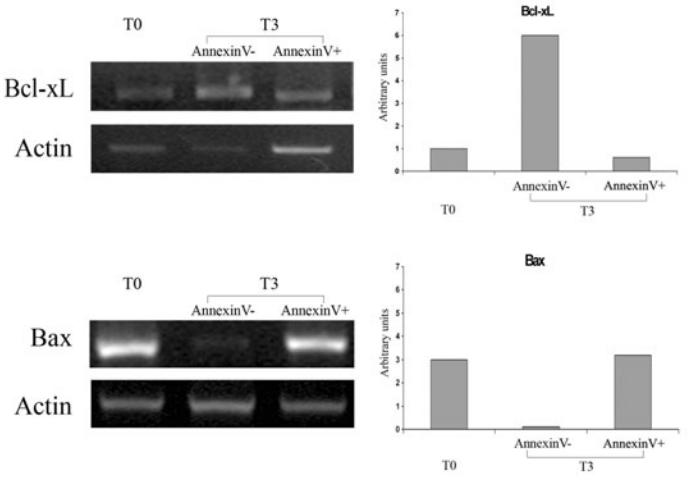

Fig. 2. RT-PCR analyses for *Bax* and *Bcl-xL* transcripts in AnnexinV-negative and -positive sorted cells. *After 3 days of culture (T3), sorted Annexin V+ oocytes showed a marked lower Bcl-xL/Bax transcript ratio than Annexin V- oocytes.*

totic cells. The number of cells morphologically identified as oocytes showing AnnexinV binding increased from 2-5% at T0 to 40%±5.5 (mean ± SE) at T3. A few PI stained oocytes were present at T0 (< 10%); at T3 these cells increased to 15-25% and about half of them were AnnexinV positive.

Separation of AnnexinV positive and negative oocytes was carried out at T3 following the procedure described in Materials and Methods. The results of three independent experiments are reported in Table 1. It appears that this procedure resulted effective in separating cells in two distinct populations of AnnexinV positive (about 52%±5.8, mean ± SE) and negative (about 48%±5.8) cells. The former showed apoptotic morphologies (reduced size, cell fragmentation and condensed chromatin) and were 72±7.2% and 82±3.5% GCNA-1 and TUNEL positive, respectively, while the latter showed healthy morphologies and were 65±8.4% GCNA-1 positive and 92±3.8% TUNEL negative (Fig. 1).

Moreover, RT-PCR analyses of mRNA of the anti apoptotic *bcl-xL* and the pro apoptotic *bax* genes showed that at T3 AnnexinV-positive cells had a marked lower Bcl-xL/Bax ratio transcripts than Annexin V-negative Cells (Fig. 2). Such difference appears mainly due to a marked increase of the levels of Bcl-xL transcripts in the AnnexinV-negative oocytes rather than to changes of transcripts of the two genes in AnnexinV-positive oocytes in comparison to T0. Thus suggesting that the low Bcl-xL/Bax ratio found also in freshly isolated 15.5 dpc oocytes at T0 is not sufficient at this time for apoptosis and that, at least in our culture condition, high Bcl-xL expression is necessary for the maintenance of oocyte survival. We conclude that the present method is able to separate fetal oocytes in two distinct populations. Annexin V-positive oocytes showing several features typical of apoptotic cells and Annexin V-negative oocytes comprising for the most part viable not apoptotic cells. According to several reports fetal oocyte degeneration takes place in the form of apoptosis (Pesce *et al.,* 1993; Coucouvanis *et al.,* 1993; Pesce and De Felici, 1994; Del Pol *et al.,* 1997; Abir *et al.,* 2002; Modi *et al.,* 2003). The process of oocyte apoptosis remains, however, incompletely characterized and alternative forms of cell death for fetal germ cells remain possible (Wartemberg *et al.,* 2001). Furthermore the causes and the molecular mechanisms underlying oocyte apoptosis are little understood (for reviews, see Morita and Tilly, 1999; Tilly, 2001; De Felici *et al.,* 2005). An impediment in the investigation of the molecular mechanisms of the fetal oocyte apoptosis has been the lack of an appropriate system for experimental analyses. The procedure of oocyte separation described here can be useful to further characterize cell death occurring in fetal oocytes and to investigate the molecular pathways underlying this crucial process of the early oogenesis.

TABLE 1

ANNEXIN V SORTING OF 15.5 DPC OOCYTES CULTURED FOR THREE DAYS

	AnnexinV+ (%)	AnnexinV- (%)
Exp.1	60	40
Exp.2	42	58
Exp.3	56	42

In each experiment, roughly 2-6 x10^3 oocytes were recovered in the sorted cell populations.

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