Improved apoptosis detection in ovine neutrophils by annexin V and carboxyfluorescein diacetate staining

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Abstract Neutrophil apoptosis is critical for final resolution of the inflammation in the tissues and for maintenance of neutrophil homeostasis under normal condition. An early hallmark of apoptotic cells is translocation of phosphatidylserine (PS) residues, normally located in the inner leaflet of cellular membrane, to the external cell surface; exposed PS is recognized by specific PS receptors on disposing cells. Here we report an improved procedure to detect neutrophil apoptosis by simultaneous staining for exposed PS with Cy3-labeled annexin V (Cy3) and for membrane integrity with the vital dye 6-carboxyfluorescein diacetate (6-CFDA) based on the APOAC apoptosis detection kit (Sigma). Spontaneous apoptosis was evaluated in ovine neutrophils cultured *ex vivo* for 18 h. We investigated the multiple parameters involved in the assay, i.e. the type of fixative (methanol, paraformaldehyde, or no fixation) and the type of slide (coated with Vectabond, polylysine or Parafilm®). Results indicated that both the adhesion to the slide and the fixation can modify neutrophil functional status and morphology, which result in misleading apoptosis detection. In order to minimize these artifacts, we have developed an improved APOAC assay procedure, staining cells while in suspension and using Parafilm® coated slides.

Keywords Apoptosis · Annexin V · 6-CFDA · Microscopy · Neutrophils

Abbreviations

RBC	Red blood cells
PMN	Polymorphonuclear neutrophilic leukocytes
PS	Phosphatidylserine
6-CFDA	6-Carboxyfluorescein diacetate
6-CF	6-Carboxyfluorescein
HBSS	Hanks' buffered salt solution
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
PFA	Paraformaldehyde
L-Gln	L-glutamine
Cy3	Annexin-Cy3

Introduction

Neutrophils form the first line of defence against invading bacteria and fungi, and possess a powerful array of cytotoxic enzymes, reactive oxidants, and associated processes. Neutrophils have high rates of spontaneous apoptosis and a corresponding very short half-life of only 6–12 h in blood (Akgul et al. 2001). However, the half life can be considerably prolonged *in vitro* by cytokine treatment, and *in vivo* during inflammation (Lee et al. 1993).

Constitutive neutrophil apoptosis is a mechanism to facilitate normal cell turnover and immune system

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homeostasis (Weinmann et al. 2003); during inflammation, apoptosis of exhausted neutrophils ensure their removal by phagocytes and the rapid resolution of inflammation (Savill 1997).

Apoptosis is characterized by morphological and biochemical features occurring at different stages, as condensation of cytoplasm and intracellular organelles, aggregation and subsequent cleavage of nuclear chromatin, and formation of apoptotic bodies with or without nuclear remnants (Maianski et al. 2004). A critical step involves the acquisition of surface changes by apoptotic cells that eventually results in the recognition and the uptake of these cells by phagocytes.

In living cells, phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membrane with phosphatidylcholine and sphingomyelin exposed on the external leaflet of the lipid bilayer, and PS predominantly observed on the inner surface facing the cytosol (Zwaal and Schroit 1997). Cells undergoing apoptosis break up this phospholipid asymmetry and expose PS, which is translocated to the outer layer of the membrane. PS exposure occurs in the early phases of apoptotic cell death during which the cell membrane itself remains intact. Translocation of PS to the external cell surface occurs also during necrosis, but is accompanied by the loss of cell membrane integrity and leakage of cellular constituents into the environment. Therefore measurement of PS exposure executed simultaneously with a vital test provide a sensitive assay to detect apoptotic cells and to discriminate between apoptosis and necrosis.

Annexin V preferentially binds to negatively charged phospholipids like PS in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyelin, which are constitutively present in the outer leaflet of plasma membrane (Van Engeland et al. 1998). The non-fluorescent compound 6-CFDA enters vital cells, is hydrolyzed by the esterase to the fluorescent compound 6-carboxyfluorescein (6-CF) and is retained in the cytoplasm without interfering with neutrophil functions (Davenport et al. 1995). Dead cells do not show uptake and have an increased efflux of the free dye (Bartkowiak et al. 1999; Dransfield et al. 1996).

In this report, we describe an improved cell apoptosis assay based upon double staining with Cy3 conjugated annexin V and the vital dye 6-CFDA, by using the APOAC apoptosis detection kit (Sigma

Chemical, St Louis, MO, USA). The assay discriminates viable cells ($\text{Cy3}^-/6\text{-CF}^+$), apoptotic cells ($\text{Cy3}^+/6\text{-CF}^+$), and dead cells ($\text{Cy3}^+/6\text{-CF}^-$). We investigated the main parameters involved in the APOAC assay, i.e. the type of fixation (methanol, PFA, or no fixation) and the type of coating of microscope slide (polylysine, Vectabond, Parafilm[®]).

Results indicate that significant improvements in the assay were achieved by staining cells while in suspension and visualization on Parafilm[®] slides, instead of staining cell adherent to microscope slides and subsequent fixation.

Material and methods

Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals and preparation of ovine neutrophils

Populations of ovine neutrophils were obtained from 3 healthy, non pregnant, non lactating sheep, fed a hay and concentrate diet. Animal health was assessed by clinical examination before and during the whole experimental period. All procedures were carried out in respect of the Italian legislation on animal care (DL n.116, 27/1/1992).

Neutrophils were isolated according to the procedure of Carlson and Kaneko (1973). Blood samples (40 mL), collected in EDTA-coated plastic tubes, were divided into 4 aliquots of 10 mL; after centrifugation for 15 min at 1,000g, plasma and buffy coat were removed and the RBC pellet was lysed by adding sterile distilled water (20 mL each tube) and mixing gently for 30 s. Isotonicity was restored by adding 10 mL of NaCl 0.39 M in phosphate buffer 0.0132 M (pH 7.2). After centrifugation for 5 min at 300g, the supernatant was discarded and remaining RBC were removed by a second hypotonic lysis. The resulting pellet of PMNs was collected into a new tube, washed two times with 40 mL of PBS (pH 7.3) and resuspended in 3 mL of HBSS.

Cell viability was determined with Neubauer hemacytometer by the Trypan blue exclusion test (Phillips and Terryberry 1957). Cell purity was

assessed by differential staining with Türk's solution as previously described (Getting et al. 1999). Mono-nuclear cells and PMN were easily identified by their different morphology and nuclear staining (Fig. 1). Cell viability and purity were routinely higher than 99% and 95%, respectively.

Freshly harvested neutrophils were suspended at 1.5×10^6 cell ml^{-1} in basic medium consisting of HEPES-containing RPMI 1640, 10%FBS (low endotoxin, heat inactivated at 56 °C for 30 min), 2 mM L-Gln and cultured at 37 °C and 5% CO_2 (Binder Incubator, Germany).

Apoptosis assay

Spontaneous apoptosis was measured on neutrophils freshly isolated (0 h) and aged for 18 h in an incubator at 37 °C and 5% CO_2 . Freshly isolated cells were considered as negative staining control and 18 h cultured cells as positive staining control (Scaife et al. 2003).

Neutrophils apoptosis rate was determined by using the APOAC Annexin V-Cy3 apoptosis detection kit (Sigma).

In preliminary experiments, we followed the manufacturer's instructions testing two different kinds of microscope slides (coated with polylysine or Vectabond). Neutrophils (10^6 cells) were removed from culture, washed with PBS and 50 μl of this suspension (0.5×10^6 cells) was allowed to adhere to the slide for 10 min at RT. After three washes with 50 μl of binding buffer (10 mM HEPES, pH 7.5, containing

140 mM NaCl and 2.5 mM CaCl_2), the specimen was incubated 15 min on ice, with 50 μl of double staining solution (binding buffer containing 0.1% gelatin, 0.25 μl of Annexin V-Cy3 and 0.25 μl of CFDA). At the end, the specimen was washed with 5 aliquots of 50 μl of binding buffer and fixed on ice with either methanol for 5 min or 2% PFA for 10 min. At the end, the specimen was mounted with 15 μl of binding buffer and visualized under fluorescence microscopy. The fixation step was included to the manufacturer's protocol to avoid nonspecific activation of neutrophils by prolonged contact with the charged surface of the slide (Sartorelli et al. 2000).

Subsequently we optimized the protocol to minimize artifacts due to the fixation and the contact of cells with charged surfaces. Neutrophils (10^6) were removed from culture, washed with PBS and incubated with 100 μl of double staining solution. After 15 min on ice, cells were pelleted at 200g and resuspended in binding buffer and a wet mount was placed on Parafilm[®] covered slides and visualized under fluorescent microscopy.

Microscopy

Fluorescent labels were detected by an Orthoplan microscope (Leica, Germany) equipped with standard fluorescein and rhodamine filter sets; Annexin V-Cy3 staining was visualized with a rhodamine filter (excitation = 535 nm, emission = 550 nm) and 6-CF staining with a fluorescein filter (excitation = 450 nm, emission = 490 nm). Images were recorded using a SV40 Canon camera (Canon Inc., Japan).

Cell counts of viable cells ($\text{Cy3}^-/\text{6-CF}^+$), apoptotic cells ($\text{Cy3}^+/\text{6-CF}^+$), and dead cells ($\text{Cy3}^+/\text{6-CF}^-$), were determined under 400 $\times$ magnification from 10 randomly selected fields containing 40–50 cells.

Data computation and statistical analysis

For statistical analysis, apoptotic (AI) and necrotic (NI) indexes were calculated as the percentage of each subpopulation on total number of counted cells and data were reported as mean $\pm$ SE of the three independent experiments.

Statistical analysis of the apoptotic and necrotic indexes (independent variables) was performed using the factorial model with “treatment” (methanol, PFA,

Fig. 1 Neutrophils lied on Parafilm[®] immediately after isolation. Neutrophils were stained with Türk's solution at 1:20 dilution. Scale bar 8 μm

living cells) and “time” (0 h and 18 h) as fixed effects (SPSSx 1997).

Results and discussion

In this paper we attempted to optimize the APOAC assay protocol for neutrophils apoptosis comparing the standard protocol, using either Vectabond or polylysine coated slides and methanol or PFA fixation, with a modified procedure, using Parafilm[®] coated slides with unfixed cells.

During preliminary experiments, we examined the morphological features obtained with the standard procedure varying the type of coating of the microscope slide (polylysine or Vectabond). With either type of fixative (methanol or PFA), comparison between polylysine or Vectabond coated slides showed that the latter gave better results. By light

microscopy, a higher number of cells were present on Vectabond than on polylysine slides; however the morphology of cells wasn't affected by the type of slide coating (data not showed). This findings suggested that Vectabond is preferable to polylysine.

Under light microscopy, images obtained by the standard procedure with methanol fixation, showed that fixation changed cell shape and topology of the cell surface both at 0 h and 18 h of incubation compared to unfixed cells (Figs. 2a, 3a). In agreement with previous studies (Pleskova et al. 2005), cells resulted were damaged and showed greater size than unfixed cells.

Under fluorescence microscopy, a high percentage of annexin V positive cells in freshly isolated cells was noticed (Figs. 2c, 3c). The resulting staining pattern was most irregular, since fluorescence background both for annexin V and CFDA was high, and the labelling was mostly perinuclear. Moreover, many of

Fig. 2 Apoptosis detection in neutrophils immediately after isolation (0 h). (a), (c), (e), Purified neutrophils were allowed to adhere to Vectabond slides. Cells were stained for annexin V (red) and CFDA (green) and then fixed with methanol. (b), (d), (f), Cells were stained for annexin V (red) and CFDA (green) in suspension and then visualized on Parafilm[®]. The results shown are typical of those obtained from three sheep. Scale bar: a, b, c, d, e, f 8 μ m

the annexin V positive cells were also CF positive (Figs. 2e, 3e), as well as annexin V negative cells were also CF negative, causing a high percentage ($P < 0.05$) of apoptotic and necrotic cells at each incubation time tested (Table 1).

PFA fixation improved preservation of cell shape and surface, compared to methanol and non fixed cells. However, by fluorescence microscopy, CF staining results were not comparable between individual cells, as in some cells was dotted and in others uniformly distributed. Annexin V staining was regularly arranged outside cell membrane, but showed a high fluorescence background (data not shown). In fact, the AI index at 0 h and 18 h did not significantly change (Table 1), probably because aged cells possess reduced adherence capacity.

By light microscopy, cells processed by the modified procedure preserved the morphology of

quiescent cells (Fig. 2b), as they had spherical shape and were above the substrate. By fluorescence microscopy, annexin V stained only the cell surface (Fig. 3d), whereas CF stained uniformly the whole cell (Fig. 3f). Moreover, all the freshly isolated cells (0 h) were vital (Table 1), whilst 18 h aged cells gave an AI of $29.6\% \pm 0.8\%$, confirming previous findings (Scaife et al. 2003).

These results indicate that the modified APOAC assay is significantly better than the standard procedure to detect neutrophil apoptosis. It seems that key improvements are staining cells while in suspension and visualization on Parafilm[®] slides, instead of staining cells adherent to microscope slides and subsequent fixation.

In fact, charged surfaces and fixation procedures, modifying the morphology of cell membrane, could contribute to a misinterpretation of the functional

Fig. 3 Apoptosis detection in aged neutrophils (18 h). (a), (c), (e), Purified neutrophils were allowed to adhere to Vectabond slides. Cells were stained for annexin V (red) and CFDA (green) and then fixed with methanol. (b), (d), (f), Cells were stained for annexin V (red) and CFDA (green) in suspension and then visualized on Parafilm[®]. The results shown are typical of those obtained from three sheep. Scale bar: a, b, c, d, e, f 8 μ m

Table 1 Apoptotic, necrotic, intact cell percentages (mean $\pm$ SE) obtained on ovine neutrophils processed with the different procedures

Fixation	Slide coating	Time	Apoptotic	Necrotic	Intact
Methanol	Vectabond	0 h	33.8 ± 8.4	66.2 ± 8.4	0.0 ± 0.0
		18 h	58.1 ± 33.9	41.2 ± 32.8	0.7 ± 1.2
PFA	Vectabond	0 h	23.0 ± 12.8	0.0 ± 0.0	77.0 ± 12.8
		18 h	18.2 ± 6.7	0.0 ± 0.0	81.8 ± 6.7
Unfixed	Parafilm	0 h	0.4 ± 0.7	0.0 ± 0.0	99.6 ± 0.7
		18 h	29.6 ± 0.8	0.0 ± 0.0	70.4 ± 0.8
Statistical analysis					
Main effects					
Treatment			*	**	**
Time			*	*	*
Treatment × Time			ns	**	**

* $P < 0.05$, ** $P < 0.01$

status of neutrophils; previous studies show that support surfaces can prime ovine neutrophils (Sartorelli et al. 2000) and mimic proinflammatory stimuli, inducing PS exposure with uropod formation (Frasch et al. 2004), but delaying neutrophil apoptosis (Simon 2003). Moreover apoptotic features, such as degradation of actin filaments, increased rigidity of the plasma membrane, and loss of sialic acid residues on cell surface (Hart et al. 2000) cause reduced adhesion of apoptotic cells (Dransfield et al. 1995; Greenstein et al. 2000) to microscope slides and eventually a misleading apoptotic index.

Finally, although there are several methods to identify apoptotic cells by flow cytometric analysis, they have the general problem of discriminating between necrotic and apoptotic cells; at the contrary, such distinction is immediate by morphological assay. Therefore morphological and flow cytometry techniques all are essential and contribute to the evaluation of apoptosis.

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