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Letter to the Editor

Viable, apoptotic and necrotic monocytes expose phosphatidylserine: cooperative binding of the ligand Annexin V to dying but not viable cells and implications for PS-dependent clearance

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Dear Editor,

An enigma exists with respect to the recognition and phagocytosis of phosphatidylserine (PS)-exposing cells. Vital PS-exposing cells (e.g. activated B cells[1,2] or neutrophils in Barth syndrome)[3] are swallowed neither by amateur nor professional phagocytes. In this letter, we show that a restricted lateral mobility of PS in the plasma membrane might be responsible for this.

A common method of detecting PS on a cell surface is the use of the PS-binding protein Annexin V (AxV; MW: 35 kDa). Annexins are amphipathic molecules. Their interaction with anionic phospholipids is dependent on Ca^{2+} ions and reversible in the presence of chelators of divalent cations like EDTA.[4,5] Interestingly, some annexins, like AxV, build multimeric complexes on membrane surfaces. The calcium-induced formation of trimers, hexamers and bigger aggregates has been demonstrated.[6] Therefore, the aggregation of AxV leads to the formation of planar crystals on artificial lipid bilayers. Several different shapes of the three-dimensional structure are described.[7,8] These complexes do not penetrate the membrane. AxV binds with high affinity to the negatively charged phospholipid PS. The Kd has been reported to have values between 15 and 0.03 nM.[9–11] Cooperativity has been reported for the interaction with Ca^{2+}.[12]

Freshly isolated, viable monocytes (00h-wo in Figure 1a) as shown by an intact mitochondrial membrane potential (Ψ_m; DiO_6C), no morphological changes of the nucleus (nSSC), G1/0 DNA content (PI-Triton) and no detectable caspase activity (zVAD-fitc) were analysed for their AxV binding in the presence and absence of EDTA. Interestingly, these viable cells already showed a considerable amount of AxV-binding sites (PS). Note that the exposure by freshly isolated monocytes of PS is 1–2 orders of magnitude below that of their apoptotic counterparts (after UV irradiation; 24-uv in Figure 1a). The PS amount decreased during culture of the primary monocytes (24-wo). Instead, the number of necrotic cells slightly increased, as detected by PI staining. Apoptosis in irradiated cells (24-uv) was detected by loss of the Ψ_m, high caspase activity, sub-G1 DNA content and an increase of granularity.

In an attempt to investigate the binding efficiencies of AxV to PS on cells, we chose vital and dying cells as PS bearer to simulate the *in vivo* situation. This setting also has the advantage that, theoretically, PS is mobile and not fixed to any kind of rigid surface. In a quantitative analysis of the binding capacity for CD14, CD54 and PS, typical saturation curves could be detected for all three targets at high concentrations of the ligand (Figure 1b). Surprisingly, in the range under 50 nM AxV, the binding was below expectations (Figure 1b; arrow). The AxV (target PS)-binding curve revealed a strong sigmoid character as compared to the others. Curves like this are indications for a specific attribute of allosteric proteins (consisting of several subunits) named positive cooperativity. As examples for noncooperative binding curves, we titrated mAb to CD14 and CD54 (Figure 1b).

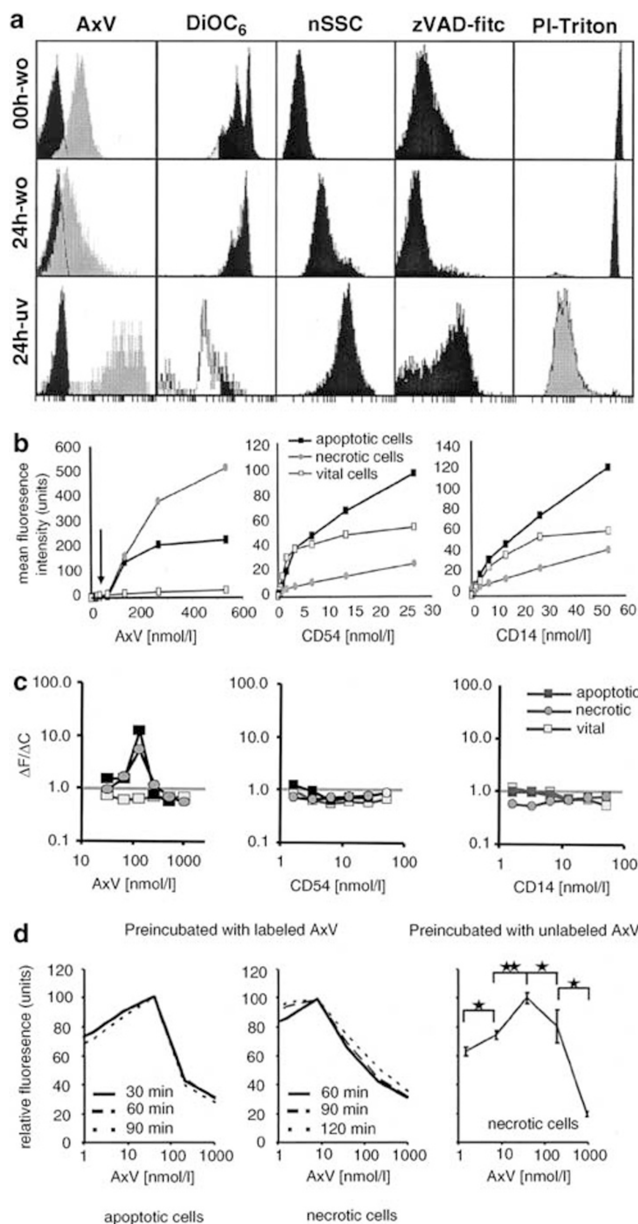
This indicates that a saturation of PS-binding sites on apoptotic cells was reached only above 250 nmol/l of AxV. For routine applications, concentrations around 500 nmol/l are used. AxV contains four PS-binding domains. Therefore, it can be calculated that approximately 10^9 binding sites for AxV were present on each apoptotic monocyte. The value for necrotic cells is up to 2.5 times higher. Vital monocytes on the other hand displayed six times less AxV-binding sites.

Thereafter, we analyzed whether AxV shows positive or negative cooperativity for PS binding on membranes. The classical way is the determination of the Hill coefficient (Figure 1c). A Hill coefficient above 1 indicates positive and below 1 negative cooperativity. A Hill coefficient of 1 indicates no cooperativity and follows the Michaelis–Menten scheme. We adjusted the Hill equation to the fact that we worked with two different units (fluorescence and concentration) that do not fit into the original equation. In analogy, we determined the factor of increase in fluorescence from one concentration of AxV, CD14 or CD54, respectively, to the doubled concentration ($\Delta F/C = \Delta F/(2C/C) = \Delta F/2$). Strikingly, only AxV binding to apoptotic or necrotic but not to vital cells showed a nonlinear increase and produced a curve with a maximum at approximately 130 nmol/l. This is a second unequivocal evidence for a positive cooperative effect. The mAb CD14 and CD54 control were always close to 1 and, therefore, displayed no signs of a cooperative effect.

Especially note that vital monocytes (as an example of PS-exposing cells that are not phagocytosed (not shown)) did not promote cooperative binding. This suggests that AxV needs a critical density or clustering of PS molecules, or that its mobility is not sufficient for cooperative behavior. It might also be that AxV needs an undefined co-factor, which is present

only on dying cells. As a matter of course, an overall binding constant cannot be determined from a cooperative interaction.

Afterwards, we analyzed the influence of increasing amounts of unlabeled AxV in competition with a fixed amount of labeled AxV to exclude that the cooperative effect was generated by the FITC label. Astonishingly, we found that competing FITC-labeled AxV with unlabeled AxV led to an increase in fluorescence up to an excess of at least 5.7 times for AxV concentrations below 100 nM. Even more surprising was the reversed experiment. Pre-incubation with varying amounts of unlabeled AxV and chasing with labeled AxV basically gave the same results. It can be concluded that unlabeled AxV cooperates with the FITC-labeled AxV and so increased the binding of free-labeled AxV. We showed that AxV binds to dying monocytes in a co-operative manner, as an example of cells that are phagocytosed (not shown).



In conclusion, we demonstrated that Annexin V displays homotrophic, positive cooperativity upon binding to membranes, with high PS amounts as found in dying cells. This means that one AxV physically interacts with another to change its conformation in such a way that it improves the binding abilities (lowers the binding energy) to PS. It has been described that AxV shows a crystal-like structure on PS liposomes.[6] We propose that those crystals consist of AxV polymers. The AxV polymers are not due to covalent interactions because on/off rates can be seen when chasing with unlabeled AxV. This has also been demonstrated with human monoclonal antiphospholipid antibodies.[13]

PS exposure on apoptotic or necrotic cells is an ‘eat me’ signal for phagocytes.[14] We demonstrated that PS is in the outer membrane leaflet of vital cells. But, in this case, it has obviously no ‘eat me’ function. We suggest that the PS density is too low to induce the ‘eat me’ signal. In analogy to the binding of AxV described in this letter, this may be due to a combination of the following reasons:

- the membrane fluidity and, therefore, the mobility of PS is too low,
- or the mechanisms for removing PS from the outer leaflet of the plasmamembrane (flip-flop) are too fast,

Figure 1 (**a**) Viable monocytes expose Annexin V-binding sites (e.g. PS). Note that freshly isolated monocytes already expose PS, but by 1–2 orders of magnitude less than their apoptotic counterparts (after UV irradiation). The analysis was performed after 0 or 24 h with untreated (wo) or UV-irradiated (uv) cells. The samples were stained for detection of PS with Annexin V-FITC (AxV-FITC) with (black) or without (gray) the addition of EDTA; for membrane potential with DiO_6C; for nuclear granularity (nSSC); for caspase activity (zVAD-FITC); and for DNA content (propidium iodide in the presence of Triton X-100 = PIT). Untouched monocytes were isolated from PBMC by depletion of other cells with magnetic beads. (**b**) Apoptotic, necrotic and vital primary human monocytes (1×10^5 cells/350 μl) from human normal healthy donor (NHD) were either stained with 14 different concentrations (534 nmol/l geometrically diluted by a factor of two (D factor 2) down to 0.07 nmol/l) of FITC-conjugated recombinant human Annexin V or 14–15 different concentrations of PE-conjugated anti-CD14 or anti-CD54 (53.2 nmol/l D factor 2 down to 0.003 nmol/l) monoclonal antibodies (mAb), respectively. After 45 min of incubation at room temperature, probes were subjected to FACS analysis. (**c**) Apoptotic, necrotic and vital primary monocytes (1×10^5 cells/350 μl) were separately stained with eight different concentrations of FITC-conjugated recombinant human Annexin V (1068 nmol/l D factor 2 down to 8.34 nmol/l) or with anti-CD14 or anti-CD54 mAb (53.2 nmol/l D factor 2 down to 0.42 nmol/l), respectively. After a 45 min incubation time, probes where measured by cytofluorometry. Note that AxV binds in a cooperative manner to apoptotic and necrotic cells but not to viable ones. (**d**, left and middle) Necrotic and apoptotic cells from human monocyte cell line U937 (1×10^5 cells/560μl) were stained with FITC-conjugated recombinant human Annexin V (6.675 nmol/l) and incubated at room temperature for 45 min. Thereafter, cells were chased with five different concentrations of unlabeled human Annexin V (951.9, 190.4, 38.1, 7.6, 1.5 nmol/l). In this experiment, we used Ringer's solution to obtain a final volume of 560 μl for each probe. Cells were analyzed by FACS immediately after staining and were re-measured after 30, 60, 90 and 120 min. The process is fast and basically completed at the first time point. One out of five experiments essentially showing the same results is displayed. (**d**, right) Necrotic U937 cells were separately preincubated for 45 min at room temperature with five different concentrations of unlabeled human Annexin V (951.9, 190.4, 38.1, 7.6, 1.5 nmol/l). Afterwards, cells were incubated with FITC-conjugated recombinant human Annexin V (6.675 nmol/l) at room temperature for 3.5 h. Cells were analyzed in triplicates by FACS. The mean value was deduced within triplicates. The experiment was repeated three times. One exemplary measurement is shown. Significant data points (Student's *t*-test) are indicated with an asterisk and highly significant ones with two. Apoptosis in human monocytes and human monocyte cell line U937 was induced by irradiation with UV-B (120 mJ/cm^2). For the induction of necrosis, cells were incubated for 30 min at 57°C

- or the amounts of PS are too low for clustering of PS receptors in phagocytes, which might be necessary for the 'eat me' signal.

The data suggest a mechanism for phagocytes to differentiate dying and vital cells by means of PS clustering and/or lateral mobility due to membrane fluidity. This might explain why vital cells are not subject to phagocytosis.

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