Molecular mechanisms of complement activation by damaged cells
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Citation for published version (APA):

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Complement activation by apoptotic cells in normal plasma occurs predominantly via IgM and is limited to late apoptotic (secondary necrotic) cells.

IgM activates complement on late apoptotic cells

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* Supported by grants from the Dutch League against Rheumatism: nr. 97.402.01, and the Dutch Platform for Alternatives to Animal Experiments: nr 98-08

Autoimmunity
2004 37: 96-102
Abstract

Apoptotic cells activate complement via various molecular mechanisms. It is not known which of these mechanisms predominate in a physiological environment. Using Jurkat cells as a model, we investigated complement deposition on vital, early and late apoptotic (secondary necrotic) cells in physiological medium, human plasma, and established the main molecular mechanism involved in this activation.

Upon incubation with recalcified plasma, binding of C3 and C4 to early apoptotic cells was similar to background binding on vital cells. In contrast, late apoptotic (secondary necrotic) cells consistently displayed substantial binding of C4 and C3 and low, but detectable, binding of C1q. Binding of C3 and C4 to the apoptotic cells was abolished by EDTA or Mg-EGTA, and also by C1-Inhibitor or a monoclonal antibody that inhibits C1q binding, indicating that complement fixation by the apoptotic cells was mainly dependent on the classical pathway. Late apoptotic cells also consistently bound IgM, which binding significantly correlated with that of C4 and C3. Depletion of plasma for IgM abolished most of the complement fixation by apoptotic cells, which was restored by supplementation with purified IgM.

We conclude that complement binding by apoptotic cells in normal human plasma occurs mainly to late apoptotic, secondary necrotic cells, and that the dominant mechanism involves classical pathway activation by IgM.

Introduction

For adequate cell homeostasis in multicellular organisms a highly regulated turnover of cells is essential. This turnover involves proliferation and differentiation on one hand, and cell death according to an orderly process called apoptosis, on the other. The general assumption is that the majority of apoptotic cells are cleared from the body without eliciting inflammation. An early event in the apoptotic process is the exposure of phosphatidylserine [1] and phosphatidylethanolamine [2] which in vital cells are mainly confined to the inner leaflet of the membrane, a phenomenon referred to as membrane ‘flip-flop’. Exposure of phosphatidylserine is sufficient for phagocytosis of the apoptotic cell, because phagocytic cells possess specific receptors for this phospholipid [3]. However, the vitronectin receptor (CD51/CD61), the scavenger receptor type A and B, the Ox-LDL receptor (CD68), the LPS receptor (CD14) and the tyrosine kinase family receptor, MER [4-9], may contribute to the clearance of apoptotic cells as well.
Although direct recognition of phosphatidylserine exposed in the outer leaflet of the membrane by phagocytic receptors likely is important for phagocytosis of apoptotic cells, recognition in vivo may be modulated by proteins in plasma or the interstitial fluid that bind to phosphatidylserine or other structures exposed on apoptotic cells. Indeed, apoptotic cells have been shown to bind such diverse proteins as thrombospondin, pentraxins (serum amyloid P component (SAP), C-reactive protein (CRP), pentraxin-3 (PTX-3)), immunoglobulins, collectins (C1q, mannan binding lectin (MBL), surfactant protein A and D), complement (C3, C4), and apolipoproteins (β2-glycoprotein-I, clusterin) [10-22]. Consequently, receptors for these proteins, such as the thrombospondin/vitronectin receptor complex and complement and surfactant receptors contribute to the clearance of apoptotic cells as well.

Recently, attention has been focussed on the importance of complement in the clearance of apoptotic cells. The finding that C1q binds to apoptotic cells [16], in combination with the well known strong association between C1q deficiency and the development of systemic lupus erythematosus (SLE), have put forward the hypothesis that C1q deficiency could predispose to SLE because of a defect in the clearance of apoptotic cells [16, 23]. Mice deficient in C1q indeed display disturbed phagocytosis of apoptotic cells [24] and develop glomerulonephritis associated with accumulation of apoptotic cell debris [23].

Though it is now generally accepted that apoptotic cells can activate and bind complement, there is no agreement regarding the molecular mechanism of this activation. Multiple mechanisms including direct binding of C1q or MBL, indirect binding of C1q via adaptor molecules such as CRP, SAP or immunoglobulins, and also alternative pathway activation, could explain complement activation by apoptotic cells [12, 15, 19, 20]. Which of these mechanisms dominate under physiological conditions, is not well understood. Moreover, though it has been shown that complement activation to apoptotic cells is a late event and that many other features of apoptosis, such as loss of mitochondrial transmembrane potential, PS exposure, hypergranularity and nuclear fragmentation precede complement binding [25], it is also not well known at which stage of apoptosis cells can activate complement under physiological conditions. In the present study we investigated the molecular mechanisms of complement activation in a physiological medium, i.e. human plasma. In addition, we studied during which stage of the apoptotic process cells activate complement. Our results indicate that particularly late apoptotic cells activate complement, and the dominant molecular mechanism of this activation involves IgM.
Materials and Methods

Materials

Mouse monoclonal antibodies (mAbs) specific for C1q (C1q-2 and C1q-85), C4 (C4-4), C3 (C3-9), CRP (5G4), SAP (SAP-14) and MBL (5E12) were developed in our laboratory [11, 26-28]. C1-Inhibitor (Cl-Inh) and mAbs specific for IgG heavy chain (MH16) and IgM heavy chain (MH15) were obtained from the CLB (Amsterdam, Netherlands). Etoposide was purchased from Sigma (St. Louis, MO), streptavidin-allophycocyanin (strep-APC) from Pharmingen (San Diego, CA) and Annexin-V-FITC from BenderMed (Vienna, Austria). CNBr-Sepharose was purchased from Pharmacia Biochem (Uppsala, Sweden).

Cell culture and apoptosis induction

Jurkat cells were maintained in culture medium (1MDM, 5% (v/v) fetal calf serum (FCS), penicillin, streptomycin and 50 μM β-mercaptoethanol, further referred to as CM). Before apoptosis induction, cells were washed twice in CM without FCS (CM'), to prevent interaction between cells and proteins in FCS. 0.5-2x10^6 Jurkat cells per ml were resuspended in CM' and etoposide was added to yield a final concentration of 200 μM. Cells were then incubated for 6 hours which yielded mainly early apoptotic cells (Annexin-V-positive, propidium iodide-negative (PI')) or 16 hours to obtain late apoptotic cells (AnnV', PI').

Recalcified plasma and IgM depletion

Healthy donor blood was collected in vials containing sodium citrate (10 mM final concentration). Plasma was prepared by centrifugation for 15 min at 3000 x g and transferred into a glass tube. Then CaCl_2 was added (10 mM final concentration) and the mixture was incubated for 15 min at 37°C to allow clotting, followed by incubation on ice for 30 min for clot retraction. The clot was removed and the recalcified plasma was stored at -20°C until further use.

IgM-depleted plasma was prepared with an affinity column, which consisted of mAb MH15 coupled to CNBr-sepharose. Prior to IgM depletion, recalcified plasma was supplemented with EDTA (10 mM final concentration) and NaCl (0.5 M final concentration) to prevent C1q binding and subsequent complement activation. The IgM was eluted from the column with 0.1 M glycin pH 2.5 and immediately supplemented with 1 M TRIS until the pH was 7.5. Both the IgM-depleted plasma and the IgM were subsequently dialyzed against 5
mM veronal buffer pH 7.4 containing 150 mM NaCl for 16 hours at 4°C and stored at -20°C until use.

**FACS analysis**

The mixed apoptotic cell cultures were washed twice in CM and suspended at 5x10^5 - 1x10^6 cells per ml. Hundred µl of the cell suspension were then transferred into a 96-well round bottom plate. Subsequently, 100 µl of dilutions of recalcified plasma in CM were added and the mixtures were incubated for 60 min at 37°C. The cells were then washed twice in ice-cold FACS buffer (HEPES 10 mM pH 7.2 containing NaCl 150 mM, KCl 5 mM, CaCl_2 2 mM, MgCl_2 2 mM) and incubated with 2 µg/ml biotinylated mAb specific for C3, C4, C1q, CRP, SAP, IgG or IgM for 45 min at 4°C. Next, the cells were washed twice in FACS buffer, and then incubated with 250 ng/ml strep-APC and 1:200 diluted AnnV-FITC in FACS buffer for 20 min at 4°C. Finally, the cells were washed in FACS buffer and PI was added at a final concentration of 500 ng/ml and the samples were immediately evaluated with a Becton Dickinson FACSCalibur (Mountain View, CA). Vital, early and late apoptotic cells were gated according to their AnnV and PI staining properties and their corresponding protein binding was expressed as the median fluorescence intensity (MFI) of allophycocyanin on a logarithmic scale. FACS data were analyzed using WinMDI (v8) free-downloadable software (http://www.facs.scripps.edu/).

**Complement inhibition**

To investigate the mechanism of complement binding to apoptotic cells, several inhibitors of complement activation were evaluated. EDTA at a final concentration of up to 10 mM was used to inhibit all pathways of complement since activation of these requires Ca^{++} and/or Mg^{++}-ions. Mg-EGTA at a concentration of up to 10 mM was used to block both the classical and the MBL-pathway as they both require Ca^{++} and Mg^{++}-ions, whereas the alternative pathway only needs Mg^{++}-ions for activation. Cl-Inh and mAb anti-C1q-85 (IgG1-isotype), which inhibits C1q binding and C1 activation, were added to 10% plasma in CM, v/v, to yield final concentrations of 1-5 mg/ml and 5-100 µg/ml, respectively, and incubated for 20 min at room temperature. Thereafter, the preincubated plasma was added to cells and protein binding was detected as described above.
Statistics

To analyze differences in protein binding to vital cells, early apoptotic cells and late apoptotic cells, paired Student t-tests were performed. Correlation between parameters was assessed with Pearson correlation analysis. A two-sided P-value less than 0.05 was considered to represent a significant difference or correlation.

![Graphs of C1q, C4, and C3 binding to vital, early, and late apoptotic cells.]

Figure 1. Binding of complement components to vital cells, early apoptotic cells and late apoptotic cells. Apoptotic cells were incubated with several dilutions of recalcified plasma from a healthy donor for 60 min at 37°C, and triple-stained with AnnV-FITC, PI and biotinylated mAbs specific for C1q, C4 or C3 that were subsequently detected with strep-APC. Curves represent the median fluorescence activity (MFI) of complement binding.

Results

**Binding of complement to apoptotic cells**

Jurkat cells incubated with etoposide for 6 hours, yielded on average 40-60% early apoptotic cells (AnnV+/PI), 40-60% vital cells (AnnV+/PI) and less than 5% late apoptotic cells.
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(AnnV+/PI–). When cells were incubated for 16 hours 50-70% late apoptotic cells (AnnV+/PI–) were obtained whereas the remaining cells were vital. By microscopy over 95% of the PI+ cells had condensed or fragmented nuclei characteristic of apoptosis. These cells were thus considered to be late apoptotic cells.

Figure 2. The effect of Clq inhibiting monoclonal antibodies on the binding of C4 and C3 to late apoptotic cells. 10% recalcified plasma was incubated with 100 μg/ml Clq-85 for 20 min before it was added to apoptotic cells. Then cells were stained with AnnV-FITC, PI and biotinylated antibodies specific for C4, C3 and IgM that were subsequently detected with strep-APC. The histograms represent the MFI of C4 and C3 binding to late apoptotic cells in the presence (thick line) or absence (shaded histogram) of Clq-85.

When cells were incubated with various dilutions of recalcified normal human plasma, the binding of C4 and C3 to early apoptotic cells were slightly increased compared to vital cells whereas that to late apoptotic cells was about 100 times increased (fig 1). Binding of Clq to early apoptotic cells was only observed at higher plasma concentrations, whereas that to late apoptotic cells was moderately increased at all plasma concentrations tested. We decided to evaluate the mechanisms of activation at 10% plasma, unless mentioned otherwise.

Inhibition of complement binding by complement inhibitors

Complement activation by apoptotic cells has been described to occur via all three known activation pathways. To further analyze the contribution of each of these pathways to complement activation by apoptotic cells in plasma, we tested several complement inhibitors for their ability to block activation by late apoptotic cells in normal plasma. Each inhibitor was first tested at several doses to assess the optimal concentration. The overall results are summarized in table 1. EDTA completely abolished C3 binding to late apoptotic cells, whereas a residual 9% of C4 binding was observed, confirming that the observed fixation of complement fragments to the cells resulted from an activation process, rather than from aspecific binding. Mg-EGTA reduced C4 and C3 binding to 2% and 4%, respectively, ruling out a major role for the alternative pathway. Inhibition of Clq binding onto apoptotic cells
with mAb anti-C1q-85, which inhibits binding of C1q to immune complexes, resulted in an average 6% residual C4 and 3% residual C3 binding to late apoptotic cells (fig. 2). The inhibition of activation by C1-Inh yielded similar results for C4 and C3 binding which were on average 9% and 5%, respectively. Notably, in these experiments C1-Inh was used at supraphysiological concentrations (5 mg/ml) to obtain maximal inhibition. Results obtained with plasma samples from 3 separate experiments were comparable (table I). Hence, these results pointed to the classical pathway as being the dominant route of complement activation by apoptotic cells in plasma.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>C4 (%)</th>
<th>C3 (%)</th>
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<tbody>
<tr>
<td>100 μg/ml antiC1q-85</td>
<td>6 (0-18)</td>
<td>3 (1-5)</td>
</tr>
<tr>
<td>5 mg/ml C1-Inh</td>
<td>9 (0-18)</td>
<td>4 (3-5)</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>9 (0-18)</td>
<td>0</td>
</tr>
<tr>
<td>10 mM Mg-EGTA</td>
<td>2</td>
<td>4 (2-6)</td>
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Table I. Inhibition of the binding of complement to late apoptotic cells by complement inhibitors. Late apoptotic cells were incubated with recalcified plasma in the presence of complement inhibitors at the indicated final concentration. C4 and C3 binding to the cells was determined by FACS analysis as described in methods. Values represent the percentage (range) of residual complement binding as observed in 3 experiments. The MFI of complement binding in the absence of inhibitors was set at 100%, that in the absence of plasma (i.e. background staining) at 0%.

Involvement of adaptor molecules in classical pathway activation

Several proteins in normal human plasma (IgM, IgG, CRP, SAP and MBL) may act as adaptor molecules that influence complement activation by apoptotic cells. Examples of binding of adaptor molecules are shown in fig. 3. To get insight into the contribution of these molecules to the observed complement activation by apoptotic cells, we investigated binding of complement as well as adaptor molecules to the apoptotic cells in plasma samples from 10 different healthy donors. The correlation between binding of adaptor molecules and that of complement to the cells was then assessed. Analysis of the results with the 10 plasma samples revealed that binding of C3 or C4 to early apoptotic cells was not stronger than that to vital cells. As a matter of fact, vital cells bound somewhat more C3 in some plasma samples (not shown). C1q binding to vital or early apoptotic cells was negligible in all plasma samples.
Figure 3. Binding of adaptor proteins potentially involved in complement binding to vital cells, early apoptotic and late apoptotic cells. Apoptotic cells were incubated with several dilutions of recalcified plasma from a healthy donor for 60 min at 37°C, and triple stained withAnnV-FITC, PI and biotinylated mAbs specific for IgM, IgG, SAP, CRP and MBL that were subsequently detected with strep-APC. Curves represent the MFI of protein binding.

Compared to vital or early apoptotic cells, late apoptotic cells displayed increased binding of C4 and C3 (fig 4A), and of adaptor proteins IgG, IgM, SAP and CRP (fig 4B). Also a slight but significant Clq binding was observed with late apoptotic cells upon incubation with the various plasma samples, whereas MBL binding to late apoptotic cells was absent. To assess a potential link between the binding of adaptor proteins to late apoptotic cells on one hand and that of complement on the other hand, the association between complement binding and that of the adaptor molecules was studied. Of the latter, IgM binding significantly correlated to the binding of C4 and C3 ($r_p = 0.65, p = 0.04$ and $r = 0.83, p = 0.003$, respectively; fig. 5). The correlation between IgM and Clq binding was not significant ($r_p = 0.29, p = 0.41$), nor were the correlations between IgG, CRP or SAP with C4 or C3, respectively (table II).
Table II. Correlations between binding of adaptor proteins and complement to late apoptotic cells. Late apoptotic cells were incubated with recalcified plasma from 10 healthy donors, and assessed for binding of C3 and C4, and that of IgM, IgG, CRP and SAP. The correlation between binding of C3 or C4 and that of the proteins mentioned was then calculated (Pearson’s correlation coefficient).

Inhibition of complement binding in IgM-depleted plasma

The correlation between IgM binding and complement binding to late apoptotic cells suggested that IgM might play a dominant role in complement binding in normal human plasma. Therefore, we studied the effect of IgM-depletion on C4 and C3 binding. Recalcified plasma was depleted from IgM after two passages over the anti-IgM heavy chain affinity column. The residual IgM level in the IgM-depleted plasma was less than 10 μg/ml IgM, determined by IgM-ELISA (not shown). After incubation of late apoptotic cells with 10% IgM-depleted plasma, the residual C4 binding of these cells was approximately 25%, whereas no residual IgM binding was observed (fig. 6). After incubation with 40 μg/ml purified IgM no C4 binding was detected, showing that no complement activation had occurred during the IgM-depletion procedure. Reconstitution of 10% IgM-depleted plasma with purified IgM (40 μg/ml final concentration) completely restored the binding of C4 to late apoptotic cells (fig 6).
Figure 5. Correlation between the binding of IgM and C4 (A) or C3 (B) to late apoptotic cells after incubation with 10% plasma (n=10 healthy donors). IgM binding significantly correlated to C4 binding (r=0.65, p=0.04,) and C3 binding (r=0.83, p=0.003).

Discussion

In this study we investigated the mechanism of complement activation by apoptotic cells in a physiological environment, i.e. plasma. Late apoptotic cells strongly fixed complement, which fixation was virtually abolished by a mAb that inhibits Clq binding to immune complexes. Late apoptotic cells also strongly bound immunoglobulins G and M, as well as the pentraxins CRP and SAP. Depletion of IgM abrogated most of the C4 binding. In contrast, early apoptotic cells hardly bound C4. Thus, these data indicate that late, but not early apoptotic cells, activate complement in normal human plasma, and that the dominant mechanism involves binding of IgM.

Figure 6. The effect of IgM depletion and reconstitution on IgM and C4 binding to late apoptotic cells. Apoptotic cells were incubated with 10% plasma (A), 10% IgM-depleted plasma (B), 40 μg/ml purified IgM (C) and 10% IgM-depleted plasma which was reconstituted with 40 μg/ml IgM (D). Cells were stained with AnnV-FITC, PI and antibodies specific for IgM and C4. The binding of IgM (black bar) and C4 (white bar) to late apoptotic cells was evaluated. Bars represent the binding of protein expressed as a % of maximal protein binding (which was considered to be 100% in 10% IgM sufficient plasma and 0% in the absence of plasma).
Although a number of studies have demonstrated complement binding and activation by apoptotic cells [12, 15-17, 19, 20, 24, 25] there is surprisingly little data about the stage of apoptosis that cells start to activate complement. The observation that C4 and C3 binding does not occur during the early stage of apoptosis, during which stage the cell membrane is impermeable, though flip-flopped, suggests that complement is probably not important for early apoptotic cell opsonization. Hence, during this stage the exposure of PS in the outer leaflet of the cell membrane likely results in the prompt elimination of apoptotic cells by PS receptors present on professional and amateur scavenger cells [32-34].

Presumably, other mechanisms could attribute to removal of apoptotic cells as well. These mechanisms include elimination via Fc-receptors, the mannose receptor, the LRP-1 (LDL-related receptor protein-1)/calreticulin complex, LRP-2 (megalin) and surfactant receptors [17, 18, 30, 31]. Notably, these mechanisms may not elicit inflammatory reactions. Our data imply that unsuccessful removal of apoptotic cells during the early stages of apoptosis may result in complement activation by the cells during a later stage, and give rise to inflammatory reactions.

We observed that IgM binding to late apoptotic cells was strongly increased compared to early apoptotic cell binding. Recently it has been shown that phospholipids that are oxidatively modified in the apoptotic process and lysophospholipids constitute epitopes for natural IgM [14,15]. These studies did not address the stage of the apoptotic cell. Our results show that the exposure of these epitopes for IgM does not occur in early apoptotic cells, as IgM binding was largely limited to late apoptotic (secondary necrotic) cells. We assume that the formation of oxidized phospholipids and lysophospholipids causes damage to the apoptotic cell membrane, which not only results in exposure of neo-epitopes but also gives rise to apoptotic cell membrane leakage. Cell leakage results in the exposure of other intracellular epitopes that have been involved in IgM binding such as DNA and cytoskeleton as well [35,36].

C4 and C3 binding to the late apoptotic cells were almost completely dependent on C1q, as C1-Inh as well as an inhibiting anti-C1q mAb abrogated binding of C4 and C3. Previous studies have shown that purified C1q strongly bound to apoptotic cells [16,17]. We compared the binding of whole plasma C1q to purified C1q. Preliminary experiments indicated a stronger binding of purified C1q to apoptotic cells compared to plasma tested at an equimolar concentration of C1q (not shown). A possible explanation could be that C1q forms complexes with C1r and C1s in recalcified plasma, that have different binding properties to late apoptotic cells compared to purified C1q.
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The strongly increased binding of complement to apoptotic cells that we observed was in accordance with a previous study [25]. In that latter study, the mechanism of complement binding to late apoptotic cells was not determined. We observed that the binding of IgM to late apoptotic cells significantly correlated to C4 and C3 binding. In contrast, no correlation was observed between C3 and C4 binding and that of IgG, CRP and SAP. In IgM-depleted plasma C4 binding was strongly decreased and reconstitution with IgM completely restored the binding of C4. In contrast, the recovery of C3 binding after reconstitution with IgM was 60% (not shown). At present, we have no explanation for this reduced C3 binding. We conclude that a dominant mechanism of complement activation employed by late apoptotic cells apparently is fixation of IgM to intracellular epitopes and classical pathway activation by fixed IgM. Yet, IgM depletion did not completely abolish complement fixation by the late apoptotic cells. The residual C4 and C3 binding that was observed in IgM-depleted plasma was abrogated by anti-C1q mAb, and hence may involve IgG, CRP, SAP, or direct binding of C1q. We are currently investigating these possibilities.

Recently, it has been shown that MBL binds to apoptotic cells [17]. MBL complexes with the MBL-associated serine proteases 1 and 2 (MASP 1 and 2) which form a proteolytic complex that cleaves and activates C4. We presume that a role for MBL in complement binding to late apoptotic cells is limited, if any, as MBL binding to late apoptotic cells was not observed. Moreover, preincubation of plasma with 50 mM mannose, a potent inhibitor of MBL binding, had no effect on complement binding to late apoptotic cells (not shown). Finally, we did not observe enhanced binding of C3 or C4 to late apoptotic cells in MBL-deficient plasma upon supplementation with functional MBL.

With the use of a triple-staining flowcytometric method we could determine plasma protein binding to cells in various stages of apoptosis simultaneously. We demonstrated that IgM-mediated complement binding is mainly limited to late apoptotic cells. Thus, complement-independent mechanisms are likely involved in the clearance of early apoptotic cells. In contrast, both IgM and complement mainly act in a late apoptotic phase, i.e. is when cells become leaky and expose oxidized phospholipids, lysophospholipids as well as intracellular epitopes. It is tempting to speculate that IgM and complement constitute a back-up system promoting the clearance of apoptotic cells at a late stage when early clearance mechanisms have failed. Interestingly, preliminary data show that primary necrotic cells (Jurkat cells treated with 80% ethanol) bind IgM and complement to the same extent as late apoptotic cells, suggesting that loss of cell membrane impermeability triggers IgM and complement binding. Complement binding to late apoptotic cells, and presumably primary
necrotic cells, results in dead cell clearance at the expense of inflammation.

Abbreviations: SAP, serum amyloid P component; CRP, C-reactive protein; MBL, mannan binding lectin; C1-Inh, C1-esterase inhibitor; CM, culture medium; CM', culture medium without FCS; Annexin-V; PI, propidium iodide; strep-APC, streptavidin-allophycocyanin

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