

# Macrophage-mediated clearance of cells undergoing caspase-3-independent death

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## Abstract

Little is known of the functions of caspases in mediating the surface changes required for phagocytosis of dying cells. Here we investigate the role played by the effector caspase, caspase-3 in this process using the caspase-3-defective MCF-7 breast carcinoma line and derived caspase-3-expressing transfectants. Our results indicate that, while certain typical features of apoptosis induced by etoposide – namely classical morphological changes and the ability to degrade DNA into oligonucleosomal fragments – are caspase-3-dependent, loss of cell adhesion to plastic and the capacity to interact with, and to be phagocytosed by, human monocyte-derived macrophages – both by CD14-dependent and CD14-independent mechanisms – do not require caspase-3. Furthermore, both etoposide-induced caspase-3-positive and -negative MCF-7 cells suppressed proinflammatory cytokine release by macrophages. These results demonstrate directly that cell surface changes that are sufficient for anti-inflammatory clearance by human macrophages can be regulated independently of stereotypical features of the apoptosis programme that require caspase-3. *Cell Death and Differentiation* (2003) 10, 302–312. doi:10.1038/sj.cdd.4401170

**Keywords:** caspases; monocytes/macrophages; cell surface; CD14; phosphatidylserine; inflammation; phagocytosis

**Abbreviations:** PS, phosphatidylserine; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; ISEL, *In situ* end labelling; PS-R, PS-receptor; ICAD, inhibitor of caspase-activated DNase

## Introduction

The final stage in the apoptosis programme is the phagocytosis of the dead and dying cells and their membrane-bound fragments.<sup>1,2</sup> At body surfaces, apoptotic cells can be lost to the external environment, while elsewhere they are phagocytosed either by amateur phagocytic neighbours, or by macrophages, the professional phagocytes. Apoptotic cells fail to elicit proinflammatory responses and can exert anti-inflammatory effects on macrophages as evidenced by their release of soluble anti-inflammatory mediators, TGF- $\beta$ 1, PGE<sub>2</sub> and PAF, in response to apoptotic cells.<sup>3</sup> Since the persistence of apoptotic cells can have detrimental consequences such as inflammation and autoimmune disease, their efficient phagocytosis is of significant biological value.

The molecular mechanisms that underlie the recognition and phagocytosis of apoptotic cells by macrophages are not clear. Little is yet known of the changes at cell surfaces that are induced by the apoptosis programme to support apoptotic-cell clearance apart from the loss of plasma-membrane phospholipid asymmetry illustrated by the externalisation of phosphatidylserine (PS), which is normally resident in the inner leaflet of the plasma membrane.<sup>4–6</sup> Other, as yet ill-defined, cell-surface changes that support apoptotic-cell/macrophage interactions include alterations in surface carbohydrates<sup>7,8</sup> and qualitative changes in the intercellular adhesion molecule (ICAM)-3 on apoptotic leukocytes.<sup>9</sup> On the side of the macrophage, a growing body of evidence indicates that a variety of receptors of diverse structure are involved in apoptotic-cell removal. These include (i) CD14,<sup>10</sup> (ii) a novel PS-receptor,<sup>11</sup> (iii) the integrin  $\alpha_v\beta_3$ ,<sup>12,13</sup> (iv) CD36,<sup>13,14</sup> (v) the class A scavenger receptor SR-A,<sup>15</sup> (vi) the ATP-binding cassette transporter, ABC-1,<sup>16,17</sup> (vii) the receptor tyrosine kinase, MER<sup>18</sup> and (viii) the  $\alpha$ -2-macroglobulin receptor, CD91.<sup>19</sup> Additional molecules, notably thrombospondin,<sup>13</sup> Clq,<sup>19,20</sup> mannose-binding lectin, calreticulin<sup>19</sup> and C-reactive protein<sup>21</sup> may serve as intermediates that bridge macrophage and apoptotic-cell surfaces or act as opsonins. The relative importance of each of these molecules has not yet been clearly established, although mice defective in ABC-1,<sup>17</sup> MER<sup>18</sup> and Clq<sup>20</sup> have defective apoptotic-cell clearance phenotypes, with MER- and Clq-deficient animals more prone to development of autoimmunity and autoimmune disease.<sup>18,20</sup> In addition, recognition of PS by the PS-receptor appears to be essential for engulfment to proceed.<sup>11,22</sup> Intriguingly, recent genetic studies of *Caenorhabditis elegans* indicate that phagocytic events can themselves promote cell death.<sup>23,24</sup>

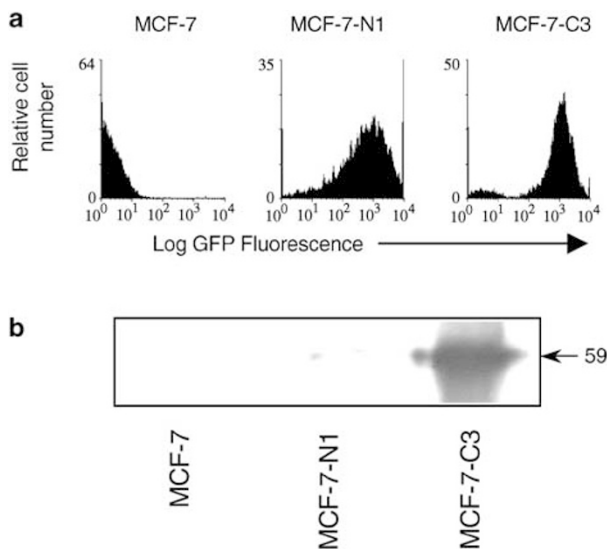
The apoptosis programme is a genetically controlled, cell-autonomous programme that probably exists in dormant form in all mammalian somatic cells. It is now widely accepted that several members of the caspase family of proteases play key roles in inducing and executing apoptosis.<sup>25,26</sup> However, little is yet known of the requirement for these proteases in causing

the changes on apoptotic-cell surfaces that are necessary for recognition and phagocytosis. One caspase in particular, caspase-3 – which is known to cleave over 40 substrates containing DxxD motifs during apoptosis<sup>26</sup> – appears critical in producing some of the most striking stereotypical features of apoptosis. Significantly, caspase-3-defective cells fail to display either the typical morphological features of apoptosis – including chromatin condensation and nuclear fragmentation – that were used originally to define the process,<sup>27</sup> or the prototypical biochemical hallmark of apoptosis, the organised degradation of DNA into oligonucleosomal fragments.<sup>28,29</sup> In this work, we investigate the role played by caspase-3 in orchestrating the changes that are required for cell engulfment by macrophages. Here we demonstrate directly that the biochemical mechanisms that cause the classical morphological features of apoptosis can be uncoupled from those that allow non-phlogistic interaction with macrophages. These findings indicate that different facets of the execution phase of the apoptosis programme are independently regulated and suggest that therapeutic strategies aimed at suppressing caspase-3 activity may require complementary measures that inhibit cell clearance by phagocytes.

## Results

### Derivation of caspase-3-expressing MCF-7 cells

The MCF-7 breast carcinoma line was chosen for these studies since it is known to be defective in caspase-3 expression.<sup>28,34</sup> Using the pEGFP-N1 expression vector, stable MCF-7 transfectants were derived with either GFP alone or a caspase-3-GFP (C-terminal) fusion protein. These transfectants were called MCF-7-N1 and MCF-7-C3, respectively (Figure 1a). GFP facilitated derivation of stable transfectants by cell sorting, permitted routine monitoring of

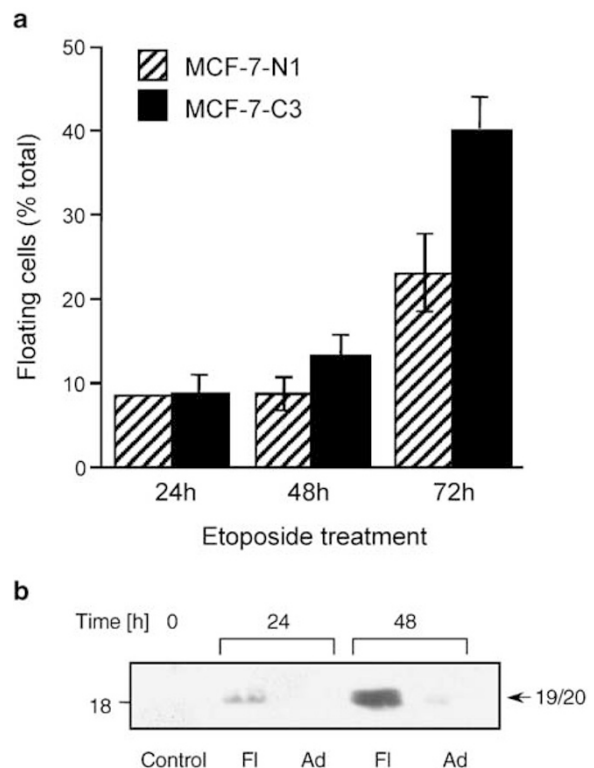


**Figure 1** GFP and caspase-3 expression in MCF-7 transfectants. (a) GFP expression assessed by flow cytometry in parental MCF-7 cells and those transfected with empty pEGFP vector (MCF-7-N1) and pEGFP vector containing caspase-3 (MCF-7-C3). (b) Lysates of MCF-7 (50  $\mu$ g), MCF-7-N1 (50  $\mu$ g) and MCF-7-C3 (25  $\mu$ g) cells were subjected to SDS-PAGE and Western blot analysis using anticaspase-3 antibody directed to the large subunit of procaspase-3

caspase-3 levels by flow cytometry (Figure 1a) and could be used to monitor plasma-membrane integrity (see Figure 4). Caspase-3-expression in MCF-7-C3 cells was confirmed by immunoblotting, which demonstrated the 59 kDa band of the caspase-3-GFP fusion protein (Figure 1b). No caspase-3 was detectable in either the MCF-7 parental line or in the MCF-7-N1 GFP transfectants.

### Caspase-3 activation accelerates, but is dispensable for, genotoxic death of MCF-7 cells

MCF-7 cells contain wild-type p53 and grow as a monolayer. Exposure to a genotoxic stimulus induces detachment from the monolayer and death. We compared the rate of detachment of MCF-7-N1 and MCF-7-C3 cells following treatment with the topoisomerase II inhibitor, etoposide. As shown in Figure 2a, the presence of caspase-3 accelerated detachment of MCF-7 cells from the monolayer, but was not a prerequisite for detachment. To assess caspase-3 activation in the etoposide-treated MCF-7-C3 cells, immunoblots of lysates of the detached, floating cells were compared with those of the adherent cells using an antibody to the large fragment of

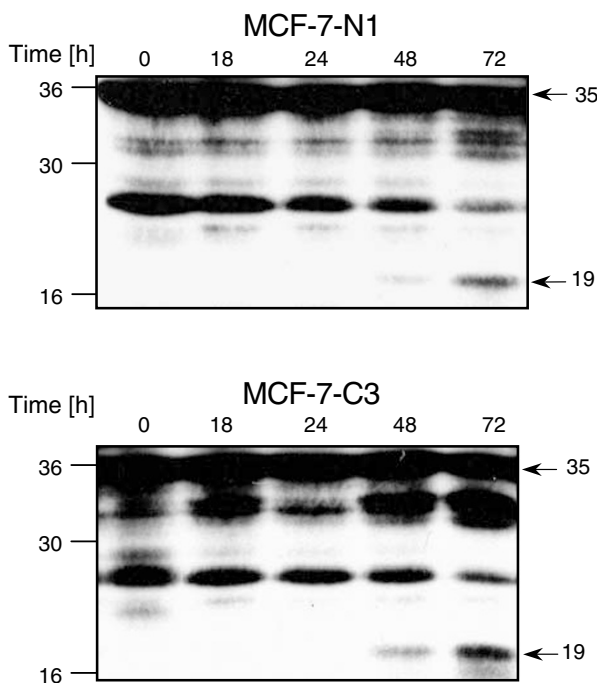


**Figure 2** Caspase-3 activation accelerates cell detachment following etoposide treatment. (a) Etoposide-induced detachment of MCF-7 cells from monolayers. MCF-7-N1 and MCF-7-C3 were treated with etoposide (100  $\mu$ M) and floating cells were counted after the indicated times and expressed as a percentage of the total. Results are means  $\pm$  S.E.M. of two separate experiments. (b) Caspase-3 activation in floating versus adherent MCF-7 cells. MCF-7-C3 cells were treated with etoposide (100  $\mu$ M) for the times indicated. Floating (FI) and adherent (Ad) cells were separated, and aliquots containing 40  $\mu$ g protein (except the 24 h floating sample, which contained 4  $\mu$ g because of constraints of low cell numbers that detach over the first 24 h) were subjected to SDS-PAGE and Western blot analysis using an anti-caspase-3 antibody directed to the large subunit of procaspase-3

caspase-3. As shown in Figure 2b, the appearance of bands of 19/20 kDa in the floating fractions of etoposide-treated cells indicated that caspase-3 was activated in the detached cells. Active caspase-3 was also detectable at times in the adherent fraction, although to a much lesser degree than in the floating fraction (see for example, the weak 19 kDa band in the 48 h adherent sample in Figure 2b). These results suggest that apoptosis, as assessed by caspase-3 activation, begins in the adherent cells and continues in the floating cells, and that the floating cells represent a relatively pure population of apoptotic MCF-7-C3 cells.

### Effector caspase activation occurs in etoposide-treated MCF-7-N1 cells

To determine whether etoposide-induced death of MCF-7 cells involved activation of an additional effector caspase that may function in the absence of caspase-3, lysates of etoposide-treated -N1 and -C3 cells were probed in immunoblots for expression and activation of the effector caspase, caspase-7 using an antibody that recognises both the 35 kDa proform of the enzyme and the p19, active large subunit. As shown in Figure 3, procaspase-7 is constitutively expressed in MCF-7 cells regardless of the expression of caspase-3. Furthermore, in lysates of both -N1 and -C3 cells, the large subunit of active caspase-7 was detectable by 48 h after treatment with etoposide and highly prominent by 72 h (Figure 3). Since caspase activation is often used as a hallmark of apoptosis, the observation that an effector caspase is activated in MCF-7-N1 cells indicates that the etoposide-induced death of N1



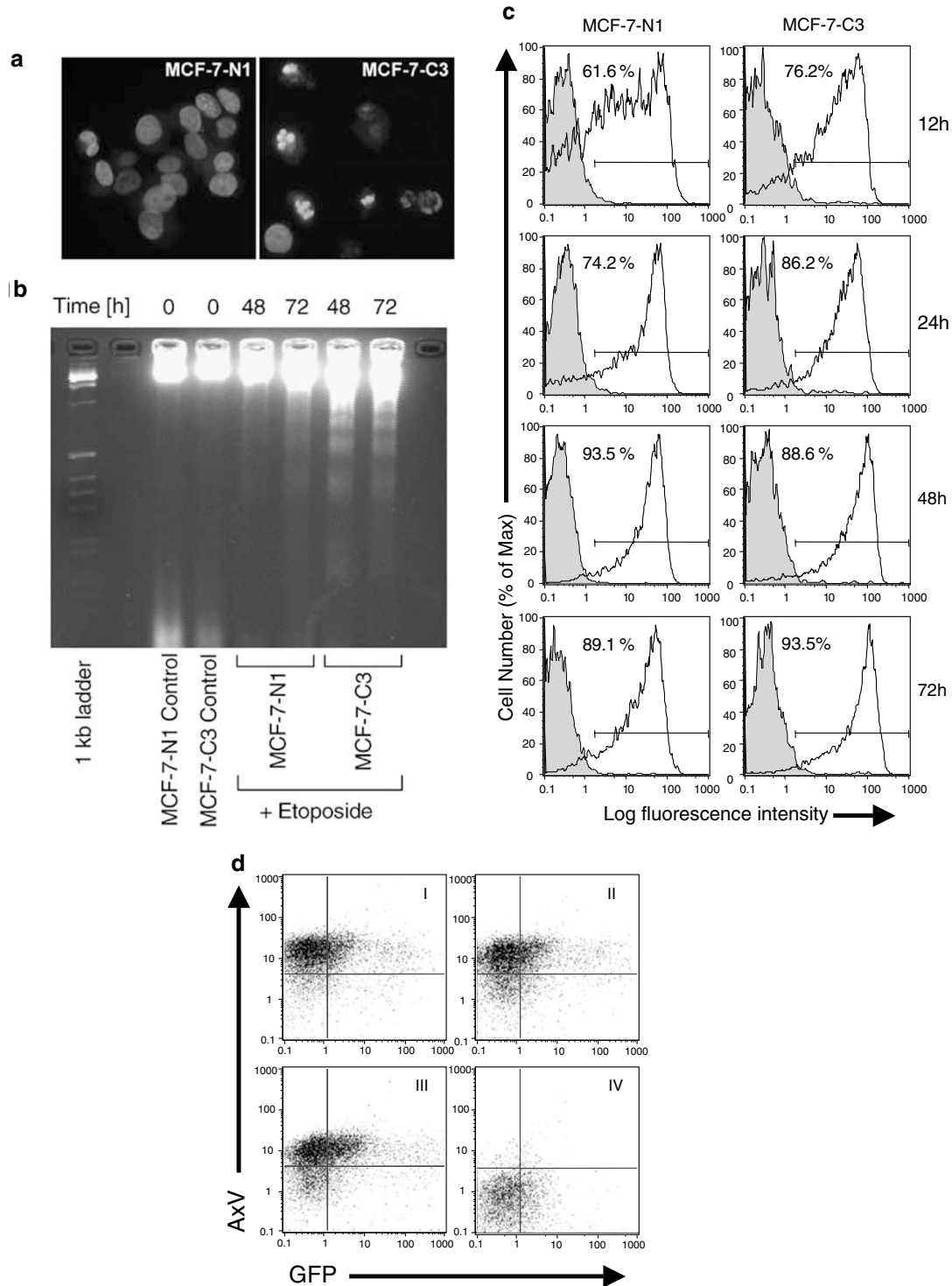
**Figure 3** Activation of caspase-7 in response to etoposide in both MCF-7-N1 and MCF-7-C3 cells. Lysates of combined floating and adherent MCF-7-N1 (upper panel) and MCF-7-C3 cells (lower panel) treated with etoposide (100  $\mu$ M) for the times indicated were subjected to SDS-PAGE and Western blot analysis using an anti-caspase-7 antibody that recognises the 35 kDa proform and the p19 active form of the protein

cells is likely to be a form of apoptosis, albeit one that lacks classical morphological features and DNA-degradative steps (see below).

### Caspase-3 is required for the acquisition of apoptotic morphology and cell-autonomous DNA degradation, but is dispensable for phosphatidylserine exposure

To study further the apoptotic features of the floating MCF-7-C3 and MCF-7-N1 cells, etoposide-treated detached cells were subjected to morphological analysis and were also assessed for their capacity to degrade DNA and to expose phosphatidylserine. As shown in Figure 4a, the morphological differences between caspase-3-positive and -negative floating MCF-7 cells as revealed by DAPI staining were striking. Caspase-3-positive cells displayed the classical nuclear features of apoptosis with condensed and fragmented chromatin whereas the nuclei of caspase-3-negative cells remained diffusely stained. Laddering of DNA into low molecular weight oligonucleosomal fragments was readily demonstrable in lysates of floating MCF-7-C3 cells, but not in -N1-derived lysates (Figure 4b). Similarly, *in situ* end labelling (ISEL) of free 3' hydroxy ends of DNA fragments with biotin-conjugated deoxynucleotides was readily demonstrable in floating, etoposide-treated MCF-7-C3 cells, but not in -N1 cells (see Figure 7c).

In stark contrast to these results, flow cytometric analysis of annexin V labelling indicated that phosphatidylserine (PS) exposure by floating cells collected after etoposide treatment was closely comparable in MCF-7-C3 and -N1 populations (Figure 4c), although at early time points the degree of PS exposure was marginally lower in the caspase-3-negative cells as compared to the caspase-3-positives. Thus in the experiment shown in Figure 4c, at 12 h post-etoposide treatment 62% of MCF-7-N1 cells were found to be annexin V-positive as compared to 76% of -C3 cells. Furthermore, a significant proportion of the positive -N1 cells was stained less brightly than the positive -C3 cells (Figure 4c). Differences between the two cell populations were less marked at 24 h, and by 48 h both the proportions of stained cells ( $\sim 90\%$ ) and the intensities of staining were comparable in -N1 and -C3 cells. Analysis of plasma-membrane integrity by trypan blue exclusion indicated that, for both -N1 and -C3 cells, the majority of the floating MCF-7 cells were similarly permeable at all time points tested. Thus, at 24 h, -N1 cells were  $68.9 \pm 4.7\%$  (mean  $\pm$  S.E.M.) trypan blue positive and -C3 cells were  $72 \pm 3.5\%$ ; at 48 h, -N1 cells were  $70.6 \pm 6.4\%$  and -C3 cells were  $80.2 \pm 8.1\%$  positive; at 72 h, -N1 cells were  $75.2 \pm 4.7\%$  and -C3 cells were  $87.0 \pm 1.9\%$  positive. However, as shown in Figure 4d, -N1 floating cells with intact plasma membranes as judged by GFP retention (see panel (I), Figure 4d) were also strongly annexin V-positive (annexin<sup>high</sup>/GFP<sup>high</sup>) at all time points tested (panels (II) – (III), Figure 4d). Similar results were obtained when -C3 cells were analysed in the same way and, furthermore, continued culture of floating cells harvested from etoposide-treated -N1 or -C3 cultures resulted in annexin<sup>high</sup>/GFP<sup>high</sup> cells joining the populations of annexin<sup>high</sup>/GFP<sup>low</sup> cells (data not shown).



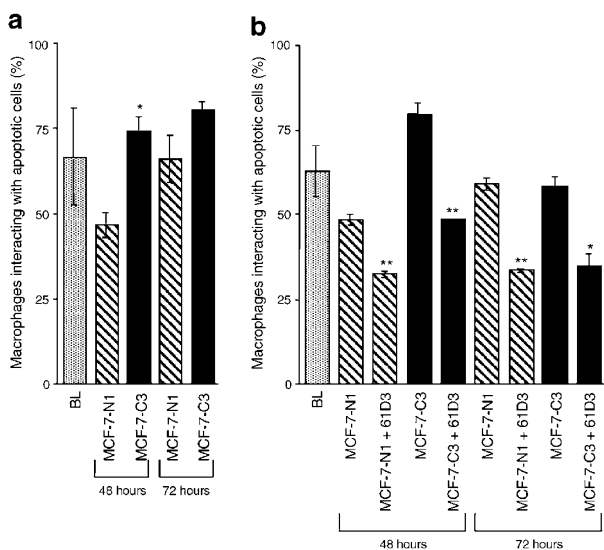
**Figure 4** Requirement of caspase-3 for morphological and DNA-degradative features of apoptosis, but not for phosphatidylserine exposure. **(a)** Morphology. Fluorescence micrographs of DAPI-stained floating MCF-7-N1 and MCF-7-C3 cells 48 h after etoposide treatment (100  $\mu$ M). **(b)** DNA degradation. Low-molecular-weight DNA from MCF-7-N1 and MCF-7-C3 untreated control cells (lanes 3 and 4) and floating cells after treatment with etoposide (100  $\mu$ M) for 48 or 72 h (lanes 5–8) was subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. Markers of 1 kb are indicated in lane 1. **(c)** Annexin V binding. Floating MCF-7-N1 (left-hand column) and MCF-7-C3 (right-hand column) cells collected after etoposide treatment for the times indicated (12–72 h) were stained with biotinylated annexin V and analysed by flow cytometry using Quantum Red-conjugated streptavidin for visualisation. Percent annexin V-positive cells are indicated. Control histograms (streptavidin-Quantum Red only) are shown in grey. Histograms are representative of three similar experiments. Data (mean % annexin V-positive cells  $\pm$  S.D.) collated from all three experiments were as follows: MCF-7-N1 24 h,  $77 \pm 6\%$ , 48 h,  $91 \pm 8\%$ , 72 h,  $92 \pm 2\%$ ; MCF-7-C3 24 h,  $90 \pm 7\%$ , 48 h,  $93 \pm 4\%$ , 72 h,  $95 \pm 3\%$ . **(d)** PS exposure. Flow cytometric dot plots of GFP fluorescence versus annexin V binding (Quantum Red fluorescence) of floating MCF-7-N1 cells (I), 24 h, (II) 48 and (III) 72 h after etoposide treatment. Annexin<sup>high</sup>/GFP<sup>high</sup> cells (upper right quadrant of each panel) represent dying MCF-7-N1 cells that have intact plasma membranes in which PS is externalised. Panel (IV), negative control for annexin V binding at 24 h (streptavidin-Quantum Red alone). Similar controls were observed at 48 h and 72 h. Dot plots representative of three similar experiments

These results indicate that PS exposure on MCF-7 cells occurs independently of caspase-3 activation.

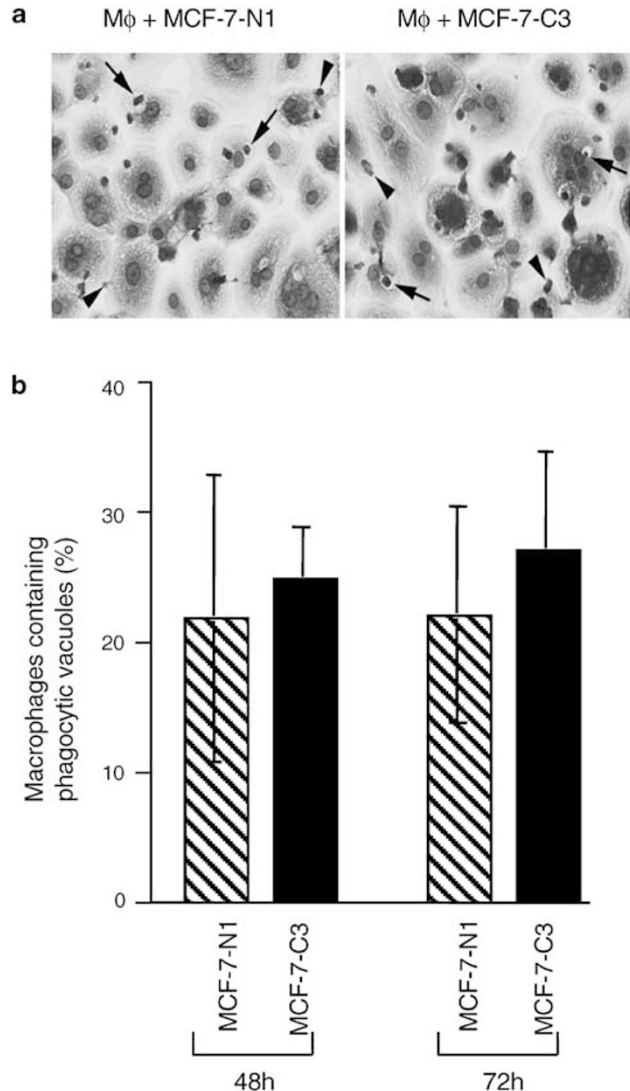
### Phagocytic clearance of apoptotic MCF-7 cells occurs in the absence of caspase-3

Since MCF-7 cells released from the monolayer following etoposide treatment fail to readhere to plastic or glass (data not shown), such cells were usable as targets in macrophage clearance assays. To determine whether the detached MCF-7 cells were recognised by macrophages, floating -N1 and -C3 cells were collected at various times after etoposide treatment and equivalent numbers of cells were exposed to 7-day-matured monocyte-derived macrophages in a well-established *in vitro* assay of recognition and engulfment of apoptotic cells by human professional phagocytes. Apoptotic Burkitt lymphoma cells, which have proven utility as targets in these assays,<sup>9,10,33</sup> were used to monitor the efficacy of the macrophage preparations to interact with apoptotic cells. As shown in Figure 5, floating MCF-7 cells were capable of interacting with macrophages independently of caspase-3 activation. While both -N1 and -C3 cells collected at 48 h were capable of interacting effectively with macrophages, -C3 cells were generally more effective at this time point (Figure 5a,b). However, this difference was not sustained, and -N1 and -C3 cells collected at 72 h were found to be equally effective in the macrophage interaction assays.

To analyse these interactions in more detail, assays were carried out in the presence of the mAb 61D3, a known inhibitor of the interaction of apoptotic cells with macrophage CD14. As

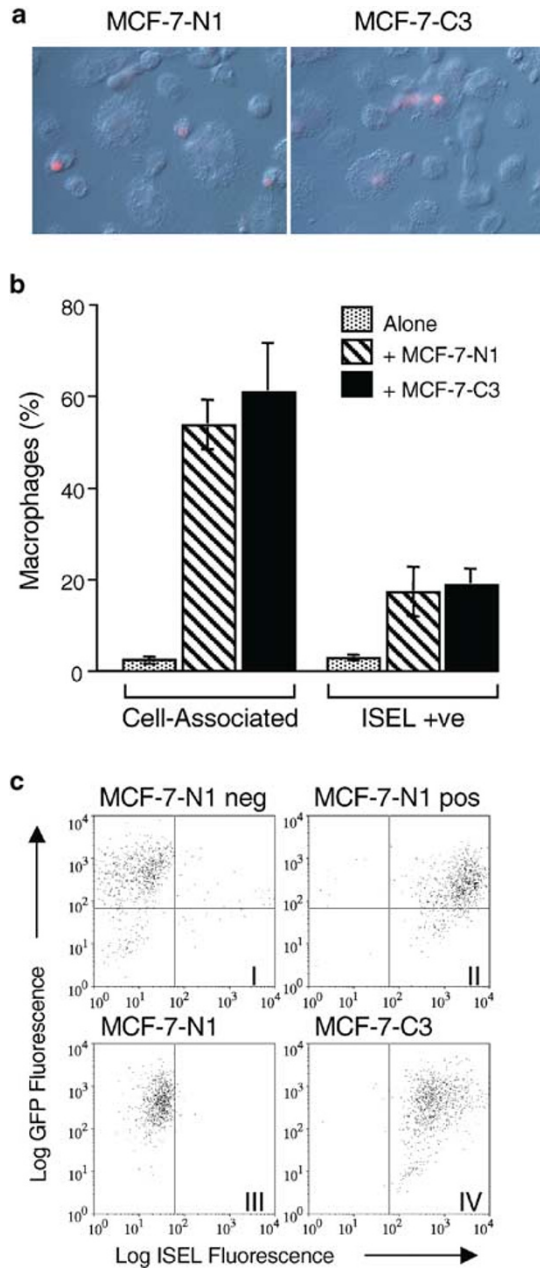


**Figure 5** Caspase-3-independent interaction of MCF-7 cells with human monocyte-derived macrophages. Quantitative analyses of macrophage-interacting properties of etoposide-treated MCF-7 cells. Floating MCF-7-N1 (striped bars) and MCF-7-C3 (black bars) cells collected after etoposide treatment for 48 and 72 h were exposed to 7-day monocyte-derived macrophages. After 1 h at 37°C, the proportion of macrophages interacting with added cells was assessed microscopically. Mutu I BL cells (stippled bar) induced into apoptosis with the calcium ionophore ionomycin for 18 h are included for comparison. In (b), the role of macrophage CD14 is assessed by inclusion of the CD14-blocking mAb 61D3 in the assays. Data (means  $\pm$  S.D.) are representative of two to five independent experiments. Student's *t*-test: (a)\*  $P < 0.05$  for interaction of MCF-7-N1 and MCF-7-C3; (b) \*\* $P < 0.01$ , \* $P < 0.05$  for level of blocking by 61D3



**Figure 6** Caspase-3-independent phagocytosis of MCF-7 cells by human monocyte-derived macrophages. (a) Morphological details of macrophage/MCF-7 interactions. Jenner-Giemsa-stained preparation showing macrophages interacting with 48 h etoposide-treated apoptotic MCF-7-N1 (left panel) and MCF-7-C3 cells (right panel). Surface-bound (arrowheads) and phagocytosed (arrows) MCF-7 cells are indicated. (b) Quantitative analysis of phagocytosis of etoposide-treated MCF-7 cells. Floating MCF-7-N1 (striped bars) and MCF-7-C3 (black bars) cells treated exactly as described in Figure 5 were subjected to more detailed microscopic analyses to assess the frequency of phagocytosis of each cell population. Data are mean percentages of interacting macrophages containing apoptotic cells in phagocytic vacuoles as defined by the arrows in a

shown in Figure 5b, significant inhibition of the interaction with macrophages of both -N1 and -C3 cells was observed with 61D3. Inclusion of nonblocking CD14 mAbs (such as 63D3, see Devitt *et al.*)<sup>10</sup> in these assays failed to cause inhibition (data not shown). These results suggest that ligands capable of interacting with the CD14-dependent clearance pathway are functional at apoptotic-cell surfaces independently of caspase-3 activation. Furthermore, the partial inhibitory effect of 61D3 in both caspase-3-positive and -negative cases (Figure 5b) suggests that apoptotic cells also acquire the capacity to interact with additional macrophage receptors regardless of caspase-3 activation.



**Figure 7** DNA degradation in free and phagocytosed etoposide-treated MCF-7 cells. (a, b) ISEL of 7-day monocyte-derived macrophages following interaction with detached (48 h etoposide-treated) MCF-7 cells. (a) Superimposed DIC and fluorescence microscopy of macrophages co-cultured with detached MCF-7-N1 (left panel) or MCF-7-C3 (right panel) cells for 1 h after which time non-associating cells were removed. Macrophages containing ISEL-positive material are labelled red (phycoerythrin read-out). (b) Frequency of macrophages containing ISEL-positive material. Macrophages treated as in (a) were subjected to quantitative microscopical analyses to determine the proportion of macrophages (1) associating with MCF-7 cells (left-hand bar triplet), and (2) containing ISEL-positive material (right triplet). Stippled bars, macrophages alone; striped bars, macrophages co-cultured with detached MCF-7-N1 cells; black bars, macrophages co-cultured with detached (apoptotic) MCF-7-C3 cells. Data (means  $\pm$  S.D.) are representative of three similar experiments. Student's *t*-test: no significant differences between MCF-7-N1 and MCF-7-C3 were apparent. (c) Flow cytometric analysis of free-floating populations from etoposide-treated MCF-7-N1 (plots I-III) and MCF-7-C3 (plot IV) cells subjected to ISEL using streptavidin-phycoerythrin as the read-out. The MCF-7-N1 cells were stained with streptavidin-phycoerythrin alone for the negative control (I) or subjected to DNase I treatment prior to ISEL for the positive control (II)

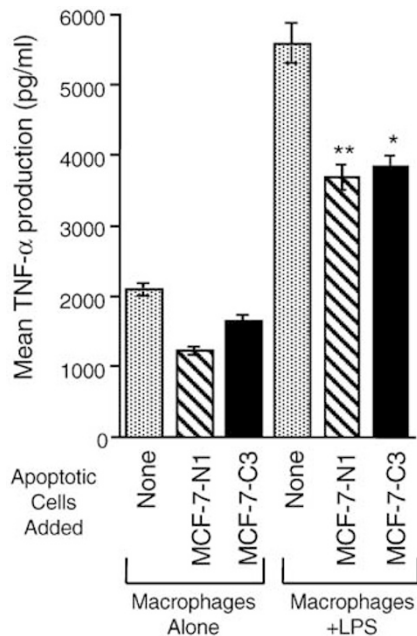
As illustrated in Figure 6a, the macrophage-interacting -N1 and -C3 cells are in each case a mixture of both bound and phagocytosed cells. Further microscopic analyses of macrophages interacting with N1 or C3 cells indicated that the frequency of phagocytic vacuoles was similar for both targets. Thus, as shown in Figure 6b, similar proportions of macrophages interacting with MCF-7 cells collected at 48 or 72 h after etoposide treatment were found to contain phagocytic vacuoles (according to morphological criteria, Figure 6a) regardless of the caspase-3 content. Notably, MCF-7-N1 cells became positive for degraded DNA (as evidenced by ISEL) following interaction with macrophages (Figure 7a,b). DNA degradation was most likely effected by DNases present in the phagocytic vacuoles as described in other systems<sup>35</sup> and provides further evidence that caspase-3-deficient cells are phagocytosed. Quantitative analysis of macrophages exposed to etoposide-treated MCF-7 cells demonstrated no difference between those interacting with caspase-3-positive and -negative cells (Figure 7a). These results are striking, given the absence of ISEL in free-floating MCF-7-N1 cells (Figure 7c, panel III). By contrast, ISEL was readily apparent in free apoptotic MCF-7-C3 cells (Figure 7c, panel IV) and was only demonstrable in MCF-7-N1 cells after DNase I treatment (Figure 7c, panel II). Importantly, there was no evidence of macrophage death induced by MCF-7-cell co-culture that could have been responsible for the positive ISEL end point in the macrophage cultures. Taken together, these results strongly argue that caspase-3-activation fails to affect binding or phagocytosis of MCF-7 cells differentially.

### MCF-7 cells dying via caspase-3-dependent or -independent mechanisms fail to elicit proinflammatory cytokine release by macrophages regardless of caspase-3 activation

Since apoptotic cells are known to suppress proinflammatory cytokine release by macrophages, experiments were carried out in order to determine whether this property was shared by MCF-7 cells undergoing caspase-3-dependent or -independent death. To this end, etoposide-treated MCF-7 cells were tested for their ability to affect LPS-induced secretion of the proinflammatory cytokine, TNF- $\alpha$  from 7-day monocyte-derived macrophages. As shown in Figure 8, TNF- $\alpha$  release by LPS-treated macrophages was markedly reduced to similar levels by etoposide-treated, floating MCF-7-N1 and -C3 cells. Basal levels of TNF- $\alpha$  release were also inhibited by etoposide-treated MCF-7 cells (Figure 8). In each case, macrophage viability remained unaffected by the presence of MCF-7 cells. These results show that caspase-3 is dispensable for the anti-inflammatory effects of dying or dead cells on the macrophage populations that can engulf them.

### Discussion

It is now widely accepted that caspase-3 acts in response to diverse stimuli of apoptosis in a wide variety of cell types as an effector or 'executioner' caspase,<sup>26</sup> which gives rise to several cellular hallmarks of apoptosis.<sup>28,29,36</sup> Using caspase-3-defective MCF-7 cells, we confirm here that caspase-3 is



**Figure 8** Suppression of TNF- $\alpha$  production by macrophages exposed to etoposide-treated MCF-7 cells. Human monocyte-derived macrophages (7 days) were either cultured alone (stippled bars) or were exposed to etoposide-treated, detached MCF-7-N1 (striped bars) or MCF-7-C3 cells (black bars) in the presence (right half of figure) or absence (left half) of LPS. The concentration of TNF- $\alpha$  in the cell-free supernatants was assayed by ELISA after 18 h. Student's *t*-test: \*\*  $P < 0.01$  for effects of MCF-7-N1 cells on LPS-stimulated macrophages and \*  $P < 0.02$  for effects of MCF-7-C3 cells

responsible for key stereotypical features of apoptosis in human cells. Thus, in the absence of caspase-3, MCF-7 cells responding to the genotoxic stimulus, etoposide die without displaying the classical morphological features of apoptosis and, at the same time, fail to develop the well-established biochemical hallmarks of DNA degradation into low-molecular-weight fragments. However, in MCF-7 cells that produce caspase-3, the same genotoxic stimulus causes caspase-3 activation and engenders both the classical morphological features of apoptosis and typical mode of oligonucleosomal DNA degradation.

The ultimate fate of apoptotic cells in tissues is their non-phlogistic engulfment by phagocytes. Using an *in vitro* system that provides a powerful human model for such clearance by macrophages, we demonstrate for the first time that constitutive absence of caspase-3 fails to alter significantly the capacity of dying and dead cells to be phagocytosed. CD14 is one of several macrophage receptors involved in apoptotic-cell clearance<sup>10</sup> and the results presented here further indicate that etoposide-induced MCF-7 cells can be cleared by macrophages both by CD14-dependent and -independent mechanisms regardless of the activation of caspase-3. Importantly, the phagocytosis of both caspase-3-deficient and caspase-3-replete cells is anti-inflammatory in nature as demonstrated by the equal abilities of caspase-3-positive and -negative cells to suppress inflammatory cytokine production by macrophages. While the present study was limited to cell populations containing mainly late-stage apoptotic cells, previous studies have suggested that such cells, including

those that have lost plasma membrane integrity can retain anti-inflammatory characteristics.<sup>37</sup> It may be predicted that the anti-inflammatory effects of the dying MCF-7 cells are mediated via an autocrine mechanism involving macrophage TGF- $\beta$ 1 release as established previously for the phagocytosis of apoptotic, caspase-3-positive leukocytes.<sup>3</sup>

What characteristics of apoptotic cells permit or support their interaction with phagocytes thereby allowing their final engulfment and safe degradation? Redistribution of phospholipids occurs during apoptosis and it is well established that the anionic phospholipid phosphatidylserine (PS), a component of the inner leaflet of the plasma membranes of viable cells, becomes exposed during apoptosis and participates in the clearance process as a new component of the external plasma-membrane leaflet.<sup>4,6</sup> Recent elegant work has shown that PS interacts with a novel macrophage PS-receptor.<sup>11</sup> Such interactions may comprise a necessary prerequisite of the process of engulfment and may also lead to the initiation of intracellular anti-inflammatory signals.<sup>11</sup> Our results show that PS is exposed on apoptotic MCF-7 cells in the absence of caspase-3, confirming observations of apoptotic thymocytes<sup>36</sup> and of embryonic fibroblasts<sup>29</sup> from caspase-3-deficient mice. While PS exposure on apoptotic cells is therefore caspase-3-independent, caspase involvement in this process has not been demonstrated directly and it is not clear how caspase activity could contribute to PS exposure, although it has been proposed that possible tethering of PS in the inner membrane leaflet through association with cytoskeletal elements such as fodrin may be lost in apoptosis as a result of caspase-mediated cleavage of fodrin.<sup>38</sup> Cleavage of fodrin, however, appears to be strictly caspase-3-dependent *in vivo*,<sup>36,39</sup> suggesting that if caspase-dependent inhibition of PS-tethering contributes to exposure of PS at the cell surface, it is unlikely to be through fodrin cleavage. The effects of caspases on other mechanisms that drive PS exposure during apoptosis, such as lipid scramblase activity,<sup>40</sup> have not been investigated.

It is probable that PS exposure, while it may be necessary, is not sufficient for apoptotic-cell recognition and engulfment by macrophages. Persuasive arguments have been made for a two-step process involving an initial tethering of the apoptotic cell to the phagocyte, followed by a PS-R-dependent engulfment phase.<sup>22</sup> Apart from qualitative changes in ICAM-3 that occur at the surface of apoptotic leukocytes,<sup>9</sup> little definitive information is yet available as to the additional cell-surface changes that function in apoptotic-cell clearance. The most popular view to date is that apoptotic cells display 'eat-me' signals or apoptotic cell-associated molecular patterns that allow the innate immune system to discriminate the plasma membrane of the apoptotic cell from that of its viable counterpart.<sup>41</sup> An alternative view is that viable cells display 'repulsive' signals for phagocytes that are lost as a consequence of apoptosis.<sup>42</sup> In either case, the present studies suggest strongly that the plasma-membrane changes that are sufficient for phagocytic clearance of apoptotic cells occur independently of caspase-3 activation. Since it is now becoming clear that, in addition to PS exposure, further changes at the surface of dying cells are required for phagocytic clearance by macrophages,<sup>43</sup> the present results indicate that caspase-3 is dispensable for the

generation of such changes. Intriguingly, however, while annexin V binding to -N1 and -C3 floating cells at 48 and 72 h were comparable, -N1 cells at 48 h interacted less effectively with macrophages than -C3 cells at the same time point. It is tempting to speculate that the additional molecular changes (or a subset of such) that are required to facilitate the interaction of apoptotic cells with macrophages are acquired more quickly when caspase-3 is active. Further work will be required to investigate this possibility.

The detailed biochemical routes to the membrane alterations that confer the capacity for clearance on an apoptotic cell remain to be established. Although our studies indicate that such alterations proceed in the absence of caspase-3, they do not address the question of overall caspase dependence. Caspase-7 is closely homologous to caspase-3 and, like caspase-3, is an acknowledged effector caspase in view of its short prodomain. In contrast to a recent report,<sup>44</sup> we found caspase-7 to be readily activated in MCF-7 cells following etoposide treatment regardless of caspase-3 activation. Some of us have previously reported caspase-7 activation in MCF-7 cells treated with TRAIL.<sup>30</sup> Although apoptosis may occur in the absence of caspase activation (see below), activation of caspase-7 helps to define death as apoptosis in MCF-7 cells lacking caspase-3. Other criteria such as PS exposure, cytoplasmic condensation and membrane blebbing<sup>45,46</sup> enhance this definition. The use of polycaspase peptide inhibitors may help to answer the question of caspase dependence of apoptotic-cell clearance. The broad-spectrum caspase inhibitor z-VAD-fmk has been shown to inhibit multiple features of apoptosis in leukocyte lines, including the capacity to interact with phagocytes, suggesting that the latter is caspase-dependent.<sup>46</sup> In the same cells, however, antimycin A and oligomycin have been used to dissociate caspase activation from PS exposure and phagocytosis of apoptotic cells by murine macrophages<sup>46</sup> indicating that the clearance phenotype is not inextricably linked to other aspects of the apoptosis programme including caspase activation. A major problem with addressing the question of the caspase-dependence of apoptotic-cell clearance using inhibitors such as z-VAD-fmk is that such approaches fail to differentiate between the caspase-dependence of the initiation of apoptosis and the caspase-dependence of the downstream pathways that are ultimately responsible for altering the apoptotic-cell surface. In the present model, MCF-7 cells were induced to undergo apoptosis through genotoxic damage, a mitochondrial pathway of apoptosis that, in light of previous studies that order the caspase activation events following release of cytochrome *c* from mitochondria,<sup>47</sup> would be predicted to generate active effector caspase-3 and -7 consequent to the activation of caspase-9. It may be of interest to compare the present results with the consequences for phagocytic clearance of initiating apoptosis through a nonmitochondrial pathway, such as death-receptor ligation. In this context, it is interesting to note that Bcl-2 can protect both lymphoid cells and neutrophils from morphologically identifiable apoptosis.<sup>48,49</sup> In the case of neutrophils, but not lymphocytes, Bcl-2 fails to prevent phagocytic clearance.<sup>49</sup> These results indicate that the biochemical pathways affected by Bcl-2 that appear to delay apoptosis in neutrophils can be uncoupled from those that endow these cells with the capacity

to be phagocytosed. Since Bcl-2 protects cells at the level of mitochondria, it is tempting to speculate, particularly in view of the present findings, that the signalling pathways that alter the cell surface following the initiation of apoptosis lie upstream of the mitochondrial phase, at least in some cell types. Within the present cell model, judicious use of the peptide inhibitor DEVD-fmk may possibly provide an opportunity to inhibit caspase-7 and thereby address the question of effector caspase involvement in apoptotic-cell clearance. However, caspase-7 activation may provide a mechanism through which apoptotic MCF-7 cells lose adhesion contacts in the absence of caspase-3 since caspase-7 is most effective in cleaving focal adhesion kinase.<sup>50</sup> In this context, we have found that the polycaspase inhibitor, z-VAD-fmk and the caspase-3 and -7 inhibitor z-DEVD-fmk can inhibit loss of adhesion of etoposide-treated MCF-7 cells (data not shown).

Evidence obtained from several cell systems has indicated that programmed death of multiple lineages of cells can occur via caspase-independent routes,<sup>51,57</sup> but the efficiency with which such cells are phagocytosed has not been investigated. Loss of function of aged platelets appears to be due to a process that closely resembles apoptosis but which is caspase-independent.<sup>58</sup> Significantly, the phagocytic clearance of such intact effete platelets proceeds normally suggesting that caspases may be dispensable for the apoptosis-driven cell surface alterations that are required for efficient phagocytosis of apoptotic cells. In view of these findings, further investigations of the phagocytosis of cells dying through caspase-independent routes are warranted.

It has been shown in other cell types, notably hepatocytes, that deficiency in caspase-3 can cause compensatory activation of alternative caspases (-2, -6 and -7) in the initiation and execution of apoptosis.<sup>59</sup> This, however, may be a tissue-specific phenomenon as compensatory caspase activation was not observed in thymocytes.<sup>59</sup> Compensatory caspase activation was not investigated directly in the present studies, but it is clear that, regardless of whether compensation occurs in MCF-7 cells receiving a pro-apoptosis stimulus, certain features of the apoptosis programme – morphological and DNA-degradative changes – are not restored. By contrast, other features – including PS exposure and the capacity to be phagocytosed non-phlogistically – proceed virtually normally. Therefore, even though caspase-7 may appear structurally and, potentially, functionally similar to caspase-3 (and, when overexpressed in active form in MCF-7 cells can even enhance apoptosis almost to the levels achieved by caspase-3<sup>60</sup>), caspase-7 activity at physiological levels in MCF-7 cells is unable to substitute for the absence of caspase-3. This may reflect the differential subcellular compartmentalisation of caspase-3 and -7.<sup>61</sup> It is noteworthy that detailed comparison of the activities of effector caspases towards a range of key substrates cleaved during apoptosis has suggested that the so-called effector caspases -6 and -7 do little to contribute to the degradation of apoptotic cells beyond the activity of caspase-3.<sup>62</sup> From the results of the present studies, we conclude that the biochemical pathways that lead to (at least some of) the hallmarks of apoptosis are nonredundant, run in parallel and can be uncoupled from one another.



The finding that the capacity to be phagocytosed can be generated in cells lacking caspase-3 suggests a mechanism by which cells lacking the typical morphological features of apoptosis can come to lie in phagocytic vacuoles. Thus, cells that have engaged the apoptosis programme may be phagocytosed as a result of the biochemical pathways that generate the capacity to be cleared being activated separately from, and prior to, caspase-3. Significantly, phagocytosed cells that appear morphologically viable have been shown to display externalised PS.<sup>63</sup> More importantly, the phagocytosis of apoptotic cells without caspase-3 activation has important implications for therapeutic strategies aimed at inhibiting caspase effector functions (see Nicholson<sup>26</sup>). The present study is limited to an *in vitro* model in which the majority of the cells analysed in macrophage assays were at a late stage of the apoptotic process. It remains possible that caspase-3 activation may be important for early-stage engulfment of apoptotic cells, although the arguments above supported by the observations of Van den Eijnde *et al.*<sup>63</sup> suggest otherwise. Clearly, it will be important to determine whether cells rescued by caspase inhibition survive long-term *in vivo* or whether they remain ultimately susceptible to phagocytic clearance mechanisms. In addition, alteration of the mechanisms by which such cells are cleared through abolition of certain pathways in the apoptosis programme may have consequences for immune responses as has been shown in other systems.<sup>64</sup> Finally, of particular relevance to the present study is the recent report that caspase-3 fails to influence the response of MCF-7 tumour xenografts to photodynamic therapy.<sup>65</sup>

Chronological ordering of the key molecular events in apoptosis is confounded by the complexities of the programme, including multiple pathways of initiation, the involvement of molecular cascades with amplification loops, subcellular compartmentalisation of effector components and variation according to cell lineage. For the moment, we can conclude that a dying cell's capacity to be phagocytosed by a macrophage can be acquired in the absence of the prototypic effector caspase, caspase-3. Defining the mechanisms that underlie the surface changes of apoptotic cells that are necessary and sufficient for phagocytic clearance and relating them temporally to other aspects of the apoptotic programme remains a significant challenge.

## Materials and Methods

### Cell lines and transfection

MCF-7 cells and Mutu I cells were grown in RPMI 1640 medium containing 2 mM L-glutamine supplemented with 10% fetal calf serum (FCS) or Serum Supreme (BioWhittaker, Wokingham, UK) and 100 IU ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin (complete medium). MCF-7 transfectants were derived by electroporation using pEGFP-N1 (Clontech, Palo Alto, CA, USA) or pEGFP-C3.<sup>30</sup> The latter was derived by cloning full-length caspase-3 cDNA into the pEGFP-N1 expression vector at the *Bam*H1 and *Xho*1 sites such that the green fluorescent protein (GFP) was located at the carboxy-terminus of the expressed caspase. This construct has been shown previously to be capable of generating active caspase-3 protease (DEVDase) activity in MCF-7 cells.<sup>30</sup> Cells were subsequently selected both by neomycin resistance and by fluorescence-activated cell sorting according to GFP expression.

### Induction of cell death

Adherent cells were trypsinised, collected into complete medium, seeded into multiwell plates or Petri dishes and incubated at 37°C overnight to allow readherence and resumption of logarithmic growth. Etoposide (Sigma, Poole, UK, 100 µM) was added to cells when at 40–50% confluency. Mutu I BL cells were induced into apoptosis by the addition of ionomycin (Calbiochem, Nottingham, UK, 1 µg ml<sup>-1</sup>) followed by incubation at 37°C for 18 h.

### Measurement of cell detachment from monolayers

Drug-treated floating cells were resuspended in complete medium and counted using a Neubauer haemocytometer. The remaining adherent cells were collected by trypsinisation, resuspended in complete medium and counted. The number of floating cells was expressed as a percentage of the total population. Plasma-membrane integrity was assessed by trypan blue exclusion.

### Determination of cellular morphology

Drug-treated floating MCF-7 cells were collected, washed twice in 1% bovine serum albumin (BSA) in PBS and fixed in 1% formaldehyde in 1% BSA in PBS and stored for up to a week at 4°C. Immediately prior to observation, fixed cells were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, 2.5 µg ml<sup>-1</sup>) and observed using a Zeiss Axioskop 2 fluorescence microscope (Carl Zeiss Ltd., Welwyn Garden City, UK).

### Measurement of DNA degradation

*In situ end labelling (ISEL)*. Floating MCF-7 cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and resuspended in 80% ethanol for storage. Staining was performed using an ISEL kit (TdT FragEL DNA fragmentation detection kit, Oncogene Research Products, CN Biosciences, Nottingham, UK) according to the manufacturer's instructions with phycoerythrin-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA) as the final read-out step. Cells were analysed immediately on the Coulter XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). In some experiments, ISEL was carried out on macrophage monolayers following interaction with apoptotic cells. In this case, the final read-out step was Texas-Red-conjugated streptavidin (Vector Laboratories) and cells were analysed under the fluorescence microscope.

### DNA laddering

The method was carried out as described,<sup>31</sup> and applied to floating and adherent cells, collected separately. Samples were subjected to electrophoresis on 2% agarose gels containing 0.5 µg ml<sup>-1</sup> ethidium bromide.

### Annexin V staining for phosphatidylserine (PS) exposure

Annexin V staining of MCF-7 transfectants, in combination with GFP fluorescence, was assessed by flow cytometry. Loss of GFP fluorescence was used as a measure of plasma-membrane disruption since the standard indicator, propidium iodide, was found to be incorporated into viable MCF-7 cells. Floating MCF-7 cells (1–2 × 10<sup>5</sup>) were collected, washed twice in binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) and incubated at 4°C with biotin-conjugated annexin V

(Alexis Corp., San Diego, CA, USA). Cells were washed twice in binding buffer then incubated at 4°C with Quantum Red-conjugated streptavidin (Sigma). Duplicate samples were prepared in the absence of annexin V to monitor background fluorescence. Samples were analysed on the Coulter XL flow cytometer. Plasma-membrane permeability was assessed simultaneously by monitoring GFP fluorescence levels, which were found to correlate directly with trypan blue exclusion as assessed microscopically.

### SDS-PAGE and Western blotting

Control adherent (no etoposide) or etoposide-treated adherent and floating cells were collected separately, washed twice in PBS and the dry pellet stored at -80°C. Cells were subsequently suspended in PBS and sonicated. For caspase-7 analyses, etoposide-treated cells were collected by scraping adherent cells into a suspension containing floating cells. The remaining procedure was carried out as previously described.<sup>30,32</sup> Blots were probed with mouse anti-caspase-3 mAb (Santa Cruz Biotechnology Inc., CA, USA), and rabbit polyclonal anti-caspase-7. Visualisation was by ECL (Amersham Pharmacia).

### Macrophage interaction assays

These were carried out using 7-day human monocyte-derived macrophages isolated as described.<sup>10,33</sup> Floating cells of etoposide-treated MCF-7 cell populations were washed in serum-free RPMI 1640, diluted in RPMI containing 0.2% BSA such that all samples contained the same concentration of cells ( $7.5 \times 10^5$  to  $1.5 \times 10^6$  cells ml<sup>-1</sup>). Macrophages on glass slides were incubated with MCF-7 or ionomycin-treated Mutu I Burkitt lymphoma cells at 37°C in 5% CO<sub>2</sub> for 1 h. Unbound cells were removed by dipping slides in cold PBS and cells fixed in methanol for 10 min. In some cases, anti-CD14 mAbs were added as described.<sup>10</sup> Cells were finally stained in Jenner-Giemsa and the proportions of free and target-cell bound macrophages were determined by light microscopy. In all, 200 macrophages were scored per well with duplicate wells per sample. Statistical analysis of the assays was performed using the Student's *t*-test for which *P* < 0.05 was significant.

### Cytokine production

7-day human monocyte-derived macrophages were cultured in 24-well trays either alone or in the presence of LPS (*E. coli* O111:B4, Sigma), 5 ng/ml and/or apoptotic MCF-7 cells ( $5 \times 10^5$  cells per well - 48 h etoposide-treated, non-adherent cells) for 18 h. Cell-free supernatants were collected and assayed for TNF- $\alpha$  by ELISA using matched-pair anti-TNF- $\alpha$  mAbs (R&D Systems Inc., Minneapolis, MN, USA).

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