Oxidative stress inhibits the phagocytosis of apoptotic cells that have externalized phosphatidylserine

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Abstract

The efficient phagocytosis of apoptotic cells by macrophages reduces the potential for an inflammatory response by ensuring that the dying cells are cleared before their intracellular contents are released. Early apoptotic cells are targeted for phagocytosis through the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. In this report, we show that the oxidant H₂O₂ inhibits phagocytosis of apoptotic cells even though the cells express functional PS on their surface. Thus, B lymphoma cells induced to undergo apoptosis by the chemotherapy drug etoposide are efficiently phagocytosed by macrophages in a process that is mediated by PS (inhibitable by PS liposomes). Exposure of the apoptotic cells to H₂O₂ inhibits phagocytosis even though the cells still express functional PS on their surface. In addition, Jurkat cells and thymocytes induced to undergo apoptosis by H₂O₂ alone are poorly phagocytosed. Inhibition of phagocytosis by H₂O₂ cannot be attributed to oxidative inactivation or redistribution of PS on the cell surface. The results indicate that PS externalization is necessary but is not sufficient to target apoptotic cells for phagocytosis. Another phagocytosis recognition factor must therefore exist to facilitate uptake of apoptotic cells, and this factor is sensitive to modification by H₂O₂.

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Abbreviations: PS, phosphatidylserine; BL, Burkitt's lymphoma; H₂O₂, hydrogen peroxide; PARP, poly-ADP ribose polymerase; 3-AB, 3-aminobenzamide; VP16, etoposide; PG, phosphatidylglycerol

Introduction

Apoptotic death is a conserved cellular process that functions to target cells for phagocytosis by macrophages. This physiological event is of fundamental importance because it ensures that cells are removed before plasma membrane integrity is lost and cytosolic molecules are released into the extracellular milieu (for review see¹). Leakage of intracellular molecules is thought to cause the induction of potentially harmful inflammatory responses.^{2,3} Indeed, some autoimmune diseases may have etiologies associated with impaired uptake of apoptotic cells.^{4–6}

A number of molecules have been identified as being involved in the timely phagocytosis of apoptotic cells by macrophages. On the surface of the macrophage, CD14,⁷ CD91,⁸ scavenger receptors,^{9,10} and a receptor specific for phosphatidylserine (PS),¹¹ have been implicated as important mediators of macrophage recognition of apoptotic cells. Integrins on the surface of macrophages appear to promote adhesion,^{12–14} and generate signals¹⁵ necessary for the phagocytosis of apoptotic cells. In addition, members of the complement system,^{16–18} thrombospondin,¹⁹ and protein β 2 glycoprotein I²⁰ in serum have been demonstrated to bind apoptotic cells and facilitate their removal.

The only phagocytosis-associated change known to occur on the apoptotic cell surface is the translocation of PS from the inner leaflet to the outer leaflet of the plasma membrane.^{21,22} This event often requires caspase activation and is observed early during apoptosis.²³ Substantial evidence indicates that PS is a key recognition moiety that targets many types of apoptotic cells for phagocytosis (for review see²⁴). Recently, Fadok and colleagues demonstrated that the introduction of PS into the external leaflet of the plasma membrane of healthy cells can target cells for phagocytosis by macrophages.²⁵ This finding suggests that PS expression on the surface of cells is sufficient for ensuring their removal by macrophages. However, this finding does not preclude the possibility that other elements on the apoptotic cell surface may also promote phagocytosis.

The complex morphological changes associated with apoptosis require the energy provided by ATP hydrolysis.^{26,27} Oxidative stress induced by H₂O₂ causes extensive DNA damage and the activation of poly-ADP ribose polymerase (PARP),²⁸ which leads to a rapid depletion of cellular ATP.²⁹ Oxidative damage can therefore inhibit apoptotic processes and convert cell death to necrosis.²⁷ From a physiologic perspective this is important since phagocytosis of necrotic cells is not initiated until after membrane integrity is lost.³⁰

The present study was initiated to determine if the externalization of PS on the surface of H₂O₂-treated cells correlated with the ability of the cells to be phagocytosed

by macrophages. Interestingly, when ATP levels in H₂O₂ treated BL cells are maintained through the inhibition of PARP, the cells undergo extensive apoptosis and PS is externalized to the plasma membrane surface. However, phagocytosis of these cells is very inefficient. In addition, Jurkat T cells and thymocytes treated with low doses of H₂O₂ become apoptotic and externalize PS, but these cells are also inefficiently phagocytosed. Thus, PS externalization does not guarantee efficient phagocytosis of apoptotic cells, and the removal of these cells requires a factor that is sensitive to H₂O₂ treatment.

Results

H₂O₂ treatment of cells prevents etoposide-induced apoptosis and PS externalization

Some BL cells fail to die by apoptosis when treated with the chemotherapeutic drug etoposide (VP16) in the presence of H₂O₂.²⁷ Instead, cell death is delayed, occurs by pyknosis

and necrosis, and cells are not phagocytosed until membrane integrity is lost.³⁰ Because of the known role of PS in mediating phagocytosis of apoptotic cells, we evaluated the effect of H₂O₂ on the ability of BL cells to externalize PS. As shown in Figure 1, BL cells were induced to externalize PS when treated with etoposide. However, if cells were treated with etoposide in the presence of 200 μ M H₂O₂, these cells failed to externalize PS. When 3-aminobenzamide (3-AB), a poly-ADP ribose polymerase inhibitor, was added to the treatment mixture, PS externalization was detected on the surface of cells treated with etoposide and H₂O₂ and was comparable to that observed with etoposide treatment alone. Annexin V positive BL cells as shown in Figure 1A were impermeable to propidium iodide, indicating that they maintained their membrane integrity. BL cells treated with H₂O₂ and 3-AB demonstrated two populations of annexin V positive cells when examined at 7 h. At 9 h these cells demonstrated a single population of annexin V positive cells, identical to cells treated with etoposide (data not shown). This suggests that BL cells undergo apoptosis with slower kinetics when treated with H₂O₂ and 3-AB. Examination

A

B

Figure 1 Effect of 3-AB on PS-externalization and ATP levels in cells treated with H₂O₂ and etoposide. (A) BL cells were treated with 200 μ g/ml etoposide and/or 200 μ M H₂O₂ for 7 h in the absence or presence of 1 mM 3-AB. Cells were then stained with annexin V-FITC and monitored by FACS analysis to assay for the extent of PS surface expression, or treated with propidium iodide to assay for membrane integrity. Data are shown on a log scale, and annexin V binding to control cells is shown in each FACS profile (light line). Heavy lines show treated cells. (B) Identical cultures were assayed for ATP content at the indicated times as described in the Materials and Method section. Results are expressed as the percentage of ATP measured in treated cultures compared to the ATP content in control cultures. Error bars denote the standard deviation

of nuclear morphology as described previously^{27,30} confirmed that the cells expressing PS had nuclear condensation characteristic of apoptosis and that the cells were impermeable to propidium iodide (data not shown). Thus, BL cells can undergo extensive apoptosis and externalize PS in the presence of H₂O₂ if PARP is inhibited.^{27,30,31}

Previous studies suggested that H₂O₂-induced apoptosis in the presence of 3-AB can be attributed to the ability of these cells to maintain adequate levels of ATP to sustain apoptosis.²⁷ This was confirmed by measuring ATP levels in cells treated with etoposide and/or H₂O₂. As shown in Figure 1B, within 1 h after exposure to H₂O₂, cellular ATP levels were reduced by 90% compared to control cells, while etoposide treated cells maintained ATP at levels similar to untreated cells. Cells treated with H₂O₂ in the presence of 3-AB maintained ATP levels of at least 50% of the levels in untreated cells. These findings are consistent with reports that ATP levels must be maintained to at least 25% of normal levels for the initiation of apoptosis.²⁶ Thus, together with the data in Figure 1, these results support the conclusion that ATP is required for the externalization of PS (and the induction of apoptosis),^{23,32} and that H₂O₂ prevents BL cell apoptosis indirectly by depleting cellular ATP.²⁷ Note that cells treated with etoposide undergo extensive apoptosis within 3 h such that the low levels of ATP seen in 6 h etoposide-treated cells are those seen near the end of the apoptotic process.

H₂O₂ treatment prevents phagocytosis of apoptotic cells that have translocated PS

The translocation of PS to the outer leaflet of the plasma membrane has been demonstrated to be an essential event that targets cells for phagocytosis.²⁴ In order to determine if PS mediates phagocytosis in our experimental system, we evaluated the ability of human macrophages to phagocytose BL cells treated with etoposide and/or H₂O₂, and correlated this with PS expression. BL cells were incubated with human monocyte-derived macrophages for 1 h at 37°C to allow for phagocytosis of the cells. Phagocytosis of apoptotic BL cells was quantified by FACscan analysis and is expressed as the fold-increase in phagocytosis. This value was determined by calculating the relative increase in the percentage of macrophages that engulfed treated-BL cells compared to the percentage of macrophages that engulfed untreated BL cells (control). This evaluation allowed for a better comparison between experiments since macrophages derived from different human donors demonstrated differences in overall levels of phagocytosis. It should be noted that identical results were obtained when phagocytosis was quantified with fluorescence microscopy as previously described,³⁰ and that nearly all of the apoptotic cells were present inside macrophages. Less than 5% of the macrophages had apoptotic cells bound to the macrophage cell surface (data not shown). As shown in Figure 2, there was a fivefold increase in the phagocytosis of etoposide-treated (apoptotic) BL cells compared to the phagocytosis of healthy cells. However, BL cells treated with etoposide in the presence of H₂O₂ were poorly phagocytosed, as were cells treated with H₂O₂ alone. This

Figure 2 H₂O₂ inhibits phagocytosis of apoptotic cells that have externalized PS. BL cells were treated with etoposide (200 µg/ml) and/or H₂O₂ (200 µM) and cultured in the absence or presence of 1 mM 3-AB for 7 h as described for Figure 1. BL cells were fluorescently labeled with the green fluorescent dye CFDA, washed and incubated with macrophages (stained with PE) derived from human peripheral blood monocytes for 1 h at 37°C. Phagocytosis of BL cells is expressed as the fold increase in engulfment of treated BL cells compared to control BL cells. Error bars denote the standard deviation

was initially attributed to the inability of these cells to externalize PS to the cell surface (Figure 1A). Surprisingly, however, when BL cells were treated with etoposide and/or H₂O₂ in the presence of 3-AB, these cells also were poorly phagocytosed by macrophages (Figure 2) even though they expressed similar amounts of PS as compared to cells treated with etoposide alone (see Figure 1). These results suggest that BL cells induced to undergo apoptosis in the presence of H₂O₂ can externalize PS, but these cells are poorly phagocytosed by macrophages.

PS exposure on the surface of apoptotic BL cells is required for phagocytosis

The observation that PS expression on BL cells does not ensure phagocytosis of apoptotic cells necessitated a re-evaluation of the role of PS in the phagocytosis of these cells. In initial studies, the kinetics of PS expression were compared with the kinetics of phagocytosis of apoptotic BL cells. As shown in Figure 3A, cells first began to express PS 2 h after etoposide treatment, and by 3.5 h, approximately 50% of the population of cells had translocated PS to the outer plasma membrane surface. BL cell membrane integrity was maintained during this time period since cells remained impermeable to staining with propidium iodide (upper right quadrant). In the same experiments, etoposide treated BL cells were assayed to determine at which time the cells became susceptible to phagocytosis. As shown in Figure 3B, an increase in phagocytosis of BL cells was first observed 1.5 h after etoposide treatment, and increased at

Figure 3 Kinetics of PS externalization and phagocytosis of etoposide-treated BL cells. **(A)** BL cells were treated with 200 μ g/ml etoposide for the indicated periods of time. The kinetics of PS externalization were determined by two-color FACScan analysis after incubating cells with annexin V-FITC and PI. Apoptotic cells with an intact plasma membrane appear in the lower right quadrant. Late apoptotic or necrotic cells that have lost plasma membrane integrity appear in the upper right quadrant. The percentage of cells present in each quadrant is indicated. **(B)** Human macrophages were incubated for 1 h with BL cells treated with etoposide for various periods of time. Phagocytosis was measured as described in the Materials and Methods section. The kinetics of PS externalization is compared to fold increase in phagocytosis of VP16 treated BL cells. Error bars denote the standard deviation

a rate similar to PS externalization. Thus, the kinetics of PS exposure on BL cells are similar to the kinetics of recognition and phagocytosis of these cells by macrophages, with the possibility that the increase in phagocytosis slightly precedes the externalization of PS.

To further evaluate the role of PS in the phagocytosis of etoposide-induced apoptotic BL cells, the ability of PS-containing liposomes to inhibit phagocytosis was measured. BL cells were treated with etoposide for various periods of time and phagocytosis was assayed in the presence of various amounts of liposomes containing PS or phosphatidylglycerol (PG). As shown in Figure 4, PS-containing liposomes reduced phagocytosis of etoposide-treated BL cells in a concentration-dependent manner, achieving approximately 75% inhibition at the highest concentrations tested. PG-containing liposomes were approximately half as effective as PS-containing liposomes in inhibiting phagocytosis of etoposide-treated BL cells. The slight inhibition of phagocytosis observed with PG-containing liposomes may be attributed to nonspecific charge effects that reduce the interaction of apoptotic cells with macrophages.³³ The inability to completely inhibit phagocytosis with PS containing liposomes has been found by

Figure 4 PS-containing liposomes inhibit the phagocytosis of apoptotic BL cells. **(A)** BL cells were treated with 200 μ g/ml of etoposide for 1.5 h or 3.0 h. Human monocyte derived macrophages were pretreated for 15 min at 37°C with various concentrations of liposomes containing either PS or PG. Liposomes were added to macrophages for 15 min, prior to the addition of BL cells. Liposomes were present throughout the phagocytosis assay. The fold increase in phagocytosis of treated cells compared to control healthy cells was calculated. The effect of liposomes on phagocytosis is shown as the percentage of control phagocytosis of apoptotic cells. Error bars denote the standard deviation

others and is most likely due to the existence of other molecules that facilitate this process.^{8,11,34} These results are consistent with numerous reports which demonstrate that PS-containing liposomes specifically inhibit phagocytosis of apoptotic cells.^{11,21} The ability of annexin V to inhibit phagocytosis has also been used to demonstrate that PS mediates phagocytosis of apoptotic cells.³⁵ In preliminary experiments we have found that annexin V inhibited the phagocytosis of apoptotic BL cells by approximately 45% (data not shown).

The H₂O₂-sensitive factor required for recognition of apoptotic cells is not PS

The ability of H₂O₂ to inhibit phagocytosis of PS-expressing apoptotic cells suggested that an essential surface molecule(s) necessary for phagocytosis is sensitive to oxidation. To further examine this possibility, BL cells were treated with etoposide for 4 h to allow for PS translocation. These cells were phagocytosed at a high rate by macrophages (Figure 5A). Co-treatment with H₂O₂ for the entire 4 h incubation

Figure 5 H₂O₂ treatment of etoposide-treated BL cells reduces phagocytosis but does not affect the binding of annexin V to cells. **(A)** BL cells were treated for 4 h in the absence or presence of etoposide (200 μg/ml) to induce PS externalization. Cells were also exposed to 200 μM H₂O₂ for either the last hour of etoposide treatment or for the entire 4 h incubation. **(B)** Cells were treated as indicated in **A** and stained with annexin V or propidium iodide. Note that cells treated with etoposide in the presence of H₂O₂ for the entire 4 h incubation are PS-negative while the cells treated with etoposide for 3 h followed by treatment with H₂O₂ for 1 h are PS positive. Error bars denote the standard deviation. **(C)** Confocal image of cells stained with annexin V-Alexa Fluor 595 after treatment with etoposide for 4 h. **(D)** Confocal image of cells stained with annexin V-Alexa Fluor after treatment with 200 μM H₂O₂ during the last hour of etoposide treatment

period inhibited phagocytosis by 90%. However, as shown in Figure 5A, when BL cells were treated with etoposide for 3 h to induce PS externalization, and then exposed to H₂O₂ for 1 h to oxidize surface molecules, phagocytosis of these cells was still inhibited by 70%. FACScan analysis indicated that these two populations of cells expressed identical levels of PS and that the cells maintained their membrane integrity since they were impermeable to propidium iodide (Figure 5B). These results suggest that the expression of PS does not guarantee efficient phagocytosis of apoptotic cells, and suggests that another factor(s) is required for recognition of apoptotic cells by macrophages.

Since it has been suggested that PS may be sensitive to oxidation during apoptosis³⁶ we considered the possibility that PS may become damaged following H₂O₂-treatment and that this may account for the inability of macrophages to phagocytose apoptotic cells treated with H₂O₂. This is unlikely since serine moieties are relatively resistant to oxidative modification by H₂O₂, requiring 10–100 mM concentrations and the presence of transition metals such as Fe²⁺ to undergo H₂O₂-induced damage.^{37,38} Our studies are carried out with 0.1–0.2 mM H₂O₂. As there are no published methods to measure selective oxidative modification of PS in the cell membrane, we performed two indirect experiments to address the possibility that H₂O₂-treatment may affect the functionality of PS. First, we evaluated the effect of H₂O₂-treatment on the binding of annexin V to apoptotic cells. FACScan analysis indicated that H₂O₂ treatment of cells does not affect the amount of annexin V that binds to apoptotic cells (Figures 1A and 5B). However, the possibility exists that oxidation affects PS distribution on the plasma membrane. To address this possibility, etoposide-treated BL cells were exposed to H₂O₂ for 1 h and examined by confocal microscopy after staining with annexin V. As shown in Figure 5C, etoposide treated BL cells demonstrated punctuate, uniform, and patchy patterns when stained with annexin V. H₂O₂ treated cells demonstrated identical annexin V staining patterns (Figure 5D). The results indicate that H₂O₂ treatment does not affect the amount of annexin V that binds PS and that H₂O₂ treatment does not affect the distribution of PS on the surface of apoptotic cells. Thus, if H₂O₂ were to modify PS it does not appear to affect the ability of PS to bind annexin V.

The second approach we took to examine whether H₂O₂ affects PS functionally was to treat PS with H₂O₂ and evaluate its ability to mediate phagocytosis of control (non-apoptotic) cells. It has been reported that PS can be incorporated into or onto the outer leaflet of the plasma membrane by mixing PS containing liposomes with healthy cells and that this leads to increased phagocytosis.²⁵ We treated PS liposomes with H₂O₂ for 30 min followed by removal of residual H₂O₂ with catalase. As shown in Figure 6A, when healthy BL cells were treated with PS-containing liposomes, 89% of the cells stained positive with annexin V, indicating that PS had been incorporated onto the plasma membrane. PS liposomes treated with H₂O₂ were incorporated onto the plasma membrane of healthy cells to a comparable degree. Note that this H₂O₂-treated PS is still recognizable by annexin V. As shown in Figure 6B, there

Figure 6 Phagocytosis of healthy BL cells following incorporation of PS from PS-containing liposomes onto the plasma membrane; absence of an effect of H₂O₂. PS-containing liposomes were incubated with or without 200 μ M H₂O₂ for 30 min, and the residual H₂O₂ was removed by treatment with 25 units of catalase for 30 min. The liposomes were then incubated with BL cells to allow for the incorporation of PS onto the plasma membrane. (A) FACScan analysis was conducted using annexin V-FITC and PI staining of cells to monitor for membrane integrity and incorporation of PS onto the plasma membrane. (B) Healthy BL cells containing surface PS derived from PS-liposomes (with or without prior H₂O₂ treatment) were incubated with human macrophages in the absence of serum as described in Figure 2. Error bars denote the standard deviation

was a threefold increase in the phagocytosis of healthy BL cells following incubation with PS-containing liposomes regardless of whether the liposomes had been treated with H₂O₂. These results indicate that the ability of H₂O₂ to inhibit phagocytosis of PS-expressing cells cannot be attributed to oxidative modification of PS on the cell surface since PS treated with H₂O₂ still maintains its functionality in terms of binding to proteins such as annexin V and promoting phagocytosis by macrophages.

Cells induced to undergo apoptosis by H₂O₂ treatment are inefficiently phagocytosed

H₂O₂ treatment of BL cells inhibits apoptosis. In other cell types, however, exposure to oxidative stress has been reported to activate apoptosis. These cell types which include Jurkat T cells³⁹ and murine thymocytes,⁴⁰ may have protective mechanisms that ensure that adequate levels of ATP are maintained for apoptosis. To determine how well cells induced to undergo apoptosis in response to oxidative stress are phagocytosed, Jurkat T cells and murine thymocytes were exposed to either H₂O₂ for 6 h or to etoposide for 3 h. Apoptosis was quantified by measuring PS externalization. As shown in Figure 7A, Jurkat T cells and murine thymocytes translocated similar levels of PS to the cell surface in response to H₂O₂ or etoposide treatments and approximately 30% of the cells stained positive with annexin V. Morphological analysis indicated that a similar proportion of cells had condensed nuclei, consistent with these cells dying by apoptosis (data not shown). Cell membrane integrity was unaffected by these treatments since cells remained impermeable to propidium iodide. Interestingly, phagocytosis of cells that had undergone apoptosis in response to H₂O₂ was only half that observed for cells undergoing apoptosis in response to etoposide. Higher concentrations of H₂O₂ did not inhibit phagocytosis by any greater degree. This may be due to incomplete oxidation of putative recognition factor(s) at the concentrations of H₂O₂ (20–100 μ M) used to induce only apoptosis. Unfortunately, at higher concentrations of H₂O₂ (200–400 μ M), Jurkat and thymocyte membrane integrity is lost and cell death occurs by necrosis. Phagocytosis of necrotic cells is not sensitive to oxidation, and it is very difficult to inhibit phagocytosis of these cells with PS-containing liposomes (data not shown). Nonetheless, these results indicate that phagocytosis of cells induced to undergo apoptosis by H₂O₂ is relatively inefficient even though the cells express PS on the cell surface.

Discussion

The main physiological difference between apoptotic and necrotic death is thought to reside in how the cells affect the surrounding tissue. Apoptotic cell death results in cells that are efficiently removed by macrophages before membrane integrity is lost, and stimulates macrophages to secrete anti-inflammatory cytokines.^{2,41} In contrast, the leakage of intracellular factors associated with necrosis serves as a potent natural adjuvant^{3,42} which can stimulate inflammatory responses as well as generate pathological immune responses to intracellular antigens.⁵

H₂O₂ induces cell death and alters cell death pathways.²⁷ It is generated in high levels by activated phagocytes (neutrophils and macrophages) during the

Figure 7 The phagocytosis of Jurkat T cells and murine thymocytes induced to undergo apoptosis with H₂O₂ is inefficient. Jurkat cells were treated with 100 μ M H₂O₂ (6 h) or 200 μ g/ml VP16 (3 h). Murine thymocytes were treated with 20 μ M H₂O₂ (6 h) or 50 μ g/ml VP 16 (3 h). (A) PS externalization and cell viability were measured by two color FACScan analysis. (B) Phagocytosis of Jurkat cells or thymocytes was measured as described in the legend to Figure 2. Error bars denote the standard deviation

inflammatory response to bacterial pathogens and tumor cells. H₂O₂ rapidly diffuses across cellular membranes to oxidize proteins, DNA, and lipids. It is a key oxidant since it has a high diffusion potential and can serve as a precursor for the more damaging reactive oxygen intermediates, including OH· and HOCl. H₂O₂ production by phagocytes is required for killing of tumor cells and pathogens, but can also cause damage to normal cells and tissues if not contained. (for review see;^{43,44}).

Death induced by H₂O₂ can occur by a variety of mechanisms. Depending upon the target cell type and concentration, H₂O₂ can induce both apoptotic and necrotic forms of cell death. In many systems, the exposure of cells to low doses of H₂O₂ induces apoptosis,⁴⁵ while higher concentrations of H₂O₂ cause necrotic forms of cell death.^{27,46} Since cells dying by necrosis are not phagocytosed until membrane integrity is lost,³⁰ these cells function as potential inducers of inflammatory responses.

The timely removal of apoptotic cells by macrophages is dependent on the externalization of PS to the outer plasma membrane leaflet.¹ Interestingly, the externalization of PS has been shown to be a change that occurs very early when apoptosis is induced.²² Consistent with a role for PS in the phagocytosis of apoptotic cells, we found that the phagocytosis of apoptotic BL cells coincides with the appearance of PS on the cell surface and is specifically inhibited by PS containing liposomes. In addition, when treated with etoposide together with H₂O₂, BL cells rapidly lose their ATP stores due to activation of PARP.²⁷ These cells do not externalize PS and are poorly phagocytosed by macrophages. Thus, PS externalization correlates with the ability of macrophages to phagocytose BL cells. These results are consistent with published reports that the externalization of PS requires ATP²⁶ and is necessary for the phagocytosis of apoptotic cells.²¹

The novel observation that we report in this study is that cells induced to undergo apoptosis in the presence of H₂O₂ are not efficiently phagocytosed even though they have externalized PS. These results have two implications. First, apoptosis induced *in vivo* by oxidative stress may be accompanied by inefficient phagocytosis, which may contribute to perpetuation of inflammatory responses. Oxidative stress is associated with pathogen infection, ischemia-reperfusion injury (i.e. myocardial infarction and stroke), emphysema, acute respiratory distress syndrome, atherosclerosis, rheumatoid arthritis, and cancer (for review see;⁴³). The inefficient phagocytosis of apoptotic cells may contribute to the inflammation associated with these conditions. Second, our results raise the possibility that at least one more factor on the surface of apoptotic cells, in addition to PS, is required to facilitate the efficient phagocytosis of the cells. This putative factor is sensitive to oxidative stress.

In addition to PS externalization, the opsonization of cells with complement,^{8,16,18} and integrin-mediated adhesion have been proposed to function as recognition signals that promote the phagocytosis of apoptotic cells.¹² The possibility therefore exists that these putative mediators may be sensitive to oxidation. However, we have been unable to detect increased binding of complement subunits

C1q, C3, or C4, to etoposide-induced apoptotic BL cells, and have been unable to induce increased phagocytosis by the addition of these factors to the medium (unpublished results). In addition, RGD-containing peptides and integrin-specific antibodies did not inhibit phagocytosis of apoptotic BL cells (J Williams and E Shacter, unpublished observations). Therefore, we hypothesize that oxidative stress probably alters some other factor required for phagocytosis of apoptotic cells.

PS externalization is the only molecular change known to occur on the surface of apoptotic cells. Our results suggest that a factor, other than PS, that is sensitive to H₂O₂ treatment is required for the phagocytosis of apoptotic cells. This putative factor is either present on the surface constitutively or may be transported to the cell surface during apoptosis. It may function either in combination with PS or independently to provide a phagocytosis recognition signal. An alternative model consistent with our data is that oxidative stress induced by H₂O₂-treatment may alter apoptotic cells in other ways to diminish their phagocytosis. For example, H₂O₂-treatment could cause apoptotic cells to release factors or increase expression of factors that inhibit phagocytosis. Our study shows that a closer analysis of the apoptotic cell surface is merited to identify molecules sensitive to oxidation that function as macrophage recognition signals for phagocytosis of apoptotic cells.

Materials and Methods

Cell lines and culture conditions

The human leukemic cell line Jurkat (clone E6-1, ATCC) and the human Burkitt's lymphoma cell line BL-41³⁰ were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 50 μ M 2-ME at 37°C in 5% CO₂ in air. Thymocytes were prepared from two C57BL/6 mice by removing thymus organs and dislodging cells by gently meshing thymus tissue with a screen. Thymocytes were washed twice in culture medium. For treatment with chemicals, cells were cultured in medium with etoposide (VP16, Sigma, St. Louis, MO, USA) or H₂O₂ (Fisher, New Jersey, USA) at concentrations indicated in the figure legends. The 3-aminobenzamide (3-AB, Sigma) was used at a concentration of 1 mM.

Phagocytosis assay and FACSscan analyses

Human monocytes were isolated from leukopaques by elutriation and cultured in 24-well plates for 4 to 7 days at approximately 0.5×10^6 cells/well in media containing 100 ng/ml rhM-CSF. For phagocytosis assays, target cells were labeled with 0.12 ng/ml of the green fluorescent dye CFDA (Molecular Probes #C-1157; 5,6-carboxyfluorescein diacetate, succinimidyl ester, mixed ester) in PBS at 37°C for 20 min. The intensity of green CFDA staining of the target cells did not diminish over the course of any of the incubations.³⁰ Cells were washed one time with Hanks' Balanced Salt Solution and incubated with human macrophages in culture medium for 1 h. The ratio of target cells to macrophages was 2:1 unless otherwise indicated. Macrophages were washed four times to remove BL cells and stained with PE-labeled anti-CD 11b and anti-CD 14 (PharMingen) for 30 min at 4°C. Macrophages were then detached from plates with 1% lidocaine (Sigma) in PBS containing 0.5% FCS. Two color FACSscan analysis was used to determine the percentage of red fluorescent macrophages that had phagocytosed

green fluorescent target cells. Phagocytosis of cells is expressed as the fold increase in the percentage of macrophages that contain apoptotic cells compared to the percentage of macrophages that engulfed untreated (healthy) cells. Typically between 5–10% of the macrophages contain target cells after incubation with control (untreated) BL-41 cells. Results were verified by fluorescence microscopy as described previously³⁰ and showed that there was very little binding of green BL cells to the outer surface of the macrophages. Hence cell staining of both the green and red in the FACScan represent macrophages that have engulfed the target cells.

To determine the percentage of cells expressing PS, cells were stained with 0.5 μ g of annexin V-FITC (PharMingen) and 0.1 mg/ml propidium iodide in 140 mM NaCl, 2.5 mM CaCl₂, 10 mM HEPES, pH 7.4, for 15 min at room temperature. Cells were then analyzed by two color FACScan. In control experiments, the percentage of apoptosis and membrane integrity were confirmed by fluorescence microscopy using Hoechst 33342 and propidium iodide as described previously.²⁷

Preparation of liposomes

1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (sodium salt), 1,2-dioleoyl-sn-glycero-3-[phospho-rac(1-glycerol)] (sodium salt), and 1,2-dioleoyl-sn-glycero-3-phosphocholine were purchased from Avanti (Alabaster, AL, USA). Liposomes were prepared as described by others.¹¹ Briefly, lipids were supplied in chloroform/methanol and were dried under nitrogen gas, resuspended in PBS, and sonicated with a Virtis sonicator. Sonication was performed on ice, 10–30 s pulses, at a 10% output level. Liposomes contained 50% (M/M) phosphatidylcholine, and either 50% (M/M) phosphatidylserine or 50% (M/M) phosphatidylglycerol. Liposomes were used within 4 h of preparation.

Assay for cellular ATP

ATP content in cells was assayed as described previously,³¹ with the following modifications. BL cells (0.5×10^6) were pelleted and then lysed in 150 μ l of 3% HClO₄, followed by neutralization with 75 μ l of 1 M KOH, and 30 μ l of potassium phosphate buffer (1 M K₂HPO₄/KH₂PO₄, pH 7.4) for a 15 min incubation on ice. Cells were then pelleted by centrifugation at $9000 \times g$ for 2 min. The supernatant was used to determine ATP content. Five μ l of sample was mixed with 45 μ l of potassium phosphate buffer containing 4 mM MgSO₄, and added to a 96 well plate containing 50 μ l of 0.8 mg/ml luciferin (Sigma) in 50 mM NaAsO₂, 20 mM MgSO₄. Chemiluminescence was determined with a luminometer. ATP was determined from a standard curve (linear range 20–800 nM). ATP levels in treated cells are expressed as a percentage of the ATP levels in control cells.

Confocal microscopy

1×10^6 apoptotic BL cells were stained with 1 μ l annexin V-Alexa Fluor 595 (Molecular Probes, Eugene, Oregon, USA) in 20 μ l of 140 mM NaCl, 2.5 mM CaCl₂, 10 mM HEPES, pH 7.4. Approximately 5 μ l of a cell suspension was then added to a glass slide and mounted with a cover slip. Annexin V-Alexa Fluor 595 was visualized using a LSM5 PASCAL scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA). Samples were visualized with a $40 \times$ objective and fluorescence was collected using 543–560 nm bandpass emission filters. Images represent a single optical section approximately 1.0 μ m in thickness.

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