Silent Cleanup of Very Early Apoptotic Cells by Macrophages

Kahori Kurosaka, Munehisa Takahashi, Naoko Watanabe and Yoshiro Kobayashi

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Silent Cleanup of Very Early Apoptotic Cells by Macrophages

Kahori Kurosaka, Munehisa Takahashi, Naoko Watanabe, and Yoshiro Kobayashi

Apoptotic cells are phagocytosed as soon as they appear in vivo. In this study, we first determined precisely at what stage apoptotic cells are phagocytosed by macrophages, and then examined the subsequent cytokine production. Phagocytosis was confirmed by flow cytometry and confocal laser microscopy, whereas the subsequent response was examined by ELISA and RT-PCR for quantitative and semiquantitative measurement of the protein and mRNA levels of cytokines, respectively. Even the cell populations containing very early apoptotic cells, such as IL-2-dependent CTLL-2 cells cultured in the absence of IL-2 for 4 h and a murine leukemic cell line, P388 cells, treated with etoposide for 5 h, were phagocytosed by macrophages. Although the cell populations containing the very early apoptotic cells used in this study were FITC-Annexin V-negative and did not show a decrease in cell size as compared with untreated cells, they showed a very small increase in phosphatidylserine on the cell surface, as detected with Cy3-Annexin V, and a decrease in mitochondrial membrane potential, indicating that the cell populations had already started the apoptotic process. Phagocytosis of such populations containing very early apoptotic cells was inhibited by phospho-L-serine much more significantly than Arg-Gly-Asp-Ser. In addition, macrophages hardly produced either proinflammatory or anti-inflammatory cytokines after phagocytosis, thus being an almost null response. These results are contrary to the generally accepted concept that the phagocytosis of apoptotic cells leads to the production of anti-inflammatory cytokines, suggesting instead that cells starting to undergo apoptosis are quickly phagocytosed by macrophages without any inflammation in vivo. The Journal of Immunology, 2003, 171: 4672–4679.

It is widely accepted that macrophages in the phagocytosis of apoptotic cells exhibit an anti-inflammatory response. For example, there has been a representative study in which IL-10 production was detected when human monocytes and apoptotic PBMC were cocultured for 16 h (1). Fadok et al. (2), on the other hand, reported that TGF-β was detected when human monocytederived macrophages (MDM) were cocultured with apoptotic polymorphonuclear cells for 10 h.

Because phosphatidylserine (PS) exposure on apoptotic cells is believed to be involved in the uptake of apoptotic cells by macrophages (3), we have examined the response of macrophages after phagocytosis using annexin V-positive/propidium iodide (PI)-positive cells as apoptotic cells (4). Although such apoptotic cells are at a late stage of apoptosis, there was no release of cytoplasmic proteins, as assessed as to lactate dehydrogenase, indicating that secondary necrosis had not occurred. In addition, it was indicated that the recognition was mediated through PS, because the uptake was suppressed by phospho-L-serine (PLS). Unexpectedly, a large amount of IL-8 was produced when the response of macrophages was examined upon phagocytosis of apoptotic cells. Although the production of IL-8 was always significant irrespective of the stage of apoptosis, the more advanced the stage of apoptosis was the greater the amount of IL-8 produced by macrophages in the phagocytosis of the cells. As our subsequent study revealed, however, when human serum was included during phagocytosis by human macrophages, the level of IL-8 was decreased, whereas the levels of anti-inflammatory cytokines TGF-β and IL-10 were increased, although the phagocytosis efficiency did not change (5). Furthermore, the anti-inflammatory cytokine production by macrophages also increased as the stage of apoptosis advanced. Of note was that the suppressive effect of human serum was not observed with necrotic cells. Therefore, this effect of human serum seems to be specific to apoptotic cells. Overall, phagocytosis of apoptotic cells by macrophages appears to be associated with the production of anti-inflammatory cytokines in vitro.

Although we do not discount these results concerning the production of anti-inflammatory cytokines during coculture with apoptotic cells, we considered whether such production might be physiologically irrelevant. For example, the body has one hundred million apoptotic neutrophils every day. The phagocytosis of apoptotic neutrophils would produce large amounts of IL-10 and TGF-β in the blood. Because apoptotic cells are always phagocytosed not only in the blood but also in other places, high levels of IL-10 and TGF-β should be produced throughout our body. Consequently, unknown mechanisms must operate in vivo to prevent such production. Besides, apoptotic cells are hardly detected in normal tissues. Taken together, we hypothesized that upon receipt of an apoptotic signal, cells at a very early stage of apoptosis are quickly phagocytosed without any adverse effects. To test this hypothesis, in this study, we shorten the induction time for apoptosis to determine by flow cytometry and confocal laser microscopy at what stage apoptotic cells are phagocytosed by macrophages. We also examine the macrophage response upon phagocytosis of apoptotic cells.
Materials and Methods

Induction of apoptosis

An IL-2-dependent CTL line, CTLL-2, was washed with PBS (20 mM PBS, pH 7.4, containing 14 mM Na₂HPO₄ and 6 mM KH₂PO₄) by centrifugation at 1000 × g for 10 min at 4°C, followed by incubation in an IL-2-free RPMI 1640 medium containing 10% FCS (Life Technologies, Gaithersburg, MD) for 0–12 h at 37°C in a culture dish with a cell density of 5 × 10⁵ cells/ml. A murine leukemia cell line, P388s, was adjusted to the cell density of 10⁶ cells/ml in the culture medium, followed by the addition of 1 mg/ml of etoposide (WAKO, Osaka, Japan) to a final concentration of 1 µg/ml, and then the cells were incubated for 5 h at 37°C.

Preparation of cells and phagocytosis

THP-1 cells were maintained in RPMI 1640 medium containing 10% FCS (Life Technologies). The cells were washed with PBS three times, followed by incubation in 10% FCS-RPMI and 160 mM PMA for 72 h at 37°C with a cell density of 5 × 10⁶ cells/ml. THP-2 cells were maintained in RPMI 1640 medium containing 10% FCS, 5 × 10⁻⁵ M 2-ME and 100 µM of recombinant human IL-2 (kindly provided by Takeda Pharmaceutical, Osaka, Japan). To induce apoptosis, CTLL-2 cells were washed with PBS three times, followed by incubation in an IL-2-free medium for various times at 37°C in a cell density of 5 × 10⁵ cells/ml. PMA-treated THP-1 cells were washed with PBS three times, followed by the addition of apoptotic CTLL-2 cells and incubation for 1, 2, or 3 h at 37°C in RPMI 1640 medium containing 10% FCS. As a positive control for cytokine production, PMA-treated THP-1 cells were stimulated with 0.1 µg/ml LPS (Escherichia coli O55B5; Difco, Detroit, MI) for 3 h. Human MDMs were obtained by culturing plastic-adherent human PBMC in RPMI 1640 medium containing 10% FCS and 1000 U/ml of human M-CSF derived from human urine (Denka, Tokyo, Japan) for 3 days, replacing half of the medium with fresh medium, and then culturing them for an additional 4 days. Mouse thiglycollate broth-induced peritoneal exudate cells (PEC), Kupffer cells, and alveolar macrophages were obtained according to the standard methods previously described (6, 7). Irrespective of the kind of macrophages used, phagocytosis assay was performed in RPMI 1640 medium containing 10% FCS.

Flow cytometric analysis

The cell size and PS exposure were analyzed by flow cytometry using a FACScan and Lyssys software (BD Biosciences, San Jose, CA). PS exposure was estimated by FITC- or Cy3-conjugated Annexin V staining. FITC- and Cy3-Annexin V were purchased from Bender MedSystems (Vienna, Austria) and Biovision (Mountain View, CA), respectively. PMA-treated THP-1 cells were cocultered with apoptotic CTLL-2 cells for 3 h at 37°C in RPMI 1640 medium containing 10% FCS. The cells were then washed with PBS three times and placed on ice. Adherent cells were recovered by gentle flushing with prechilled PBS and tapping of the bottom of the plate.

Confocal microscopy

CTLL-2 cells and P388 cells were stained with PKH26 dye (red fluorescence) (Sigma-Aldrich, St. Louis, MO), followed by culturing with macrophages for 3 h at 37°C in RPMI 1640 medium containing 10% FCS in a slide chamber. The cells were washed, and adherent cells were recovered. The cells were then stained with FITC-conjugated mouse mAb to human HLA-ABC class I (Serotec, Oxford, U.K.) for 15 min at 4°C. The cells were examined by confocal microscopy using a Fluoview system (Olympus, Tokyo, Japan). Phagocytosis was confirmed by means of z-axis scanning every time. The phagocytic cells are defined as cells containing at least one PKH26-positive cell. The phagocytosis, as a percentage, was determined by dividing the number of phagocytic cells by the number of macrophages (adherent cells) (at least 200).

MitoTracker staining

To measure the membrane potential of mitochondria, a cationic lipophilic fluorochrome, chloromethyl X-rosamine (CMXRos) (8), was purchased from Molecular Probes (Eugene, OR). CTLL-2 cells were incubated at 37°C for 15 min in the presence of CMXRos (0.1 mM), followed by immediate analysis of fluorochrome incorporation with a flow cytometer. In control experiments, cells were labeled in the presence of uncoupling agent carbonyl cyanide mClCCP (100 µM; Sigma-Aldrich). CMXRos fluorescence was recorded in FL3.
Results

Kinetics of early apoptosis and phagocytosis

Growth factor-dependent cells are known to become apoptotic when cultured in the absence of a growth factor. In this study, we used IL-2-dependent CTLL-2 cells as growth factor-dependent cells. As shown in Fig. 1A (left and middle panels), CTLL-2 cells exhibited a decrease in cell size after 9-h culture in the absence of IL-2. In addition, the exposure of PS was also detected with FITC-Annexin V after 9-h culture (10.2% positive), and the extent of exposure became more significant as the cells were incubated for longer times (for instance 87.5% positive after 12-h culture). The cells were not stained by PI even after 12-h culture. DNA ladder formation and nuclear condensation, conversely, were detected only after 12-h culture and 24-h culture, respectively. Moreover, these apoptotic cells did not release cytoplasmic enzymes, as assessed as lactate dehydrogenase activity.

The human monocytic leukemia-derived cell line THP-1 is known to exhibit increased phagocytic activity on treatment with PMA. Consequently, we have examined the phagocytosis of apoptotic cells by using PMA-treated THP-1 cells as macrophages. In our previous work, there was an increase in cell size when macrophages phagocytosed apoptotic cells, and phagocytosis started after 1-h coculture and reached a plateau after 3-h coculture (4). In this study, therefore, macrophages adhering to the plastic bottom were cocultured for 3 h with CTLL-2 cells incubated in the absence of IL-2 for 0 (control), 3, 4, 6, 9, or 12 h, followed by removal of nonadherent cells by washing. Then, the adherent cells, namely macrophages, were recovered and analyzed by flow cytometry (Fig. 1A, right panel). Phagocytosis was also confirmed by confocal microscopy (Figs. 1B and 2) in which, after coculturing for 1, 2, or 3 h, macrophages were stained with FITC-conjugated mouse mAb to human HLA class I.

When macrophages were cocultured for 3 h with CTLL-2 cells after 4-h culture in the absence of IL-2 (4-h apoptotic CTLL-2 cells), there was an increase in cell size of macrophages (Fig. 1A), suggesting that phagocytosis occurs. The phagocytosis was confirmed by confocal microscopy (Figs. 1B and 2). Fig. 2 demonstrates that 4-h apoptotic CTLL-2 cells (red) were phagocytosed by coculturing with macrophages (green) for 3 h. Although we do not have direct evidence that the phagocytosed cells were really very early apoptotic CTLL-2 cells, the data in Figs. 1B and 2 strongly suggested that macrophages recognized and phagocytosed CTLL-2 cells at such an early stage of apoptosis. Of note was that CTLL-2 cells after 3-h culture in the absence of IL-2 (3-h apoptotic CTLL-2 cells) were hardly or very slightly phagocytosed by macrophages even after 3-h coculture (Figs. 1, A and B, and 2). The phagocytosis after 3-h coculture of 3-h apoptotic CTLL-2 cells was significantly smaller than that after 2-h coculture of 4-h apoptotic CTLL-2 cells, although the total time length of culturing was the same, namely 6 h (Fig. 1B). Furthermore, even when the time length of coculturing was shortened to 1 or 2 h, 4-h apoptotic CTLL-2 cells were also phagocytosed to a smaller extent (Fig. 1B). Taken together, macrophages appear to differentiate between 3-h apoptotic CTLL-2 cells and 4-h apoptotic ones.

Although the data are not shown, we observed the phagocytosis of FITC-Annexin V-negative very early apoptotic P388 cells treated with etoposide for 5 h, suggesting that such phagocytosis is not restricted to CTLL-2 cells.

We then characterized such an early stage of apoptosis as to various criteria including an increase in cell surface PS, lowering of the membrane potential of mitochondria, and the activation of caspase-3-like protease. We first analyzed cell surface PS with Cy3-Annexin V, because Cy3-Annexin V is more sensitive than

![FIGURE 1. A. Kinetics of early apoptosis and phagocytosis. CTLL-2 cells were cultured in the absence of IL-2 for the indicated times, followed by analysis with a flow cytometer for cell size (left panel) and for staining with FITC-conjugated Annexin V (middle panel). The experimental profiles are filled and the control profile is outlined. PMA-treated THP-1 cells, macrophages (MΦ), were cocultured with IL-2-free CTLL-2 cells for 3 h at 37°C in RPMI medium containing 10% FCS, followed by evaluation of cell size (right panel). The experimental profiles are filled and the control profile of macrophages only is outlined. The x-axes are shown linearly for cell size or logarithmically for fluorescence. B. Confocal microscopic analysis of kinetics of phagocytosis. PMA-treated THP-1 cells were cocultured for 1, 2, or 3 h with CTLL-2 cells cultured in the absence for 3, 4, 6, or 9 h, followed by evaluation of phagocytosis under confocal microscopy. The phagocytosis, as a percentage, was determined according to the method described in Materials and Methods. The data are expressed as the mean ± SE of three independent cultures. The differences between 3-h apoptotic CTLL-2 cells and others after coculturing for 1, 2, or 3 h, respectively, were statistically analyzed by one-factor ANOVA followed by Fisher PLSD test. The asterisks indicate significant differences <0.05.](http://www.jimmunol.org/Downloadedfrom/...H11021)
FITC-Annexin V. As shown in Fig. 3, a very small increase in cell surface PS was detected with a flow cytometer. When 3-h apoptotic CTLL-2 cells were analyzed, cell surface PS was the same as found in normal CTLL-2 cells, measured by mean fluorescence intensity (MFI) for normal unstained cells, $17 \pm 1.7$; MFI for normal cells stained with Cy3-Annexin V, $29 \pm 1.1$; MFI for unstained 3-h apoptotic cells, $17 \pm 1.8$; MFI for 3-h apoptotic cells stained with Cy3-Annexin V, $30 \pm 0.7$. When we analyzed very early apoptotic P388 cells after treatment with etoposide for 5 h, essentially the same results were obtained (MFI for control unstained cells, $17 \pm 0.4$; MFI for control cells stained with Cy3-Annexin V, $25 \pm 1.0$; MFI for etoposide-treated unstained cells, $18 \pm 2.2$; MFI for etoposide-treated cells stained with Cy3-Annexin V, $35 \pm 0.8$). Then, we analyzed the membrane potential of mitochondria of very early apoptotic cells by staining with MitoTracker. The MFI decreased from 4351 for live cells to 2540 for the very early apoptotic cells, indicating lowering of the membrane potential of mitochondria (Fig. 4A). The lowering of the membrane potential of mitochondria was also detected in 3-h apoptotic CTLL-2 cells (data not shown). Finally, we examined kinetics of the activation of caspase-3-like protease. As shown in Fig. 4B, a slight but significant activation was observed at 3 and 6 h, whereas the activation peaked at 12 h. Taken together, only the increase in cell surface PS among examined appears to differentiate between 3-h apoptotic CTLL-2 cells and 4-h apoptotic ones, although the differences were very small.

### Cytokine production upon coculturing of macrophages

We then examined the cytokine production upon coculturing of macrophages with apoptotic cells at various stages. First, the mRNA levels of various cytokines in each coculture were determined by semiquantitative RT-PCR (Fig. 5A). We examined proinflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, and TNF-α.
the macrophage response to very early apoptotic cells changed in the presence of human serum. Although the inclusion of human serum did not affect the efficiency of phagocytosis, it led to an increase in the production of anti-inflammatory cytokines IL-10 and TGF-β, and suppression of proinflammatory IL-8 production (Fig. 6). When IgG was added at a final concentration of 5 mg/ml instead of human serum, it also led to an increase in the production of IL-10 (<11 vs 43.5 ± 16.8 pg/ml) and TGF-β (11.7 ± 2.6 pg/ml vs 146.5 ± 32.4 pg/ml), and suppression of IL-8 production (0.53 ± 0.01 ng/ml vs 0.21 ± 0.01 ng/ml). Such changes were similar in the case of macrophages that phagocytosed FITC-Annexin V-high positive cells, but the cytokine production by macrophages phagocytosing very early apoptotic cells was significantly low, irrespective of the presence of human serum. The same was true for etoposide-treated very early apoptotic P388 cells (data not shown).

Inhibition of IL-8 production and phagocytosis

We then examined by what mechanism very early apoptotic cells were phagocytosed by macrophages. First we added inhibitors known to inhibit the phagocytosis of apoptotic cells, namely RGDS and PLS, the former blocking phagocytosis via the vitronectin receptor and the latter blocking phagocytosis through PS (Fig. 7, A and B). PLS showed greater suppression than RGDS when the IL-8 production of macrophages on coculturing was examined (Fig. 7A). Macrophages produced undetectable levels of IL-8 (i.e., <10 pg/ml) without coculturing with apoptotic cells, and these inhibitors did not affect the response. PLS also showed

![Figure 5](image_url)

**FIGURE 5.** Cytokine production upon coculturing of PMA-treated THP-1 cells with CTLL-2 cells cultured in the absence of IL-2 for various times. A, PMA-treated THP-1 cells were cocultured for 3 h with CTLL-2 cells after culturing in the absence of IL-2 for various times. Total RNA was extracted and subjected to RT-PCR as described in Materials and Methods. The relative amount of each mRNA was determined in triplicate as described in Materials and Methods, and expressed as the mean ± SE of the percentage of β2-m. Dash represents macrophages only. The data are representative of three independent determinations. B, The levels of IL-8 protein in the supernatants of 3-h cocultures of PMA-treated THP-1 cells with CTLL-2 cells cultured in the absence of IL-2 for various times were determined in triplicate by means of a specific ELISA, as described in Materials and Methods and expressed in nanogram per milliliters. Dash represents macrophages only. The data are representative of three independent determinations and expressed as the mean ± SE.

and anti-inflammatory cytokines IL-1 receptor antagonist, IL-10, and TGF-β. Among those tested, only the expression of IL-1β, IL-8, IL-10, and TGF-β mRNA was increased. It was commonly observed irrespective of the cytokine that the expression level increased as the stage of apoptotic cells advanced. In addition, no expression of cytokine mRNAs except for that of IL-8 was detected in macrophages upon coculturing with very early apoptotic cells after 4-h culture in the absence of IL-2. Of note was that even IL-8 mRNA was expressed at a remarkably low level. IL-8 protein level was then measured by a specific ELISA (Fig. 5B). IL-8 protein began to be detected in a coculture with very early apoptotic cells after 4-h culture in the absence of IL-2 and it increased with the stages of apoptosis. In our previous work, IL-8 protein was detected as early as the 1-h coculture and reached a plateau after 3-h coculture (5). Consequently IL-8 protein should have also been produced in a coculture with CTLL-2 cells after 3-h culture in the absence of IL-2, although the level was undetectable, i.e., <10 pg/ml (Fig. 5B).

In our previous work, we found that the macrophage response changed from proinflammatory to anti-inflammatory when human macrophages phagocytosed apoptotic cells in the presence of human serum and that one of the responsible components in human serum was identified as IgG (5). Consequently we examined how

![Figure 6](image_url)

**FIGURE 6.** Potentiation by human serum of cytokine production in response to apoptotic cells. The levels of IL-8, IL-10, and TGF-β in the supernatants of cocultures of PMA-treated THP-1 cells with CTLL-2 cells, which were incubated for 4 or 12 h in the absence of IL-2 in the presence of 10% FCS (■) or 10% human serum (HS) (○), were determined in triplicate by means of a specific ELISA, as described in Materials and Methods. CTLL-2 cells after culturing in the absence of IL-2 for 4 h are Cy3-Annexin V low positive. CTLL-2 cells after culturing in the absence of IL-2 for 12 h are FITC-Annexin V high positive. The data are representative of three independent determinations and expressed as the mean ± SE.
greater suppression than RGDS when phagocytosis was examined by flow cytometry (Fig. 7B) and confocal microscopy (Table I), indicating that phagocytosis itself was significantly inhibited by PLS.

Then we examined the effects of PLT, as another phosphoamino acid, and DXS, as an acidic compound, on phagocytosis and IL-8 or MIP-2 production. We also examined phagocytosis by using various macrophages, including PMA-treated THP-1 cells and two kinds of very early apoptotic cells, CTLL-2 cells cultured in the absence of IL-2 and etoposide-treated P388 cells. In this study, phagocytosis was examined by confocal laser microscopy. As shown in Table I, all the macrophages among those tested phagocytosed the cell populations containing very early apoptotic cells, CTLL-2 cells cultured in the absence of IL-2, and etoposide-treated P388 cells, with the percentage of phagocytosis varying from 4% to 50%. Of note was that alveolar macrophages phagocytosed apoptotic cells inefficiently. In contrast, late apoptotic cells such as CTLL-2 cells cultured in the absence of IL-2 for 12 or 28 h and P388 cells cultured with etoposide for 24 h were phagocytosed by macrophages, with the percentage of phagocytosis being close to 90% (see also Figs. 1B and 2). PLS inhibited phagocytosis most significantly, although other reagents tended to also inhibit phagocytosis. PLS also inhibited IL-8 or MIP-2 production most significantly except for the combination of PEC and apoptotic CTLL-2 cells (Table I). When phagocytosis was suppressed, IL-8 or MIP-2 production would tend to be reduced (Table I).

Discussion

This study demonstrated that PMA-treated THP-1 cells, human MDM, mouse Kupffer cells, mouse alveolar macrophages, and thioglycolate broth-induced PEC all recognized and phagocytosed the cell populations containing cells at the very early apoptotic stage (FITC-Annexin V-negative (Fig. 1A) and Cy3-Annexin V low-positive (Fig. 3)), although alveolar macrophages phagocytosed apoptotic cells inefficiently. Such an inefficient phagocytosis of alveolar macrophages was compatible with the published results (13). The recognition and phagocytosis by macrophages of the cell populations containing very early apoptotic cells were confirmed by an increase in cell size, as judged with a flow cytometer (Figs. 1A and 7B), and by confocal laser microscopy (Figs. 1B and 2, and Table I).

This study also demonstrated that such phagocytosis is not accompanied by the production of either anti-inflammatory or proinflammatory cytokines, leading to “silent cleanup” (Figs. 5 and 6). Because cells with apoptotic features are hardly detected in normal tissues, such phagocytosis of very early apoptotic cells without the production of cytokines should occur throughout the body, thereby maintaining an inflammation-free status under healthy conditions.

When the cell populations containing very early apoptotic P388 cells were cocultured with PMA-treated THP-1 cells, somewhat higher levels of IL-8 were produced in comparison with other combinations of apoptotic cells and macrophages (Table I), raising the possibility that silent cleanup may be restricted to particular cell types. Of note, however, was that production of IL-8 after a coculture with the cell populations containing very early apoptotic P388 cells was approximately one-eighth the production of P388 cells treated with etoposide for 28 h (this study and Ref. 14) (see below).

Although the very early apoptotic cells were Cy3-Annexin V low-positive, they showed neither a decrease in cell size (Fig. 1A), DNA ladder formation, chromatin condensation, nor ability to stain with PI. Such a small increase in surface PS, however, was not observed in 3-h apoptotic CTLL-2 cells. Although these data were reproducible, we really need more sensitive assay methods for detection of surface PS and are currently trying to develop it. It has been recognized that apoptotic signals induce lowering of the membrane potential of mitochondria leading to the activation of caspase (15–19). Conversely, PS exposure is controlled by caspases (20) other than caspase-3 (21). Morphological changes, which are key features of apoptosis, are also controlled by caspase-3 (21). To characterize the very early apoptotic cells, we also analyzed the membrane potential of mitochondria and caspase-3-like protease activity. As a result, we found lowering of the membrane potential of mitochondria as well as the activation of caspase-3-like protease in 3-h apoptotic CTLL-2 cells. This demonstrated differences between live cells and very early apoptotic cells, clearly indicating that such very early apoptotic cells have already started the apoptotic process.

We then examined the cytokine production by macrophages on coculturing with apoptotic cells at various stages (Fig. 5A). Of note was that no expression of cytokines except for IL-8 was detected in macrophages upon coculturing with the cell populations containing very early apoptotic cells. The expression level of IL-8 mRNA after a coculture with the cell populations containing the very early apoptotic cells was approximately one-hundredth of that with apoptotic cells after 12-h culture in the absence of IL-2.
(FITC-Annexin V high-positive, PI-negative). When the IL-8 protein level was measured by a specific ELISA, the production after a coculture with the cell populations containing the very early apoptotic cells was approximately one-eighth of the production with apoptotic cells after 12-h culture in the absence of IL-2 (FITC-Annexin V high-positive, PI-negative). Although it is widely accepted that an anti-inflammatory response is associated with the phagocytosis of apoptotic cells, neither IL-10 nor TGF-β was detected at the protein or mRNA level upon coculture with the cell populations containing the very early apoptotic cells. When macrophages phagocytosed the cell populations containing the very early apoptotic cells in the presence of human serum or IgG, we obtained a similar result with FITC-Annexin V high-positive apoptotic cells, in which the production of anti-inflammatory cytokines was much lower than those with FITC-Annexin V high-positive apoptotic cells. IgG was thus principally responsible for the change in anti-inflammatory cytokine production.

Table I. Inhibition of phagocytosis and chemokine production by various reagents

<table>
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<th>THP-1</th>
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<td>–</td>
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<tr>
<td>DXS</td>
<td>0.41 ± 0.1*</td>
<td>0.22 ± 0.01*</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.30 ± 0.1</td>
</tr>
<tr>
<td>P388 5 h</td>
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<tr>
<td>–</td>
<td>13.1 ± 0.5</td>
<td>0.40 ± 0.06</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.3 ± 0.07</td>
</tr>
<tr>
<td>PLT</td>
<td>5.5 ± 0.3*</td>
<td>0.20 ± 0.1*</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.2 ± 0.02*</td>
</tr>
<tr>
<td>PLS</td>
<td>13.5 ± 0.3</td>
<td>0.30 ± 0.1*</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.7 ± 0.02</td>
</tr>
<tr>
<td>DXS</td>
<td>13.0 ± 0.3*</td>
<td>0.22 ± 0.01*</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.7 ± 0.07</td>
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</table>

aMacrophages were cocultured with the cell populations containing Cy3-Annexin V-low positive very early apoptotic cells (CTL-L-2 cells cultured in the absence of IL-2 for 4 h, or P388 cells treated with etoposide for 5 h) at a ratio of 1:2 for 3 h at 37°C. The experiments were carried out once or twice in triplicate (three sets of culture in one experiment).

bPhagocytosis was analysed by confocal microscopy, and expressed as the mean ± SE of the percentage of phagocytosis.

The level of IL-8 or MIP-2 was determined by specific ELISAs. The results were expressed as the mean ± SE. PLT and PLT were used at 1 mM, whereas DXS was used at 500 μg/ml. N.D., not detectable. –, no inhibitor.

* p < 0.05, as compared with no inhibitor.

That the macrophages recognized very early apoptotic cells through cell surface PS, for which the increase was only detected with Cy3-Annexin V (Fig. 3). Although it is possible that a change in the localization of PS on very early apoptotic cells is specifically recognized by macrophages, we could not detect such a change even with Cy3-Annexin V. Because it was recently reported that the phagocytosis of apoptotic cells promotes programmed cell death in Caenorhabditis elegans (22, 23), there is another possibility that recognition of very early apoptotic cells by macrophages dramatically hastens PS exposure. Although we examined possible changes in PS exposure during coculture, we could not detect any. On the other hand, an acidic substance may be concerned with the mechanism of phagocytosis through scavenger receptors, because DXS inhibited phagocytosis by Kupffer cells significantly. Further research is indeed necessary to elucidate the mechanism of phagocytosis of very early apoptotic cells.

Several mechanisms for the phagocytosis of apoptotic cells have been reported so far, as follows: 1) changes in the carbohydrate structure on the cell membrane are recognized by a lectin-like molecule (24), 2) unknown molecules on apoptotic cells are recognized by receptors on macrophages (vitronectin receptor and CD36) through thrombospondin (25–29), 3) PS exposed on the cell surface is recognized by receptors including scavenger receptor LOX-1 and SR-BI (30–32), and 4) apoptotic cells are recognized by CD14, SR-AI/II, and ABC class 1 molecules on macrophages (33–35).

Among the mechanisms previously described, interaction between PS and receptors has been extensively analyzed and supported by many researchers. In the normal cell membrane, most phospholipids are distributed asymmetrically between the double layers, which are thought to be regulated by multiple enzymatic actions to control the transfer of phospholipids (36, 37). Generally, over 90% of PS is localized in the internal layer of the cell membrane. Apoptosis induces changes in these enzymatic activities to...
abolish the asymmetry of the phospholipid distribution and to expose PS on the cell surface (38–40). Although it was found that exposure of PS is controlled by caspases, it has not yet been clarified how enzymatic activities are altered to expose PS during the progress of apoptosis. In contrast, a PS receptor on macrophages was reported in 2000 (30). In addition, Ogden et al. (41) newly discovered another mechanism for phagocytosis through CD91, in which C1q and mannose-binding protein in serum bind to normal cells as well as blebs and calreticulin with altered localization on apoptotic cells, causing phagocytosis. Determination of whether or not such mechanisms are involved in the recognition of Cy3-Annexin V low-positive very early apoptotic cells by macrophages awaits further investigation.

In summary, upon triggering of apoptosis, very early apoptotic cells appear to be silently cleaned up by macrophages as if nothing also happens, contributing to maintenance of homeostasis. Conversely, when the timing for appropriate phagocytosis is retarded for some reason, inflammation may occur through unbalanced production of proinflammatory cytokines under some conditions, such as the absence of human serum in the vicinity of macrophages.

References
