

Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells

Fumitake Takizawa^a, Shoutaro Tsuji^{a,b}, Shigeharu Nagasawa^{a,*}

^aFaculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

^bFaculty of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422, Japan

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Abstract Apoptotic cells activate homologous complement and are opsonized with iC3b. We assessed the effect of iC3b opsonization upon phagocytosis of apoptotic Jurkat cells by macrophages, which were differentiated from THP-1 cells by treatment with retinoic acid. Macrophage phagocytosis of apoptotic Jurkat cells was enhanced upon incubation of the apoptotic cells with normal human serum. The enhanced macrophage phagocytosis of normal serum-treated apoptotic cells was decreased by anti-human C3 F(ab')₂ and anti-CR3 and anti-CR4 mAbs to the level of phagocytosis of those treated with complement-blocked serum. These results suggest that interaction between iC3b on apoptotic cells and complement receptor type 3 (CR3) and/or complement receptor type 4 (CR4) on macrophages could play an important role for the clearance of apoptotic cells by macrophages *in vivo*.

Key words: Apoptosis; Complement receptor; iC3b; Phagocytosis; Cycloheximide

1. Introduction

One of the characteristics of apoptosis, a kind of programmed cell death, is the rapid phagocytosis by macrophages of apoptotic cells before they are lysed to induce inflammatory responses [1]. Apoptosis is known to be accompanied by surface changes that allow recognition and removal of these cells by macrophages. Three types of molecules on macrophages have been proposed to be involved in recognition and processing of apoptotic cells: asialoglycoprotein receptors [2], thrombospondin receptors [3], and phosphatidylserine receptors [4]. Asialoglycoprotein receptors recognize side chain sugars, which become exposed by the release of sialic acid. Thrombospondin receptors, such as vitronectin receptors and CD36, recognize thrombospondin bound to the thrombospondin-binding moiety on apoptotic cells. Phosphatidylserine receptors bind phosphatidylserine, which becomes exposed from the inner membrane to the outer membrane upon apoptosis. Although complement receptors such as CR3 and CR4 are known to play an important role in macrophage phagocytosis [5], little attention has been paid to the role of these molecules in phagocytosis of apoptotic cells.

Complement is a humoral immune system composed of two cascades termed the classical and alternative pathways [6]. Nascent C3b which has a labile binding site is produced upon activation of both complement pathways and binds covalently to the target cell membrane [7]. Most C3b on the target cells is rapidly cleaved by the serum protease factor I into iC3b [8], which is the ligand of the complement receptors, CR3 and CR4. Phagocytes such as neutrophils and macro-

phages rapidly ingest the iC3b-opsonized target cells through CR3 and CR4 [5].

We have reported that apoptotic human cells can activate the alternative pathway of human complement and become opsonized with iC3b [9,10]. It was expected that iC3b–CR3 interaction would promote the binding of iC3b-opsonized apoptotic cells to macrophages and subsequently enhance macrophage phagocytosis of iC3b-opsonized apoptotic cells. Here, we report that iC3b deposited on apoptotic Jurkat cells, a human T lymphoma cell line, can induce CR3-dependent phagocytosis by macrophages, which were differentiated from THP-1 cells by retinoic acid treatment.

2. Materials and methods

2.1. Materials

Anti-Fas mAb (clone CH-11) was obtained from Medical and Biological Lab. Fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ anti-mouse IgG was obtained from American Qualex International. Goat F(ab')₂ anti-human C3 and goat F(ab')₂ anti-mouse IgG were obtained from Organon Teknika. Anti-CR3 mAb (clone 44), anti-CR4 mAb (clone 3.9), FITC, and retinoic acid were obtained from Sigma. Anti-iC3b mAb (clone C5-G) was a gift from Dr. K. Iida, Discovery Research Laboratories, Takeda Chemical Industries. Actinomycin D and cycloheximide were obtained from Wako Pure Chemicals and ICN Biomedicals, respectively.

2.2. Cell culture

Jurkat cells (E6-1), a human T lymphoma cell line, were obtained from ATCC, and the human monocytic leukemia cell line, THP-1, was obtained from JCRB. These cell lines were cultured in RPMI-1640 medium, containing 5% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin G and 100 µg/ml streptomycin.

2.3. Induction of apoptosis and FITC-labeling of apoptotic cells

Jurkat cells (5×10^6 /ml) were induced to undergo apoptosis by incubation with cycloheximide (30 µM), anti-Fas (50 ng/ml), or anti-Fas (50 ng/ml) plus actinomycin D (100 nM) for 16 h at 37°C. The cells were washed with phosphate-buffered saline containing 10 mM EDTA (EDTA–PBS), and apoptotic cell death was confirmed by agarose gel electrophoresis of the DNA fraction. For FITC labeling of apoptotic cells, the cycloheximide-treated apoptotic cells were washed with 50 mM HEPES-buffered saline (HBS), pH 8.5, and the cells (1×10^7 /ml) were incubated in HBS (pH 8.5) containing 4 µg/ml FITC and 0.04% dimethyl sulfoxide for 10 min at 37°C. The FITC-treated cells were then washed with HBS (pH 7.0) and veronal buffered saline, containing 0.15 mM CaCl₂ and 1 mM MgCl₂ (VB⁺⁺), and used as FITC-apoptotic cells.

2.4. Differentiation of THP-1 cells into macrophage-like cells

THP-1 cells (5×10^6 /ml) were incubated with RPMI-1640 containing 5% FBS and retinoic acid (1 µM) for 4 days at 37°C. Retinoic acid-treated THP-1 cells were collected and washed with EDTA–PBS and FBS-free RPMI-1640. The washed cells were suspended in FBS-free RPMI-1640 (2.5×10^6 /ml) and used as macrophages.

2.5. Measurement of phagocytosis by flow cytometry

FITC-apoptotic cells were incubated for 20 min at 37°C with normal human serum, heat-inactivated (56°C for 30 min), or EDTA-

*Corresponding author. Fax: (81) (011) 706-4990.

treated serum (10 mM EDTA in 90% human serum). These cells were washed with VB⁺⁺, suspended in RPMI-1640, and incubated with macrophages (5×10^5 cells) in 24-well plates in RPMI-1640, containing 10% heat-inactivated human serum, at 37°C. Then, each well was washed with PBS containing 0.1% BSA and 0.1% sodium azide to remove the FITC-apoptotic cells that did not bind with macrophages. Macrophages adhering to the 24-well plates were then harvested by pipetting with the same buffer and suspended in 50 mM acetate buf-

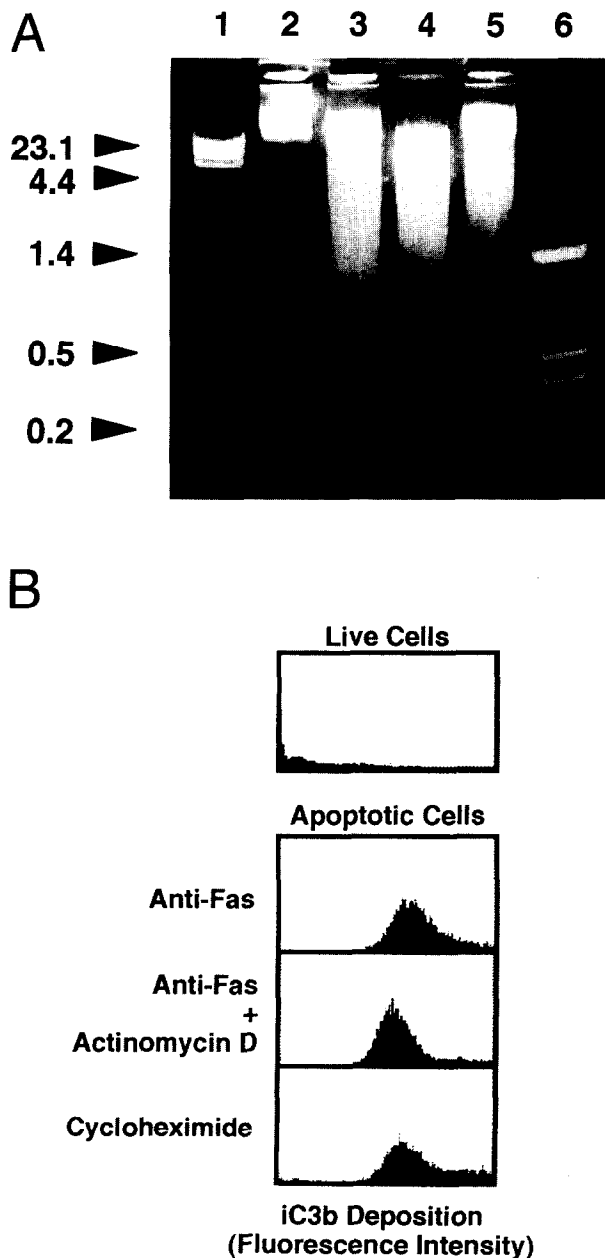


Fig. 1. A: DNA fragmentation in Jurkat cells treated with anti-Fas mAb or cycloheximide. Jurkat cells were incubated with anti-Fas mAb (lane 3), anti-Fas mAb plus actinomycin D (lane 4), or cycloheximide (lane 5) for 18 h at 37°C. Lane 2 was non-treated Jurkat cells. DNA was extracted from these cells and the DNA (5 µg) was resolved by electrophoresis in an agarose gel. Lanes 1 and 5 show molecular weight markers (Lambda/HindIII digest and pUC/HinfI digest), and molecular weight is expressed in terms of kilo base pairs. B: iC3b deposition on apoptotic Jurkat cells upon treatment with human serum. Live cells and apoptotic cells in (A) were incubated with normal human serum for 30 min at 37°C. iC3b deposition was measured with anti-iC3b mAb (clone C5-G) by flow cytometry. The vertical axis shows the cell number and the horizontal axis shows fluorescence intensity.

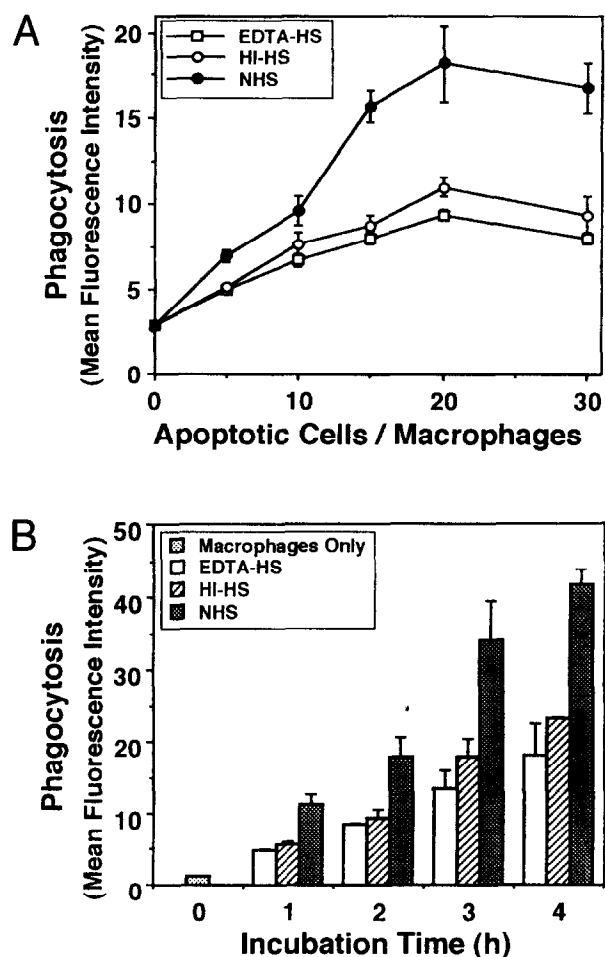


Fig. 2. A: Effect of apoptotic cell/macrophage ratio upon macrophage phagocytosis of apoptotic cells. Macrophages (5×10^5 cells) were incubated in medium (0.4 ml) for 2 h with apoptotic cells (2.5 – 15×10^6 cells) treated with EDTA-serum (EDTA-HS; $\square$), heat-inactivated serum (HI-HS; $\circ$), or normal serum (NHS; $\bullet$). Values of phagocytosis are expressed as mean fluorescence intensity and as the means $\pm$ SD of triplicate determinations. These experiments were each repeated three times with similar results. B: Time course of macrophage phagocytosis of serum-treated apoptotic cells. Macrophages (5×10^5 cells) were incubated for 0, 1, 2, 3, and 4 h with apoptotic cells (8×10^6 cells) treated with EDTA-serum (EDTA-HS), heat-inactivated serum (HI-HS), or normal serum (NHS). Values of phagocytosis are expressed as mean fluorescence intensity and as the means $\pm$ SD of triplicate determinations. These experiments were each repeated three times with similar results.

fered saline (pH 4.5). To quench fluorescence due to FITC-apoptotic cells, which were not internalized by macrophages, trypan blue (2 mg/ml) was added, and the fluorescence due to the internalized-FITC was rapidly determined by flow cytometry (EPICS-CS; Coulter) [11,12]. Fluorescence microscopic analysis confirmed that the fluorescence of FITC-apoptotic cells binding at the macrophage surface was completely quenched by the pretreatment with trypan blue. To examine the effect of anti-human C3 F(ab')₂ upon phagocytosis, serum-treated FITC-apoptotic cells in PBS containing 0.1% BSA and 0.1% sodium azide were preincubated with 10 µg/ml goat anti-human C3 F(ab')₂ for 30 min at 4°C, washed, and incubated with macrophages.

3. Results

Previously, we used anti-Fas mAb to induce apoptosis of Jurkat cells [10]. mAb-Treated apoptotic cells can be phagocytosed through FcR and thus seemed to be unfavorable for

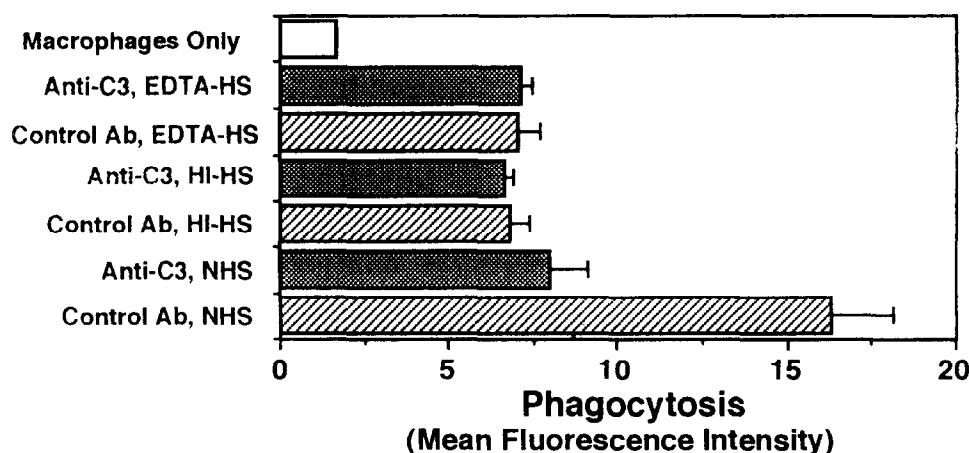


Fig. 3. Inhibition of the enhanced phagocytosis of normal serum-treated apoptotic cells by anti-C3 F(ab')₂. Macrophages (5×10^5 cells) were incubated for 2 h with serum-treated apoptotic cells (8×10^6 cells) incubated with goat F(ab')₂ anti-C3 (Anti-C3) or goat F(ab')₂ anti-mouse IgG (control Ab). Values of phagocytosis are expressed as mean fluorescence intensity and as the means $\pm$ SD of triplicate determinations. These experiments were each repeated 3 times with similar results.

the investigation of CR3-dependent macrophages phagocytosis. Thus, we searched chemical reagents to induce apoptosis of Jurkat cells. Fig. 1A shows the results of gel electrophoresis of the DNA fraction of Jurkat cells which died following treatment with anti-Fas or cycloheximide. DNA fragmentation characteristic of apoptosis was observed with cycloheximide-treated as well as anti-Fas-treated Jurkat cells, suggesting that cell death induced by cycloheximide is mediated by apoptosis. Fig. 1B shows iC3b deposition on these apoptotic cells upon treatment with human serum. iC3b deposition was not observed with serum-treated live cells, suggesting that cycloheximide-induced apoptotic Jurkat cells can also activate homologous complement similarly to Fas-induced apoptotic Jurkat cells. Therefore, we used the cycloheximide-treated Jurkat cells as apoptotic cells in this study.

THP-1 cells differentiate into macrophage-like cells on retinoic acid treatment [13]. Monocytes are known to increase their levels of expressions of CR3 and CR4 upon differentiation into macrophages [14,15]. When THP-1 cells were cultured with 1 μ M retinoic acid, CR3 and CR4 levels were

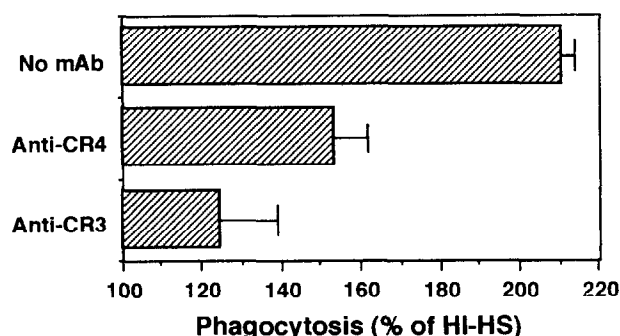


Fig. 4. Inhibition of the enhanced phagocytosis of normal serum-treated apoptotic cells by anti-CR3 and anti-CR4 mAb. Macrophages (5×10^5 cells) were incubated for 2 h with apoptotic cells (8×10^6 cells) treated with normal or heat-inactivated serum in medium containing 10 μ g/ml anti-CR3 mAb (Anti-CR3), 10 μ g/ml anti-CR4 mAb (Anti-CR4), or no mAb (No mAb). Values are expressed as percentages of the phagocytosis of heat-inactivated serum-treated apoptotic cells and as the means $\pm$ SD of triplicate determinations. These experiments were each repeated twice with similar results.

found to increase by 10–20-fold after 4 days then reached a plateau (data not shown). Therefore, THP-1 cells, which had been cultured with retinoic acid for 4 days, were used as macrophages in this study. To a constant number of macrophages, increasing numbers of FITC-apoptotic cells, which had been incubated with normal human serum, were added and the fluorescence incorporated into macrophages was determined by flow cytometry (Fig. 2A). Macrophage phagocytosis increased with increasing number of apoptotic cells and reached plateau at an apoptotic cells/macrophages ratio of 15:1. Although apoptotic cells treated with EDTA- or heat-inactivated serum were also phagocytosed by macrophages, the enhancement of phagocytosis by complement-blocked serum was less prominent than that observed with normal serum. Living cells and the serum-untreated apoptotic cells were only poorly phagocytosed by macrophages (data not shown). As shown in Fig. 2B, phagocytosis of apoptotic cells by macrophages increased time-dependently, but the enhancement of phagocytosis by treatment with normal serum was always by about 2-fold compared to treatment with heat-inactivated serum. These results suggested that phagocytosis of apoptotic cells was enhanced by complement activation on apoptotic cells.

To assess whether this enhanced phagocytosis was due to the opsonic effect of iC3b deposited on apoptotic cells, serum-treated apoptotic cells were incubated with anti-human C3 F(ab')₂. As shown in Fig. 3, the enhanced phagocytosis of normal serum-treated apoptotic cells decreased to the level of phagocytosis of those treated with heat-inactivated serum. Moreover, the phagocytic response enhanced by normal serum treatment was inhibited in medium containing anti-CR3 mAb or anti-CR4 mAb (Fig. 4). These results suggested that the enhanced phagocytosis of normal serum-treated apoptotic cells was caused by interaction between iC3b deposited on apoptotic cells and CR3 and/or CR4 on macrophages.

4. Discussion

Rapid removal of apoptotic cells from tissue is important to prevent tissue damage by toxic substances released from apoptotic cells. At present, three ligand–receptor interactions

have been described for macrophage recognition of apoptotic cells [1]. These studies focused on direct recognition of ligands exposed on apoptotic cells by macrophage and did not take into consideration that apoptotic cells may come into contact with interstitial fluids and thus may be opsonized with serum proteins. Thus, most studies of phagocytosis by macrophages of apoptotic cells were performed in the absence of serum [2–4]. We have reported previously that apoptotic cells can activate homologous complement and are opsonized with iC3b [9,10]. It seemed likely that the interaction between iC3b and CR3 and/or CR4 would augment the recognition and processing of apoptotic cells by macrophages. The present study demonstrated a fourth mechanism for removal of apoptotic cells: phagocytosis by macrophages of iC3b-opsonized apoptotic cells through CR3 and/or CR4.

In the present study, we used cycloheximide to induce apoptosis of Jurkat cells. Cycloheximide has been reported to show dual effects on apoptosis: it inhibits fibroblast growth factor-induced apoptosis of endothelial cells [16], while it augments anti-Fas-induced apoptosis of the sarcoma cell line A673 [17]. We observed that cycloheximide can induce apoptosis of Jurkat cells and that the cycloheximide-induced apoptotic Jurkat cells acquired the ability to activate the human alternative complement pathway, similarly to those induced by anti-Fas mAb.

Macrophages, which were differentiated from THP-1 cells by retinoic acid treatment, phagocytosed cycloheximide-induced apoptotic Jurkat cells only poorly, unless they were treated with normal or heat-treated serum. The enhanced phagocytosis by macrophages of normal serum-treated apoptotic cells was decreased by anti-C3 F(ab')₂ or anti-CR3 and anti-CR4 mAbs to the level of phagocytosis of apoptotic cells, which had been treated with complement-blocked serum. Thus, the interaction between CR3 and/or CR4 and iC3b seems to be operative in phagocytosis of normal serum-treated apoptotic cells by macrophages. However, this finding appeared to be contradictory to the reports that blockage with monoclonal antibodies to macrophages CR3 and CR4 failed to affect macrophage phagocytosis of apoptotic cells [18,19]. However, the difference in the effect of anti-CR3 antibody upon macrophage phagocytosis of apoptotic cells can partly be explained by the difference in the experimental conditions in that the apoptotic cells were treated with diluted serum in the previous studies (< 10%). We observed that effective iC3b deposition on apoptotic cells through the alternative pathway can occur in the presence of the serum concentrations higher than 20% [9]. Thus, effective complement activation did not proceed on apoptotic cells, and consequently CR3–iC3b interaction was not operative in the recognition of apoptotic cells by macrophages.

The present findings do not exclude the roles of other re-

ceptors on macrophages in phagocytosis of serum-treated apoptotic cells. We observed that macrophage phagocytosis of apoptotic cells was also enhanced upon treatment of apoptotic cells with complement-blocked serum, i.e., that treated with heat or EDTA. The opsonic effect of complement-blocked serum may represent the presence of additional opsonic factors.

We reported that CR3 and FcRII on neutrophils act cooperatively in the phagocytosis of iC3b-opsonized immune complexes [20]. Thus, it remains to be clarified whether the interaction between CR3 and iC3b itself can induce macrophage phagocytosis or augments the phagocytic response through other ligand–receptor interactions.

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