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Activation of the alternative pathway of complement by apoptotic Jurkat cells

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Abstract Jurkat T cells die of apoptosis upon exposure to anti-Fas mAb. Here we show that although the alternative complement pathway generally does not attack homologous cells, anti-Fas-induced apoptotic Jurkat T cells were attacked antibody-independently by the alternative pathway of human complement and opsonized with iC3b, which is a ligand of the complement receptor type 3 (CR3) of phagocytes. These results suggest that apoptotic cells become the targets of the homologous alternative complement pathway, which facilitates the clearance of apoptotic cells by phagocytes.

Key words: Apoptosis; Complement; Fas antigen; Jurkat T cell; Phagocytosis

1. Introduction

Apoptosis, a form of programmed cell death, plays an important role in immune responses, such as the negative selection of T lymphocytes in thymus and target cell killing by cytotoxic T lymphocytes [1,2]. The Fas antigen (Fas), is expressed in immature T cells and transduces signals leading to apoptosis [3]. The cross-linking of Fas by anti-Fas mAb has been used for investigating the molecular mechanism leading to the apoptosis of T cells [4]. The physiological significance of apoptosis is the rapid processing of apoptotic cells by phagocytes to protect normal tissues from damage by the harmful contents of dying cells [5]. Although the molecular mechanism leading to apoptosis has been throughly investigated, little is known about the mechanism by which a phagocyte discriminates between apoptotic and normal self cells.

Complement is a humoral immune system and composed of two independent enzyme cascades, termed the classical and the alternative pathways. The classical complement pathway is mainly activated by immune complexes, whereas the alternative pathway is activated antibody-independently by activator molecules on heterologous cells, such as lipopolysaccharides and proteoglycans [6]. The activation of the alternative complement pathway does not proceed on homologous cells, because they lack activator molecules and they express the complement regulatory membrane proteins, decay-accelerating factor (DAF) and membrane cofactor protein (MCP) on their membrane [7,8]. We reported that apoptotic human umbilical vein endothelial cells (HUVEC) can activate the alternative pathway of human complement [9]. These findings prompted us to investigate whether apoptosis also allows T cells to activate homologous complement. Here, we report that anti-Fas-induced apoptotic Jurkat cells, a malignant human T cell line, activate the homologous alternative complement pathway and are deposited with iC3b, the ligand of the complement receptor type 3 (CR3) of phagocytes.

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2. Materials and methods

2.1. Materials

The anti-Fas mAb (CH-11 and 2D1) were obtained from Medical & Biological Lab., Nagoya and Dr. S. Takahashi, Sapporo Medical University, respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG and FITC-labelled goat anti-mouse IgG $F(ab')_2$ were obtained from TAGO. Goat anti-C3 antibody was obtained from Cappel. Anti-C3b mAb (C5-G) was donated by Prof. T. Fujita, Fukushima Medical School. Rabbit polyclonal anti-C3d antibody was prepared in our laboratory.

2.2. Cell culture

Jurkat cells, E6–1, were obtained from the ATCC and cultured in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum.

2.3. Induction of apoptosis

Jurkat cells, 1×10^6/ml, were incubated with anti-Fas (50 ng/ml) for various periods at 37°C, collected and stained with Trypan blue to estimate the ratio of non-viable cells.

2.4. DNA gel electrophoresis

The DNA fraction of Jurkat cells was prepared by the method described by Takahashi et al. [10] and resolved on 1% agarose gel containing 0.05 μg/ml ethidium bromide. DNA bands were visualized by UV light.

2.5. Flow cytometry

Cells exposed to anti-Fas (1×10^6) were washed twice with phosphate-buffered saline (PBS), containing 0.1% bovine serum albumin (BSA) and 0.1% NaN_3, then suspended in 80% human serum. After incubation for 30 min at 37°C, the cells were washed twice with PBS-BSA buffer, suspended in the same buffer, then incubated with anti-C3b mAb (10 μg/ml) for 30 min at 4°C. After two washes with the PBS-BSA buffer, the cells were incubated with FITC-conjugated goat anti-mouse IgG (25 μg/ml) for 30 min at 4°C. Cell-bound C3b was then analyzed in a Coulter Epics-C cytofluorometer (Coulter Electronics, FL)

2.6. Analysis of cell-bound C3b

Jurkat cells (1×10^8) were incubated with anti-Fas (50 ng/ml) for 15 h at 37°C and mixed with 2 ml of Mg^{2+}-EGTA-90% human serum for 30 min at 37°C. The cells were washed twice with EDTA-PBS and solubilized with 1 ml of 1% NP-40, containing 10 mM Tris buffer, pH 7.4, 140 mM NaCl, 0.1% NaN_3, 10 mM EDTA, 1 mg/ml of iodoacetamide, and 1 mM phenylmethylsulfonylfluoride (Wako) by vigorous agitation for 1 h at 4°C. After centrifugation at 100,000 × *g* for 1 h, the supernatants were collected, mixed with goat anti-C3 antibody (2 μg/ml), and immunosorbed by mixing with protein G-plus/protein A-agarose (Oncogene Science, Co.) overnight at 4°C. The immuno-

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sorbed proteins were washed firstly with 20 mM Tris buffer, pH 7.4, containing 2 M NaCl and secondary with 20 mM Tris buffer, pH 7.4, containing 0.5% NP-40 and 0.5 M NaCl, and finally with 10 mM Tris, pH 7.4. The immunosorbents were mixed with 10 mM Tris buffer, pH 6.8, containing 1% SDS and 2% 2-mercaptoethanol, then solubilized by boiling for 5 min. The sample was divided into two aliquots. One was directly resolved by SDS-PAGE according to the method reported by Laemmli [11]. Another was mixed with an equal volume of 2 M hydroxylamine-0.2 M sodium carbonate, pH 10 for 2 h at room temperature, dialyzed against 10 mM Tris buffer, pH 6.8, containing 0.5% SDS at room temperature, then resolved by SDS-PAGE. Gels were transferred to PVDF membranes (Millipore Co.), blocked with 5% non-fat milk and reacted with rabbit anti-C3d polyclonal antibody. After washing, the blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and developed with ECL (Amersham).

3. Results

Fig. 1A demonstrates the time dependent death of Jurkat cells after treatment with anti-Fas (CH-11). Cell death was detectable 3 h after exposure to anti-Fas and reached a plateau after 12 h. The rate of cell death was accelerated when Jurkat cells were treated with higher concentrations of anti-Fas (data not shown). In DNA gel electrophoresis (Fig.1B), a DNA ladder pattern was detected 2–3 h after exposure to anti-Fas, suggesting cell death by apoptosis. Similar results were obtained when Jurkat cells were treated with another anti-Fas mAb, 2D1. Next, we examined whether the apoptotic Jurkat cells can activate homologous complement. C3 contains an intrac hain thioester bond on the α chain (110 kDa) and upon activation to C3b, forms an ester or amide bond with hydroxyl or amino groups on the cell membrane expressing complement activators. Thus, complement activation on target cells is accompanied by the deposition of C3b on their surface. Jurkat cells exposed to anti-Fas were periodically withdrawn and mixed with human serum. Flow cytometry of human serum-treated Jurkat cells demonstrated that C3b deposition became evident 6 h after anti-Fas, treatment and reached a plateau after 12 h (Fig. 2). This result was in accord with the time course of anti-Fas-induced cell death. Evidence that anti-Fas-treated live Jurkat cells cannot activate homologous complement indicates that the complement activation by Jurkat cells is not induced simply by binding with anti-Fas antibody but requires cellular events accompanying with apoptotic cell death. The C3b deposition increased linearly with serum concentrations and reached a plateau at about 50% human serum (data not shown), excluding the possibility that the C3b deposition is due to the non-specific deposition of plasma proteins.

Complement is activated by two independent cascade systems, termed the classical and alternative pathways. The classical complement pathway is generally activated by immune complexes and requires both Ca^{2+} and Mg^{2+} ions, whereas the alternative complement pathway can be activated antibody-independently by various molecules on heterologous cells and requires only Mg^{2+} ions. Apoptotic Jurkat cells were incubated with human serum mixed with EDTA (EDTA-serum) or Mg^{2+}-EGTA (Mg^{2+}-EGTA-serum). Fig. 3 shows that C3b was deposited only when apoptotic Jurkat cells were incubated with Mg^{2+}-EGTA human serum, suggesting that the activation of alternative, but not the classical pathway is responsible for the C3b deposition on apoptotic Jurkat cells.

To verify that the C3b deposited on apoptotic Jurkat cells is covalently bound, C3b deposits were immunoprecipitated with anti-C3b, reduced, and Western blotted with anti-C3d antibody. Since the α chain of C3b is 105 kDa, immunostained bands larger than 105 kDa represent covalent complexes of the α′ chain of C3b and acceptor molecules on apoptotic cells (Fig. 4). Alpha′ chain-acceptor complexes larger than 200 kDa were cleaved by hydroxylamine, indicating the binding of C3b via an ester bond. Two α′chain-acceptor complexes of 130 and 140 kDa did not dissociate after treatment with hydroxylamine, indicating covalent binding via an amido bond. Western blots of the hydroxylamine-treated sample revealed a major band of 65 kDa with a faint band of 105 kDa. C3b is cleaved by the complement regulatory enzyme, factor I, at its α chain (105 kDa) to form iC3b, which is composed of three disulfide chains, α1 (65 kDa), α2 (40 kDa), and β (70 kDa) [12]. The C3d domain is located in the α1 chain. Thus, the release of the 65 kDa fragment as the major band by hydroxylamine indicated that most of C3b deposited on apoptotic cells is converted into iC3b.

4. Discussion

The alternative complement pathway is not usually activated by homologous cells unless some activator molecules are ex-

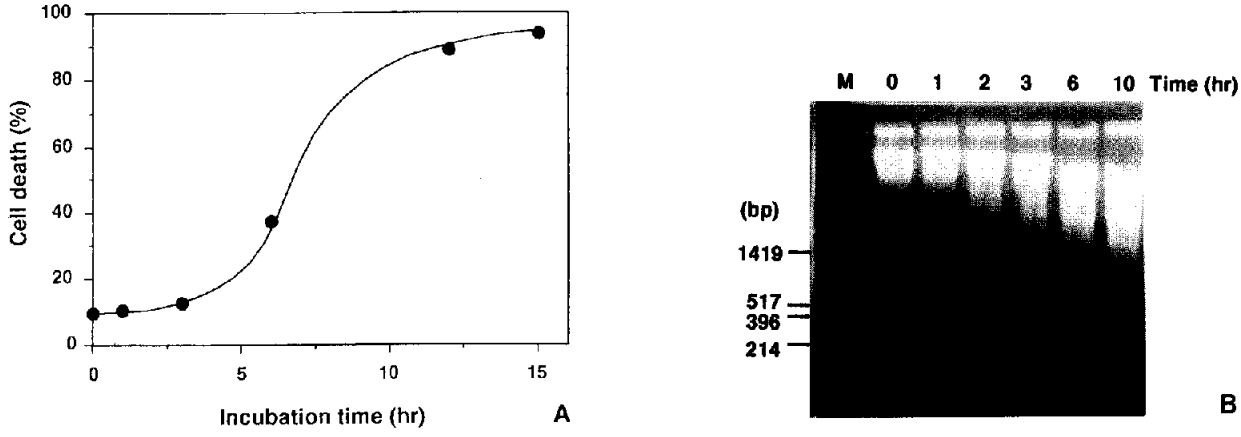

Fig. 1. (A) Time-dependent death of Jurkat cells upon exposure to anti-Fas mAb. Jurkat cells (1×10^6 /ml) were incubated in complete medium containing anti-Fas (CH-11, 50 ng/ml) for various periods at 37°C. Cell death (%): the number of dying cells was estimated by staining with Trypan blue. (B) DNA agarose gel electrophoresis of Jurkat cells. Total DNA of Jurkat cells incubated with anti-Fas for indicated period was processed and analyzed as described section 2. Molecular sizes in bp are indicated at the left of the gel.

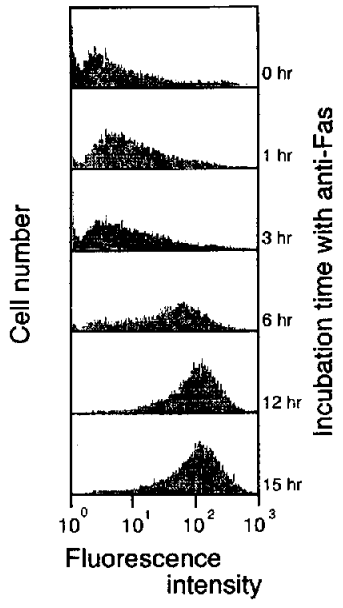

Fig. 2. Flow cytometry of C3b deposition on apoptotic Jurkat cells. Jurkat cells (1×10^6/ml) were incubated in complete medium containing anti-Fas (CH-11, 50 ng/ml) at 37°C. After incubation for various periods, the cells were incubated with 80% normal human serum for 30 min at 37°C. C3b deposition was measured by EPICS analysis as described in section 2. The vertical and horizontal axes express the cell number and the fluorescence intensity, respectively.

pressed on the surface of homologous cells. We found that apoptotic HUVEC can activate the alternative pathway of human complement, suggesting that apoptosis is accompanied by the expression of the complement activator molecule on apoptotic cell surface [9]. Apoptosis of T cells has attracted considerable interest because it plays a pivotal role in the negative selection of self-reactive T cells. In this study we examined whether apoptotic T cells, such as HUVEC, can activate the homologous alternative complement pathway.

Jurkat cells, a malignant human T cell line, induce apoptosis upon exposure to a monoclonal antibody against human Fas antigen [10]. We treated Jurkat cells with anti-Fas mAb, then assessed the cell death and C3b deposition, which represents the degree of homologous complement activation. Although mAb-treated Jurkat cells can be regarded as a type of immune complex, they failed to activate human complement unless they died of apoptosis. These results suggest that the homologous complement activation is not induced simply by the binding of anti-Fas mAb to Jurkat cells. In addition, the C3b deposition on apoptotic Jurkat cells seems to occur via the alternative complement pathway, as is true of apoptotic HUVEC [9].

The activation of the alternative complement pathway requires activators, which allow the amplification loop of complement activation on target cells. Malignant human leukemia B cell lines, such as Daudi and Raji cells, can activate the alternative pathway of human complement. CR2 of these B cell lines are responsible for the homologous complement activation via the alternative pathway [13,14]. Evidence that the degree of C3b deposition on anti-Fas-treated Jurkat cells correlates with the increase in their death, suggests that an activator molecule is expressed on apoptotic Jurkat cells. Whether putative activator molecules are de novo synthesized upon apoptosis or simply migrate from the cytosol to the cell surface, remains to be elucidated.

Most of the C3b deposited on apoptotic Jurkat cells is converted into iC3b, which is a ligand for the CR3 of phagocytes. This information suggests that with deposits of iC3b, apoptotic

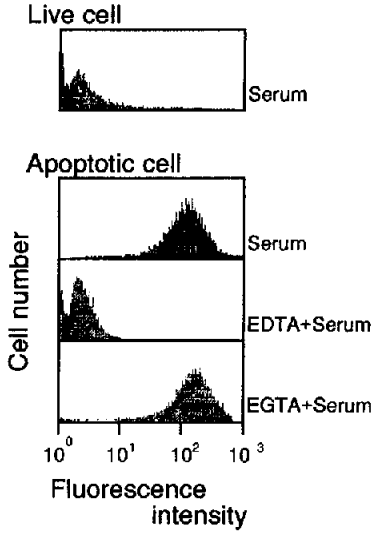

Fig. 3. Effect of EDTA and EGTA upon C3b deposition on apoptotic Jurkat cells. Jurkat cells were incubated with anti-Fas (CH-11, 50 ng/ml) for 15 h at 37°C, then were incubated with 80% human serum containing 20% saline (normal serum), 80% human serum containing 20% saline and 10 mM EDTA (EDTA + serum), or 80% human serum containing 20% saline, 30 mM EGTA, and 35 mM $MgCl_2$ (EGTA + serum). Normal Jurkat cells were similarly incubated. C3b deposition was assessed by flow cytometry as described in section 2.

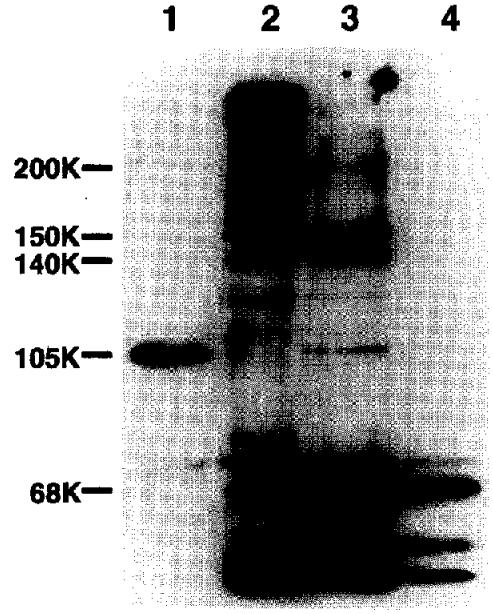

Fig. 4. Western blot of the C3b deposition on apoptotic Jurkat cells. Apoptotic cells prepared as described in the legend to Fig. 3 were incubated with 90% EGTA-serum. C3b deposition was collected by immunoprecipitation, resolved by SDS-PAGE, and Western blotted as described in section 2. Lane 1, reduced C3b (0.2 μg); lane 2, immunoprecipitates of EGTA-serum-treated apoptotic cells; lane 3, hydroxylamine-treated lane 2 sample; lane 4, reduced iC3b (0.3 μg).

cells are processed promptly by phagocytes. We found the that phagocytosis of immune complexes by neutrophils becomes enhanced when C1q is deposited upon immune complexes and that active oxygen formed during the processing of C1q-immune complexes in phagosomes is not released extracellularly (Ohkuro et al., submitted). It is of interest to assess whether iC3b-deposition accelerates the processing of apoptotic cells by phagocytes without releasing harmful cellular components causing secondary tissue damage.

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