Accelerated Fas-Mediated Apoptosis of Monocytes and Maturing Macrophages from Patients with Systemic Lupus Erythematosus: Relevance to In Vitro Impairment of Interaction with iC3b-Opsonized Apoptotic Cells

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Accelerated Fas-Mediated Apoptosis of Monocytes and Maturing Macrophages from Patients with Systemic Lupus Erythematosus: Relevance to In Vitro Impairment of Interaction with iC3b-Opsonized Apoptotic Cells

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Impaired handling of apoptotic cells has been suggested as an important factor in the development of systemic lupus erythematosus (SLE), and a role in complement in the removal of apoptotic cells was shown recently. We studied the in vitro function of macrophages from 40 patients with SLE and their matched controls in the removal of heterologous apoptotic cells opsonized by iC3b. Interaction index of apoptotic cells opsonized by iC3b was significantly lower in patients with SLE and averaged 71% ± 37 of that of healthy individuals (p < 0.002) and 69% ± 35 of patients with rheumatoid arthritis (p < 0.007). SLE patients had increased apoptosis of both freshly isolated monocytes (p < 0.001) and maturing macrophages (p < 0.04) that led to decreased density of monocyte-derived macrophages. Apoptosis was inhibited by adding soluble Fas receptor indicating Fas-mediated apoptosis. As demonstrated in both healthy controls and patients with SLE, decreased macrophage density by itself caused significant decreased uptake of apoptotic cells by the remaining macrophages. Maintaining normal density in SLE patients either by an increased initial density or by using soluble Fas restored the interaction capacity of the individual macrophages in the majority of patients. We concluded that impaired in vitro interaction of iC3b-opsonized apoptotic cells with macrophages from patients with SLE was mainly associated with Fas-dependent accelerated apoptosis of the monocytes/macrophages. Accelerated apoptosis of phagocytes may represent a novel in vitro mechanism of impairment of interaction with apoptotic cells that, apart from reducing the number of professional phagocytes, alters the function of the remaining macrophages. The Journal of Immunology, 2001, 167: 5963–5969.

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of apoptotic cells such as αβ, CD14, CD36, and phosphatidylserine receptor is not dominant. We show that a decrease in interaction characterized most patients with SLE and was associated with accelerated Fas-mediated apoptosis of monocytes and maturing macrophages.

Materials and Methods

Patients

Forty SLE patients who fulfilled the revised American Rheumatism Association criteria for SLE from an outpatient rheumatology clinic participated in the current study, which was approved by the institutional ethics committee, Ministry of Health, Jerusalem, Israel. Patients on cytotoxic drug therapy, cyclophosphamide, azathoprine, or methotrexate, as well as those under treatment with prednisone >30 mg/day were excluded. After signing an informed consent, the patients were evaluated for SLE disease activity index and 30–45 ml of peripheral venous blood was drawn. Forty sex- and age-matched (±4.2 years) healthy controls were recruited from the hospital staff on the same day, and 26 sex- and age-matched (±3.5 years) patients with rheumatoid arthritis (RA) comprised the disease control group.

Cell isolation and culture

Human mononuclear cells were isolated from heparinized peripheral blood by density gradient centrifugation and adherence to plastic (17), from which 10⁴/ml mononuclears were plated, unless indicated otherwise. The cells were cultured with 10% AB human serum (Sigma-Aldrich, MO) in Iscove’s medium (Biological Industries, Kibbutz Bet-Haemek, Israel). After 7 days of culture, the cells matured into macrophages (>95% pure, as assessed by CD14 staining) and were used for interaction assays.

Apoptosis

Apoptosis of thymocytes from BALB/c or C3H/N/J was induced by irradiation (600 rad) or serum deprivation (3). Apoptosis was confirmed by morphology as well as by two flow cytometry methods: propidium iodide (PI) staining of fragmented DNA (23) and phosphatidylserine staining by annexin V conjugated to FITC (24) (R&D Systems, Minneapolis, MN). More than 70% of the cells were apoptotic and had an intact membrane, as evidenced by 0–2% trypan blue-positive cells and 2–4% PI-positive cells. Apoptosis of monocytes was assessed both by staining of fragmented DNA following 20 min of trypsin-EDTA treatment and by 24-h interval counting of marked areas on the slides. Triplicates of marked areas contained at least 200 adhered monocytes at day 0. Eight SLE patients (20%) had low lymphocyte counts, and two (5%) had low monocyte counts on the day of blood donation. However, survival counting was started 1 h after adherence and applied to adhered monocytes only.

Interaction of apoptotic cells with macrophages

Interaction assays were performed simultaneously on peripheral blood monocyte-derived macrophages obtained from healthy individuals and the SLE study patients, and when possible simultaneously with patients with RA, as described elsewhere (17). Briefly, to examine the behavior of the iC3b-opsonized apoptotic cells and the complement receptors, CD11b/CD18 and CD11c/CD18, specifically, a heterologous system was used with murine apoptotic thymocytes and human monocyte-derived macrophages. In this system, >90% of apoptotic cells are opsonized by iC3b and interact via CD11b/CD18 and CD11c/CD18 (17). Macrophages (~25,000) were incubated for 1 h at 37°C/5% CO₂ with 10⁷ apoptotic cells (approximate ratio of 1:40) in triplicate in the presence or absence of 10% AB serum (Sigma-Aldrich) or complement-deficient sera (Sigma-Aldrich). The interaction was stopped by dipping the slides in ice-cold PBS to remove the nonbound apoptotic cells. In some experiments, we added trypsin-EDTA (Biological Industries) for 20 min and cytosprined the cells. The slides were fixed with 100% ethanol and Wright stained. The proportion of free and macrophage-bound target cells was established by counting 400 monolayer cells per test sample. The interaction index was defined as the number of apoptotic thymocytes that were attached to 100 macrophages. The term “interaction index” was used to describe association between apoptotic cells and macrophages without further kinetic evidences for clearance. Some authors used trypsin-EDTA to distinguish between binding or tethering and phagocytosis. Following the interaction assay, we added several samples trypsin-EDTA for 20 min and reevaluated interaction index. We observed a 30–40% decrease in the index, suggesting that >60% of opsonized apoptotic cells were internalized or trypsin resistant bound to macrophages. However, we were not sure that this method distinguishes between bound or internalized apoptotic cells, so it was not applied to the whole study. Accuracy was considerably enhanced by video imaging the high-power microscopic fields. Two independent observers who were blinded to conditions evaluated the samples. Receptor-blocking reagents were added before the incubation with apoptotic cells in some experiments. Interaction index was also evaluated as a function of macrophage densities. Although plated monocyte numbers were comparable, the actual numbers per one square millimeter could vary according to the area chosen (usually between 200 and 300 monocytes at 1 h after adherence). Absolute numbers could have been misleading and were used only for single donor evaluation to compare the survival rate between different donors, we used percentage density of macrophages instead of total numbers. For example, if donor A and donor B had 100 cells per one square millimeter by day 7, their mean percentage density would depend on their number at day 0. If, for instance, by day 0 donor A had 200 cells per one square millimeter and donor B had 300, their mean percentage density at day 7 would be 50 and 33% for A and B, respectively. The term “mean” was used because we averaged three counts.

Validation of interaction index was obtained by staining apoptotic cells with PKH-27 (Sigma-Aldrich), and using flow cytometry for measuring uptake of apoptotic cells (16). Chinese hamster ovary (CHO) transfected with CD11b/CD18 (25) was used to further show the role of CD11b/CD18 in this system (17).

Blocking mAbs and peptides

The following reagents were used to determine the relative contribution of each receptor. Macrophage lectin receptor was inhibited by 20 mM N-acetyl glucosamine (Sigma-Aldrich); αβ, the vitronectin receptor, was blocked with 2 mM tetra peptide RGDS (Sigma-Aldrich) or control tetra peptide RGES (Sigma-Aldrich) for 15 min/37°C/5% CO₂. CD36 was blocked with the anti-CD36 mAb (FA6, kindly provided by R. Silverstein, Cornell University, New York, NY) or an isotype-matched control Ab. CD14 was blocked with anti-61D3 (kindly provided by C. Grey, Cornell University, New York, NY) or anti-CD14 (63D3, control mAb; American Type Culture Collection, Manassas, VA), and the complement receptors, CD11b/CD18 and CD11c/CD18, were blocked by mAbs, anti-CD11b/CD18 (1 domain, MN-41, a gift from V. Vetticka, Louisville, KY), and anti-CD11c/CD18 anti-CD11c/CD18 (anti-CD11c/CD18). Soluble Fas/Fc chimera (R&D Systems) and Fc fragment (Cheminon International, Temecula, CA) were used for blocking Fas-mediated apoptosis.

Flow cytometry

Mononuclear cells were gated using forward and side scatter to distinguish between lymphocytes and monocytes. The gating was verified by using CD14-APC (IQ Products, Groningen, The Netherlands) staining for monocytes. Apoptosis was determined using PI and hypodiploid staining or annexin V FITC and PI for nonfixed cells. Flow cytometry analysis was performed on a FACScan (BD Biosciences, Mountain View, CA).

Statistics

Experiments were performed in duplicate or triplicate, and average results were compared among healthy donors, SLE patients, and patients with RA. Student’s t test was used to compare results.

Results

Opsonization with iC3b enabled interaction of apoptotic thymocytes with human macrophages via the complement receptors

As shown in Fig. 1A, complement factors were required for interaction of >90% of apoptotic thymocytes with human macrophages. More than 90% of the apoptotic thymocytes were coated with iC3b (data not shown). The interaction index of healthy individuals that had been 541 ± 74 in the presence of human serum, decreased dramatically both by heat inactivation and by C1q, C2, C3, and C4 depletion. Moreover, adding the missing factors, but not C9, restored the uptake. To verify that CD11b/CD18 and CD11c/CD18 were important in the interaction with iC3b-opsonized cells, 20 mM N-acetyl glucosamine and mAbs to CD14 (61D3), CD36, CD11b/CD18, CD11c/CD18, and RGDS, inhibitory tetra peptide to vitronectin receptor, were examined for inhibition (Fig. 1B). Significant inhibition of interaction was seen by using mAbs to CD11b/CD18 and CD11c/CD18 (p < 0.001 and p < 0.006, respectively). Mild inhibition (p < 0.08) was seen
using RGD peptide. To further establish the role of complement receptors in this system, a CHO cell line transfected with human CD11b/CD18 was examined for uptake of iC3b-opsonized apoptotic murine thymocytes. Adhered cells with no transfection showed an interaction index of 3 ± 1, whereas transfected CHO cells had 166 ± 19 (p < 0.0001). Taken together, these results confirm that in this assay the complement system is activated by apoptotic thymocytes is seen using 20 mM N-acetyl glucosamine (Lectin) and mAbs to CD14–61D3 (CD14) or CD36 (CD36). Mild inhibition (p < 0.08) is seen using RGD peptide (RGD).

**Impaired interaction between apoptotic thymocytes opsonized by iC3b and monocyte-derived macrophages from SLE patients**

Of 40 blood samples from SLE patients, 30 interaction assays were performed using monocyte-derived macrophages from SLE patients. The assays were performed simultaneously with 30 healthy and disease (RA) controls. Eight additional sets were excluded due to an extremely low survival rate of macrophages from SLE patients (data not shown). Although the same number of mononuclear cells from SLE, RA, and healthy donors was plated on slides, the slides from SLE patients contained fewer macrophages per area when compared with healthy individuals. To further explore this observation, 19 SLE patients were evaluated for survival and apoptosis. The numbers of monocyte-derived macrophages per one square millimeter were significantly reduced in SLE patients (p < 0.01) (Fig. 3). However, two subpopulations could be identified, and 11 of 19 counted (58%) SLE patients had significantly reduced survival, while the rest were in normal range. In addition, eight of 38 patients with SLE and none with RA were excluded from the assays due to

**Decreased survival of monocyte-derived macrophages in SLE**

These results indicate that at least one-third of SLE patients had specific impairment in interaction of iC3b-opsonized apoptotic cells with macrophages. No significant differences were noted between the subgroup with severe impairment and the rest of the group with regard to SLE disease activity index or disease duration (data not shown).

**FIGURE 2.** Impaired interaction of iC3b-opsonized apoptotic cells with macrophages from SLE patients. The interaction index of patients with SLE or RA is presented as percentage of healthy controls. Whereas interaction with apoptotic cells by RA patients (97.1%) was not significantly different from controls, interaction with apoptotic cells by SLE patients (71.1%) was significantly reduced (p < 0.002, healthy controls; p < 0.007, RA patients).

**FIGURE 3.** Decreased survival of monocyte-derived macrophages from SLE patients. Mononuclear cells were plated and counted following adhesion and washing of nonadherent cells. The decrease in numbers of monocytes/macrophages from day 1 to day 6 is presented. Six SLE patients and six age-matched healthy donors (Controls) are presented. Macrophages from SLE patients had an average survival of 55.4 ± 14.4% compared with that of healthy donors, 77.4 ± 2.4% (p < 0.01).
extremely reduced survival and low numbers of macrophages at day 7. Taken together, at day 7 in culture, approximately two-thirds of SLE patients had decreased survival of adherent monocyte-derived macrophages.

Monocytes and macrophages from patients with SLE show accelerated Fas-mediated apoptosis

To verify that the decreased numbers of macrophages were due to accelerated apoptosis, we examined first the percentage of apoptosis in freshly isolated monocytes. An increased apoptosis rate of freshly isolated lymphocytes and mononuclear cells had been reported by others (26, 27). However, no data were provided regarding freshly isolated monocytes in these studies. To determine whether the decreased numbers of monocyte-derived macrophages are due to accelerated apoptosis of freshly isolated monocytes, we examined 14 samples from SLE patients and compared them with healthy and RA controls. As shown in Fig. 4A, apoptosis of monocytes was significantly increased in the SLE patients when compared with healthy (p < 0.001) or RA (p < 0.004) controls. Furthermore, monocyte apoptosis was much more significant than lymphocytes, as shown in Fig. 4B. The next question to be examined was whether accelerated apoptosis characterizes freshly isolated monocytes only or is seen during macrophage maturation as well. We tested the rate of apoptosis of the monocytes upon their maturation to macrophages on a daily basis. As shown in Fig. 5A, the percentage of apoptosis was significantly (p < 0.04) increased in patients with SLE during monocyte/macrophage maturation. Thus, in patients with SLE, both freshly isolated monocytes and adhered monocytes that mature to macrophages show increased rate of apoptosis. Since apoptosis of monocytes during maturation to macrophages was shown to be Fas (CD95) dependent (28, 29), we next asked whether the acceleration in apoptosis of monocytes in patients with SLE is Fas dependent. As shown in Fig. 5B, monocytes exposed to recombinant human Fas/Fc chimera, but not to control, had restored numbers of macrophages at day 7.

An increased rate of apoptosis of monocyte-derived macrophages was the major determinant for impaired interaction between iC3b-opsonized apoptotic cells and macrophages

No significant differences were seen in the expression of CD11b/CD18 or CD11c/CD18 on macrophages from SLE patients when compared with healthy controls (data not shown). Although the impaired clearance could have been due to intrinsic malfunction of the receptors, we examined the role of macrophage density in relation to interaction with apoptotic cells. Our assumption was that the reduction of macrophages should not, by itself, have changed the interaction index, since it expresses the ability of the remaining macrophages only to bind/engulf apoptotic cells. To verify that the increased death of monocytes is not a major contributor to the decreased uptake observed in the remaining macrophages, we prepared gradually decreasing densities of macrophages from healthy individuals and evaluated their interaction index as an expression of their density (Fig. 6A). We were surprised to see that a decreased density by itself caused significant decreased uptake of apoptotic cells, meaning that the function of the remaining macrophages was altered by the absence of other macrophages that underwent apoptosis. This is further emphasized by the fact that the number of apoptotic cells was not reduced, allowing higher ratios of apoptotic cells/macrophenes. To further examine whether the low density of monocyte-derived macrophages in SLE patients influences the altered uptake in these patients, we used macrophages with added Fas/Fc chimera at day 0 for evaluation of interaction index. As shown in Fig. 6B, a dramatic improvement of the impaired interaction index was observed when macrophages were in densities similar to those of healthy controls (p < 0.002). We performed a similar set of experiments in which we plated at day 0 up to 80% higher concentrations of monocytes from patients with known accelerated apoptosis. On day 7, just before the phagocytic assay, we counted macrophages per area and included patients with ≥15% of densities of healthy donors and compared them with both healthy donors and their own macrophages being plated at normal densities. Again, the interaction index improved dramatically (data not shown). We named this phenomenon a loss of community effect, indicating that macrophages need to be in a close contact with one another to better bind/engulf apoptotic cells.

Discussion

Handling of apoptotic cells was suggested to have an important role in the development of autoimmunity (1, 3, 6, 17, 30–33).
Noninflammatory presentation of constituents of apoptotic cells by APCs maintains homeostasis and prevents autoimmune reaction (34–36). However, secondary necrosis may occur if phagocytosis is inefficient. As shown recently (32), phagocytosis of necrotic cells by dendritic cells caused maturation with expression of CD83 and costimulatory markers CD40 and CD86, as opposed to phagocytosis of apoptotic cells that do not mature dendritic cells in the absence of conditioned media. Using a unique heterologous system in which complement activation is the dominant factor leading to interaction with apoptotic cells (17), we were able to show that the majority of SLE patients had decreased in vitro interaction between macrophages and apoptotic cells. This is in accordance with a previous observation of Hermann et al. (30). However, in this specific system in which association of apoptotic cells with macrophages was complement dependent, the role of other important receptors for the uptake of apoptotic cells such as α, β2, CD14, CD36, and phosphatidylserine receptor was not examined. In this system, we demonstrated that accelerated apoptosis of both freshly isolated monocytes and monocytes maturing to macrophages was complement dependent, the role of other important receptors for the uptake of apoptotic cells such as α, β2, CD14, CD36, and phosphatidylserine receptor was not examined. In this system, we demonstrated that accelerated apoptosis of both freshly isolated monocytes and monocytes maturing to macrophages was associated with in vitro impairment in interaction between apoptotic cells and macrophages. The accelerated apoptosis dramatically influenced the efficiency of clearance of the remaining macrophages. Indeed, upon restoration of the numbers of macrophages per one square millimeter, many SLE macrophages were able to interact with apoptotic cells similarly to normal and RA controls. Interestingly, a community effect is seen both in patients with SLE and healthy controls and may explain conflicting results reported from different studies examining uptake of apoptotic cells without careful comparison of their densities. A loss of community effect could be secondary to physical contact by pseudopods or ingredients of the microenvironment like cytokines, chemokines, or serum proteins (31). A community effect may contribute to each step of migration toward an apoptotic cell, i.e., protrusion, adhesion, traction, and deadhesion (37).

The next question that arises is why do monocytes/macrophages from SLE patients have accelerated apoptosis? Sera from SLE patients were suggested to inhibit adhered monocyte motility and, as a consequence, decreased phagocytosis of yeast (38) or to induce apoptosis to monocytes (39). In this study, monocytes were maturing in the presence of AB serum from healthy donors for 1 wk, and the interaction assays were done in the presence of AB serum. Furthermore, the factors suggested to mediate these effects were low complement factors and the presence of autoantibodies or immune complexes, none of which was present in the serum used. Another possibility is expression of proapoptotic molecules following activation, as suggested for lymphocytes (40). In contrast to ced-9 in C. elegans, mammalian homologues have both anti- and proapoptotic genes (inhibitors such as Bcl-2 and promoters such as Bax), and proapoptotic equilibrium of bcl-2 family may appear in lymphocytes from SLE patients (41–43). Alternatively, the withdrawal of growth factors and cytokines may have a paracrine and/or autocrine role, as shown for lymphocytes (43, 44). However, recent reports explored death mechanisms appearing specifically in monocytes and monocytes maturing to macrophages in healthy individuals. Human monocytes undergo spontaneous apoptosis due to Fas upon being cultured by an autocrine or paracrine pathway (28, 29). The death receptor, Fas, a member of the TNFR family, efficiently activates caspases and induces apoptosis following binding and trimerization. Fas-Ig fusion protein, an antagonistic anti-Fas mAb, and a rabbit anti-Fas ligand Ab all greatly reduced the onset of apoptosis (29). In this study, adding soluble Fas avoided accelerated apoptosis of monocytes, indicating that the accelerated apoptosis is at least partially Fas mediated and may be related to activation-induced cell death (45).

Acceleration of
additional non-Fas-dependent apoptosis pathways was not excluded in this study. We observed in a few patients severe acceleration in apoptosis during maturation, but most death occurred at days 0–2, and macrophages that survived showed normal morphology. The reason that the vast majority of death occurred during the first day of maturation may be related to the late expression of Fas-associated death domain-like IL-1β-converting enzyme-inhibitory protein (FLIP) activity (46). Monocyte-derived macrophages, despite the expression of both Fas and Fas ligand, do not undergo spontaneous apoptosis and are not sensitive to stimulation by an agonistic anti-Fas IgM. Recently, a protective mechanism in monocyte-derived macrophages that exist at a site downstream of the receptor-ligand interaction was identified. Monocyte differentiation into macrophages was recently shown to correspond to up-regulation of FLIP, in association with a decrease in Fas-mediated apoptosis (47). Thus, the remaining maturing macrophages may be protected from Fas-dependent apoptosis due to expression of FLIP. The last question is whether this in vitro phenomenon has any relevance to in vivo situations. Phagocytosis was suggested to be defective in lupus patients due to rare genetic defects such as complement deficiencies (48) or specific alleles such as FcγRIIa (49). Studies of handling of immune complexes showed that both FcR and complement receptors are saturable (50–53). Taken together, it is suggested that what is demonstrated in rare individuals with genetic deficiencies that develop SLE or SLE-like disease may be found in the larger population of SLE patients as a common endpoint pattern of altered phagocytosis. In the example of this study, accelerated apoptosis characterized most patients and was the main cause of reduced in vitro interaction with apoptotic cells. The relevance to the in vivo state is not known, but several findings may support altered phagocytosis of apoptotic cells in SLE. First, in this study, both freshly isolated and maturing macrophages showed accelerated apoptosis with a reduced capacity of the remaining mature macrophages to clear apoptotic cells. Second, increased levels of nucleosomes were found in plasma of patients with SLE (54). Nucleosomes are formed in the process of programmed cell death, but in efficient phagocytosis, nucleosomes are generally created within a phagocyte following ingestion of cells undergoing early apoptosis (55). In this way, nucleosomes are not released to the plasma, where another safety mechanism in the form of nucleases exists. The pathogenicity of nucleosomes was demonstrated both by the finding that nucleosomes are the preferred targets of anti-DNA autoantibodies (56) and by their ability to induce immunoproliferative response (57) and IL-6 activity (58). Recently, it was shown that SLE-like disease develops in mice deficient in DNase I, which is the major nuclease present in the serum (59). Third, in animal models, mice deficient in C1q (60), C4 (61), ABC1 cassette transporter (62), and mer (19) are deficient in receptors or factors required for interaction with apoptotic cells, and in their absence develop autoimmunity or lupus-like disease. Fourth, in lupus patients, serum factors may have additional inhibitory (38, 39, 63) or proinflammatory (64) effects. In summary, iC3b-opsonized apoptotic cell interaction with macrophages is altered, in vitro, in a significant number of SLE patients. It may be related to several genetic, environmental, or disease-driven factors, but in this study it was associated with accelerated apoptosis of monocytes/macrophages. Altered interaction with apoptotic cells could contribute to induction or persistence of systemic autoimmunity. However, at present, the in vivo biological significance of in vitro data remains to be determined.

References


