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Cutting Edge: SDS-Stable Fas Microaggregates: An Early Event of Fas Activation Occurring with Agonistic Anti-Fas Antibody but Not with Fas Ligand

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Fas or CD95 is a transmembrane receptor of the TNFR superfamily of apoptosis inducers. Fas activation via its natural ligand FasL or agonistic mAbs leads to the formation of the death-inducing signaling complex (DISC) at its intracellular death domain (1). The DISC contains the Fas-associated death domain-containing protein and the zymogen procaspase-8, which once cleaved initiates signal transduction through the activation of downstream effector caspases such as caspase-3. This pathway is used by so-called type I cells. In other cells known as type II cells, the mitochondria play a prominent role in the amplification of the apoptotic signal, via the activation of caspase-9 which in turn activates caspase-3, and Bcl-2 can protect from cell death (2–4).

Fas preexists on the cell surface as noncovalently associated homotrimers of the 45-kDa Fas protein (5, 6). Several reports described the appearance following Fas activation of high-molecular mass forms of Fas which migrate at 200–250 kDa in SDS-PAGE, which are not dispersed by boiling in SDS (7–11) and which resist to reducing agents such as 2-ME (7, 10). These microaggregates appear very rapidly after Fas triggering (1), since they precede the formation of the DISC in the ordering of the initial signaling events of Fas activation (1, 11). These events are followed by the capping of Fas molecules into large clusters at the cell surface.

So far, all the experiments describing the appearance of the SDS-stable Fas microaggregates have used agonistic anti-Fas mAbs, and no results have been reported using the natural ligand FasL. This is an important point, because their occurrence with FasL would demonstrate that they have a physiological relevance in Fas signaling. In contrast, if these microaggregates do not form, it would demonstrate the limitation of using agonistic mAbs only to define the Fas pathways. This must be considered, given that subtle differences in the apoptotic mechanisms triggered by FasL and anti-Fas Abs are suspected to exist (12–15). To answer this question, we used four different human cell lines belonging to either the type I or the type II classification of Fas-mediated apoptosis, as well as normal blood lymphocytes.

Materials and Methods

Cells and reagents

The human T lymphoma cell line Jurkat 77 was obtained from Dr. P. Anderson (Brigham and Women’s Hospital, Boston, MA). The human T lymphoma cell lines CEM and H9, and the human B cell line SKW6.4 were a kind gift from Dr. M. Peter (Ben May Institute for Cancer Research, Chicago, IL). The human FasL-expressing murine 1A12 cell line and the nontransfected WR19L parent cell line were obtained from Prof. S. Nagata (Osaka Bioscience Institute, Osaka, Japan) (16). The cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine and 8% FCS. PBLs were obtained from normal blood donors. After isolation by Ficoll centrifugation and removal of monocytes by a 1-h plastic adherence step, they were kept in culture in the same medium as above for 48 h, or incubated in the presence of the activators PHA (1 μg/ml) and IL-2 (1000 IU/ml) for 1 wk, before being analyzed. The agonistic anti-Fas mAb 7C11 was from Immunotech (Marseille, France), the anti-Fas rabbit polyclonal antiserum C20 was from Santa Cruz Biotechnology (Santa Cruz, CA). The isotype-matched IgM-negative control mAb 10C9 was homemade. Functional soluble Fasl was purchased from Alexis Corporation (Cergy, Paris, France), and was used as recommended in the presence of its cross-linking...
“enhancer reagent”. Our chimeric IgFasL construct is a biologically active chimera between the Ig-like module of the LIF receptor gp190 as previously described (17) fused at its C terminus to the full FasL extracellular domain. The size of the monomer is 32 kDa by SDS-PAGE, but IgFasL exists as polymers of 400–700 kDa by gel filtration, and is biologically active on all the FasL sensitive cell lines tested without requiring cross-linking (P. Legembre, S. Dubron, and J.-L. Taupin, manuscript in preparation).

**Measurement of apoptosis**

The cytotoxic activity of soluble FasL or of the agonistic anti-Fas mAb 7C11 was measured using the MTT viability assay. In brief, cells (4 \times 10^5 per well) were cultured for 20 h in flat-bottom 96-well plates with the indicated concentrations of the apoptosis inducer. Then 0.015 ml of MTT (5 mg/ml in PBS) was added to each well and incubated for 4 h at 37°C. The precipitates were dissolved by adding 0.115 ml of isopropanol containing 1% formic acid (v/v), and the absorbance was measured at 570 nm (Titertek Laby systems Multiskan reader, Turku, Finland).

The cytotoxic activity of the membrane FasL-expressing 1A12 cell clone was determined by a 51Cr-release assay as described previously (18). Target cells were labeled with 50 μg of 51Cr (ICN, Irvine, CA) per 10^6 cells, for 1 h at 37°C. These cells were dispensed in round-bottom 96-well plates (4 \times 10^3 cells per well, in triplicate), and mixed with the effector cells (1A12 cells) at indicated ratios, in a total volume of 0.1 ml. After 4 h at 37°C, 0.025 ml of supernatants were mixed with 0.15 ml of scintillation liquid and the released radioactivity was quantitated using a MicroBeta Trilux beta counter (Wallac, Turku, Finland). To measure the cell DNA content by the propidium iodide assay, FasL- or 7C11 mAb-treated cells (10^6) were washed once in PBS and resuspended in 0.5 ml of ice-cold 70% ethanol. After overnight incubation at –20°C and centrifugation, the cell pellet was washed once in PBS and resuspended in PBS containing propidium iodide (25 μg/ml) and RNase A (50 μg/ml) for 1 h at 37°C before analysis on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). The cells with the morphology of living cells were gated. In addition, in experiments using 1A12 effector cells, the cell mixture was stained with a FITC-labeled anti-human CD45 (BD Biosciences) before the ethanol fixation, and analysis was performed on the CD45-positive subpopulation. This allowed the gating of the Jurkat cells and the exclusion of the murine 1A12 cells.

**Immunoblot experiments**

The cells were incubated with the anti-Fas mAb 7C11 or with the IgFasL, then lysed in HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 7.4) containing protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin) and 1% Triton X-100 for 30 min on ice. After centrifugation (10 min, 4°C, 15000 rpm), the supernatant was harvested. Protein concentration in cellular extracts was determined using the bicinchoninic acid method (Sigma-Aldrich, Irvine, CA, France). Proteins (15 μg per lane) were boiled 5 min and separated by SDS-PAGE on 8% gels in reducing conditions (0.1 M DTT) and transferred to a PVDF membrane (Amersham, Orsay, France). The membrane was blocked overnight at 4°C with TBST buffer (50 mM Tris, 160 mM NaCl, 0.1% Tween 20, pH 8) containing 5% dried skimmed milk. The indicated specific Ab was then incubated for 90 min at room temperature. After three washes with the TBST buffer, the HRP-labeled polyclonal anti-mouse (Amersham), anti-goat (Vector Laboratories, Burlingame, CA), or anti-rabbit (Zymed Laboratories, San Francisco, CA) secondary antiserum was added for 90 min. The proteins were visualized with the ECL kit (Amersham).

**Results and Discussion**

**Formation of SDS-stable Fas microaggregates is induced by agonistic mAb 7C11 but not by soluble or membrane bound FasL in the Jurkat cell line**

We analyzed the apoptotic response of the type II Jurkat 77 cell line to the stimulation of the Fas receptor. Cell death was triggered with the agonistic mAb 7C11, with membrane-bound FasL on effector 1A12 cells, and with two distinct functional forms of recombinant soluble FasL, one commercially available consisting in an epitope tagged sFasL, polymerized via an epitope-specific mAb and one consisting in a recombinant biologically active soluble FasL (IgFasL) (see Materials and Methods) (Fig. 1A). The cell line was highly responsive to mAb 7C11 and to both forms of soluble FasL, since nearly 100% of the cells were killed within 24 h with the three apoptosis inducers, as analyzed by MTT viability assay. Similarly, the Jurkat cells were rapidly killed by the 1A12 effector cells as measured by the 4-h chromium-release assay.

We analyzed the kinetics of formation of the SDS-resistant Fas microaggregates following the triggering of the Jurkat 77 cells via the anti-Fas mAb 7C11, soluble IgFasL and membrane-bound FasL on 1A12 cells (Fig. 1B). The cells were incubated with these inducers at concentrations which trigger

![FIGURE 1. Analysis of the Fas microaggregates in the Jurkat cell line. A, Sensitivity of the Jurkat cell line to Fas-induced apoptosis. In the left graph, Jurkat cells were incubated for 20 h with the anti-Fas mAb 7C11 (Δ), the commercially available FasL with its enhancer (○) or the homemade IgFasL (■) at the indicated concentrations, and viability was measured using the MTT assay. In the right graph, 51Cr-labeled target Jurkat cells were incubated for 4 h with the FasL-expressing 1A12 cells (▲) or its nontransfected counterpart (△) at the indicated ratios, and cell death was measured with the 51Cr-release assay. B, Time-course appearance of the Fas microaggregates. Jurkat cells were incubated with the anti-Fas mAb 7C11 (or negative control mAb) at 100 ng/ml (left panel), with IgFasL (or without, – condition) at 200 ng/ml (middle panel), or effector 1A12 cells (or nontransfected control WR191L cells) at a 2:1 ratio (right panel) for the indicated times. Then cells were lysed, total proteins (15 μg/lane) were separated by SDS-PAGE, and Fas was immunoblotted with the anti-Fas C-20 polyclonal antiserum. The position of the molecular mass markers in kilodaltons is shown on the left. Fas appears as two bands in the Jurkat cell line. Below each blot is the measurement for each condition of cell death expressed as the percentage of cells (mean of two experiments for 7C11 and IgFasL, mean ± SD of three experiments for 1A12) containing decreased amount of DNA (<G1 peak), as determined by flow cytometry with the propidium iodide assay.](http://www.jimmunol.org/)

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maximal cell death, i.e., 100 ng/ml for mAb 7C11 and 200 ng/ml for IgFasL, or at a 2:1 cellular ratio for membrane FasL. The CEM cell line is a type II cell, like the Jurkat, whereas the H9 and SKW6.4 are type I (3, 4). For the detection of the Fas microaggregates (Fig. 2B), the inducers were used at a concentration which triggered maximal cell death, i.e., 100 ng/ml for mAb 7C11 and 200 ng/ml for IgFasL. The Fas microaggregates could be detected for the three cell lines in the presence of mAb 7C11, as early as 0.5 h for the SKW6.4 and H9 cell lines, but only after 2 h for the CEM cell line. It could always be detected before cell death could be measured via propidium iodide staining. As observed with the Jurkat cells, the Fas microaggregates were never observed when IgFasL was used as a death inducer, even at the 5 h time point, for which cell death was much more significant than with the anti-Fas mAb. To ascertain that the difference in the formation of the microaggregates between the two inducers was not due to differences in the formation of the DISC, it was analyzed by immunoprecipitation of Fas in the type I SKW6.4. At a time of 15 min after the triggering of Fas receptors with each of the inducers at 1 μg/ml, comparable amounts of caspase-8 and Fas-associated death domain were recruited (results not shown). In addition, similar
amounts of activated caspase-8 cleavage fragments were evidenced by western blotting of cell lysates at 30 and 90 min. (results not shown). Therefore, these early events of Fas activation occurred with comparable kinetics in the presence of Ig-FasL and anti-Fas agonistic mAb.

The aggregation of the Fas receptor was also analyzed in lymphocytes from normal human blood. Although resistant to Fas triggering by the agonistic mAb 7C11 and IgFasL when in a resting state, PBLs become sensitive to both inducers upon activation via PHA and IL-2, as shown with the propidium iodide staining in Fig. 3. However, the triggering of resting PBLs via mAb 7C11 led to the formation of microaggregates, despite the absence of cell death. This result was not observed when IgFasL was used as the trigger. The Fas microaggregates were also formed in the presence of mAb 7C11 in activated PBLs, whereas they did not appear in the presence of IgFasL, although cell death was triggered to a similar extent with both inducers (Fig. 3). Therefore, this experiment showed that normal human cells behaved like the laboratory cell lines we tested in our study.

Our results demonstrate that the microaggregation of Fas into SDS-stable polymers is not a necessary step in the triggering of cell death via FasL, in cell lines and normal blood lymphocytes. In contrast, microaggregates always form in response to an agonistic anti-Fas mAb, in Fas-sensitive cell lines as well as in Fas-resistant resting PBLs. In this latter case, our results confirm that Fas microaggregation and Fas-mediated apoptosis should not be correlated, as was previously suggested in a work conducted on T and B cell lines with an anti-Fas mAb (10). Therefore, the microaggregation of Fas receptors most likely reflects a property of the agonistic anti-Fas Abs.

Our findings also suggest that anti-Fas agonistic mAb and FasL may not trigger cell death exactly via the same mechanisms. A previous report also highlighted this point, using a deletion mutant of Fas lacking the last 15 amino acids of the intracellular domain and a chimera of Fas where the intracellular region had been replaced with that from the TNFR1 p55 subunit (12). When expressed in cells, both constructs conferred a higher sensitivity than the wild-type receptor to agonistic anti-Fas mAbs, but not to FasL, both as a soluble polymeric and a membrane bound form. Similarly, the distinction between the type I and type II pathways of Fas-mediated apoptosis has also been put into question. Indeed, one group reported that it was limited to the agonistic mAb and irrelevant for the FasL, because the weaker formation of the DISC and the protective effect of Bcl-2 were not retrieved in type II cells stimulated with FasL (13). However, this has been and still remains a matter of controversy (14, 19, 20, 21). As a conclusion, one may remember that the results obtained with agonistic mAbs should not be straightforward extrapolated to the natural ligand.

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