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Research Paper

CD95 Rapidly Clusters in Cells of Diverse Origins

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ABSTRACT

We have shown that CD95-mediated cell death requires a clustering of the receptor in distinct sphingolipid-rich domains of the cell membrane (Grassmé et al., 2000, Cremesti et al., 2000). These domains form in response to acid sphingomyelinase (ASM)-induced ceramide generation. However, recent studies challenged the finding of early CD95 clustering (Algeciras-Schimnich et al., 2002). Here, six independent groups tested clustering of CD95 in diverse cell type including primary cells ex vivo and established cell lines. The studies show clustering of CD95 within seconds to minutes in all cell types tested by the different groups. In addition, clustering of CD95 was detected after stimulation of cells using three agonistic anti-CD95 antibodies (CH11, APO-1-3 and JO2), CD95 ligand and stimuli that induce an upregulation and activation of the endogenous CD95/CD95 ligand system. The data confirm our previous studies and suggest rapid, i.e., within seconds to minutes, CD95 clustering as a general phenomenon occurring in many cell types.

INTRODUCTION

Evidence suggests that the plasma membrane contains small distinct sphingolipid- and cholesterol-rich domains, named rafts that seem central to transmembrane signaling via specific cell surface receptors. Rafts exist because cholesterol and sphingolipids, mostly sphingomyelin, bind tightly to each other through hydrogen-bonds between the C3 hydroxyl group of cholesterol and the sphingosine headgroup of sphingomyelin. This tight interaction makes the regions containing these lipids more ordered and less fluid than the rest of the phospholipid bilayer, effectively creating a discrete microdomain within the bulk of membrane phospholipid.[1-3]

We have recently suggested a mechanism that transforms these rafts into larger signaling platforms that are suitable for initiation of receptor signaling.[4-7] Activation of CD95 induced translocation of the acid sphingomyelinase (ASM) from an intracellular vesicular compartment onto the cell surface into the rafts.[4,5] The activity of the ASM converted sphingomyelin into ceramide, which possesses the intrinsic capacity to fuse these small rafts into larger platform macrodomains. These macrodomains are of sufficient size to be visualized in living cells.[4-6] Platform formation appeared pre-requisite for trapping ligated CD95 molecules, apparently serving as a scaffold for the formation of large CD95 clusters. The significance of these studies is indicated by the finding that disruption of rafts using reagents that extract cholesterol from the plasma membrane, or prevention of raft reorganization into larger platforms by inactivation of ASM, rendered cells resistant to CD95 clustering, an event required for apoptosis induction.[4-6] The notion of CD95 receptor clustering in distinct membrane domains is further supported by our finding that CD95 is present in biochemically-purified detergent resistant membrane macrodomains of stimulated JY or Jurkat cells (A. Cremesti and R. Kolesnick, unpublished data; J. Bock and E. Gulbins, unpublished data). This finding is consistent with recent findings by Hueber et al.[8] and Gajate et al.[9] that indicated the presence of CD95 in detergent resistant domains of thymocytes and Jurkat cells. In addition, Garofolo et al. demonstrated a recruitment of CD95 and caspase 8 into the detergent-resistant membrane fraction upon stimulation.[10] Further, destruction of detergent-resistant domains by extraction of cholesterol prevented CD95-induced apoptosis in thymocytes[8] and human lymphoblastoid CEM cells[10] supporting our previous findings[4-6] using cyclodextrin, nystatin and filipin to disrupt membrane rafts.

Based on an article recently published by Algeciras-Schimnich et al.,[11] however, a controversy has developed regarding the role of sphingolipid-mediated membrane re-organization in CD95 clustering. While Grassmé et al.[4] and Cremesti et al.[6] demonstrated that clustering occurred within seconds to minutes of CD95 ligation and preceded formation of

A

CD95 clusters on a variety of cells of different origin

Cells	Jurkat	H9	K50	JY	SKW 6.4	Mouse granulosa cells	Human PBL	Murine splenocytes	WI-38	Chang	Hepatocytes
Percent clustering (protocol)	23 ± 3, pr.1 55 ± 5, pr.2 30 ± 3, pr.3 48 ± 1, pr.4 43 ± 8, pr.5 45 ± 5, pr.6	37 ± 5, pr.1	25 ± 5, pr.5 35 ± 5, pr.6	45 ± 2, pr.5 50 ± 4, pr.6	28 ± 8, pr. 5 45 ± 7, pr.6	43 ± 6, pr.4	55 ± 8, pr.6	35 ± 6, pr.6	90 ± 6, pr.6	92 ± 10, pr.6	25 ± 4, pr.6

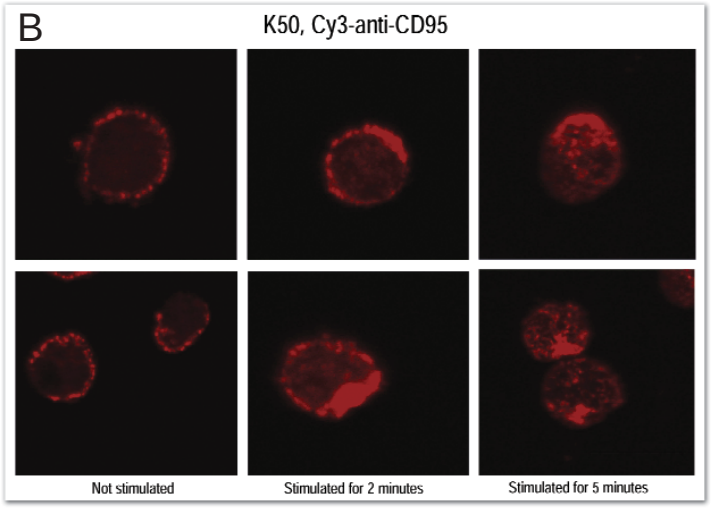

Figure 1. CD95 rapidly clusters. Shown is the percent of cells demonstrating CD95 clusters on the cell surface 5 min after stimulation. The number in parenthesis refers to the group´s affiliation. In brief, human PBLs were stimulated with recombinant CD95 ligand, all other human cells with anti-CD95 CH11, all murine cells with anti- CD95 JO2. WI-38 and Chang cells were infected with *P. aeruginosa* for 15 min, conditions that are known to trigger apoptosis via the endogenous CD95/CD95 ligand system.[13] All cells were fixed, immobilized, stained with FITC- or Cy3-coupled anti-CD95 antibodies and analyzed by fluorescence microscopy. Panel B displays clustering of CD95 in K50 cells 2 and 5 min after stimulation. Cells were stained with Cy3-labeled anti-CD95 CH11 antibodies and analyzed by confocal microscopy.

the death-inducing signaling complex (DISC), an event absolutely required for apoptosis to initiate, Algeciras- Schimnich et al.[11] showed that CD95 clustering is delayed and questioned its involvement in activating the DISC. Since the raft hypothesis of CD95 signaling is contingent on the rapid lateral redistribution of CD95 into these structures, at least in some cell types, it is crucial to precisely define the kinetics of CD95 clustering.

Here, we present data from 6 independent groups that demonstrate CD95 clustering on diverse cell types that occur within seconds to minutes of receptor ligation.

MATERIALS AND METHODS

Cells and Stimulation. JY, Jurkat, WI-38, Chang and H9 cells were from ATCC. Mouse granulosa cells were collected from mouse ovaries 42 hrs after treatment with 5 IU pregnant mare serum gonadotropin and plated in MEM containing 10% FCS. SKW 6.4 and K50 cells were kindly provided by Dr. M. E. Peter, University of Chicago, IL. Murine splenocytes were obtained from C57/BL6 *lpr-cg* mice or normal control mice (kindly provided by Dr. T. Möröy, University of Essen, Germany). Human peripheral blood lymphocytes (PBL) were from healthy volunteers. All primary murine or human lymphocytes were purified via a Ficoll gradient (Histopaque; Sigma, St. Louis, MO). Murine splenocytes were cultured for 6 days with 10 μg/ml PHA (Sigma) and 10 μ/ml murine IL-2 (Roche, Mannheim, Germany) to trigger CD95 expression. Human PBLs were treated with 10 μg/ml PHA and 10 μ/ml human IL-2 (Roche) for 5 days. All cells were cultured in phenol red-free RPMI-1640 supplemented with 10% fetal calf serum (FCS), 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin (all Life Technologies) and 50 μM β-mercaptoethanol.

Anti-human CD95 antibodies CH11 were from UBI (Lake Placid, N.Y.), anti-CD95 APO-1-3, recombinant CD95 ligand and anti-Flag antibodies were from Alexis (Cornerstone, PA), and anti-murine CD95 antibodies JO2 were from Pharmingen (San Diego, CA).

Staining Protocols and Fluorescence Microscopy

Protocol 1 (Jessica C. Fanzo and Alessandra Pernis). Jurkat and H9 cells (5×10^5) were incubated with anti-CD95 antibody CH-11 (UBI) at 1 μg/ml for 60 min at 4°C followed by an incubation with Alexa-Fluor anti-mouse IgM conjugated secondary antibodies (1:100 dilution) for 60 min at 4°C. Cells were then incubated at 37°C to induce capping for 30 sec, 2 min, 5 min, and 30 min and immediately fixed with 3.7% formaldehyde for 20 min on ice. Unstimulated control cells were exposed to primary anti-CD95 antibody CH11 and secondary antibody at 4°C, but no capping was induced at 37°C. The percentage of cells displaying caps, in which the Alexa-fluor condenses on to less than 25% of the cell surface was determined by counting 150–250 cells/sample. Visualization of cells and images were collected with a ZEISS Axioplan II microscope (Carl Zeiss, Oberkochen, Germany) using a Plan-Apochromat 100X/1.4 N.A. objective lens, and a cooled CCD camera (Orca-100, Hamamatsu Photonics, Bridgewater, NJ).

Protocol 2 (Marc Hyer and James Norris). Jurkat cells (a kind gift from Dr. Y. Hannun, Medical University of South Carolina, Charleston, SC) were resuspended in H/S (132 mM NaCl, 20 mM HEPES (pH 7.4), 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM MgCl2, 0.8 mM $MgSO_4$) supplemented with 2% FCS. Cells (0.8×10^6) were sequentially incubated with anti-CD95 CH-11 (1 μg/ml) and FITC-conjugated secondary antibody (Roche Molecular Biochemicals, 1:100 dilution) at 4°C for 1 hr. Capping was induced by warming at 37°C for 0, 0.5, 2, 5, 10, 15, 30, and 60 minutes. Cells were immediately fixed using 2% paraformaldehyde (PFA) and scored for capping. A positive capping event was counted when the fluorescence detected on the cell surface condensed onto less than 50% of the cell surface. Cells were examined under 400x oil immersion optics (Zeiss Axioskop) and images captured using a Spot digital camera (Diagnostic Instruments, Inc.).

Protocol 3 (Hyewon Phee and Mark Coggeshall). Jurkat T cells were washed, resuspended in H/S and stimulated at 37°C with 100 ng/ml of anti-CD95 monoclonal antibody CH11 (UBI). Stimulations were stopped by addition of fixative solution (1% formaldehyde in PBS containing 0.1% NaN3). Unstimulated cells were incubated at 37°C in H/S only and fixed before addition of primary anti-CD95 antibody. Fixed cells were washed in 3% FCS and 0.1% sodiumazide (NaN_3) in PBS and incubated with Cy5-conjugated F(ab')2 fragments of rabbit anti-mouse Ig. The stained cells were added to poly-L-lysine (0.1%, w/v; Sigma)-coated coverslips and mounted onto slides. Cells were visualized with a Leica TCS laser scanning confocal microscope for image acquisition, as previously described.[12]

Protocol 4 (Maureen Lynch and Bo Rueda). Mouse granulosa cells were plated at 4×10^4 cells per well in 4 well chamber slides and allowed to adhere overnight. Granulosa or Jurkat T cells were washed twice with H/S containing 2% FCS and allowed to equilibrate in this buffer for 8 minutes at 37°C. Cells were then incubated with 200 ng/ml anti-CD95 CH11 antibody (Jurkat cells) or with 200 ng/ml anti-CD95 JO2 (granulosa cells) in H/S, 2% FCS for 0 min, 30 sec, 2 min, 5 min and 10 min at 37°C, washed with ice cold H/S, 1% FCS and fixed in 1% paraformaldehyde for 20 minutes

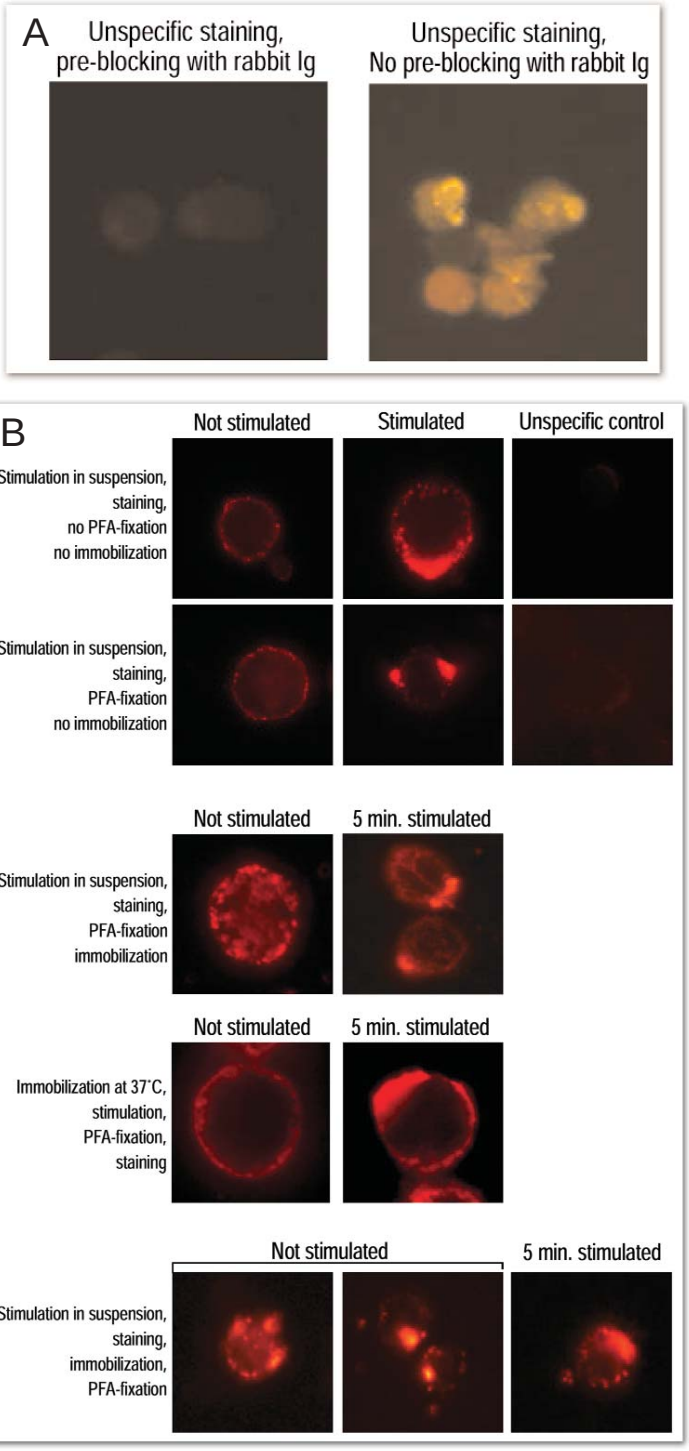

Figure 2. Technical issues of CD95 clustering. Blockade of Fc-receptors is required to prevent unspecific binding of Cy3-labeled mouse IgG3. CD95 clustering is similar when cells were neither fixed nor immobilized (1st row), fixed but not immobilized (2nd row), stained and fixed prior to immobilization (3rd row), or immobilized with poly L-lysine at 37°C for 15 min prior to stimulation, fixation and finally staining (4th row). In contrast, cells that were first stained, then immobilized and finally 1% PFA-fixed showed severe artifactual alterations of CD95 clustering, and even in unstimulated cells, small cluster-like structures were detected (5th row).

before staining. For staining, cells were incubated with anti-CD95 CH11 (Jurkat cells) or JO2 (granulosa cells) at 500 ng/ml for 1 h, washed in H/S plus 1% FCS followed by staining with secondary FITC-conjugated goat anti mouse IgM (Roche) (for Jurkat cells) or FITC-conjugated anti-hamster antibody (Serotec) (for granulosa cells) at each 1:100 dilution. Jurkat cells were mounted on slides and coverslipped for viewing on a Nikon 300 TE equipped with a SPOT digital camera. Cells in several fields per slide were counted for each time point and percent of cells displaying clustering of CD95 was determined.

Protocol 5 (Aida Cremesti and Richard Kolesnick). Jurkat, JY, SKW or K50 cells (0.8 x 10^6 cells/sample) were blocked with H/S and 2% FCS for 15 min, followed by incubation with 1 mg/ml anti-CD95 CH11 antibody in the presence of 2% FCS for 45–60 min on ice. Unbound anti-CD95 CH11 was washed off two times with H/S. Cells were then incubated on ice for 45-60 min with Texas Red-conjugated secondary anti-mouse IgM antibodies (1:100 dilution, Jackson ImmunoResearch). To initiate capping, cells were warmed to 37°C with mild agitation. Control cells were not warmed. Stimulation was terminated at multiple times between 30 sec and 20 min by the addition of 2% PFA and cells were fixed for 20 min at room temperature. Cells were washed twice, mounted in fluorescent mounting medium (Dako) and analyzed on a S-100 Zeiss Axiovert Fluorescence Microscope equipped with a SPOT Digital camera as described.[6]

Protocol 6 (Heike Grassmé and Erich Gulbins). Cells were washed in H/S, resuspended in the same buffer, rested for 8 min at 37°C and stimulated with 50 ng/ml anti-CD95 CH11, 100 ng/ml anti-CD95 JO2, or 1 mg/ml anti-CD95 APO 1-3. Unstimulated samples were incubated with irrelevant isotype IgM, IgG3 or hamster antibodies (Sigma), respectively. CD95 ligand (Flag-tagged, Alexis Inc.) was added to cells at 20 ng/ml for 10 min at 4°C, cells were washed and cellular activation initiated by addition of 100 ng/ml anti-Flag F(ab')2 antibodies in prewarmed H/S at 37°C. Stimulation was stopped by addition of 1 ml ice cold PBS, 1% FCS and 0.1% NaN3, cells were then washed in PBS and fixed in 1% PFA (w/v) in PBS (pH 7.3) for 15 min. Samples were washed in PBS and blocked with 1% FCS in PBS, 1% NaN3 for 20 min to reduce unspecific antibody binding. To neutralize Fc-receptors all B lymphocytes were incubated with an irrelevant rabbit Ig (20 mg/ml, Sigma) for 45 min prior to addition of the primary antibodies. Control experiments revealed marked binding and even capping of normal Ig to cells without Fc-receptor blockade. Cells were then washed in PBS and incubated for 45 min at 4°C with 500 ng/ml anti-CD95 CH11 or JO2, respectively, in PBS, 1% FCS and 0.1% NaN3. Control samples were incubated with an irrelevant murine IgM or hamster IgG, respectively (both from Sigma). Samples were washed three times in PBS and stained for 45 min with cyanine 3.18 (Cy3)-coupled F(ab')2 fragments of donkey anti-mouse IgM or IgG antibodies, respectively, or FITC-labeled $F(ab')_2$ fragments of goat anti-hamster antibodies (all from Jackson ImmunoResearch, West Grove, PA) in PBS, 1% FCS and 0.1% NaN3. Cells were again washed and immobilized on glass cover slips coated with 1% (v/v) poly-L-lysine for 10 min. The cover slips were embedded in Moviol and viewed with a Zeiss Axioplan fluorescence microscope (Jena, Germany) or a Leica TCS NT scanning confocal microscope (Munich, Germany). Clustering was defined as one or several intense spots of fluorescence on the cell surface, whereas unstimulated cells displayed a homogenous distribution of the fluorescence throughout the membrane. In each experiment, the presence or absence of clustering in samples of 200 cells was scored by two independent observers. The results are given as percentage of cells showing a cluster. For studies using *P. aeruginosa*, WI-38 or Chang cells were grown on the cover slips, infected for 15 min with the *P. aeruginosa* strain ATCC 27853 (ATCC, Manassas, VA) at a cell:bacteria ratio of 1:500 as previously described (13), washed with ice-cold PBS, and fixed and stained with anti-CD95 CH11 as above. Bacteria were grown to mid-logarithmic phase prior to infection.

RESULTS AND DISCUSSION

In the present study 6 independent groups assessed clustering of CD95 in 11 cell types of diverse origins, some of which were primary cultures. A number of different agonistic antibodies for CD95, recombinant CD95 ligand and physiological stimuli of CD95 were used to initiate the process. Each group used a different

combination of cell types and agonists except for the use of Jurkat cells by all groups to assess consistency in measuring clustering. The studies revealed rapid clustering of CD95 within 30 sec—5 min after stimulation in all cells tested (Fig. 1A), indicating that rapid clustering may be a generic event upon CD95 ligation. Figure 1B displays a representative clustering of CD95 in K50 cells analyzed by confocal fluorescence microscopy.

To resolve the differences between these data and those of Algeciras-Schimnich,8 we also carried out CD95 clustering experiments using different conditions including those reported by Algeciras-Schimnich et al. The experiments revealed potential technical problems in the detection of receptor clustering, explaining the differences in clustering detection. Since IgG3 isotype antibodies (e.g., the anti-CD95 antibody Apo-1-3) or intact fluorescent secondary antibodies, might bind Fc-receptors present on human B lymphocytes, inadvertent staining of cells through this mechanism must be excluded. In fact, incubation of JY B cells with an irrelevant murine IgG3 antibody (Sigma) followed by an $F(ab)_2$ fragment of a Cy3-coupled rabbit anti-mouse Ig revealed a cluster-like binding (Fig. 2A), that was abrogated by a blockade of Fc-receptors using rabbit Ig (20 μg/ml), or by omission of the IgG3 antibody (not shown). The use of IgM molecules (CH11) and $F(ab)_2$ fragments of the secondary fluorescent-labeled antibodies employed for staining circumvented this problem, since these proteins do not bind to Fc-receptors.

Further, the sequence of the immobilization of cells on poly-L-lysine and staining prior to PFA fixation, as used in the Algeciras-Schimnich study,[11] was also found by us to alter CD95 membrane distribution, inducing cluster-like structures even in unstimulated cells (Fig. 2B).

Hence, technical issues associated with failure to block Fc-receptors, and fixation of the cells after staining and immobilization on poly-L-lysine, resulted in failure to detect early CD95 clustering,[11] and

In summary, the present data obtained from 6 independent groups demonstrate rapid CD95 clustering using diverse cells and CD95 agonist stimuli. These data confirm previous studies from us[4-6] and others[8-10] and identify reasons that a previous investigation[11] may have failed to do so.

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