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Imaging of Fas–FasL membrane microdomains during apoptosis in a reconstituted cell–cell junction

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ABSTRACT

The Fas death receptor interacts with its ligand FasL and induces apoptosis. The Fas–FasL interaction occurs at the cell–cell interface *in vivo*, since both proteins are expressed in cell membranes. However, most studies on the Fas signal pathway have been performed in a nonphysiological manner by using soluble molecules (antibody or crosslinked FasL proteins) to stimulate Fas. The Fas–FasL interaction at the cell–cell contact site has only been studied recently, but the information derived from cell–cell interaction studies is still rather limited and not necessarily consistent with the past results obtained by using soluble Fas-stimulatory molecules. Therefore, we develop a novel reconstituted system that mimics the Fas–FasL interaction at cell–cell contact sites for further examination of the physiological Fas–FasL signaling system. By conjugating FasL extracellular domains to planar lipid bilayers, we created a model cell membrane to activate Fas-expressing cells. Using this system, we generated an image of Fas–FasL interactions at the cell–membrane interface at high resolution. We observed that the Fas–FasL interaction between two membranes creates submicron membrane microdomains. Shortly after microdomain formation, the cells exhibit various features of apoptosis. These results suggest that our reconstituted system provides a useful platform to dissect Fas–FasL apoptosis signaling at near physiological conditions.

1. Introduction

Fas (CD95/APO-1) is a TNF receptor family protein that can trigger apoptosis by binding to its physiological ligand, FasL (CD95L or APO-1L) [1]. The Fas–FasL apoptosis signal is a critical component of the immune system, and a deficiency or mutation of either gene can result in lymphadenopathy, systemic lupus erythematosus (SLE)-like autoimmune diseases, or autoimmune lymphoproliferative syndrome (ALPS) [2]. It is well established that Fas stimulation induces the recruitment of Fas-associated death domain protein (FADD) and caspase-8 (a cysteine protease) to bind to Fas [1,3]. These proteins interact via the death domain (DD) and the death effector domain (DED), forming the death-inducing signal complex (DISC) [3]. In the DISC, inactive procaspase-8 dimerizes and autocleaves to generate active caspase-8 molecules. The molecular proximity of procaspase-8 in DISC is believed to play an important role in inducing caspase-8 activation [4,5].

Although Fas, FADD, and caspase-8 are ubiquitously expressed molecules, and caspase-8 activation is common in Fas signaling of many cell types [2], diverse downstream signaling events are observed. In one apoptosis pathway, caspase-8 activates caspase-3, and activated caspase-3 cleaves lamin and other vital cellular proteins [6]. In another pathway, caspase-8 cleaves the BH3-only family protein BID, and truncated BID translocates to the mitochondria where the release of cytochrome c, and subsequent apoptotic events are initiated [7]. Some cell lines (the so-called type I, e.g., lymphocytes) or restimulated primary T cells appear to use the caspase-3-dependent pathway, whereas other cell lines (type II) or recently activated T cells largely use the BID-mitochondria pathway [2,6]. Moreover, caspase-8 activation by Fas also results in non-apoptotic functions, including cellular proliferation and tumor growth via NF-kB and MAPK pathways [8–10].

The mechanism implicated in the regulation of such diversity of the Fas signal is still poorly understood. One complexity stems from the fact that different manners of Fas activation result in rather diverse molecular and cellular responses. Since FasL is a transmembrane protein, Fas activation *in vivo* is primarily regulated by cell–cell interaction. It has also been shown that membrane-bound FasL is essential in the immune system, while soluble FasL generated by proteolysis is not [11]. Accordingly, soluble FasL monomer or trimers have much weaker effects on Fas-induced apoptosis than anti-Fas antibodies, heavily cross-linked Fas, or membrane-bound forms of FasL [12,13]. Direct imaging of DISC in cells also generates

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diverse results. One initial study reveals that Fas forms microscopic clusters or cap structures in cells activated with anti-Fas antibody, and these structures depend, at least partially, on caspase-8 activity and lipid compositions [14]. A more recent imaging study of cell–cell contact demonstrates that Fas and FasL expressed in two apposed cells form macroscopic adhesion sites at cell–cell interfaces [15]. The Fas–FasL adhesion site created between two cells is independent of caspase-8 activity, FADD, lipid rafts, and even the cytoplasmic domains of Fas and FasL [15]. Note that these factors were shown to be important in the DISC formation induced by anti-Fas [16]. These Fas–FasL clusters at cell–cell contact sites are also cell-autonomous (formed either in type I or type II cells) and are not rapidly internalized, contrary to the previous observation of Fas internalization after the stimulation with anti-Fas or soluble FasL [17]. All these results suggest that the use of cell–cell interaction system is important to identify the true factors that regulate Fas signal diversity, although the information obtained through the imaging of cell–cell interaction remains rather limited.

To gain a greater insight into Fas–FasL signaling in cell–cell interaction, we developed a novel experimental system that reconstitutes the Fas–FasL interaction between live cells and planar lipid bilayers. The FasL (extracellular domain) molecules are bound to planar lipid membranes, and fluidity is maintained. Fas proteins in the cells interact with the membrane-bound FasL as it does *in vivo*. Moreover, we can examine Fas–FasL interaction alone in our system, separate from the interactions of other cellular adhesion molecules or lipid rafts in FasL-expressing cells. The feasibility of the system is also supported from the fact that the extracellular domain of FasL is sufficient to generate Fas–FasL clusters at the cell–cell interface [15]. In this planar geometry, we can readily image Fas and other signal molecules as well as various apoptotic events at a much higher resolution compared to the cell–cell interface. By using this system, we were able to resolve submicron-scale Fas–FasL spatial organizations in the plasma membranes of apoptotic cells.

2. Materials and methods

2.1. Cells and reagents

Jurkat T cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Nichirei Biosciences, Tokyo, Japan). The gene for human Fas was cloned using a template of cDNA prepared from Jurkat cells and ligated into pAcGFP-N1 vector (Clontech Laboratories, Inc., Mountain View, CA, USA). 1×10^6 Jurkat cells were transfected with 2.5 μg of Fas-GFP plasmid diluted in 250 μL of Opti-MEM I-reduced serum medium and 7.5 μL of trans IT-LT1 reagent. Transfected cells were analyzed 24 h later. All lipids were acquired from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Other reagents used in experiments are EZ-Link NHS-Biotin (Thermo Fisher Scientific Inc., Waltham, MA, USA), recombinant soluble human FasL protein (Enzo Life Sciences International, Inc. Farmingdale, NY, USA), Cy3-conjugated streptavidin (Invitrogen Corp. Carlsbad, CA, USA), Alexa Fluor 488-conjugated annexin V (Molecular Probes, Inc. Grand Island, NY, USA), and propidium iodide (PI; Dojindo, Kumamoto, Japan). A375 and Hela cells were cultured in DMEM supplemented with 10% fetal bovine serum.

2.2. Preparation of planar lipid bilayer

Phospholipid vesicles were prepared as follows. The desired lipids were mixed in a chloroform solution, dried under a stream of nitrogen, and hydrated in distilled water to a final concentration of 4 mM. The hydrated lipids were probe-sonicated to clarity on ice. Small unilamellar vesicles were separated from the debris and multilamellar vesicles by ultracentrifugation for 2.5 h at 65000*g*, and then stored at 4 °C. Fluid bilayers were made from vesicles containing 99.9% DOPC [1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphorcholine] and 0.1% biotinyl-cap-PE [1,2-di-(9Z-octa-decenoyl)-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt)]. Vesicles were mixed at room temperature, and then deposited on a glass surface cleaned by piranha solution (H_2SO_4 and H_2O_2 mixed in a 3:1 ratio). The resulting lipid bilayer-functionalized substrate was sealed in an Attofluor cell chamber (Invitrogen Corp.) and rinsed with imaging buffer (20 mM HEPES buffer pH 7.4, 10 mM glucose, 135 mM NaCl, 4 mM KCl, 1mM Na_2HPO_4 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$).

2.3. Surface functionalization

The lipid bilayer was incubated for 45 min with 40 mg/mL bovine serum albumin (BSA; Sigma–Aldrich, Inc., St. Louis, MO, USA) solution to minimize non-specific protein adsorption, and then incubated for 30 min with Cy3-conjugated streptavidin. After rinsing with imaging buffer, the lipid bilayer was incubated for 45 min with a solution of monobiotinylated FasL. Recombinant human soluble FasL was monobiotinylated.

2.4. Microscopy and image analysis

The interaction surface was imaged on a Leica AF6000LX system (Solms, Germany) equipped with Cascade II EMCCD camera (Roper Scientific, Trenton, NJ). The system had a magnification of 100×, and an objective lens with a numerical aperture of 1.46 for TIRF illumination. Suspension cells were washed and resuspended in imaging buffer to a concentration of 1×10^7/mL several minutes before imaging. Adherent cells were collected by rubber scraper after washing and then resuspended in imaging buffer to a concentration of 1×10^7/mL. Ten microliters of the cell suspension was then added onto the functional substrate to interact with membrane-linked proteins. For staining live cells, 5 μL of Alexa Fluor 488-conjugated annexin V and 0.1 μL of PI (1 mg/mL) were mixed with 5 μL of imaging buffer, and added onto the substrate. Time lapse was performed 15 min later. The images were recorded by Leica LAS AF 6000 software and analyzed using ImageJ software (NIH, Bethesda, MD, USA).

3. Results

3.1. Fas–FasL membrane microdomain formation at a cell–lipid bilayer interface

The reconstituted model membrane was formed in the following manner (Fig. 1A). Lipid vesicles incorporated with biotinylated lipid are deposited on clean, etched coverslips under the buffer solution, resulting in the formation of fluid lipid bilayers. After blocking with BSA, fluorescently labeled streptavidin and biotinylated FasL extracellular domains are sequentially added to the lipid bilayers. As a result, freely diffusing FasL proteins are tightly bound to planar lipid bilayers. The concentration of biotinylated lipids in the bilayers is ~1400 molecules/μm^2 that is the upper bound of the concentration of FasL linked to the bilayers. In the planar geometry of the system, various microscopy techniques, including total internal reflection fluorescence (TIRF) microscopy, can be used to image the cell membranes. Another advantage of the reconstituted system is that expression of exogenous fluorescent Fas protein is not required, since we can image the FasL–Fas interaction at the cell-bilayer junction through the fluorescence of labeled streptavidin.

We first introduced Jurkat cells into the system, since this cell line was used as the standard model in the past Fas signal studies.

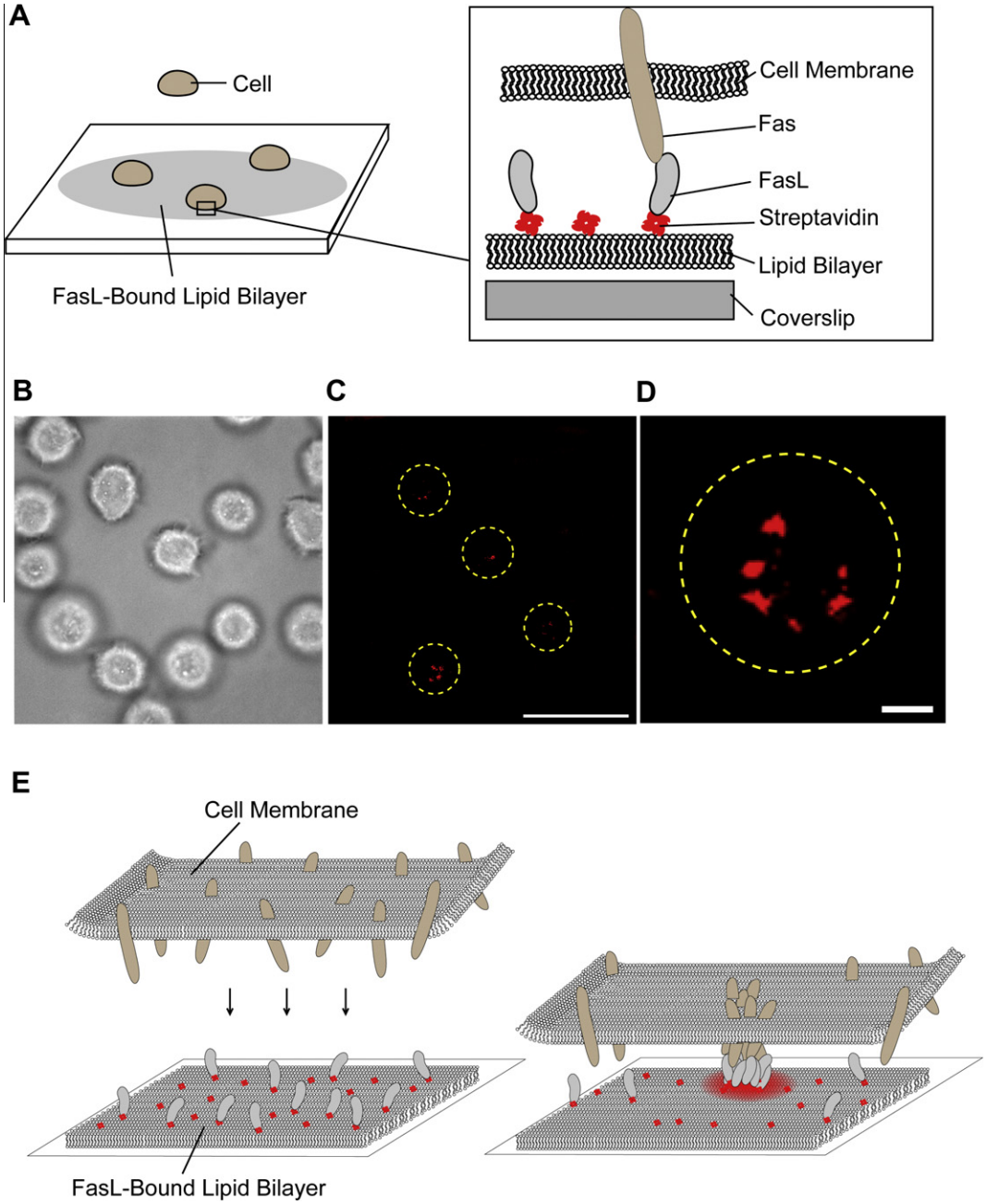

Fig. 1. Reconstitution of Fas–FasL interaction at cell–lipid bilayer interface. (A) Schematic illustration of the reconstituted cell–bilayer system. Cells were adhered to planar lipid bilayers containing fluid FasL (extracellular domain) bound to fluorescent (Cy3) streptavidin, which was attached to biotinylated phospholipids (see Materials and methods). (B–E) Bright-field (B) and fluorescence (C) images of cell-FasL-bilayer interaction. Red fluorescence from condensed streptavidin at the cell-bilayer interface indicative of the Fas–FasL interaction, highlighted with dot circles. Bar is 25 μm. A blowup image of single cell (D) resolves the membrane microdomains on a submicron scale. A schematic illustration of the microdomain formation is included in E. Bar is 5 μm.

We observed clear fluorescent spots formed at the junction between the Jurkat cells and lipid bilayers, suggesting that the Fas–FasL interaction was successfully reconstituted in the artificial cell–bilayer system (Fig. 1B–E). The fluorescence intensity of the spots corresponds to tens of Fas–FasL protein complexes (data not shown), suggesting that the Fas–FasL interaction at the cell-bilayer junction creates condensed membrane microdomains. Thus, microdomains organize molecules at a scale much larger than a single trimer–trimer Fas–FasL complex that is considered as the minimum interaction unit (Fig. 1E) [2]. The overall morphologies of these microdomains are similar to the adhesion sites of Fas–FasL observed at cell–cell contact sites [15]. However, at higher resolution imaging, we are able to resolve submicron-scale organization of the membrane microdomains (Fig. 1D). The Fas–FasL microdomains emerged within ~10 min after the cells were incubated with FasL-bound bilayers. Note that caspase-8 activation in the DISC of Jurkat cells activated with soluble anti-Fas occurs more slowly (about an hour after the cell activation) [6].

3.2. Fas–FasL membrane microdomain induces apoptosis in cells

We subsequently confirmed that the Fas–FasL interaction in the reconstituted system induces apoptosis in cells. To monitor the onset of apoptosis of Jurkat cells interacting with FasL-linked lipid

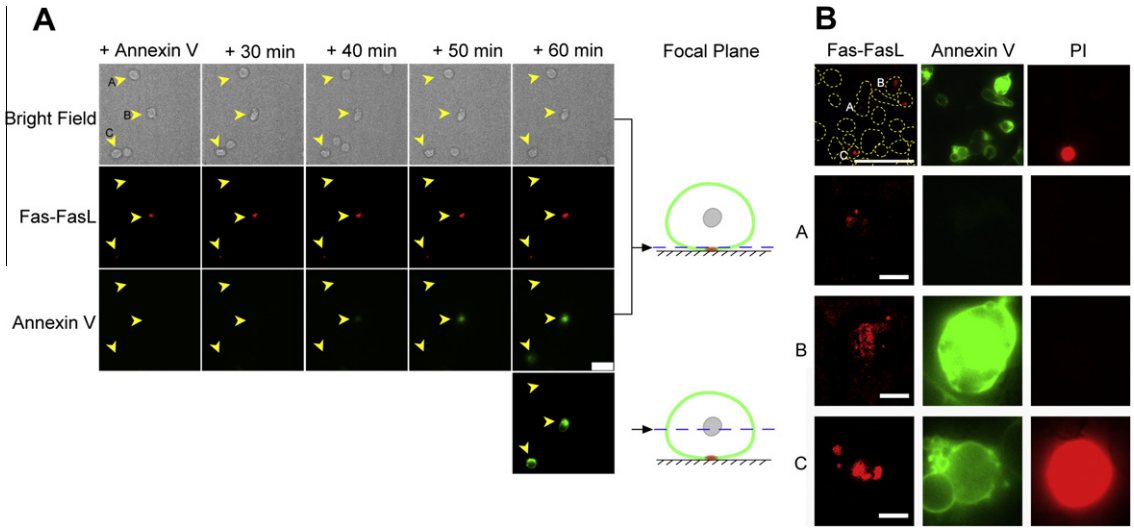

Fig. 2. Imaging of apoptosis induced by membrane microdomains. (A) Dynamic apoptosis imaging by annexin V staining. After the cells were plated on FasL-bound bilayers, they were incubated with Alexa 488-labeled annexin V (at time = 0, panels of the left-side column). Binding of annexin to the cell membranes was monitored by time-lapse imaging. Cells that form Fas–FasL microdomains (labeled B and C) were stained with annexin, whereas the cell A without a visible microdomain was not. Two different focal planes (one for imaging membrane microdomains and the other for the cross section of the cell to clarify the annexin binding to the plasma membranes) were used as illustrated at the right-side panels. Bar is 25 μm. (B) Late apoptotic events induced by Fas–FasL microdomains. Cells were plated on Fas-bound bilayers, and after 1 h, cells were stained with annexin V and PI. Locations of all the cells (from bright-field image; not shown) are indicated with yellow dot lines. Cell A formed microdomains, but at a very early stage in apoptosis. No annexin and PI staining were observed. Cell B was stained with annexin V. Cell C exhibited membrane blebbing (clearly shown by annexin staining) as well as uptake of PI. Bar is 5 μm.

bilayers, we plated the cells on the bilayers and incubated the cells with Alexa 488-labeled annexin V, a marker for the early phase of apoptosis. We observed dynamic binding of annexin V to the cells that formed Fas–FasL microdomains within 30–60 min by using time-lapse microscopy (cells B and C in Fig. 2A). This result demonstrates that the FasL-bound bilayer induces apoptosis in cells and the progress of apoptosis can be monitored in real time by using our system. Annexin did not bind to a cell without any visible Fas–FasL microdomains (cell A in Fig. 2A). At the contact site of the cells B and C in Fig. 2A, microscopically visible aggregates of Cy3-conjugated streptavidin emerged, which are regarded as Fas–FasL microdomains. The area that organizes Fas–FasL microdomains in cell B is apparently larger than that of cell C. That might explain why apoptosis of cell B occurs more rapidly (characterized by more rapid annexin V staining) than that of cell C (Fig. 2A). No obvious morphological changes in the Fas–FasL microdomains were observed for at least several hours, and the cells B and C had been adhered to their initial locations (data not shown). Note that the cell A in Fig. 2A did not exhibit any visible cluster, however either very weak Fas–FasL interaction or other nonspecific interactions might restrict cell movement, and that other cells diffused randomly.

Annexin V specifically detects early apoptosis signal by binding to phosphatidylserine accumulated in the outer leaflet of plasma membranes. To investigate whether the FasL-bound bilayers can induce cells to enter later stages of apoptosis, we monitored cells for a longer time period, and also examined the uptake of PI that stains the nuclei in late apoptotic cells. Within approximately an hour of microdomain formation, we also observed morphological changes in the cell membrane (membrane blebbing) that is indicative of late apoptosis as well as PI uptake (cell C in Fig. 2B). Therefore, we conclude that our FasL-bound bilayer can induce cellular apoptosis toward a very late stage in the process.

3.3. High concentration of Fas proteins promote the formation of stable microdomains that induce apoptosis without internalization

Dual-color imaging was performed to gain greater insights into the Fas–FasL microdomain. Jurkat cells expressing Fas-GFP interacted with FasL-bound bilayers, and the cells formed microdomains enriched with Fas-GFP and FasL (Fig. 3A). All Jurkat cells (including GFP-negative cells) expressed endogenous Fas protein. However, it is evident from Fig. 3A that Fas-GFP-expressing Jurkat cells formed microdomains more frequently than cells with endogenous Fas protein alone. All cells were exposed to a similar number of FasL proteins that were homogeneously distributed on bilayers. Therefore, a specific threshold of Fas concentration may be required to induce Fas–FasL interaction and microdomain formation at the cell–cell interface. We can also monitor Fas localization by imaging Fas-GFP proteins via wide-field and TIRF microscopy. The morphology of Fas–FasL microdomain was unchanged for hours, and no significant internalization of Fas proteins was observed. The majority of Fas-GFP localized in the microdomains and plasma membranes, and persisted there during the observations (data not shown). This result is consistent with the previous observations regarding cell–cell contact, where Fas–FasL clusters are stable for hours without any detectable Fas internalization [15].

Two additional cell lines (HeLa cells and A375 human melanoma cells) also formed very similar microdomains as Jurkat cells, so Fas–FasL microdomain formation is likely to be cell-autonomous in nature (Fig. 3B). The anti-Fas antibody that has been used in many biochemical studies of Fas signaling was examined for the ability to substitute for FasL at the cell–lipid bilayer junction. The anti-Fas antibodies were biotinylated and linked to lipid bilayers, and we observed very similar Fas microdomain formation in a variety of cell lines that interact with the anti-Fas-linked bilayers (Fig. 3B). We conclude that Fas–FasL microdomain formation is primarily dependent upon Fas receptor clustering, and the anti-Fas antibody-bound bilayers can also be a useful tool to study Fas membrane microdomain formation and signaling.

4. Discussion

By developing a novel reconstituted membrane-cell junction system, we can image Fas protein organization in live cells at high resolution without expressing fluorescent proteins exogenously.

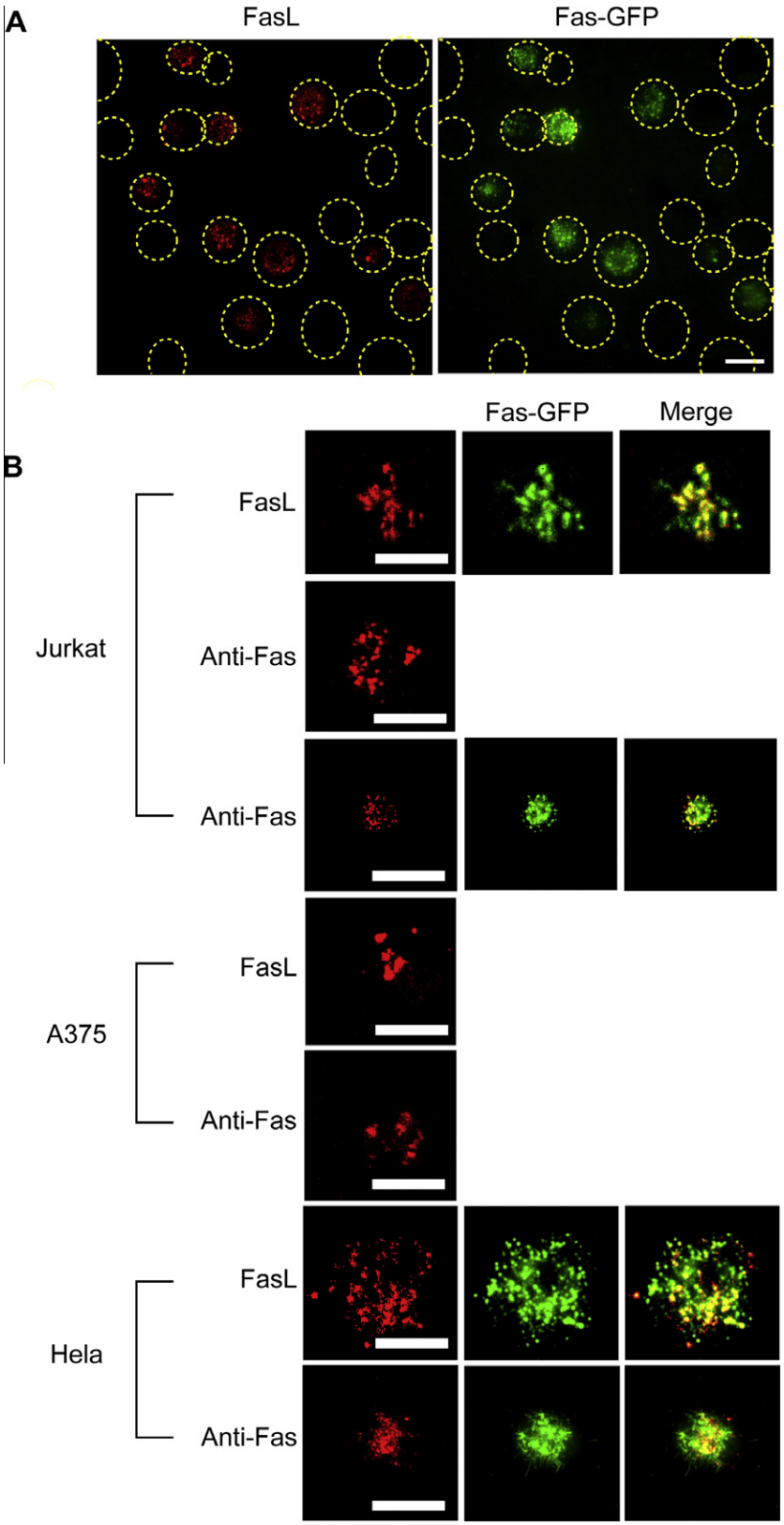

Fig. 3. Characterization of Fas microdomains. (A) Jurkat cells expressing Fas-GFP were plated on FasL-bound bilayers. The locations of all the cells (both GFP-positive and GFP-negative) are shown by yellow dot lines (from bright-field image; not shown). Images of FasL (fluorescent streptavidin, red), Fas (GFP, green, by TIRF microscopy) are paneled. Bar is 25 μm. (B) Single-cell images of Jurkat cells (cells expressing Fas-GFP and cells only expressing endogenous Fas), A375 cells, and HeLa cells (expressing Fas-GFP) interacting on either FasL-bound or anti-Fas antibody-bound bilayers. Images of FasL (red) and Fas-GFP (green, by TIRF microscopy) were shown. Bar is 5 μm.

The Fas–FasL organizations formed at cell–lipid bilayer junctions are similar to the Fas–FasL adhesion/clusters observed at cell–cell interfaces, but our high-resolution imaging is able to resolve structures in greater detail. We confirmed that FasL-bound lipid bilayers induce apoptosis in cells. Therefore, we report that our reconstituted membrane system is a useful model for the dissection of Fas–FasL membrane organizations and apoptotic signals *in vivo*. We now discuss key features of microdomain formation and Fas signaling.

4.1. Fas membrane microdomains and signal transduction

Our observations suggest that a relatively high-level of Fas expression is required for microdomain formation at the cell–membrane interface, and that microdomains are formed with either FasL or anti-Fas antibody. A previous cell–cell imaging study showed that the extracellular domain of Fas alone is necessary and sufficient for Fas–FasL membrane microdomain formation [15]. Such a macroscopic aggregation of membrane proteins lacking cytoplasmic domains was also observed in immune receptors [18]. Collectively, these observations suggest that the integrity of microdomains is maintained primarily by the extracellular domain of Fas. In contrast, structural studies demonstrate that Fas and FADD can pack together into ordered structures [19]. Such a packed organizational pattern may be an infrequent event that occurs only transiently in the microdomains. There are related observations: (1) FADD-GFP does not exclusively localize in Fas-FasL microdomains but rather diffuses across the entire cytoplasm (data not shown), and (2) Fas molecules in Fas-FasL adhesion clusters at cell-cell interface are fluid [15]. Therefore, an important question is by what mechanism is caspase-8 activated in the Fas–FasL microdomains. Further studies using the reconstituted cell–lipid bilayer system will elucidate how the Fas–FADD–caspase-8 interaction occurs in the microdomains.

Recent studies reveal that various types of protein organizations are generated in the cell membranes. These organizations include scaffolding structures such as tight junctions and focal adhesions, endocytosis machinery, and signal protein microdomains in immune cells [20]. The Fas microdomains are also likely to organize signal proteins to facilitate signal transduction, as the microdomains of immune receptors do. Signal microdomains of immune receptors trigger quick immune responses by detecting a small number of ligand molecules. In contrast, Fas may not be quite so sensitive to its ligand that it can help avoiding unexpected cell death, which may explain the marked increase of microdomain formation in cells overexpressing exogenous Fas-GFP (Fig. 3A). The Fas–FasL microdomains do not appear to use actin polymerization as a scaffold to generate and maintain microdomain structures as is the case for immune receptors [21], since we did not observe any cell spreading or membrane protrusion. The lack of an actin scaffold may also explain the relatively high threshold in the number of Fas required for microdomain formation. In summary, we suggest that Fas–FasL microdomain formation requires relatively high concentrations of Fas and FasL proteins to satisfy the threshold requirements for microdomain formation, but once the microdomain forms, an efficient apoptosis signal appears to be transduced in cells.

4.2. Fas internalization and different apoptosis pathways

Various cell types exhibit quantitative differences in DISC formation kinetics when stimulated with anti-Fas antibodies; more efficient and rapid DISC formation occurs in type I cells compared to type II cells [6]. Type II cells may only form a small DISC, but they may use the BID-mitochondrial pathway to amplify the apoptosis signal. By contrast, in the type I cells stimulated with anti-Fas or soluble FasL proteins, Fas appears to internalize and organize signals with FADD and caspase-8 in endosomes to provide more active caspase-8 molecules and circumvent the mitochondrial pathway to induce apoptosis [17]. However, cell–cell imaging of the Fas–FasL structure does not resolve any substantial differences in the organization of Fas–FasL membrane between type I and type II cells, nor does it detect any substantial Fas internalization [15]. In our cell–membrane junction system, no internalization of Fas molecules was observed over a period of several hours, although we observed efficient microdomain formation within 10 min and rapid induction of apoptosis within an hour in type II cells. The mechanism of Fas signal regulation *in vivo* remains unclear, however it may be the combination of the two cases. When soluble FasL molecules are generated, they can induce endocytosis of Fas, resulting in the amplification of the apoptosis signal in endosomes. However, when the Fas signal is mediated by membrane-bound FasL, stable Fas–FasL organizations are formed at the cell–cell interface and they transduce the apoptosis signal. Another question is whether these stable Fas–FasL membrane organizations transduce signals with the BID-mitochondrial pathway, a hypothesis that can be examined by imaging the mitochondrial membrane potential in our reconstituted system.

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