A Novel Signaling Mechanism for Soluble CD95 Ligand

SYNERGY WITH ANTI-CD95 MONOCLONAL ANTIBODIES FOR APOPTOSIS AND NF-κB NUCLEAR TRANSLLOCATION

Sheng Xiao‡¶, Satoshi Jodo‡¶, Sun-sang J. Sung**, Ann Marshak-Rothstein‡‡, and Shyr-Te Ju¶§§¶§§

Received for publication, June 19, 2002, and in revised form, September 16, 2002
Published, JBC Papers in Press, October 21, 2002, DOI 10.1074/jbc.M206093200

From the ‡Department of Medicine, the ¶Department of Pathology and Laboratory Medicine, and the §§Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118, and the **Department of Medicine, Division of Rheumatology and Immunology, University of Virginia, Charlottesville, Virginia 22908

Soluble CD95 (Fas) ligand (sFasL) is known to be deficient in transducing signals upon engagement with membrane Fas. Here we report that sFasL tranduces, in synergy with non-cytotoxic anti-Fas monoclonal antibody (mAb), signals for apoptosis and nuclear translocation of the NF-κB (p65/p50) heterodimer. Activation of the specific signaling pathways correlates with target Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein expression. Synergy with anti-Fas mAb was demonstrated with a trimeric unit of sFasL bearing a single binding site for Fas trimer. In contrast, membrane-bound FasL, as expressed on cell-derived vesicles was fully competent in transducing Fas-mediated signals for apoptosis and NF-κB nuclear translocation. We propose a model in which the trimeric sFasL signaling requires target expression of a high focal density of Fas, which is induced by the signal-incompetent anti-Fas mAb. Membrane-bound FasL induces powerful Fas-mediated signals because it possesses both Fas-focusing and signal-transducing functions.

CD95 (Fas)1 is a type I transmembrane protein expressed by a variety of nucleated cells (1). The physiological ligand for Fas (FasL) is a type II transmembrane protein expressed by activated T cells and non-T cells under a variety of conditions (2–10). Cross-linking of Fas by membrane FasL or by agonist anti-Fas mAb induces apoptosis in specific Fas+ target cells (10–12). The FasL-induced apoptosis has been implicated in immune privileged sites (1–6, 13–19). Cross-linking of Fas has also been shown to induce NF-κB nuclear translocation (20, 21). NF-κB translocation can be induced in certain target populations with anti-Fas mAb or cross-linked recombinant soluble FasL (rsFasL). This activation pathway may be responsible for Fas-dependent co-stimulation of T cell response (22), FasL-induced chemokine production, infiltration of neutrophils, and rejection of tumor cells (23–27). However, the relative capacity of the physiologically derived full-length FasL or sFasL to induce NF-κB nuclear translocation has not been reported. This is an important question because sFasL displays distinct properties and can regulate various functions of membrane FasL.

We have shown recently (28) that cells transfected with human FasL gene produce not only the expected transmembrane FasL and sFasL but also microvesicles (FasL VP) that express the transmembrane protein. Cleavage of the membrane FasL at position 130 yields a 152-amino acid polypeptide with a molecular mass of 26,000–27,000 daltons (24, 30, 31). The released sFasL exist as a trimeric unit with a molecular mass of 81,000 daltons (31). In most cases, sFasL not only fails to induce apoptosis of Fas+ targets but, when in great excess, can actually inhibit the cytotoxicity mediated by the cell-bound membrane FasL (30, 32). By contrast, FasL VP remains cytotoxic against the same targets that are sensitive to the parental FasL-expressing cell lines from which the FasL VP is prepared. Interestingly, sFas is able to kill certain transfected target populations that overexpress Fas (31). In addition, thymocytes, which express a high level of Fas (33), are moderately sensitive to sFasL (31).

To explain this conundrum, we hypothesize that sFasL directly induces signal transduction only in targets whose focal Fas expression (local density) is very high such as can be found on thymocytes and Fas-transfected cell lines. To test this hypothesis, we took advantage of the ability of noncytotoxic anti-Fas mAb to create micro-clusters of Fas, and we demonstrated that such treated cells become highly responsive to sFasL. Target cells that are resistant to killing by either agent alone became highly sensitive in a fashion that indicates a synergy for the induction of apoptosis signal had occurred. In addition, the synergy between sFasL and anti-Fas mAb was extended to the NF-κB signaling pathway in target cells expressing high levels of FLIP that inhibits the apoptosis pathway. We demonstrated that nuclear translocation of NF-κB p65/p50 heterodimer could be induced by FasL VP but not by sFasL or anti-Fas mAb alone. However, NF-κB nuclear translocation was effectively induced when both sFasL and anti-Fas mAb were used. Based on these observations, we propose a model in which each sFasL trimer can signal through anti-Fas-induced micro-clusters of Fas and that membrane-bound FasL pos-
ses the ability to form micro-clusters of Fas and the ability to transduce the Fas-mediated signals. The dual functions of membrane-bound FasL could explain its effectiveness in inducing Fas-mediated signaling pathways.

MATERIALS AND METHODS

Production of FasL-expressing Cell Lines—We have generated NIH-3T3 cells expressing the human FasL (hFasL-3T3) and Neuro-2a cells expressing mouse FasL resistant to metalloproteinase (mFasL-N2a) (34). We have also generated a tumor T cell line (pL-2-hFasL-EL-4) in which the hfas gene is put under the control of a 1.9-kb IL-2 promoter (28). The production and characterization of these cell lines have been described (28, 34).

Preparation of Vesicles (VP) and sFasL—FasL-expressing 3T3 cells (80% confluence) were maintained in 150 × 25-mm culture dishes in 25 ml of culture medium for 2 days. For pL-2-hFasL-EL-4 cells, phorbol 12-myristate 13-acetate (20 ng/ml) and ionomycin (0.5 μM) (Sigma) were used to induce FasL expression. Cells were cultured at 10^6/ml for 24 h. Supernatants were centrifuged at 13,000 rpm in a Sorvall RC-5B refrigerated centrifuge (Newton, CT) at 5 °C for 30 min to remove cell debris. The cell-free supernatants were then centrifuged for 3–5 h at 5 °C at 25,000 rpm in a Beckman ultracentrifuge (model L8-55, Beckman Coulter, Fullerton, CA) using an SW25 rotor. The vesicle-containing pellets were suspended with culture medium to 7% of the original culture supernatant and passed through a 0.45-μm sterile filter (FasL VP) for use. To prepare sFasL, cell-free supernatants were centrifuged at 25,000 rpm for 16 h, and the top 10% volume was collected for analysis. In some experiments, FasL-expressing 3T3 cells were cultured in serum-free medium for 2 days. After ultracentrifugation, supernatants were dialyzed against distilled water, lyophilized, and dissolved in phosphate-buffered saline (PBS, pH 7.2) using 1/10 of the original volume. The concentrated sFasL was passed through a Centri-CON-Plus 20 filtration apparatus (cut-off point of 100,000 daltons, Millipore, Boston, MA) to obtain the sFasL consisting of a single trimeric binding unit with an estimated molecular mass of 78,000–81,000 daltons (35). Western blot analysis demonstrated that FasL VP is composed of full-length FasL of 40,000 daltons and sFasL contains only sFasL of 26,000–27,000 daltons (35). FasL concentrations in various preparations were determined using a capture ELISA kit (Oncogene, Boston) as described previously (28).

Tumor Cell Lines and Antibodies—The tumor cell line IIA1.6 (36), an FcεR-negative derivative of the B lymphoma line A20, was obtained by Dr. C. A. Janeway (Yale University, New Haven, CT). LF/H9253, a T lymphoma cell line overexpressing Fas (37), was obtained from Dr. P. Golstein (INSERM, France). Jo2 mAb, PE-Jo2, and NOK-1 anti-human FasL mAb were purchased from BD Biosciences. RK-8, RMP2, and RMP6 were obtained from MBL Co. (Watertown, MA). Rabbit anti-IFN-γ antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. The generation and characterization of K3/H28 (K3) T cell hybridoma and Fas-lg fusion protein have been described (6).

Cytotoxicity Assay—Target cells (3 × 10^6/0.5 ml) were mildly labeled with 150 μCi of Na_2^31CrO_4 in a 37 °C incubator for 35 min with periodic shakings every 10 min. Cells were washed two times with medium and used as targets. The effectors analyzed included various anti-Fas mAbs, FasL-expressing cells, FasL VP, and sFasL preparations. Because LF/H9262 cells express FcγR that could influence analysis when anti-Fas mAbs were used, we added saturating 2.4G2 mAb (1 μg/ml, Pharmingen) to block any potential FcγR-mediated influence when assays were conducted with this target. Various amounts of each sample were cultured with 2 × 10^4 target cells in a total of 0.2 ml in individual wells of a 96-well plate. In some experiments, NOK-1 or Fas-lg was added to the culture mixture as inhibitors. After 5 h of culture, supernatants were removed, and radioactivity (counts/min) was determined with a γ-countilation counter (Amersham Biosciences). Background release was determined by culturing target cells in the absence of the effectors. Target cells, treated with 0.5% Nonidet P-40, were used to determine total release, which represented 100% cell death. Cytotoxicity is expressed as % specific ^31Cr release, which is determined by the formula: 100% × (experimental release − background release)/total release − background release). All experiments were carried out in duplicate and were conducted two times or more. The Fas-dependent nature of the cytotoxic cells, vesicle preparations, and sFasL has been rigorously demonstrated in previous studies (28, 34).

RT-PCR and EMSA—RNA extracts of K3 and IIA1.6 cells were reverse-transcribed to cDNA, followed by RT-PCR as described (38, 39). EMSA for activation-induced nuclear translocation of NF-κB was carried out as described previously (39, 40).

RESULTS

Comparison of Cytotoxicity Mediated by Anti-Fas mAb, sFasL, FasL VP, and FasL-expressing Cells, and Anti-Fas mAb—Various amounts of sFasL, FasL VP, and FasL-expressing cells were cultured in a 6-well plate under various treatment conditions as described in the figure legends. FasL VP and sFasL prepared from FasL-3T3 cells were used. At various times after treatment, nuclear extracts were prepared. EMSA was conducted using the NF-κB consensus probe (5′-CGCTTGTAGT- CAGCCGGAA-3′, Promega, Madison, WI) and the Sp1 probe (5′-ATT- TCTGGGCGGATTCCTCTTTGGA-3′) that we have described previously (39, 41). The specificity of binding was determined by antibody-mediated supershift analysis using various anti-NF-κB antibodies (39, 40).

Confocal Microscopic Analysis of Fas Micro-clusters and Apoptosis—To visually examine surface Fas micro-cluster formation and cell apoptosis, IIA1.6 cells (10^4) were incubated with 1 μg of PE-Jo2 with or without sFasL (10 nm) in 150 μl of PBS containing 4% bovine serum albumin on ice for 1 h and then incubated in a 37 °C water bath. At various times after incubation, cells were washed with PBS and then fixed with 2% paraformaldehyde for 20 min at room temperature. Fixed cells were observed using a Carl Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, NY).

Comparison of Cytotoxicity Mediated by Anti-Fas mAb, sFasL, FasL VP, and FasL-expressing Cells—The ability of various anti-Fas mAbs and various forms of FasL to induce apoptosis was determined by a 5-h cytotoxicity assay using as targets the B cell lymphoma A20-derived, FcγR^− variant IIA1.6 and the T cell-derived tumor target LF. The sFasL and FasL VP were prepared from FasL-3T3 cells that constitutively express FasL. The results shown in Fig. 1a indicate that both FasL VP and FasL-3T3 cells could effectively induce apoptosis in both targets. By contrast, no cytotoxicity was detected with the same molar concentration of sFasL. The results demonstrate that sFasL but not FasL VP failed to kill tumor targets that are sensitive to the parental FasL-expressing cells. Interestingly, none of the anti-Fas mAbs induced apoptosis of the two targets under the 5-h cytotoxicity assay, which detects the
synergy is a general phenomenon applicable to various preparations of sFasL, tumor targets, and anti-Fas mAb.

A Single Trimeric Unit of sFasL Is Able to Induce Killing in Synergy with Anti-Fas mAb—To exclude the possibility that the synergy is caused by aggregated sFasL, we further purified sFasL by passing it through a Millipore filtration apparatus (Centricon-Plus-20) to obtain sFasL, whose molecular mass was less than 100,000 daltons. It has been shown that the physiologically derived sFasL, which is cytotoxic against transfected targets, has a molecular mass of 81,000 daltons corresponding to a homotrimeric protein structure (31). The concentration of the fractionated sFasL preparation was determined by capture ELISA, as described under “Materials and Methods.” The purified fraction and the original sFasL preparation were compared on a molar basis for the ability to act in synergy with Jo2 mAb (Fig. 3). The result shows no difference between the purified sFasL and the original sFasL preparation in the ability to synergize with Jo2 mAb in killing IIA1.6 (Fig. 3b). The data suggest that a single trimeric unit of sFasL is able to synergize with anti-Fas mAb, and this is an intrinsic property of sFasL acquired as the result of matrix metalloproteinase-mediated cleavage.

NF-κB Nuclear Translocation Is Induced by FasL VP—A recent study (20) has demonstrated that polymerized rsFasL induces NF-κB nuclear translocation via a FLIP/TRAF2-mediated pathway. However, whether membrane-bound FasL could activate this pathway was not determined. It has been demonstrated that FLIP, an inhibitor of Fas-mediated apoptotic pathway, acts as a swing molecule to direct the Fas-mediated signal toward the NF-κB activation pathway (20). We compared IIA1.6 B lymphoma cells and K3 hybridoma T cells for mRNA level of Fas and FLIP. In addition, we compared them for the induction of apoptosis and the ability to translocate NF-κB upon engagement with FasL VP. The results showed that K3 cells but not IIA1.6 cells express high levels of FLIP mRNA, whereas IIA1.6 cells but not K3 cells express high levels of Fas (Fig. 4a). The differences in FLIP and Fas mRNA levels correlate with their susceptibility to FasL VP-induced apoptosis. Thus, IIA1.6 but not K3 are strongly killed by FasL VP (Fig. 4b). In contrast to apoptosis, the ability of FasL VP to induce NF-κB nuclear translocation is observed in K3 but not IIA1.6.
Membrane Regulation of Fas-mediated Signals

Fig. 4. FasL VP induction of apoptosis correlates with Fas and FLIP mRNA levels in target cells. a, Fas and FLIP mRNA expression levels in K3 hybridoma T cells and IIA1.6 B lymphoma cells. Fas, FLIP, and β-actin mRNA levels were determined by RT-PCR as described under “Materials and Methods.” b, IIA1.6 but not K3 is sensitive to FasL VP in 5-h cytotoxicity assays as described under “Materials and Methods.” Two different FasL VP preparations were tested with similar results.

Fig. 5. FasL VP induction of the p65/p50 NF-κB nuclear translocation in K3 but not IIA1.6 cells. FasL VP (10 nM), prepared from FasL-3T3 cells, was used to treat target cells (5 × 10⁶) in 5 ml of culture medium. Cells cultured without VP were used as controls. Nuclear extracts were prepared at 2 h for IIA1.6 and 3 h for K3. Under the experimental conditions, a low level of apoptosis was observed for IIA1.6, and <5% apoptosis was observed for K3. Longer incubation resulted in significant apoptosis of IIA1.6 cells. The major activated NF-κB member in the FasL VP-treated K3 cells is p65/p50 heterodimer (solid arrow) as determined by Ab-mediated supershift assay. Note that IIA1.6 and K3 display different binding patterns, and they differ in the major species of NF-κB members as indicated by Ab-mediated supershift assay.

Fig. 6. Trimeric sFasL with a single binding site for Fas acts in synergy with anti-Fas mAb to induce nuclear translocation of NF-κB in K3 hybridoma T cells. K3 cells (5 × 10⁶) were cultured in 5 ml of culture medium for 3 and 5 h, respectively. Cells cultured in medium only were included for comparison. *Solid arrow indicates p65/p50 heterodimer; open arrow indicates p50/p50, and asterisk indicates Sp1.

Mechanism of Synergy between sFasL and Anti-Fas mAb—It is generally believed that Fas cross-linking is sufficient to induce apoptosis, but this is based on a 24-h cytotoxicity assay that detects a weak apoptotic signal. Apparently, anti-Fas mAbs do not generate sufficient signal for apoptosis following Fas cross-linking for 5 h. Confocal microscopy study was conducted to prove that anti-Fas mAb indeed had cross-linked Fas under the experimental conditions and that synergy with sFasL to induce apoptosis occurred only after Fas was cross-linked. As shown in Fig. 7, binding of PE-Jo2 to IIA1.6 resulted in a time-dependent formation of micro-clusters, which can be detected as early as 30 min after the addition of PE-Jo2. Despite prolonged incubation and extensive formation of Fas clusters, apoptosis was not induced (Fig. 7A). However, the presence of sFasL in the culture strongly induced apoptosis in IIA1.6 cells (Fig. 7B). Remnants of lysed cells and aggregates of apoptotic cells were observed. Apoptosis seems to affect the...
extent of Fas aggregation. Micro-clusters of Fas were not formed when IIA1.6 cells were incubated with PE-Jo2 mAb on ice, and the addition of sFasL also failed to induce apoptosis under these conditions (Fig. 7B). The data suggest a mechanism for synergy in signal transduction for apoptosis by the combined action of sFasL and Jo2. Based on the data presented in this study, we propose that anti-Fas mAb can effectively cross-link membrane Fas but are ineffective in signal transduction, whereas sFasL is an effectively signal transducer through aggregated Fas but could not cross-link membrane Fas. Synergy occurs because the cross-linking of Fas by anti-Fas mAb permits sFasL to bind to the clustered Fas and transduce signal effectively. A scheme for this hypothesis is depicted in Fig. 7C.

DISCUSSION

In this study, we describe a novel property of sFasL, namely that it can act in synergy with anti-Fas mAb in activating the Fas-mediated signal transduction pathways. We demonstrate that sFasL acts in synergy with several anti-Fas mAbs in killing Fas" targets even though neither agent alone induces apoptosis of the target cells. In addition, we demonstrate that nuclear translocation of the p65/p50 NF-kB can be induced by a mixture of sFasL and anti-Fas mAb but not by either agent alone. We have demonstrated this synergy with sFasL prepared from two different FasL-expressing cell lines and four different anti-Fas mAbs. The targets we have examined include tumor cell lines and freshly obtained normal lymphocytes from thymus, spleen, and lymph node. These observations indicate that the synergy between sFasL and anti-Fas mAbs is a general phenomenon applicable to various anti-Fas mAbs and cell populations. Although the synergy between sFasL and anti-Fas mAbs induces only a moderate signal for p65/p50 NF-kB nuclear translocation, synergy for apoptosis is very potent. Not only a high percentage of target cells were killed but the killing was also acutely induced within a short period of 5 h. Our analysis supports a model in which sFasL with a single binding site for Fas could induce activation and apoptotic signals in cells that express a high focal density of Fas through antibody-mediated focusing.

Cross-linking of Fas on the cell membrane induces apoptosis of sensitive targets. However, there are many exceptions and variations to this over-generalized paradigm. Under the experimental conditions of the 5-h cytotoxicity assay, FasL-expressing cells and FasL VP could effectively kill IIA1.6 and LF targets, whereas sFasL or anti-Fas mAbs could not (Fig. 2). In addition, both sFasL and anti-Fas mAbs inhibit both cell-mediated and the FasL VP-mediated cytotoxicity, indicating that sFasL and anti-Fas mAbs competed with membrane FasL for Fas binding. Thus, it was not predicted that a combination of sFasL and anti-Fas mAbs would deliver a strong Fas-mediated apoptotic signal to the very same target cells (Fig. 3). The synergy is interesting because both sFasL and anti-Fas mAbs...
bind to Fas, a situation under which competition for binding might be expected. Competition between sFasL and anti-Fas mAbs has not been addressed in previous studies, although citing unpublished observations, Nishimura et al. claimed inhibition of sFasL binding by Jo2 but not by RK-8 (44). However, by using target cells that are sensitive to sFasL, we have been unable to inhibit killing with several anti-Fas mAbs to which the target cells are resistant (data not shown). Therefore, the synergy between sFasL and anti-Fas mAbs is compatible with the interpretation that they bind to different regions of the Fas molecule. We favor this interpretation also because synergy in killing was observed with a wide dose range of sFasL and anti-Fas mAbs (Fig. 2).

Physiologically derived sFasL trimers have been shown to induce apoptosis in certain Fas-transfected target populations (31, 45). We showed that purified sFasL bearing a molecular mass of <100,000 daltons can act in synergy with Jo2 to kill IIA1.6 targets. This indicates that the synergy between sFasL and anti-Fas mAbs is not solely due to enhanced cross-linking of the anti-Fas-Fas complex. Indeed, apoptosis was not observed when IIA1.6 cells were cultured with combinations of various anti-Fas mAbs (data not shown). In addition, synergy between anti-Fas mAb was not observed with either FasL VP or FasL-expressing cells (in limiting dose); the latter are multivalent and independently capable of both cross-linking Fas and engaging the physiological binding site. A recent study (46) suggests that sFasL may bind to matrix proteins such as fibronectin, vitronectin, and collagen IV, which could be found as trace components in serum. However, cross-linking by serum components was unnecessary in our system because synergy between sFasL and anti-Fas mAbs was also observed when assays were conducted in serum-free medium using target cells adapted to growing in such medium (data not shown).

The synergy in Fas-mediated signaling between sFasL and anti-Fas mAbs is not restricted to apoptosis because sFasL and anti-Fas mAbs also act in synergy to induce nuclear translocation of p65/p50 NF-κB (Fig. 6). It has been demonstrated that Fas cross-linking by FLAG-tagged rsFasL in the presence of anti-FLAG antibodies could induce a signaling complex that contains Fas-associated death domain and FLIP. Aggregated FLIP attracts Raf-1 and TRAF1/2 to the complex to activate the extracellular signal-regulated kinase and NF-κB pathways, respectively (20). As FLIP inhibits apoptosis, FLIP expression level could dictate which pathway is activated upon cross-linking Fas. Such a correlation was observed in the present study between apoptosis-prone, FasL VP-sensitive IIA1.6 and apoptosis-resistant, FasL VP-resistant K3 targets. Indeed, the FLIP mRNA levels in K3 are significantly higher than IIA1.6, and NF-κB translocation to nucleus could be induced by FasL VP in K3 hybridoma T cells but not in IIA1.6 cells. As with the synergy between sFasL and anti-Fas mAbs for the induction of apoptosis of IIA1.6 cells, the p65/p50 NF-κB nuclear translocation in K3 hybridoma T cells could not be induced by anti-Fas mAbs or sFasL alone but required the simultaneous presence of both agents. These observations indicate that the synergy between sFasL and anti-Fas mAbs occurs at the step before the FLIP-regulated bifurcation of the apoptotic pathway and the NF-κB activation pathway. The data suggest that the synergy occurs at the level of membrane binding and its immediate signaling event. The data do not support the hypothesis that sFasL and anti-Fas mAbs induce separate signals, and synergy occurs as a result of merging of the two independently induced signals.

It should be noted that many receptor/ligand systems such as IL-2 and IL-4 transduce signals without the apparent cross-linking of their receptors. Indeed, even the soluble tumor necrosis factor-α, a close family member of FasL, transduces a signal without the need for cross-linking. It will be of interest to determine whether synergy in killing could be demonstrated with tumor necrosis factor-α and anti-TNFRII mAbs, although this could be complicated by the influence of TNFRII signaling (47). The reason why the sFasL trimer that displays a single binding site for Fas can induce apoptosis in certain targets has not been addressed in previous studies. The targets that are sensitive are usually derived by transfection with the fas gene or naturally express a very high level of Fas (as is the case with thymocytes). Based on the present study, we propose a model to explain how membrane Fas binding activates the Fas-dependent downstream signaling pathways (Fig. 7). We hypothesize that physiologic engagement of a micro-cluster of Fas trimers is required to activate effectively the Fas-mediated signal transduction pathways. The engagement of Fas by sFasL may lead to a conformational or biochemical change of Fas, but only a cluster of such modified molecules can lead to the formation of an effective signaling complex, and anti-Fas mAb can bring about such clusters on target cell membranes. Because anti-Fas antibodies do not engage Fas in a manner comparable with physiological FasL, they can effectively facilitate cluster formation but are less effective in their capacity to induce signal transduction. Conversely, sFasL is ineffective in signal transduction because it does not have the ability to generate micro-clusters of Fas. Our hypothesis is supported by studies using confocal microscopy, which demonstrated micro-cluster formation by anti-Fas mAb and synergy in apoptosis by the co-presence of anti-Fas mAb and sFasL (Fig. 7). A similar hypothesis based on the synergy observed between recombinant sFasL and ceramide has been proposed (29). In this case, the presence of ceramide around the Fas-associated micro-domain allows the recombinant sFasL to cap Fas and transduce an apoptotic signal (NF-κB signaling was not determined). Interestingly, anti-Fas cross-linking activates acidic sphingomyelinase to generate ceramide. Regardless whether ceramide is involved in our model, under physiological conditions membrane FasL, expressed on cells or vesicles, possesses both the ability to form micro-clusters of Fas and the ability to transduce signals. Thus, membrane FasL is the most effective physiologically derived ligand for the activation of Fas-mediated signaling pathways.

In conclusion, four major points are made in this study. First, we show that sFasL acts in synergy with noncytotoxic anti-Fas mAbs in Fas-mediated signal transduction. Second, the synergy is demonstrated for both the apoptotic signaling pathway and the p65/p50 NF-κB nuclear translocation pathway. Third, the synergy in signaling for NF-κB nuclear translocation or apoptosis correlates with the FLIP mRNA levels in target cells. Fourth, FasL VP, a physiological form of vesicles bearing full-length FasL, is capable of activating both signaling pathways depending on the FLIP level of target cells. The information has relevance to our understanding of Fas-mediated signaling transduction pathway at the membrane level.

REFERENCES

1. Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992) Nature 356, 314–317
2. Takahashi, T., Tanaka, M., Brannan, C. I., Jenkins, N. A., Copeland, N. G., Suda, T., and Nagata, S. (1994) Cell 76, 369–376
3. Lynch, D. H., Watson, M. L., Alderson, M. L., Baum, P. R., Miller, R. E., Tough, T., Gibson, M., Davis-Smith, T., Smith, C. A., Hunter, K., Bhat, D., Din, W., Goodwin, R. G., and Seldin, M. F. (1994) Immunol. Rev. 131, 151–156
4. Dhein, J., Walczak, H., Baumer, C., Debatin, K. M., and Krammer, P. H. (1995) Nature 373, 438–441
5. Brunner, T., Mogil, R. J., LaFace, D., Yoo, N. J., Mahboubi, A., Echeverri, F., Martin, S. J., Force, W., Lynch, D. H., Ware, C. F., and Green, D. R. (1995) Nature 373, 441–444
6. Su, S.-T., Panka, D. J., Cui, H., Ettinger, R., El-Khatib, M., Sherr, D. H., Stanger, B. Z., and Marshak-Rothstein, A. (1985) Nature 314, 444–448
7. French, L. E., Hahne, M., Viard, I., Radigruher, G., Zanone, R., Becker, K., Muller, C., and Tschopp, J. (1996) J. Cell Biol. 133, 335–343
8. French, L. E., Wilson, A., Hahne, M., Viard, I., Tschopp, J., and MacDonald,
Membrane Regulation of Fas-mediated Signals

50913

H. R. (1997) J. Immunol. 159, 2196–2202
9. Bonfoco, E., Stuart, P. M., Brunner, T., Lin, T., Griffith, T. S., Gao, Y., Nakajima, H., Henkart, P. A., Ferguson, T. A., and Green, D. R. (1998) Immunity 9, 711–720
10. Ju, S.-T., Cui, H., Panka, D. J., Ettinger, R., and Marshak-Rothstein, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4185–4189
11. Trauth, B. C., Kias, C., Peters, A. M., Matzku, S., Moller, P., Falk, W., Debatin, K.-M., and Krammer, P. H. (1989) Science 245, 301–305
12. Yonehara, S., Ishii, A., and Yonehara, M. (1989) J. Exp. Med. 169, 1747–1756
13. Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitanura, Y., Itoh, N., Suda, T., and Nagata, S. (1995) Nature 364, 806–809
14. Ettinger, R., Panka, D. J., Wang, J. L. K., Stanger, B. Z., Ju, S.-T., and Marshak-Rothstein, A. (1995) J. Immunol. 154, 4302–4308
15. Rothstein, T. L., Wang, J. K. L., Panka, D. J., Foste, L. C., Wang, Z., Stanger, B. Z., Cui, H., Ju, S.-T., and Marshak-Rothstein, A. (1995) Nature 374, 163–165
16. Bellgrau, D., Gold, D., Selawry, H., Moore, J., Franzusoff, A., and Duke, R. C. (1995) Nature 377, 630–632
17. Hahne, M., Rimoldi, D., Schroter, M., Romero, P., Schreiter, M., French, L. E., Schneider, P., Bornand, T., Fontana, A., Lienard, D., Cerottini, J.-C., and Tschopp, J. (1996) Science 274, 1363–1366
18. O’Connell, J., O’Sullivan, G. C., Collins, J. K., and Shanahan, F. (1996) J. Exp. Med. 184, 1075–1082
19. Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R., and Ferguson, T. A. (1995) Science 270, 1189–1192
20. Kataoka, T., Budd, R. C., Holler, N., Thome, M., Martinon, F., Irmler, M., Buerz, K., Hahne, M., Kennedy, N., Kovacevic, M., and Tschopp, J. (2000) Curr. Biol. 10, 640–648
21. Ponton, A., Clement, M.-V., and Stamenkovic, I. (1996) J. Biol. Chem. 271, 8991–8995
22. Alderson, M. R., Tough, T. W., Braddy, S., Davis-Smith, T., Roux, E., Schooley, K., Miller, R. E., and Lynch, D. H. (1994) Int. Immunol. 6, 1799–1806
23. Arai, H., Gordon, D., Nabel, E. G., and Nabel, G. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13862–13867
24. Kayakagi, N., Kawasaki, A., Ebata, T., Ohmoto, H., Ikeda, S., Inoue, S., Yoshino, K., Okumura, K., and Yagita, H. (1995) J. Exp. Med. 182, 1777–1783
25. Hohlbaum, A. M., Gregory, M. S., Ju, S.-T., and Marshak-Rothstein, A. (2001) J. Immunol. 167, 6217–6224
26. Chen, J.-J., Sun, Y., and Nabel, G. J. (1998) Science 282, 1714–1717
27. Hohlbaum, A. M., Moe, S., and Marshak-Rothstein, A. (2000) J. Exp. Med. 191, 1209–1220.
28. Jodo, S., Xiao, S., Hohlbaum, A. M., Strehlow, D., Marshak-Rothstein, A., and Ju, S.-T. (2001) J. Biol. Chem. 276, 39938–39944
29. Cremesti, A., Paris, F., Grassmé, H., Holler, N., Tschopp, J., Fuku, Z., Gubins, E., and Kolesnick, R. (2001) J. Biol. Chem. 276, 23954–23961
30. Tanaka, M., Itai, T., Adachi, M., and Nagata, S. (1998) Nat. Med. 4, 31–36
31. Tanaka, M., Suda, T., Takahashi, T., and Nagata, S. (1995) EMBO J. 14, 1129–1135
32. Suda, T., Hashimoto, H., Tanaka, M., Ochi, T., and Nagata, S. (1997) J. Exp. Med. 186, 2045–2050
33. Drappa, J., Brut, N., and Elkon, K. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10340–10344
34. Jodo, S., Hohlbaum, H., Xiao, S., Chao, D., Strehlow, D., Sherr, D. H., Marshak-Rothstein, A., and Ju, S.-T. (2000) J. Immunol. 165, 5487–5494
35. Jodo, S., Strehlow, D., and Ju, S.-T. (2000) J. Immunol. 164, 5062–5069
36. Jones, B., Tite, J. P., and Janeway, C. A. (1986) J. Immunol. 136, 348–356
37. Rouvier, E., Luciani, M. F., and Golstein, P. (1993) J. Exp. Med. 177, 185–200
38. Refaeli, Y., Van Parijs, L., London, C. A., Tschopp, J., and Abbas, A. K. (1998) Immunity 8, 615–623
39. Xiao, S., Marshak-Rothstein, A., and Ju, S.-T. (2001) Eur. J. Immunol. 31, 3339–3348
40. Cui, H., Matsui, K., Onura, S., Schauer, S. L., Matulka, R. A., Sonenshein, G. E., and Ju, S.-T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7515–7520
41. Xiao, S., Matsui, K., Fine, A., Zhu, B., Marshak-Rothstein, A., Widom, R. L., and Ju, S.-T. (1999) Eur. J. Immunol. 29, 3456–3465
42. Nishimura, Y., Ishii, A., Kobayashi, Y., Yamasaki, Y., and Yonehara, S. (1995) J. Immunol. 154, 4385–4403
43. Budd, R. C. (2002) J. Clin. Invest. 109, 437–442
44. Nishimura, Y., Hirabayashi, Y., Matsuuyki, Y., Musette, P., Ishii, A., Nakauchi, H., Inoue, T., and Yonehara, S. (1997) Int. Immunol. 9, 307–316
45. Schneider, P., Holler, N., Bodmer, J.-L., Hahne, M., Frei, K., Fontana, A., and Tschopp, J. (1998) J. Exp. Med. 187, 1205–1213
46. Aoki, K., Kurooka, M., Chen, J.-J., Petryniak, J., Nabel, E. G., and Nabel, G. J. (2001) Nat. Immunol. 2, 353–357
47. Jupp, O. J., McFarlane, S. M., Anderson, H. M., Littlejohn, A. F., Mohamed, A. A. A., Mackay, R. H., Vanderabeele, P., and MacEwan, D. J. (2001) Biochem. J. 359, 525–535
