Oligomerization of Soluble Fas Antigen Induces Its Cytotoxicity*  

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The abbreviations used are: TNF, tumor necrosis factor; FasL, Fas ligand; sFas, soluble Fas; rhFas, recombinant human Fas; TM, transmembrane; ELISA, enzyme-linked immunosorbent assay; RA, rheumatoid arthritis.

Soluble Fas antigen can protect cells against Fas-mediated apoptosis. High level soluble Fas antigen characteristic for blood of patients with autoimmune disease or cancer is believed to prevent the elimination of autoimmune lymphocytes or tumor cells. Here we report that human recombinant FasTM, i.e. soluble Fas generated by alternative splicing of the intact exon 6, is capable of inducing death of transformed cells by “reverse” apoptotic signaling via transmembrane Fas ligand. FasTM, as well as transmembrane Fas antigen, can be either monomeric or oligomeric, and both its forms are efficient in blocking Fas-mediated apoptosis, although the cytotoxic activity is exhibited solely by the latter. An in vivo analysis of soluble Fas antigen showed that unlike in healthy controls, nearly the total FasTM present in sera of rheumatoid arthritis patients was oligomeric. This resulted in suppression of cell proliferation in the experimental sera and in its promotion in controls. Thus, oligomerization/depolymerization of soluble Fas antigen can regulate its activity and contribute to the pathogenesis of autoimmune diseases and cancer.

Receptors and their ligands from the families of TNF1 receptors and TNF ligands are crucial for cell proliferation, differentiation, and death. Some of these functions are realized through ligand-induced activation of transmembrane receptors with subsequent signal transduction to the receptor-expressing cell. The molecular mechanisms of this kind of transduction are well studied and described in many reviews (1–3). Other functions performed by the TNF receptor-ligand system are realized through the so-called “reverse signaling”, with transmembrane ligand acting as a receptor, i.e. a molecule that receives and delivers the signal, and the receptor (soluble or transmembrane) serving as a ligand, i.e. the signaling molecule. The reverse signaling has been shown for many members of the TNF ligand family (4–11). However, the activation modes and pathways of the reverse signal are virtually unexplored and rarely reported on (11–17).

Fas antigen (Fas) and Fas ligand (FasL) play a key role in maintaining homeostasis of the immune system. FasL-induced activation of transmembrane Fas resulting in death of Fas-bearing cells underlies selection of T- and B-lymphocytes and target elimination by T- and natural killers (3, 18). FasL-mediated reverse signaling was first revealed independently by two research teams (9, 10) and shown to participate in growth control of CD8+ and CD4+ T-lymphocytes. Suzuki and Fink (9, 19, 20) reported that cross-linked FasIgG enhanced proliferation of CD8+ T-lymphocytes. Desbarats et al. (10) showed that activation of transmembrane Fas ligand on CD4+ T-lymphocytes inhibited expression of IL-2, thereby suppressing cell proliferation and causing cell death. Presumably, signal transduction via Fas ligand involves kinases Erk 1 and 2, phospholipase A2, and some proteins containing SH3 and WW domains, specifically, Fyn kinase (14–16).

The current study deals with the possibility of occurrence of FasL-mediated reverse signaling in transformed cells of various origin. It was shown that human recombinant soluble Fas (FasTM) is capable of inducing cell death by apoptosis with an efficiency dependent on the level of FasL expression on the target cells. Anti-Fas antibodies blocked FasTM-induced cell death. The cytotoxic activity of recombinant soluble Fas proved to result from Fas oligomerization. Moreover, soluble Fas in sera of rheumatoid arthritis patients was shown to be oligomeric. This finding suggests that in blood serum, soluble Fas antigen activity is regulated not only by the level of Fas expression but also by its oligomerization/depolymerization.

EXPERIMENTAL PROCEDURES

Cell Cultures and Reagents—The following cell cultures were used: human histiocyte lymphoma cell line U-937, human cervical adenocarcinoma HeLa cells, and human Burkitt’s lymphoma Raji cells. All cell lines were from Specialist collection of continuous cell lines of vertebrates (Institute of Cytology, Russian Academy of Sciences, St.-Petersburg, Russia). Antibodies to Fas antigen and Fas ligand were from Bioasenal (Moscow), to caspase-3 and poly(ADP-ribose) polymerase from Pharmingen BD. Fas ligand exhibiting cytotoxic activity and inactive FasL-FLAG were a gift from Prof. D. Wallach.

Generation of rhFasTM and FasFc—The cDNA fragment FasTM was a product of reverse transcription followed by PCR in the presence of specific primers. mRNA isolated from human HeLa cells was used as a template. The amplified cDNA FasTM was cloned into vector pET30b(+)1. Expression and purification of the protein from Escherichia coli BL21(DE3) were performed using the Talon metal affinity resin (Clontech) in accordance with the manufacturer’s instructions.

The cDNA fragment of the Fas corresponding to amino acids 1–156 was amplified with specific primers and cloned into the vector Signal pLg pl2 (Ingenius). COS-1 cells were transfected with the obtained vector, the FasFc-containing incubation medium was collected, and the protein was purified according to the manufacturer’s instructions (Ingenius).

Western Blot Analysis—Protein expression was studied using cell extracts yielded by cell lysis in buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethyloxycarbonyl fluoride, and 10 μg/ml solutions of leupeptin, pepstatin, and aprotinin. The protein concentration was aligned according to Bradford (21). After electrophoresis in 12% PAGE in the presence of sodium...
dodecyl sulfate (22), the samples were transferred onto nitrocellulose membrane. For immunoblotting, murine monoclonal antibodies were used as primary antibodies, and anti-mouse antibodies conjugated with peroxidase were used as secondary antibodies. The antigen-antibody complex staining was performed using the ECLplus kit or diaminobenzidine in the presence of hydrogen peroxide.

**Cytotoxic Assays**—Cells were seeded in 96-well microtiter plates (2–0–5×104 cells/ml). After treatment with cytotoxic agents the cells were incubated for 24 h. Viability of suspension cells was determined by staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (23). Adherent cell viability was determined by the neutral-red uptake method (24). The cell viability value was estimated as percentage of viable cells after treatment (untreated cells were taken as 100%).

**DNA and RNA Isolation and Reverse Transcripase PCR Analysis**—Total DNA and RNA were isolated essentially as described earlier (25, 26). Expression of mRNA in the cells was determined by the reverse transcriptase PCR analysis (27). The following set of primers was used (forward and reverse, respectively) to estimate gene expression: for Fas antigen, 5′-ACTGTATGTGAACACTGTGACCCT-3′ and 5′-CAGGATTATTTAAGGTGG-3′; for Fas ligand, 5′-AGAAGGCCGTGTCA-AAGGGGCGG-5' and 5′-TTAGACCTTATAAAGCCGAAAACG-3'; for β-actin, 5′-TATCGGTGAGAGGCCCAGAG-3′ and 5′-TACTCTCTGTCTGGTGTACATC-3′. PCR products and DNA fragments of apoptotic cells were analyzed by 1% agarose or 8% acrylamide electrophoresis.

**Serum Analysis**—Blood was drawn from 28 patients with rheumatoid arthritis and from 11 healthy controls at the Moscow Medical Academy Hospital. Sera were harvested by centrifugation at 1500 g and thereafter stored at −70 °C until further processing. A commercially available ELISA kit (Bioasanal, Moscow, Russia) recognizing recombinant and natural sFas was used for quantification of sFas according to the manufacturer's instructions. Briefly, serum samples were diluted 10-fold and thereafter applied to a polystyrene multil (24 h). The cell viability value was estimated as percentage of viable cells after treatment (untreated cells were taken as 100%).

**Results**

**Cytotoxic Activity of Soluble Fas Antigen**—In the cell, there are transmembrane and soluble forms of Fas (28–30). Among the latter, the highest expression is characteristic of FasΔTM (generated by alternative splicing of the intact exon 6) that encodes the Fas transmembrane domain. For the purpose of cloning, FasΔTM mRNA isolated from HeLa cells was used. The recombinant protein was expressed and purified as described under “Experimental Procedures” and in accordance with the instructions by equipment manufacturers.

The molecular mass of FasΔTM isolated by SDS-PAGE electrophoresis was about 40 kDa, and it could be recognized by specific anti-Fas antibodies (Fig. 1A). When added to various human cell cultures, FasΔTM exhibited the cytotoxic activity (Fig. 1B) to which the cells were sensitive to a variable degree. Antibodies to the extracellular, but not intracellular, portion of Fas blocked the FasΔTM-dependent apoptosis (Fig. 1C), thereby demonstrating specificity of FasΔTM as a cell killer.

No effect of FasΔTM on cell distribution over the cell cycle phases was observed (data not shown). As revealed by Western blotting and analysis of DNA from apoptotic cells, FasΔTM induced cell death by apoptosis (Fig. 2A). Caspase-3 that was formed in the cells 4 h after treatment cleaved poly(ADP-ribose) polymerase to trigger internucleosome fragmentation of the DNA (31). Interestingly, prior to the DNA fragmentation in apoptotic cells, FasL expression decreased with increasing amount of Fas mRNA (Fig. 2B). Because inhibition of RNA synthesis by actinomycin D enhances FasΔTM-induced apoptosis (Fig. 1B), it can be believed that a change in expression of FasL and Fas mRNA is a protective response to the treatment by target cells.

A reason for variable degrees of FasΔTM sensitivity shown by cell cultures could lie in different levels of expression of acceptor molecules mediating the action of FasΔTM. Among potential FasΔTM targets, there could be Fasl, Fas receptor, or probably other proteins (30, 32, 33). An analysis of expression of FasL and Fas receptor in the studied cell cultures showed that the level of expression of Fas antigen was nearly the same in all the samples, whereas FasL expression was higher in HeLa and U-937 cells as compared with Raji (Fig. 3A). However, HeLa and U-937 cells were more sensitive to FasΔTM cytoxicity than Raji cells (Fig. 1B). To verify the correlation between the FasΔTM expression level and efficiency of FasΔTM-induced cell death, FasΔTM cytotoxicity was assayed using HeLa cells transiently transfected with Fasl cdna-bearing vector in the antisense orientation. As found, inhibition of FasL expression led to an increased cell resistance against FasΔTM (Fig. 3B).

A direct evidence for Fasl contribution to FasΔTM-induced cell death came from an analysis of the specificity of interactions between recombinant FasΔTM and Fasl in the experiment. Protein extracts of FasΔTM-sensitive cell cultures passed through a FasΔTM-containing column. After eluting, the FasΔTM-bound proteins were subjected to Western blotting with specific anti-Fasl antibodies (Fig. 3C). Fasl was detected in the protein fraction bound to FasΔTM. Hence, the obtained FasΔTM was able to bind Fasl.

Taken together, these results suggest that it is Fasl that serves as a target for FasΔTM.

**Oligomerization of Soluble Fas Antigen**—Further studies of the FasΔTM sample showed that after its separation by gel filtration, the protein was contained by the fraction corresponding to a molecular mass of about 150 kDa (Fig. 4). This demonstrated the ability of FasΔTM to be oligomeric, which was in good agreement with earlier findings by other researchers (32, 33). It is noteworthy that only samples containing a high molecular fraction of FasΔTM displayed the FasΔTM cytoxic activity.

To verify the suggestion that the cytotoxic activity of FasΔTM depends on its oligomerization, we used FasFc, a COS cell-derived combination of the extracellular portion of Fas and the Fc fragment of human IgG. When added to cell cultures, FasFc exhibited no notable cytotoxicity, although its cross-linking with the use of anti-Fc antibodies induced cell death (Fig. 5A). Note that both monomeric and cross-linked FasFc efficiently blocked Fas ligand-induced apoptosis (Fig. 5B). Thus, interactions of both cross-linked FasFc and recombinant oligomeric FasΔTM with transmembrane Fasl ligand induce apoptosis of target cells. Binding of both oligomeric and monomeric Fas to soluble Fasl ligand suppresses the cytotoxic activity of the latter.

Thus, cytotoxicity of FasΔTM comes from its oligomerization. The reasons for FasΔTM oligomerization are still unclear. It may be believed that spontaneous self-association of FasΔTM results from interactions between CRD1/PLAD regions of the extracellular portion of Fas and/or the "death domains" of the
Fas intracellular region at high concentrations of Fas/H9004TM (33–35).

Oligomeric Fas Antigen in the Sera of Patients—The fact of oligomerization of recombinant Fas/H9004TM suggested existence of natural oligomeric soluble Fas. Specifically, a high concentration of soluble Fas was reported for blood drawn from patients with autoimmune disease (36, 37). Because rheumatoid arthritis (RA) is known to be characterized by a higher concentration of soluble Fas in the blood of a patient, a question arises as to whether Fas is monomeric or oligomeric in sera from RA patients. For this purpose, the sera were separated by gel filtration, and the obtained fractions were assayed for the Fas presence using sandwich ELISA.

In RA sera with 3–5-fold Fas expression (up to 10 ng/ml) as compared with controls, the major part of Fas antigen was Fas intracellular region at high concentrations of FasΔTM (33–35).

Oligomeric Fas Antigen in the Sera of Patients—The fact of oligomerization of recombinant FasΔTM suggested existence of natural oligomeric soluble Fas. Specifically, a high concentration of soluble Fas was reported for blood drawn from patients with autoimmune disease (36, 37). Because rheumatoid arthritis (RA) is known to be characterized by a higher concentration of soluble Fas in the blood of a patient, a question arises as to whether Fas is monomeric or oligomeric in sera from RA patients. For this purpose, the sera were separated by gel filtration, and the obtained fractions were assayed for the Fas presence using sandwich ELISA.

In RA sera with 3–5-fold Fas expression (up to 10 ng/ml) as compared with controls, the major part of Fas antigen was

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found to belong to high molecular fractions with a molecular mass of 150–200 kDa (Fig. 6A). Confirmation of the presence of Fas antigen in these fractions came from their immunoprecipitation with anti-Fas antibodies (Fig. 6B). In healthy controls, no soluble Fas was detected in high molecular fractions, and no other Fas but monomeric was observed (data not shown). However, it cannot be ruled out that this was a result of a low concentration of the receptor.

Since oligomeric recombinant Fas exhibited the cytotoxic activity, we assayed some sera containing oligomeric soluble Fas at high concentrations (6–10 ng/ml) for such an activity. As was expected, addition of 20% healthy control to HeLa cell culture resulted in a higher proliferation of the cells. Note that non-cytotoxic FasL-FLAG capable of interacting with Fas produced no effect on the proliferation-stimulating activity of normal sera (Fig. 6C). Unlike cultivation with controls, that with RA sera containing soluble Fas at a high concentration resulted in a suppressed proliferation of HeLa cells, and the cytotoxicity was notably down-regulated by FasL-FLAG. Hence, cytotoxicity of RA sera is determined, at least partially, by oligomeric soluble Fas antigen. Besides, the suppression of proliferation of transformed cells by sera from autoimmune disease patients suggests that Fas is a key signaling molecule providing a link between cancero- and autoimmune genesis.

**DISCUSSION**

Here we report that (a) FasTM is capable of inducing death of transformed cells; (b) in vivo there exists oligomeric soluble Fas antigen, e.g. in RA sera; and (c) cytotoxicity of soluble Fas antigen results from its oligomerization.

We believe that FasTM cytotoxicity is indicative of a novel functional role of soluble Fas antigen. Taken together, the literature data and our results suggest that functionally, soluble Fas antigen not only inhibits Fas ligand cytotoxicity and hence, Fas-mediated apoptosis (28), but also triggers cell death through reverse signaling via transmembrane Fas ligand. This requires more attention to the cases of higher expression of soluble Fas with distinction as to its apoptosis-inhibiting and apoptosis-stimulating activities. Specifically, inhibition of apoptosis of peripheral blood lymphocytes by soluble FasL in hepatocellular carcinoma patients described by Nakamoto et al. (38) may be a result of neutralization of cytotoxic soluble Fas antigen whose high level of expression in hepatocellular carcinoma samples has been reliably documented (39, 40). Sup-
pressed cytotoxic soluble Fas may also underlie the therapeutic effect of antibodies to Fas antigen reported in a number of papers (41–43). Besides, the effects of metalloprotease (that cleaves membrane-bound FasL) and its inhibitors can also be considered from the viewpoint of reverse signaling as events inhibiting or promoting signal transduction via transmembrane FasL (44–46).

The cytotoxicity-oligomerization relationship of soluble Fas seems to offer novel interpretations of previous findings. First, the illusory contradiction concerning the FasFc effect upon lymphocytes reported by Suzuki and Fink (f) on the one hand and by Desbarats et al. (10) on the other could arise from the difference between FasFc samples used. Probably, the former research team used monomeric non-cytotoxic FasFc, while the latter dealt with dimeric FasFc capable of inducing death of T-lymphocytes (9, 10). Next, it can be believed that certain mutations of Fas antigen that are likely to underlie autoimmune diseases (47–49) affect not only cytotoxic signal transduction via membrane Fas but also oligomerization of soluble Fas and, hence, realization of reverse signaling as well. Biologically, Fas\textsuperscript{TM} oligomerization can shed light on the reason for

**Fig. 5.** Cross-linked FasFc, but not monomeric FasFc, induces cell death. A, HeLa cells were incubated with the indicated concentrations of FasFc in the absence (filled bars) or presence (hatched bars) of anti-Fc antibodies for 24 h. The cytotoxic activity of anti-Fc antibodies alone is shown as open bars. Cell death was determined by the neutral-red assay. The results are given as percentage of dead cells against the entire cell population. Error bars represent S.D. values of data from three independent experiments. B, monomeric and cross-linked FasFc suppress FasL-dependent cell death. HeLa cells were incubated with soluble FasL (1 μg/ml) alone (open bars), or with soluble FasL combined with 2.5 μg/ml monomeric or cross-linked FasFc (hatched bars), or with soluble FasL combined with 2.5 μg/ml monomeric or cross-linked FasFc (filled bars) for 24 h. Cell death was determined by the neutral-red assay. Error bars represent S.D. values of data from three independent experiments.

**Fig. 6.** Soluble Fas from RA patients is oligomeric and determines the anti-proliferation activity of host sera. A, samples (100 μl) of sera from RA patients 2-fold diluted with phosphate-buffered saline were applied to a Superose 12 gel filtration column. The absorption of the eluent was monitored at 280 nm (solid line). Fractions were collected, and the elution of sFas was determined by sandwich ELISA (dashed line). The inset shows the results of Western blotting with anti-Fas antibodies for fractions 15 and 30 yielded by RA serum separation. B, HeLa cells were incubated with 20% solutions of sera from RA patients (columns 1–3) and from healthy donors (columns 4–6) in the absence (open bars) or presence (filled bars) of non-cytotoxic FasL-FLAG for 24 h. Cell proliferation was determined by the neutral-red assay. Typical results of one of the four experiments are presented.
the existence of several forms of soluble Fas antigen (29, 30), which probably lies in synthesis of molecules that interact with Fas ligand but are unable to form homooligomers and, hence, to trigger the cytotoxic reverse signaling. Moreover, the ability of soluble Fas antigen to make high molecular associations presupposes rigid regulatory mechanisms of its oligomerization/depolymerization.

Finally, one can leave room for possible associations of soluble Fas with heterologous proteins that bear a CRD1-like motif and provide a signal exchange between the Fas-Fas ligand system and cytokines of other families.

Thus, the cytotoxic properties of oligomeric soluble Fas antigen reported here contribute to our knowledge of functioning of the Fas-Fas ligand system and open up new opportunities in the treatment of tumors and autoimmune diseases.

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Additions and Corrections

Vol. 278 (2003) 36236–36241

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Page 36240: The two parts of Fig. 5 are the same. The correct figure is shown below:

![Correct Figure](image)

**FIG. 5**

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Molecular modeling correctly predicts the functional importance of Phe^{594} in transmembrane helix 11 of the multidrug resistance protein, MRP1 (ABCC1).

Jeff D. Campbell, Koji Koike, Christophe Moreau, Mark S. P. Sansom, Roger G. Deeley, and Susan P. C. Cole

Dr. Sansom’s name was listed incorrectly. The correct listing is shown above.