Generation of a FasL-based Proapoptotic Fusion Protein Devoid of Systemic Toxicity due to Cell-surface Antigen-restricted Activation*

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We describe the construction of a FasL fusion protein devoid of systemic toxicity, inducing apoptosis only on cell-surface antigen-positive cells. The fusion protein consists carboxyl-terminally of the extracellular domain of FasL and amino-terminally of a fibroblast activation protein (FAP)-specific single chain antibody fragment (sc40-FasL). The latter allows immobilization-dependent conversion of the inactive soluble FasL fusion protein into an entity with membrane FasL-like activity. Thus, sc40-FasL efficiently induced apoptosis only in FAP-expressing cells. In accordance with a strict target-selective activity of sc40-FasL, the intravenous application of this reagent in mice revealed no signs of systemic toxicity and prevented growth of xenotransplanted FAP-expressing tumor cells. The principle described here for the first time, in which cell-surface antigen-mediated activation of Fas permits local activation of Fas in vivo, opens novel avenues for the use of Fas signaling in cancer therapy.

Fas is the prototype death receptor of the tumor necrosis factor (TNF) receptor superfamily, which, upon activation by its cognate ligand (FasL) or by agonistic antibodies, induces apoptosis in a wide variety of cell types via a direct (type 1) or a mitochondria-dependent (type 2) caspase cascade (for review see Ref. 1). As most other members of the TNF ligand family, Fas ligand is a trimeric type 2 membrane protein (2), from which a soluble molecule can be derived by proteolytic processing or in mice by alternative splicing (3, 4). Soluble homotrimmeric FasL (sFasL) is biologically inactive in vitro and in vivo and may even antagonize the function of membrane-bound FasL (5–7). However, complexes of secondarily aggregated sFasL trimers obtained experimentally by cross-linking antianalogs (or at least hexameric fusion proteins of FasL) are potent Fas activators (7, 8).

Ex vivo, isolated tumor cells and cell lines derived from many different tumors express Fas; in vitro, several of them are sensitive to treatment with agonistic antibodies or recombinant multimeric FasL constructs (for review see Ref. 9) whereby some cell types require pre- or cosensitization by various chemotherapeutics. Sensitization to Fas-induced apoptosis may be effective at distinct levels acting at a receptor-proximal, mitochondrial, and/or effector-caspase level(s) along the apoptotic signal pathway and typically involves down-regulation of the function or amount of intracellular anti-apoptotic proteins. Moreover, the anti-tumoral effects of chemotherapeutic drugs have been attributed partly to p53-mediated up-regulation of Fas and FasL (10–12). Thus, direct induction of Fas-mediated apoptosis in principle is a powerful strategy for anti-cancer therapy particularly in combination with chemotherapy. However, attempts to exploit Fas agonists for cancer therapy have revealed severe systemic toxicity of such reagents with liver failure being the major determinant for acute lethality (13, 14). Systemic toxicity was observed, although to various degrees, with all reagents capable of activating Fas, such as agonistic antibodies, multimeric recombinant FasL preparations, and cells expressing membrane FasL or membrane vesicles derived therefrom (9). Nevertheless, upon prevention of systemic action of FasL either by the use of mice lacking a functional Fas/FasL system (gld/ipl) or by the use of human Fas-specific antibodies and xenotransplants, the principle feasibility of Fas activation as an anti-tumoral strategy has been shown (15, 16). Thus, anti-cancer strategies based on Fas activation have to meet, first and foremost, the criterion of strictly localized action, i.e. at the tumor site.

We have shown previously that soluble fusion proteins comprised of the extracellular domain of TNF or TRAIL and comprised of a single chain antibody variable-region fragment exert superior functional capabilities compared with the corresponding soluble ligands (17, 18). From these studies, it is clear that signaling of the TNF and TRAIL single chain fusion proteins via their cognate type 2 (but not type 1) receptors was dependent on specific binding of the single chain moeity to a cell membrane-expressed target antigen (17, 18). However, the mechanisms by which these fusion proteins exert their superior signal capacity remain to be elucidated. Thus, it seems that artificial cell-surface immobilization of soluble TNF ligands is sufficient to convert these molecules into fully active ligands. Here we have exploited this principle to construct FasL chimeric molecules that reconstitute bioactivity only upon binding to defined cell-surface molecules, thereby creating reagents with high local apoptotic potential that could be safely applied in vivo without systemic action.
EXPERIMENTAL PROCEDURES

Animals, Cell Lines, Plasmids, and Reagents—Female C57Bl/6 x CBA/Ca, CD1, and NMRI nu/nu mice were purchased from Elevage Jacques Le Genest St, Isle, France. The human HeLa and T24 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The HT1080 and the fibroblast activation protein (FAP)-expressing clones derived therefrom as well as the anti-FAP mAb F19 were a kind gift from W. Rettig (Boehringer Ingelheim Pharma, Vienna, Austria). HeLa, HEK293, and HT1080 cells, as well as stable transfectants thereof, were maintained in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 5% fetal calf serum. The medium of stable transfectants was occasionally (4–7 days per month) supplemented with 200 μg/ml G418. Fas-Comp, Fas-Fc, TRAIL-R2-Fc, FLAG-tagged FasL, and the pP5089 vector were generously supplied by P. Schneider and J. Tschopp (University of Lausanne, Switzerland). The caspase-8-specific mAb was a kind gift of Klaus Schulze-Osthoff (University of Düsseldorf, Germany). Anti-FLAG mAb M2, FLAG peptide, and secondary antibodies were from Sigma.

To generate the sc40-FasL expression plasmid, the human FAP-specific single chain antibody fragment “40” including the leader sequence (19) was amplified by proofreading PCR with a primer pair containing a KpnI (forward primer) and a NotI site (reverse primer) in their 5’-overhang to allow insertion in the corresponding sites of pcDNA3.1 (Invitrogen). The reverse primer, in addition, contained a FLAG-tag encoding sequence upstream of the NotI overhang. Subsequently, an ampiclon corresponding to amino acids 139–281 of human FasL was generated with a primer pair containing a NotI (forward primer) and a NheI site (reverse primer) in their 5’-overhang and inserted into the NotI/NheI-compatible site of the pcDNA3.1(+) vector containing sc40 to deliver the sc40-FasL construct. Note that there is a FLAG epitope between the amino-terminal sc40 and the carboxy-terminal FasL part.

Production of sc40-FasL—The expression plasmid encoding sc40-FasL was stably introduced into HEK293 cells. After G418 selection, clones (~250) were pooled and expanded. Finally, a clone (293-40-FasLA) producing 5–15 μg/ml sc40-FasL was obtained by limiting dilution. For large scale production of sc40-FasL, clone 293-40-FaslA was expanded in RPMI 1640 medium and 0.5% fetal calf serum, and supernatants of confluent-grown cells were collected. sc40-FasL-containing supernatants (or control supernatants) were either concentrated or purified sc40-FasL (50 μg/ml) was obtained after 2 weeks of selection with G418 (500 μg/ml) were electroporated (4-mm cuvette; 250 V, 1800 microfarads, maximal resistance) with appropriate expression constructs encoding FAP and the neomycin resistance gene in medium with 5% fetal calf serum. Stable clones (>40) obtained after 2 weeks of selection with G418 (500 μg/ml) were pooled, expanded, and enriched for expression of FAP by three cycles of cell sorting (see above).

Determination of Alamine Transmembrane and Caspase-3 Activities—As an indicator of liver damage, the plasma activity of alamine aminotransferase was assessed by measuring enzyme activities according to Bergmeyer (20) using an automated procedure. Plasma samples were prepared as follows: Mice were killed by inhalation of Etherane, and blood (500–800 μl) was taken by heart puncture with syringes containing 80 μl of heparin. After centrifugation (5 min, 13,000 rpm, 4°C), plasma supernatants were taken and stored at −80°C until analysis. To determine the activation of caspase-3 in the liver tissue of mice, homogenates (50% w/v) were prepared in lysis buffer containing 10 mM HEPES, pH 7.4, 1 mM CHAPS, and 1 mM diethiothreitol and analyzed using the colorimetric caspase-3 assay kit (Sigma) according to the manufacturer’s instructions.

Animal Studies—Animal care and all experiments performed were in accordance with federal guidelines and have been approved by university and state authorities. Subcutaneous tumor growth of parental HT1080 and FAP transfectants was assessed by subcutaneous injection of 1.5 × 106 cells suspended in 100 μl of 0.9% NaCl solution into the back of narcotized (with Etherane) nu/nu mice. Treatment of groups of 2–9 animals with the reagents and doses as indicated in the legend of Fig. 3 started the following day with four daily subcutaneous injections of concentrated or purified sc40-FasL (50 μg) into the area of tumor cell medium in Groups 1 and 2. sc40-FasL-treated mice were also treated with 20 μg of cycloheximide. Tumor growth was monitored as described (21) and documented photographically. Tumor-bearing animals were sacrificed after 4 weeks or when the tumor size exceeded 250 mm2. To investigate systemic toxicity of sc40-FasL and secondarily cross-linked sc40-FasL, groups of 2–6 animals were treated with the indicated doses of reagents (Table I and Fig. 3) by two subsequent intravenous injections within 20 min. Survival and phenomenological signs of sickness were monitored for 48 h. For additional biochemical and histological analyses, groups of five animals were sacrificed 45 min and 6 h, respectively, after injection of reagents. Blood was collected for determination of alamine aminotransferase activity. Macroscopic liver damage was documented photographically, and liver biopsies were taken for determination of caspase-3 activity.

RESULTS AND DISCUSSION

A FAP-specific Single Chain Antibody (sc40)-FasL Fusion Protein Selectively Induces Apoptosis in FAP-expressing Cells—The observation that scFvFasL trimers possess virtually no apoptosis-inducing capacity, whereas secondarily aggregated sFasL trimers are potent apoptosis inducers (7, 8), suggests (i) that interaction between Fas and scFasL is too weak to allow the formation of signaling competent receptor complexes and (ii) that multivalent binding of scFasL is sufficient to fully activate Fas. In this context, binding of one scFasL trimer to three Fas receptor molecules is considered a monovalent interaction. We therefore reasoned that recruitment of an a priori inactive scFasL fusion protein to a cell surface by a Fas/FasL-independent protein–protein interaction domain could induce multivalent binding and/or aggregation of the FasL fusion protein subsequently mimicking a fully signaling competent membrane FasL acting on the same or adjacent cells. Of course, if this hypothesis is correct, the appropriate choice of the protein–protein interaction domain should allow the generation of sFasL fusion proteins that selectively act on cells expressing cell-surface structures recognized by the chosen protein–protein interaction domain or on Fas-sensitive cells adjacent to the target cell.

We generated a fusion protein (sc40-FasL) consisting carboxyl-terminally of the extracellular domain of FasL and amino-terminally of a single chain antibody recognizing FAP (Fig. 1A), a membrane-expressed tumor stroma marker (22). The sc40-
FasL fusion protein was expressed in HEK293 cells and purified by affinity chromatography using a FLAG tag placed between the FasL and the sc40 domain. Gel-filtration analyses indicated a homotrimeric organization of sc40-FasL (data not shown), which was expected from the trimeric structure of FasL. Bioactivity of sc40-FasL was analyzed on HT1080 and HeLa cells (which express no FAP) and on transfectants of both cell lines stably expressing FAP (Fig. 1B). In both cases, the parental cell lines (and the FAP transfectants derived therefrom) displayed identical sensitivity to Fas-mediated apoptosis as revealed from dose-response plots of anti-FLAG mAb cross-linked with FLAG-specific mAb M2 (C) and by sc40-FasL (D), respectively. HT1080 (left panel) and HeLa cells (right panel) (open symbols) and the corresponding FAP-expressing transfectants (closed symbols) were challenged with the indicated concentrations of FasL-M2 complex or sc40-FasL overnight in the presence of 1 μg/ml cycloheximide. Finally, cell viability was determined by staining adherent cells with crystal violet. E, Western blot analyses of procaspase-8 processing in untreated (−) or sc40-FasL-stimulated (+) HT1080, HeLa, and the corresponding FAP transfectants.

Fig. 1.

sc40-FasL induces cell death only in FAP-expressing cells. A, scheme of sc40-FasL. B, FACS analysis of FAP expression on parental HT1080 and HeLa cells and FAP-expressing transfectants derived therefrom. C and D, induction of apoptosis by sFasL cross-linked with FLAG-specific mAb M2 (C) and by sc40-FasL (D), respectively. HT1080 (left panel) and HeLa cells (right panel) (open symbols) and the corresponding FAP-expressing transfectants (closed symbols) were challenged with the indicated concentrations of FasL-M2 complex or sc40-FasL overnight in the presence of 1 μg/ml cycloheximide. Finally, cell viability was determined by staining adherent cells with crystal violet. E, Western blot analyses of procaspase-8 processing in untreated (−) or sc40-FasL-stimulated (+) HT1080, HeLa, and the corresponding FAP transfectants.
A

![Diagram of cell-surface antigen-restricted activation of Fas](image)

**Fig. 2.** sc40-FasL-induced apoptosis is dependent upon binding to FAP and is mediated by Fas. A, FAP-expressing HT1080 cells were left untreated, or apoptosis was induced by treatment with sc40-FasL in the absence or presence of Z-VD-fmk (20 μM), Fas-Comp (2 μg/ml), Fas-Fc (10 μg/ml), TRAIL-R2-Fc (10 μg/ml), or anti-FAP (5 μg/ml). Cells (Z-VD-fmk, anti-FAP) or sc40-FasL (Fas-Comp, Fas-Fc, TRAIL-R2-Fc) were preincubated for 1 h before starting the experiment. B, scheme of sc40-FasL action.

The complete lack of in vivo toxicity observed with sc40-FasL prompted pilot experiments assessing a potential antitumoral activity of sc40-FasL. Nu/nu mice were injected subcutaneously with FAP-expressing HT1080 cells, and the development of local fibrosarcomas was monitored for several weeks (Fig. 3, A–C). These data clearly show that sc40-FasL is per se inactive and is well tolerated by the organism but, nevertheless, has latentently retained the pronounced proapoptotic features of conventional Fas agonists.

TABLE I

| Treatment (intravenous injection)** | Mouse strain | Surviving animals | Animals dead within 6 h |
|-----------------------------------|--------------|-------------------|------------------------|
| 90 μg sc40-FasL                   | CD1          | 6                 | 0                      |
| 2 μg sc40-FasL                    | M2           | 6                 | 0                      |
| 4 μg sc40-FasL                    | CD1          | 5                 | 1                      |
| 8 μg sc40-FasL                    | CD1          | 6                 | 0                      |
| 50 μg sc40-FasL                   | M2           | nu/nu             | 0                      |
| 5 μg sc40-FasL                    | M2           | 2                 | 0                      |
| 50 μg sc40-FasL                   | Saline       | nu/nu             | 2                      |
| 5 μg sc40-FasL                    | Saline       | nu/nu             | 0                      |
| 50 μg sc40-FasL                   | C57BL/6xB6/ABJ | 0               | 2                      |
| 50 μg sc40-FasL                   | C57BL/6xB6/ABJ | 2           | 0                      |

*The second intravenous injection was given within 30 min after the first. Note that sc40-FasL contains a FLAG epitope allowing secondary cross-linking with the FLAG-specific mAb M2.

**The mice strains used differ in average body weight (CD1, 30–40 g; nu/nu and C57BL/6xB6/ABJ, 20–30 g).

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a J. Park and P. Garin-Chesa, personal communication.

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addional analyses of various other anti-FAP single chain FasL fusion proteins differing in the FAP-specific single chain domain. Some of these constructs displayed a strong FAP-independent apoptotic capacity, which in gel-filtration analyses eluted in fractions corresponding to high molecular weight aggregates (data not shown).

sc40-FasL Lacks Systemic Toxicity and Efficiently Inhibits Growth of Xenotransplanted Tumors—The conversion of trimeric sFasL fusion proteins to membrane FasL-like entities by cell-surface immobilization, exemplified above with sc40-FasL, has the apparent potential to direct the devastating systemic actions of FasL in vivo to wanted sites of action, i.e. the tumor cell itself or tumor-associated structures and thus would enable a safe clinical application. To prove the feasibility of such FasL fusion proteins for potential clinical use, we first tested the in vivo toxicity of sc40-FasL. Mice were injected intravenously with a single dose of up to 90 μg of sc40-FasL/animal, and overall performance and survival were monitored for several days. All animals survived and showed no apparent signs of sickness (Table I). In contrast, upon cross-linking of sc40-FasL via its internal FLAG-tag with the FLAG-specific mAb M2, dose-dependent toxicity was noted, with all animals dying from a single injection of 5–8 μg of sc40-FasL within 45–90 min (Table I). Similar to other Fas agonists (9), the M2-cross-linked sc40-FasL complex induced liver cell apoptosis and acute liver failure leading to death, which was preceded by a sharp rise in biochemical hallmarks of liver damage. Thus, serum levels of alanine aminotransferase (Fig. 3A) and intrahepatic caspase-3 activity (Fig. 3B) were strongly increased and the livers of mice challenged this way were highly hemorrhagic (Fig. 3C). All of these parameters remained normal upon sc40-FasL application (Fig. 3, A–C). These data clearly show that sc40-FasL is per se inactive and is well tolerated by the organism but, nevertheless, has latentently retained the pronounced proapoptotic features of conventional Fas agonists.

The second intravenous injection was given within 30 min after the first. Note that sc40-FasL contains a FLAG epitope allowing secondary cross-linking with the FLAG-specific mAb M2.

**The mice strains used differ in average body weight (CD1, 30–40 g; nu/nu and C57BL/6xB6/ABJ, 20–30 g).**
detectable after a further 3 weeks, whereas all other animals remained tumor-free during an observation period of at least 9 weeks. Because of the instability of FAP antigen in vivo, the few animals developing tumors under sc40-FasL treatment might have lost the target required for immobilization-dependent activation of Fas, thus becoming resistant to the action of sc40-FasL. However, alternative explanations for treatment failure (e.g. insufficient dosing) cannot be ruled out. In support of this reasoning, we have preliminary evidence that reduction in the number of sc40-FasL applications was associated with an increased number of tumor-bearing animals (data not shown).

Concerning the in vivo function of the FasL-fusion protein, several mechanisms have to be considered. First, our in vitro data imply the possibility of a direct apoptosis-inducing action of sc40-FasL on the tumor cells themselves. Second, and not excluding a direct action on the tumor cells, FasL-mediated tumor rejection could be caused by recruitment of cellular effector functions of the innate immune system reported to prevent growth of FasL-positive tumors in both wild-type and T-cell-deficient mice (23–26). In fact, in the latter case, the application of an sc40-FasL-like fusion protein would also be an amenable treatment strategy for tumors a priori resistant to Fas-mediated apoptosis.

Future studies will be required to reveal to which extent direct Fas-mediated killing of tumor cells and immune cell-mediated effector mechanisms contribute to the selective antitumoral action of sc40-FasL. Nevertheless, the pilot animal studies presented here already point out a feature of immense importance to this novel anti-tumoral FasL reagent, namely the complete lack of systemic toxicity even under repeated high dose application, yet effective action at the targeted tissue, the tumor cells. This feature discriminates sc40-FasL from all other presently available Fas-specific reagents. These include novel Fas-specific antibodies with apparently reduced hepatotoxic activity (for a review see Ref. 27) and other cytokines with potential anti-tumoral activities, all of which typically possess unwanted treatment limiting side effects upon systemic administration.

In conclusion, our data show that homotrimeric FasL-based
fusion proteins can be generated that are devoid of bioactivity but upon cell-surface binding, via their antibody module, regain full activity. Such fusion proteins thus fulfill essential prerequisites for a safely applicable anti-cancer reagent, namely target-restricted highly specific activity (e.g. tumor cell apoptosis induction) and lack of systemic toxicity. Of note, cell-surface immobilization-dependent activation of Fas by trimeric FasL-fusion proteins is not restricted to antibody derivatives but can also be achieved with other selective protein-protein interactions, for example, with a fusion protein of the extracellular domain of CD40 and the extracellular domain of FasL, which selectively binds to and acts on CD40L-expressing cells (data not shown). The promising results presented here warrant further studies in animal tumor models to elucidate both the in vivo mechanisms involved in this process and the full potential of this new class of anti-cancer protein therapeutics.

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