[Frontiers in Bioscience 1, d12-18 January 1, 1996]

SOLUBLE FAS/APO-1 SPLICING VARIANTS AND APOPTOSIS

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1. ABSTRACT

In addition to the full length mRNA, activated human peripheral blood mononuclear cells (PBMC) and T cell tumor lines express several alternatively spliced Fas variants. At least five of these code for soluble Fas (CD95) molecules. *In vitro* studies suggest that these soluble Fas isoforms inhibit apoptosis induced by agonistic antibodies and, more importantly, by the natural Fas ligand in Fas-bearing sensitive cells. Interestingly, this functional property can be assigned to the first 49 amino acids of the mature protein, the only region shared by the soluble Fas molecules.

2. INTRODUCTION

Development and homeostasis of multicellular organisms are controlled not only by cell proliferation and differentiation but also by the elimination of cells that are unnecessary or deleterious. This is achieved by a process referred to as programmed cell death or apoptosis (Reviewed in Ref. 1). Apoptosis leads to chromatin condensation and margination, nuclear fragmentation, cell shrinkage, membrane blebbing and in the majority, but not in all cells, enzymatic internucleosomal fragmentation of nuclear DNA (2,3).

Apoptosis is the result of an active cellular response that can be elicited by a variety of stimuli such as growth factor deprivation, a molecular damage that does not cause severe loss of integrity, or by triggering of specific cellular receptors such as the tumor necrosis factor receptor type 1 (TNFR1) or Fas/Apo-1. The Fas/Apo-1 molecule, also designated as CD95, (4,5) belongs to the TNFR family (6). Fas positive cells treated either with specific agonistic antibodies (7,8) or following interaction with the natural Fas ligand (FasL) (9-13) show the characteristic morphologic features of cells undergoing apoptosis (2,3).

Recent evidence suggests that dysregulation of apoptosis contributes to the pathogenesis of several human diseases including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and AIDS (Reviewed in Ref. 14).

In the immune system, Fas and FasL are involved in the down-regulation of immune reactions as well as in T cell mediated cytotoxicity. (Reviewed in Ref. 15). Spontaneous loss-of-function mutations of Fas and FasL have been identified respectively in *lpr* (16-20) and *gld* (12,13) mice. These mutations cause the accumulation of activated lymphocytes in tissues and accelerate the autoimmune disease processes. Fas gene mutations associated with T cell apoptosis defects have also been reported in children with a rare autoimmune lymphoproliferative syndrome (ALPS) (21,22).

There is growing evidence that not all Fas positive cells are susceptible to apoptosis induction. Several mechanisms of Fas-mediated apoptosis resistance have been postulated. These include a defective expression of hematopoietic cell protein tyrosine phosphatase (HCP) in lymphoid cells (23); high expression of FAP-1, a protein tyrosine phosphatase that associates with Fas (24); low expression of bax-α, a bcl-2 family member (25); mutations of the Fas gene in ALPS lymphocytes (21,22) and expression of a truncated Fas receptor

Received 12/01/95; Accepted 01/11/96.

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lacking the intracellular death-signaling domain (FasExo8Del) in tumor resistant clones (26, 27).

In addition to the above mechanisms, it must be considered that Fas-mediated apoptosis undoubtedly involves a delicate balance of receptor/ligand interactions and that these may be modulated by soluble proteins. In fact there is accumulating evidence for the natural occurrence of soluble forms of cell surface receptors produced either by proteolytic cleavage of membrane-bound receptors or by alternative splicing. In this article, we summarize current knowledge on the human Fas splicing variants that have so far been reported (28-31). Next, we discuss the possible role of Fas soluble isoforms in the physiological and pathological fine tuning of apoptosis and consequently in the regulation of the immune responses.

3. HUMAN FAS SPLICING VARIANT MRNAS IN ACTIVATED PBMC AND IN CELL LINES

Our group and others have reported that normal human PHA-activated lymphocytes express, in addition to the full length mRNA, less abundant, shorter Fas mRNA species (28-31). The genomic intron/exon organization of the regions surrounding the deleted sequences demonstrated that the transcripts derive by alternative splicing of the Fas gene. A more descriptive nomenclature of the Fas variants has been proposed based on the Fas/Apo-1 gene structure (32, 33): FasExo6Del (previously called FasTMDel, ref. 28,29); FasExo3,4Del (previously called FasDel2, ref. 29), FasExo3,4,6Del (previously called FasDel3, ref. 29); FasExo4Del and FasExo4,6Del (30) and FasExo4,7Del (31). With the exception of FasExo6Del, which is characterized by an in frame deletion of exon 6, in all of these variants, the deletions result in a different reading frame with premature termination codons. Thus, the variants should code for smaller mature Fas proteins with a C-terminal end of 21 or 38 amino acids that differ from those of the membrane-bound form of Fas (29). Fas (CD95) is widely expressed on both hematopoietic and non hematopoietic tumor cells (34-37). Information on the expression of these splicing variants is far from complete. Besides in PBMC, several variants are known to be expressed in tumor cells also. For example, FasExo6Del expression has been reported in human hepatoma (38) and osteosarcoma cells (39).

Two variants have been detected only in pathological conditions and correspond to mutations in splicing recognition sites. However, it must be pointed out that there is no definitive proof that the expression of the last two variants is strictly confined to pathological situations.

FasExo8Del has been reported in apoptosis-resistant clones derived from a human lymphoma cell line. This variant codes for a truncated Fas molecule that lacks the intracellular death-signalling domain. The involved mutation was identified as a deletion-insertion in the intron 7/Exon 8 region of the Fas gene. Notably, this mutation affects the phenotype in a dominant negative fashion, i.e. in the presence of the normal receptor (26, 27).

FasExo3Del has been reported in a patient with ALPS, in addition to normal-sized Fas mRNA and to the previously described FasExo3,4Del. This patient showed a mutation in the 5' splice site of intron 3 (22).

The human Fas isoforms so far described in physiological or pathological conditions are schematically represented in Fig. 1.

4. SEVERAL FAS SPLICING VARIANTS CODE FOR SOLUBLE FAS PROTEINS

Several of the variants depicted in Fig. 1 retain the hydrophobic leader peptide but lack the hydrophobic transmembrane domain. This suggests that they might be expressed as soluble forms and secreted. Moreover they share the 5' portion (exons 1 and 2) corresponding to the N-terminal 49 amino acids of the mature protein. This may indicate that this is a physiologically important domain and that, in turn, these splicing variants must have some defined functional activity. However, before considering this possibility, it was important to demonstrate that these mRNA variants could be translated as proteins and, more importantly, that they could be secreted in the extra-cellular fluid. This initially presented some problems because several Fas monoclonal antibodies i.e. CH-11 (Upstate Biotechnology Inc., Lake Placid, NY) and DX2 (42) reacted with Fas and FasExo6Del but did not react with the other variants (29). The most likely explanation is that these antibodies must recognise a sequence contained in exons 4 and/or 5, since these are the only two extracytoplasmic regions that are missing in all the Ab-negative variants. After a rather extensive search, three antibodies, M24, M1 (43) and FasN18 (Santa Cruz, Biotechnology,CA) which are capable of recognizing the 49 amino acids at the N-terminal region were identified (30). A sandwich ELISA using M24 and FasN18 antibodies was used to detect the Fas variants in both cell lysates and supernatants of transfected cells.

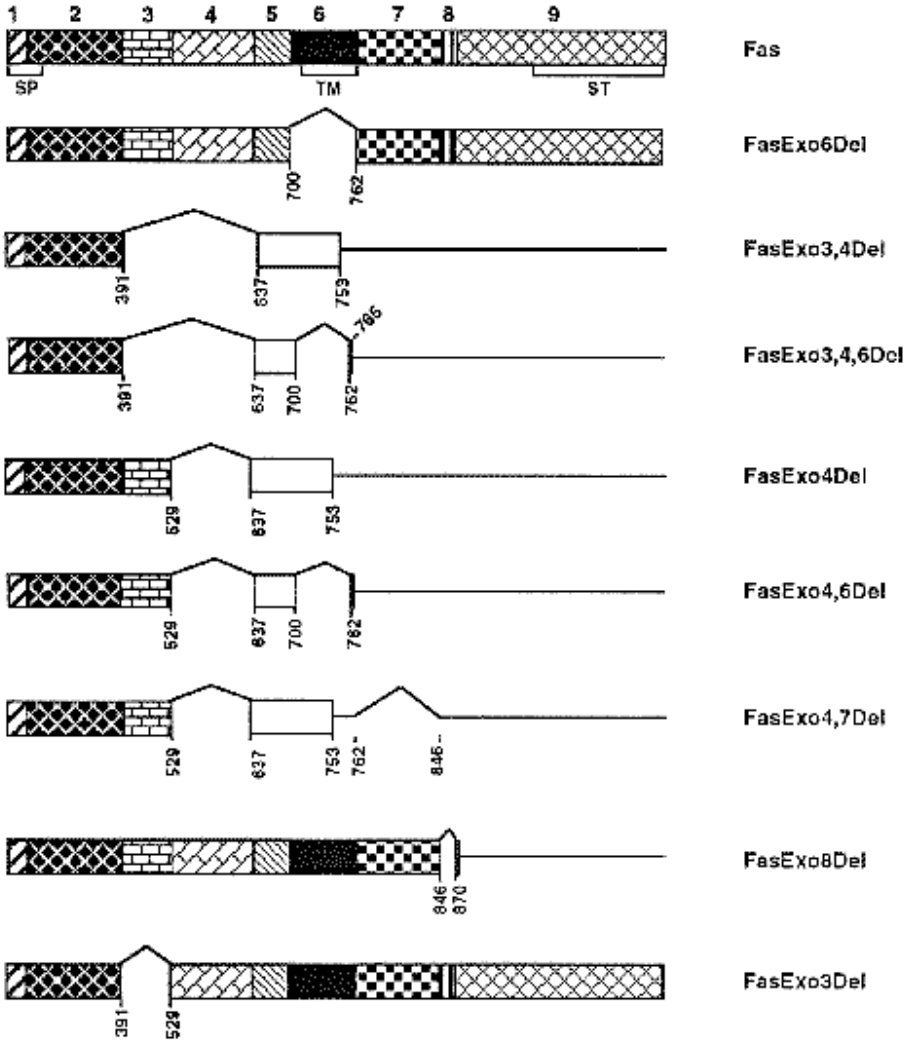

Fig. 1 Schematic representation of human Fas cDNAs. A. The coding regions are represented as boxes. Sequences generated by a different reading frame are indicated as open boxes (29). SP indicates signal peptide; TM indicates the transmembrane region, and ST represents the signal transducing domain (40,41). Lines indicate untranslated regions. Numbering is according to Itoh et al. (4). The coding regions corresponding to exons 1 to 9 are indicated.

Fas proteins were found to be present in the supernatants at a concentration two orders of magnitude higher than that found intracellularly, suggesting that the Fas isoforms code for Fas soluble proteins and that these are secreted by the cells and accumulate in the medium (30).

5. DO SOLUBLE FAS (SFAS) MOLECULES HAVE A BIOLOGICAL FUNCTION?

As yet, there is no a definitive answer to this question, but a number of considerations suggest that this is at least a likely possibility. Evidence for a biological effect of sFas derives from two sets of data. A) *In vivo* observations of quantitative variations of sFas under physiological and pathological conditions.

1. Intrahepatic T lymphocytes in the mouse have been shown to possess different sensitivity to apoptosis according to the mRNA expression of a soluble Fas isoform (Fas β) (44).

2. Sera of patients with systemic lupus erithematosus (28) and sera of patients with different high- and low-grade malignant B- and T-cell leukemias and lymphomas (45) have been reported to have an increased level of FasTMDel (FasExo6Del).

The results in the mouse system appear clear-cut, except for the fact that they are limited to RNA expression and lack data on Fas proteins. The data concerning sera of autoimmune patients are more open to criticism because the cells producing sFas

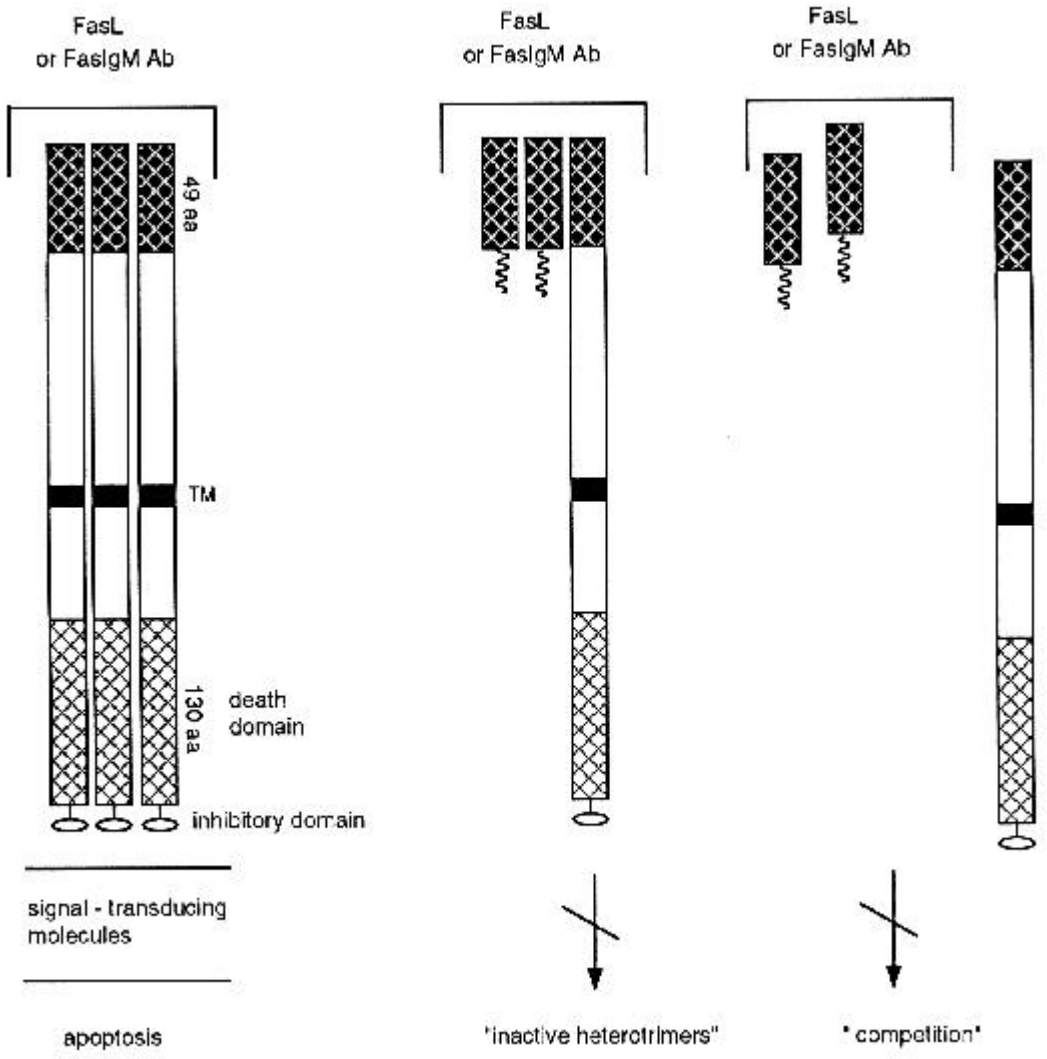

Fig 2. Hypothetical models of apoptosis inhibition.

have not been characterized. Direct proof of a role of sFas in those diseases remains to be established. Moreover, the ELISA used in the above studies detected only FasTMDel (FasExo6Del) but not the other variants with deletions in the extracytoplasmic regions. This problem can now be addressed by an ELISA allowing the detection all Fas isoforms so far identified (30). A correlation between the expression pattern of the different sFas in human T cells and sensitivity to apoptosis remains to be determined. Preliminary data from our group show marked qualitative and quantitative variations in the contribution of each variant to the "soluble Fas-pool" in different cell lines and in PBMC from different individuals.

It will be interesting to test whether these variations are associated with a differential regulation of apoptosis.

B) *In vitro* apoptosis inhibition studies.

Antibody to Fas and recombinant soluble FasL (rFasL) induce apoptosis in some tumor cell lines (Reviewed in Ref. 15). Early observations of an

apoptosis-inhibition function of sFas molecules were based on Fas antibody- induced apoptosis that is far from being a physiological mechanism (28, 29). However several isoforms also block Fas activation due to interaction with its natural ligand suggesting a more likely potential regulatory function (30).

The mechanism of the inhibitory effect is still unknown. Following Fas/FasL interaction, the signal is transmitted to the death domain and from this to the apoptosis machinery. A necessary requirement for this process is supposed to be the trimerization of the Fas molecules, as suggested by the homology to TNFα and TNFβ (46-50), the fact that IgM anti-Fas antibodies have agonistic activities (8) and the report that FasL forms homotrimers (51). Based on these data, two models can be postulated, called the "competition" model and the "inactive heterotrimers" model. These are shown schematically in Fig. 2.

The "competition" model assumes that sFas molecules compete with Fas for binding. This possibility is unlikely for Ab-induced apoptosis since sFas isoforms, with the exception of FasExo6Del, are not recognized by the CH-11 agonistic IgM Ab. However, this possibility may be valid for FasL-induced apoptosis. In this case, this would imply different mechanisms for apoptosis inhibition in the two *in vitro* systems. In the "inactive heterotrimer" model, we propose that the sFas forms are still able to trimerize with Fas but that they are not able to form active trimers because of the lack of other domains including the death domain. As a consequence, the signal provided by FasL or by Fas antibody is prevented from reaching the inside of the cell and results in inhibition of apoptosis. The fact that all variants exhibit a marked inhibition points toward the N-terminal domain as being responsible for this effect. The "inactive heterotrimer" model, even though more appealing is, at the present, only a working hypothesis that may help to organize further experimentation. A possible analogy with sFas apoptosis inhibition can be found in the inhibition of tyrosine kinase activity of the epidermal growth factor receptor (EGFR). This growth factor receptor was found to be regulated by a truncated receptor not by simple competition for available EGF but by specific association with the EGFR (52).

In conclusion, alternative splicing may be an important event in the regulation of Fas/FasL interaction and thus in the regulation of immune responses by these receptor-ligand pairs. However many important questions remain to be answered. Interestingly, other apoptosis genes like Ich-1, an Ice/ced3 related gene and bclx, a bcl2 related gene, can be expressed as splicing variants that either prevent or cause cell death depending on how the mRNA is processed (53, 54).

6. ACKNOWLEDGMENTS

We are very grateful to R. Tosi and R. Butler for the stimulating discussion and critical reading of the manuscript. This work was partially supported by grant n. 8206-12 AIDS, from National Institute of Health, by a grant from Associazione Italiana per la Ricerca sul Cancro and by the EEC Human Capital and Mobility Program grant CHRX-CT94-0537.

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