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Award Number: DAMD17-01-1-0482

TITLE: Function of FasL Reverse Signaling in Survival and Growth of Breast Cancer Cells

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REPORT DATE: September 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Final (15 Aug 01 -14 Aug 02)

4. TITLE AND SUBTITLE
Function of FasL Reverse Signaling in Survival and Growth of Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-01-1-0482

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

20030226 094

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
We have investigated a possible role of Fas ligand (FasL) in reverse signaling to mediate cell survival and growth signals through its intracellular proline-rich domain to protect FasL-expressing breast cancer cells from cell death. We have generated expressing constructs containing either a wild-type or the cytoplasmic proline-rich domain-deletion mutant cDNAs of FasL and transfected them into NIH 3T3 cells. We found that the cells expressing the wild-type FasL are more resistant to Fas-induced cell death than the mutant, suggesting that the cytoplasmic proline-rich domain of FasL indeed promote cell survival. We also generated bacteria-expressed GST-FasL fusion proteins and used these proteins to identify proteins that physically interact with the cytoplasmic domain of FasL. Two protein bands from 35S-Methioline-labled 3T3 cell lysates were found to specifically bind to FasL. We are currently characterizing these proteins. These results suggest a novel function of FasL in promoting survival and growth of breast cancer cells. The complete understanding of function and mechanism of FasL in tumor cells is necessary for developing effective diagnostic and therapeutic reagents to treat breast cancer patients.

14. SUBJECT TERMS:	15. NUMBER OF PAGES
cancer, FasL, apoptosis, reverse signaling	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Function of FasL Reverse Signaling in Survival and Growth of Breast Cancer Cells

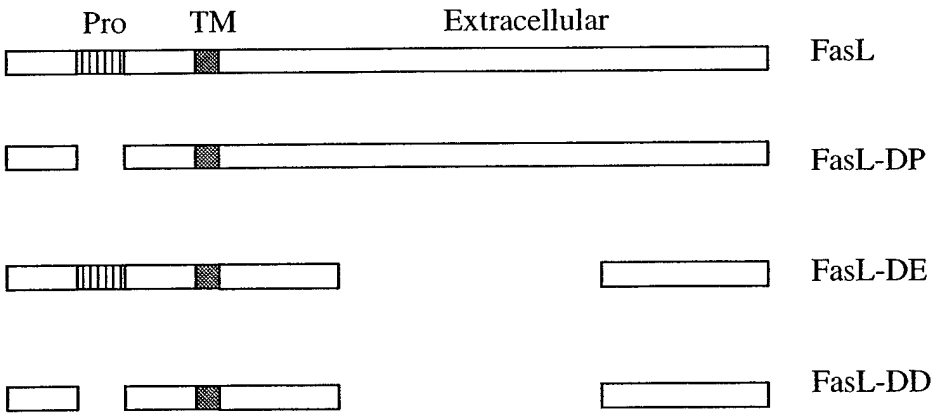
INTRODUCTION:

Fas ligand (FasL) is a type II transmembrane protein. It is a key molecule in regulating cell death and is detected in majority of breast carcinomas. Current studies on FasL focus on its role as a signaling ligand in inducing cell death through its receptor, Fas. FasL also contains an intracellular domain, whose function has not been understood. The cytoplasmic domain of FasL is rich in proline residues (40% over 80 amino acids), which are potential sites to interact with SH3 domains of cell survival and growth signaling molecules. FasL expressions are detected in more than 85% of breast carcinomas, majority of which overexpress FasL (1-3). Furthermore, the level of FasL expression correlates with decreased patient's disease-free survival and increased mortality (1). Therefore, FasL may play an important role in selection of highly aggressive breast tumor variants during tumor development. The precise function and mechanism of FasL in tumor cell growth has not been clearly understood. It has been suggested that cancer cells that express high levels of FasL may have the privilege to escape immune surveillance by inducing apoptosis of infiltrating immune cells (4). This hypothesis however does not explain why the cancer cells that express FasL as well as its receptor Fas do not die by suicide (3). FasL may have additional function in promoting tumor cell survival and growth through reverse signaling. We hypothesize that FasL may function as a signaling receptor to mediate cell survival and growth signals in breast cancer cells through reverse signaling. This reverse signaling, mediated by interactions between the cytoplasmic domain of FasL and SH3 domains of certain signaling molecules, may suppress apoptosis and promote cell growth. This hypothesis is supported by the observation that maximal proliferation of $CD8^+$ T cells requires reverse signaling through FasL (4). The proposed research is to reveal the function and mechanism of FasL-mediated reverse signaling in survival and growth of breast cancer cells, to define the role of the cytoplasmic domain of FasL in Fas-mediated apoptosis and in regulation of cell survival and growth signal transductions, and to identify proteins that physically interact with the cytoplasmic domain of FasL and to understand the function of these interactions.

BODY:

Cytoplasmic proline-rich domain of FasL protects cells from Fas-mediated cell death.

Function of cytoplasmic domain of FasL in Fas-mediated cell death were investigated by introducing into FasL sensitive NIH3T3 cells with cDNA expression constructs containing either a wild-type full-length, or deletion mutant cDNAs of FasL in transient transfection assays. Three mutant cDNAs were generated. One has the cytoplasmic praline-rich domain (Pro) deleted (FasL-DP); one has part of the extracellular domain deleted (FasL-DE); and one has both domains deleted (FasL-DD):



Cell death caused by expression of the four cDNAs were examined and compared. Transient transfection of the wild-type FasL (FasL) caused about 60% of cell death, whereas transfection of the proline domain-deletion mutant (FasL-DP) caused more than 90% of cell death. Transfection of the two extracellular domain-deletion mutants did not induce cell death. When exogenous FasL were added into the two extracellular domain-deletion mutant transfected cell cultures, cell death was about 60% in the FasL-DE-transfected cell culture, but was more than 90% in the FasL-DD cell culture. These results strongly suggest that the cytoplasmic proline-rich domain of FasL has a function to protect cells from FasL-induced cell death.

Effect of overexpression of the proline-rich domain of FasL on cell survival and growth signal transduction.

Function of the FasL cytoplasmic domain in regulation of known cell survival signaling pathways was investigated by examining the activation/phosphorylation of key cell survival signaling molecules, Akt, Erk, JNK, p38MAPK, and Bad, in NIH3T3 cells transfected with the above mentioned FasL cDNAs. No conclusive data has been obtained yet.

Identification of FasL cytoplasmic domain-interacting proteins.

The interaction of the proline-rich sequence of FasL with SH3-containing signaling molecules was investigated by GST fusion protein pull-down assays. A GST-FasL cytoplasmic domain fusion protein was generated and used to affinity purify proteins from NIH3T3 and breast carcinoma cells. Cells were metabolically labeled with 35S-Methioline. Total cell lysates were incubated with the GST-FasL Proline domain fusion protein or GST alone. The GST-FasL fusion protein and the GST precipitates were then resolved by SDS-PAGE. Comparing to the GST alone control, there were two protein bands specifically precipitated by the GST-FasL proline domain fusion protein. We are

currently characterizing the two proteins by western blot analysis using antibodies against known SH3-containing signaling proteins.

KEY RESEARCH ACCOMPLISHMENTS:

1. We have generated 4 expression plasmids containing wild-type and mutant forms of FasL and 2 GST-FasL fusion plasmids.

2. We have analyzed the function of the proline-rich domain of FasL in regulating cell apoptosis and found that the proline-rich domain of FasL can protect cells from FasL-induced apoptosis.

3. We have identified two potentially FasL proline domain-interacting proteins.

REPORTABLE OUTCOMES:

1. 4 expression plasmids containing wild-type and mutant forms of FasL and 2 GST-FasL fusion plasmids.

2. Cell lines expressing the wild-type and mutant forms of FasL.

CONCLUSSIONS:

In summary, we have demonstrated that the intracellular proline-rich domain of FasL can function to protect cells from FasL-induced cell death. We have preliminary data to suggest that there were at least two proteins physically interacting with the proline-rich domain of FasL. Identification of these proteins is crucial to understand the function and mechanism of FasL in promoting survival and growth of breast cancer cells. These findings are important for developing effective diagnostic and therapeutic reagents to treat breast cancer patients.

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