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TRAIL is a tumor necrosis factor family member that can specifically induce apoptosis of cancer cells but not of normal cells. However, some cancer cells are resistant to TRAIL-induced apoptosis. The purpose of this proposed study is to clone and characterize such inhibitory genes of TRAIL-induced apoptosis. Using cDNA subtraction and retroviral cDNA-based expression cloning approaches, we have obtained more than 50 candidate clones of TRAIL-inhibitory genes in the first year. In the second year, we have identified and validated Casper-S as a major cellular inhibitor of TRAIL-induced apoptosis. In the third year, we have further characterized the other candidate clones. The project has been approved for a one-year extension without additional funds.

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Reply to previous review

We thank the reviewer for his/her helpful comments on our original report. We have now modified the report as the reviewer suggested. In the revised progress report, we have added literature references; we have marked the report for unlimited distribution; we have provided a more comprehensive presentation of data. We hope the revised report is now satisfactory.

INTRODUCTION

TRAIL is a tumor necrosis factor family member that can specifically induce apoptosis of cancer cells but not of normal cells (1-5). However, some cancer cells are resistant to TRAIL-induced apoptosis (3, 4, 6-13). The purpose of this proposed study is to clone and characterize such inhibitory genes of TRAIL-induced apoptosis. To this end, following specific aims are proposed: #1. To clone genes that inhibit TRAIL-induced apoptosis of MCF7 cells by a subtractive hybridization screening approach and an expression/functional cloning approach using retroviral cDNA libraries; #2. To functionally characterize genes identified from specific aim #1.

BODY

We proposed the following Tasks and time frames in the original proposal:

Task 1. To clone genes which inhibit TRAIL-induced apoptosis of MCF7 cells by a subtractive hybridization screening approach and an expression/functional cloning approach using retroviral cDNA libraries (months 1-24)

a. Isolate and amplify TRAIL resistant (MCF7-R) and sensitive (MCF7-S) MCF7 cells (months 1-2)
b. Identify genes that inhibit TRAIL-induced apoptosis of MCF7 cells by a subtractive hybridization screening approach (months 3-12)
c. Confirm that identified candidate genes are differentially expressed in MCF7-S and MCF-R cells Northern blot analysis (months 13-15)
d. Identify genes that inhibit TRAIL-induced apoptosis of MCF7 cells by an expression/functional cloning approach using retroviral cDNA libraries (months 3-20)
e. Clone full length cDNAs for genes that are identified from Task 1 (months 21-24)

Task 2. To functionally characterize genes identified from *Task 1* (months 25-36)

a. Determine tissue distribution of expression of the genes cloned in *Task 1* (months 25-26).
b. Test whether these genes can inhibit TRAIL-induced apoptosis of MCF7-S cells in apoptosis assays (months 25-28)

c. Determine the molecular mechanisms responsible for the inhibition of TRAIL-induced apoptosis of MCF7-S cells by the genes identified in *Task 1* (months 29-36)

In the first year, we successfully performed Task 1, a,b,and partially performed Task 1d. In the second year, we continued to perform Task 1d. We sequenced a total of 52 TRAIL resistant clones obtained from retroviral cDNA library based functional cloning approach. Very interestingly, among the 52 sequenced clones, 17 encode for Casper/c-FLIP. Further sequencing analysis suggests that all 17 Casper clones represent the short splice form of Casper (Casper-S). Because of this striking observation, we decided to slightly modify our original plans to finish this project. Rather than simultaneously cloning and functionally characterizing all the candidate genes obtained from the first year, we decided to focus on the short splice form of Casper first. Using transient transfection and stable transfection approaches, we firstly confirmed that Casper-S could confer resistance to TRAIL sensitive cells. Furthermore, we found that Casper deficient embryonic fibroblasts (EFs) were highly sensitive while their wild-type counterparts were completely resistant to TRAIL-induced apoptosis. Retroviral-mediated transduction of Casper-S into Casper(-/-) EFs restored resistance to TRAIL. These data suggest that Casper-S/c-FLIPs is a major cellular inhibitor of TRAIL-induced apoptosis. Our studies on Casper-S in TRAIL resistance have been published (14).

Our results on Casper-S indicated that our approaches for identification of TRAIL resistant genes worked properly, and we validated the first TRAIL resistant genes identified in this project. So, in the second year of this project, we finished Task 1a, 1b, 1d, and partially finished Task1c, 1e, and Task 2.

Work done in the third year

In the third year, we further analyzed the other candidate genes obtained from the expression cloning. We made retroviral expression plasmids for 12 candidate genes. To do this, we amplified cDNAs for these genes by PCR using a mixed cDNA libraries or the isolated clones as templates. The cDNAs were inserted into the pFB-Neo retroviral plasmid (Stratagene). The identities and related information are summarized in Table I.

Candidate genes	Known functions	References
PP2Ac	Protein phosphatase	15
CL100/DUSP1	Dual specific phosphatase	16-17
FIP-1/RagA	Ras-related GTPase	18
RAN	Ras family member	19
NALP1/CARD7	CARD domain-containing protein	20
MKK4	MAP kinase kinase	21
α1-antiproteinase	Serine proteinase inhibitor	22
NOS3	Nitric oxidase synthase	23
SPTLC1	Serine palmitoyltransferase	24
C10	Novel	
C11	Novel	
C13	Novel	

We transduced these genes into TRAIL sensitive cell clone C1 by retroviral-mediated gene transfer. Two days after infection with the retrovirus containing these genes, cells were treated with TRAIL for overnight. We used 200 ng/ml of TRAIL for the treatment, a concentration we used in the previous expression cloning. We found that retroviral mediated transfer of the tested genes did not confer resistance to TRAIL-induced apoptosis, as judged by observation under a microscope.

To exclude the possibility that the genes were not expressed or the efficiency of retroviral-mediated gene transfer was low in the experiments, we are making retroviral vectors in which a Flag epitope tag is added to the candidate proteins. We will transduce these vectors into C1 cells and establish stable cell lines by selection with G418. We will then detect the expression of the transduced proteins by Western blot analysis with anti-Flag antibody. If protein expression is detected, we will then evaluate whether the cells are resistant to TRAIL. If necessary, we will quantitate TRAIL-induced apoptosis by MTT assays.

KEY RESEARCH ACCOMPLISHMENTS

Cloned 12 candidate genes into retroviral vector and determined whether twelve of the candidate genes could inhibit TRAIL-induced apoptosis.

REPORTABLE OUTCOMES

None

CONCLUSIONS

In the third year of the proposed study, we have cloned 12 candidate genes into retroviral vector and determined whether they can inhibit TRAIL-induced apoptosis. Although no positive genes are identified, we have worked on this project as proposed. The project has been approved for one-year extension without additional funding. We hope to validate the other candidate genes in the coming year.

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APPENDICES

None