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13. ABSTRACT (Maximum 200 Words) Prostate cancer is the most frequently diagnosed cancer among men in the United States. It represents approximately 7% of all cancer deaths and ranks as the second leading cause of cancer death in males. The majority of prostate cancer patients succumb to their malignancy as a result of metastatic invasion, while few patients die from their primary neoplasm. Despite significant improvement in surgical techniques and chemotherapies, none of the current medical technologies "cure" the metastatic disease. The KAI1 gene was originally isolated as a prostate-specific tumor metastasis suppressor gene. Based on our preliminary data, we hypothesize that the KAI1 protein on tumor cells interacts with gp-Fy on the endothelial cells, which activates a signal pathway of the KAI1 molecule, and that this activation eventually leads to cell growth arrest of tumor cells. To test this hypothesis, we will examine whether the interaction of KAI1 and gp-Fy leads to suppression of tumor metastasis in vivo (Task 1), and identify specific peptide sequences that activate KAI1 and to assess the efficacy of the peptides on tumor growth in an animal model (Task 2). Our long-term goal is to elucidate the molecular mechanism of tumor suppression by the KAI1 gene and to develop an effective therapeutic method which restores the function of the KAI1 gene in the metastatic tumor cells.				
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INTRODUCTION

The KAI1 gene was originally isolated as a prostate-specific tumor metastasis suppressor gene, using the microcell-mediated chromosome transfer method (1). Ample evidence from both clinical data and the results of in vitro as well as animal experiments overwhelmingly support the notion that KAI1 is a metastasis suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis (2). Based on our preliminary data, we hypothesize that the KAI1 protein on tumor cells interacts with gp-Fy on the endothelial cells, which activates a signal pathway of the KAI1 molecule, and that this activation eventually leads to cell growth arrest of tumor cells. To test our hypothesis, we will examine whether the interaction of KAI1 and gp-Fy leads to suppression of tumor metastasis in vivo (Task 1), and identify specific peptide sequences that activate KAI1 and assess the efficacy of the peptides on tumor growth in an animal model (Task 2). Our long-term goal is to elucidate the molecular mechanism of tumor suppression by the KAI1 gene and to develop an effective therapeutic method which restores the function of the KAI1 gene in the metastatic tumor cells.

BODY

Task 1-a.

Prepare endothelial cells from a gp-Fy knockout and wild type mouse and test their ability to bind and kill tumor cells that express KAI1

As we mentioned in the last year's report, we have nearly completed this task. GFP-tagged prostate carcinoma cells, AT6.1 and their KAI1 overexpressing derivatives, were mixed with HBME or HUVEC cells (gp-FY⁺) in suspension and plated on a 12-well plate followed by measurement of DNA synthesis and colony formation of GFP⁺ tumor cells. We found that the ability of tumor cells to form colonies significantly decreased when KAI1⁺ cells (AT6.1/Flag-KAI1), as compared to KAI1⁻ cells (AT6.1), interacted with HBME or HUVEC. In order to confirm whether this effect is mediated by gp-Fy in the endothelial cells, similar experiment was performed where the tumor cells with or without KAI1 expression (AT6.1/Flag-KAI1 or AT6.1) were mixed with cells with or without gp-Fy expression (AT6.1/Flag-Fy or At6.1). We found that the prostate tumor cells expressing KAI1 (AT6.1/Flag-KAI1) formed significantly fewer number of colonies than the KAI1⁻ cell line (AT6.1) when mixed with cells expressing gp-FY. On the other hand, there was no significant difference in the number of colonies between the two cell lines when allowed to interact with AT6.1(gp-FY⁻). We further confirmed these results by adding anti-Kai1 antibody in the assay system, and we found that the interaction between Kai⁺ tumor cells and endothelial cells was significantly suppressed by the antibody and that the ability of colony formation of Kai⁺ cells was rescued by the antibody treatment. Therefore, the interaction between epithelial cells and endothelial cells indeed requires both gp-Fy and Kai1 molecules and this interaction leads to cell growth arrest. The next important question we are asking is what signal molecules are activated by this interaction. We are using a phospho-specific signal array as well as a gene microarray to identify a specific target which ultimately blocks the metastasis process in response to a signal activated by the interaction of gp-Fy and Kai1.

Task1-b.

Examine the effects of siRNA against the gp-Fy gene on the binding and killing ability of KAI1 to endothelial cells

The experiment for this task is in progress. We have synthesized si-RNA (Dharmacon Co) which targeted to the gp-Fy gene. The si-RNA is double-stranded RNA and consists of four different species that target different sites of the gp-Fy gene. We have confirmed the knockdown of the gp-Fy expression in our cell line which is over-expressing the HA-tagged gp-Fy gene. However, we currently have a problem of using this si-RNA for the HUVEC cells due to poor transfection efficiency of the cells. We also noticed that the expression of gp-FY in commercially available HUVEC cells has some variations from batch to batch. To overcome these problems, we are currently constructing adenovirus vector which expresses si-RNA and also preparing HUVEC ourselves.

Task 1-c.

Examine the metastatic ability of a spontaneously developing tumor in gp-Fy knockout mice by constructing a hybrid animal between a transgenic prostate tumor mouse, TRAMP, and the gp-Fy knockout mouse

We are nearly completing this task as we reported in the past report. We utilized a gp-Fy knockout (KO) mice to accomplish this task. Since this task is the most vital part of the project, we have added another layer of evidence. The KO mice were indistinguishable from wild-type in terms of size, development, and health, anatomy of various organs and immune system. The syngenic metastatic tumor cell line, B16BL6, was chosen in order to establish xenograft tumors

Table 1. Spontaneous metastasis assay in gp-Fy knockout mice

Clone #	KAI1 expression	Tumor volume (mean \pm S.E.)			Incidence of pulmonary metastasis			
		Fy+/+	Fy-/-	Fy+/-	Fy+/+	Fy-/-	Fy+/-	P value
2	positive	4.9 \pm 0.03	4.5 \pm 0.02	4.5 \pm 0.01	2/15(13.3%)	9/15(60%)	1/15(6.7%)	0.02*, 0.008**
9	positive	4.6 \pm 0.05	4.5 \pm 0.03	4.9 \pm 0.04	1/15(6.7%)	6/13(46.2%)	1/15(6.7%)	0.05*, 0.05**
18	positive	4.5 \pm 0.05	4.2 \pm 0.03	3.9 \pm 0.04	0/13(0%)	6/12(50%)	0/13(0%)	0.04*, 0.04**
empty vector	negative	4.9 \pm 0.05	4.8 \pm 0.05	4.9 \pm 0.03	6/15(40%)	5/14(35.7%)	5/14(35.7%)	0.8*, 0.89**

*comparison between Fy^{-/-} and Fy^{+/+}

** comparison between Fy^{-/-} and Fy^{+/-}

Table 2. Experimental metastasis assay in gp-Fy knockout mice

Clone #	KAI1 expression	Number of pulmonary metastasis			P value
		Fy+/+	Fy-/-	Fy+/-	
1	positive	4.7 \pm 2.4 (n=9)	47.86 \pm 5.9 (n=7)	2.8 \pm 0.8 (n=6)	<0.001*, <0.001**
16	positive	4.4 \pm 2.4 (n=7)	32.14 \pm 3.6 (n=7)	9.4 \pm 2.7 (n=5)	<0.001*, 0.001**
empty vector	negative	40.0 \pm 8.4 (n=5)	56.0 \pm 11.8 (n=5)	32.5 \pm 4.8 (n=6)	0.3*, 0.08**

*comparison between Fy^{-/-} and Fy^{+/+}

** comparison between Fy^{-/-} and Fy^{+/-}

in these mice. Metastatic prostate cancer cells with or without KAI1 over-expression were subcutaneously injected into the gp-Fy^{-/-} mice as well as its heterozygote and wild-type littermates (spontaneous metastases assay). The primary growth rates of the mice were monitored and the pulmonary metastases were examined at autopsy. Primary tumor developed in 100% of the mice, and the growth rate or final volume of the tumors did not significantly vary with the KAI1 level in the grafted tumor cells or with gp-Fy status of the mice (Table 1). However, the KAI1-positive clones developed significant number of pulmonary metastases in the gp-Fy^{-/-} mice, compared to the heterozygote and wild-type littermates. The tumor cells lacking KAI1 (B16BL6/vector), however, equally metastasized in all the three groups of mice. We did a similar experiment using i.v. injection (experimental metastases assay), and we obtained nearly the same results (Table 2). Therefore, these results of our animal study strongly support our notion that metastases suppression by Kai1 requires the interaction with gp-Fy. To further confirm these results, we are currently trying to quantitate the number of cancer cells attached to the endothelial cells in both wild type and gp-Fy knockout mice.

Task 2-a.

Construct a series of deletions of gp-Fy and test their binding ability to KAI1.

As we reported in the last report, we completed this task. We tested several individual domains as well as serial deletions from the N-terminus of the KAI1 gene against full length gp-Fy target and vice versa. Our results indicate that the first 32 amino acids from the N-terminus of KAI1, spanning the first intracellular and transmembrane domains, are dispensable for the interaction, but the conformation of the protein as a whole may be important as none of the other fragments yielded a positive interaction. On the other hand, deletion of the first extracellular domain of gp-Fy at N-terminus completely abrogated the interaction, suggesting that the N terminus of gp-Fy is essential for binding to KAI1.

Task 2-b.

Screening a phage display library followed by sequencing the interacting clone

This task is in progress. We have just recently started this experiment. We have obtained a phage-display library (Biolab) and have screened the library by first adsorbing the phages to Kai-1 negative cell line followed by recovering the phages that did not attach to these cells. The recovered phages were again adsorbed to Kai+ cells, and phages adsorbed to these cells were recovered. By panning these phages, we isolated 24 different phage clones. We first need to confirm our results for all these 24 clones for their specific binding ability to Kai1+ cells, then we need to sequence the peptide region. This work is currently in progress, and the information from the results is important for pursuing Task 2-c as described below.

Task 2-c.

Synthesize small peptides corresponding to the gp-Fy binding domain and test for their tumor suppressive activity

This task is in progress. Based on the results of Task 2a, we synthesized two different peptides that cover the essential 32 amino acids region for the gp-fy-Kai1 interaction. We first tested in vitro to see if these peptides interfere with the interaction of gp-Fy and Kai1. However, our

preliminary data indicate that these peptides do not have significant effects on the interaction between Kai1+ cells and endothelial cells. Obviously there are many possibilities to explain the inability of these peptides to block the interaction, including the stability of peptides, secondary structures and other physiological conditions, and we are currently exploring these possibilities. In the meantime, we have learnt that another group (Dr. Chaudhuri at NY Blood Center) from whom we obtained the gp-Fy knockout mouse has determined a locus which is essential for attachment of Malaria parasite. This region falls in the N-terminal of gp-FY, and when a series of peptides in this region were tested, they found that a 18-amino acid peptide corresponding to the N-terminal is sufficient to block the binding. The PI has just visited this group and arranged a collaboration to use these peptides to see if they are able to bind to the Kai-1 interaction site. We are also waiting for the progress of Task 2-b so that we can synthesize other peptides of gp-Fy that would work better for inhibiting the interaction between gp-Fy and Kai1.

Task 2-d.

Test the efficacy of the specific peptides in SCID mice model of prostate cancer

We have not pursued this task because we need to wait for the results of Task 2-c.

KEY RESEARCH ACCOMPLISHMENTS

1. We have shown that cancer cells attach to the endothelial cells via interaction of Kai1 and gp-Fy molecules and that this interaction results in cell growth inhibition in vitro.
2. Using the knockout mouse of the gp-Fy gene, we have shown that the metastases suppression by Kai1 requires gp-Fy, which most strongly supports our hypothesis.
3. We have performed the screening of a phage display library and obtained positive clones that will be sequenced soon.
4. As we described in the last progress report, we also localized the essential region of gp-FY and Kai-1 for their interaction.

REPORTABLE OUTCOMES

Peer reviewed publications

1. Bandyopadhyay, S., Watabe, M., Pai, S.K., Hirota, S., Hosobe, S., Goodarzi, G., Gross, S. M. Iizumi, Pauza M., and Watabe, K. Tumor metastasis suppressor gene KAI1 interacts with the glycoprotein Fy(gp-Fy) on vascular endothelium. Submitted to Nature Medicine, Pending revision (resubmission planned in Mar.2006)

Abstract/presentation

1. Bandyopadhyay, S., Wang, Y., Pai, S.K., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Takano U., Saito, K., Commes, T., Piquemal, D., Watabe, M., and Watabe K. (2005) The metastasis suppressor gene Drg-1 downregulates the expression of ATF3 in prostate and breast carcinoma. American Association for Cancer Research. Anaheim, CA.

2. Bandyopadhyay, S., Pai, S.K., Watabe, M., Gross, S.C., Hirota, S., Hosobe, S., Tsukada T., Miura, K., Saito, K., Markwell S.J., Wang Y., Zhan R., and Watabe K. (2005) FAS expression inversely correlates with the expression of PTEN and an Akt inhibitor synergizes with FAS siRNA to induce apoptosis in prostate cancer cells. American Association for Cancer Research. Anaheim, CA.

Employment

1. Dr. Sucharita Bandyopadhaya (Postdoc) has been supported by the current grant.
2. Rui Zhan (currently in Ph.D. program) has been partly supported by the current grant.

CONCLUSIONS

The results of Task 1c strongly support our working hypothesis that gp-Fy is the essential molecule for metastases suppression by the Kai1 protein. We now have both in vitro and in vivo data that are consistent with this intriguing concept. The next important question is to understand the mechanism of the cell growth arrest. We have already asked this question by examining apoptosis of the cancer cells after exposing these cells to endothelial cells. However, all apoptosis assay resulted in negative outcome, suggesting that the growth arrest would be either due to autophagy or senescence. We are currently testing these possibilities. Another important aspect of our project is to develop a peptide drug which mimics gp-Fy in the hope that it will be utilized for anti-metastasis therapy. The progress of this part has been hampered by our initial failure of identifying such a peptide. However, we hope that the results of Task -2c, which is still ongoing, and our newly arranged collaboration will overcome this problem

So what?

We believe that the progress we have made so far to show the interaction between Kai1 and gp-Fy in our animal system strongly supports our intriguing hypothesis which will make a significant contribution to our understanding in the molecular mechanism of tumor metastases. This information is the bases of our next challenge to develop anti-metastasis peptide, which is currently underway.

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1. Dong, J.T. *et al.* KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. *Science* **268**, 884-886 (1995).
2. Yoshida, B.A., Sokoloff, M.M., Welch, D.R. & Rinker-Schaeffer, C.W. Metastasis-suppressor genes: a review and perspective on an emerging field. *J Natl Cancer Inst.* **92**,1717-1730 (2000).