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A Look at the Effects of Vitronectin and PAI-1 Binding

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UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

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PROJECT TITLE: A Look at the Effects of Vitronectin and PAI-1 Binding

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Cynthia Peterson, Faculty Mentor

Date: 05/05/04

Comments (Optional):
A Look at the Effects of Vitronectin and PAI-1 Binding
Michael Maddox
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ABSTRACT

This project combines is essentially a dual-faceted experiment combining two separate fields of science in order to gain insight into the complex mechanism of cancer in general. The first part of the experiment involves laboratory work with a concentration on cell biology, and the second part of the experiment utilizes biotechnology in order to analyze the significance of the cell biology results. The purpose of the experiment was to essentially look at specific genes that are over expressed as a result of cellular conditions that are believed to be linked to cancer but more specifically tumor metastasis. The varying conditions were produced by adding different concentrations of the proteins vitronectin and plasminogen activator inhibitor 1 (PAI-1). The interaction of PAI-1 and vitronectin alter the function of each protein in vivo, and are closely associated with the cause of tumor metastasis. The experiment used the cancerous cell line HT1080 and cell culture techniques to grow the necessarily large amounts of cells for the later steps of the experiment. Cell binding assays incorporating the different concentrations of vitronectin and PAI-1 yielded the bound cells for the RNA isolation. Once the RNA is isolated, a microarray technology is used to analyze the different gene expression patterns that resulted from the variables and controls.

INTRODUCTION

Cell Culture:
This experiment required a very large number of cells so that there would eventually be enough RNA to use for the microarray. Therefore, the first and possibly most important step to the project was to grow cells in culture flasks. The cells used, HT1080 line, grow in a minimal media with fetal bovine serum (FBS) added to it, which contains many essential growth factors for successful cell growth. Starting with a stock solution of cells, these are initially added to the media with FBS. The growth of the cells is monitored closely because the cells need to be split just before they reach confluency, at which point the cells can start to die. This typically took approximately two days. Once the cells were ready to be split, the media is removed from the flask and washed with minimal media not containing FBS in order to get rid of cells that might have died or did not adhere to the flask surface. Then the enzyme trypsin is added in a 3mL dose to the flask. This enzyme breaks the bonds between cells and the flask surface allowing the cells to come out into solution. After a few minutes the cells are off the plate, and 7mL of the FBS media is added to the flask making sure not to let the trypsin begin to degrade the cells. This 10mL is mixed and then 5mL are removed and put into another flask for growth (splitting cells). Finally, 5mL of the FBS media is added to each of the flasks and the process repeats until enough cells are grown. Contamination became a problem in the cell culture process towards the end of the experiment, which was most likely caused to some human error as a result of dealing with such a large number of cells and flasks. However, it is possible that one of the bottles of media became contaminated over the course of the project, which would also caused massive contamination.

**Vitronectin and PAI-1:**
Vitronectin, or S-protein as it is sometimes called, is a glycoprotein found in the human blood plasma and connective tissues. Plasminogen activator inhibitor I (PAI-1) is a serine protease inhibitor, or serpin, which is a large family of proteins. These two proteins separately have diverse functions in the body, but for the purpose of this experiment, the interaction between the two proteins is of importance to the project. PAI-1 is a unique serpin because it exists in two different forms, the latent and active forms. The active form is structurally unstable and thus spontaneously converts back into the more stable latent conformation. However, the interaction between vitronectin and PAI-1 stabilizes the PAI-1, which allows for a two to three fold increase in its half-life (Gibson). Vitronectin and PAI-1 are linked to many different physiological processes including the fibrinolytic pathway, cell migration, and tumor metastasis. The purpose of this experiment is to investigate why the interaction of these two proteins potentially causes cancer or at least the spread of cancer. The interaction shows the ability of vitronectin to form multimeric complexes with PAI-1 that exposes binding sites that are not available in native unbound vitronectin (Kreis). DNA microarrays will hopefully explain the resulting gene expression that is brought about by the interaction between PAI-1 and vitronectin.

MICROARRAY TECHNOLOGY:

The technology that would be used to analyze the effects of the vitronectin and PAI-1 on gene expression is a DNA microarray. This technology can be used to analyze DNA on three different levels of the central dogma. First, it is possible to analyze DNA sequences, and this method is categorized as genomics. Next, the microarray can analyze RNA expressed by a specific cell or system, which is categorized as functional genomics.
Finally, proteomics refers to an array that analyzes the expressed proteins. For the purpose of this experiment, we wanted to apply the functional genomics as we look at the RNA isolated from the HT1080 cells after being exposed to the different vitronectin and PAI-1 conditions. This array utilizes a biochip that detects hybridization of DNA. First off, a number of genes are selected because they are potentially linked to cancer. Approximately 200 microns of DNA segments from these genes are spotted on a slide. These DNA segments have a fluorescent marker attached to them as well as a quencher. The quencher and marker are on different ends of the segment, but they are attached so that the DNA is essentially folded on itself. This interaction between the marker and quencher is what prevents the spots on the slide from giving off a fluorescent signal.

After the RNA isolation of the HT1080 cells, reverse transcriptase is employed to get DNA segments from the cells. Then, the DNA that was extracted from the cells is applied to the slide with premarked spots on it. Finally, if any of the genes that are spotted on the slide are being expressed in the HT1080 cells, then there should be hybridization of the DNA. The hybridization effect causes the quencher and the marker to release giving off a fluorescent signal that is detected by the biochip and read on a computer program (Grigorenko).

**METHOD:**

This is the protocol for the cell binding assay experiment that was performed. The larger experiment uses the same preparation, but because of the great need for more cells, large six well plates were used and the 100 microliters is changed to 1800 microliters. Also, once the cells are bound to the ECM, rubber scrapers would be used to
scrape the cells off of the plates. Then, using an RNA isolation kit, the RNA would be extracted from the cells for use in the microarray described in the introduction. Those calculations are shown in the next section. The Elisa plate was coated with ECM (BD Bioscience) in 1X PBS, in a concentration of 5µg of ECM in each one ml of PBS. The stock of supplied ECM was 1mg/ml. In each well of plate 100µl of coating solution was added. The plate was kept overnight at 4 °C before use.

**Blocking of plate**

Before starting the experiment the coated plate was washed by 1X PBS three times and then blocked by 5% BSA (made in 1X PBS) and kept at 37 °C for one hour.

**Making solution Coating of the plate with ECM of VN and mixture of VN/PAI-1**

All the solutions were made in 0.5% BSA (made in 1X PBS). The concentration of the stock solution of VN and PAI-1 were 28 µM and 60 µM respectively. The solutions were kept in 37 °C for one hour. Incubation time for these solutions was strictly followed.

**Addition of solution of VN and VN/PAI-1 to the plate**

The blocking solution was removed from the plate and washed three times by 0.5% BSA. Then different solutions of VN and VN/PAI-1 were added to the plate. Then the plate with solution was incubated for 1 hour at 37 °C. VN was obtained by dialysis and PAI-1 came from Molecular Innovation.

Note: Solution should be added as soon as possible after its incubation is complete. So washing should be done before the time for incubation of the solutions is finished.

**Labeling of the cells**
Cells (grown in MMEM using 10% FBS) were trypsinized and collected and then were washed with serum free MMEM. The cells were then resuspended in required volume of MMEM and counted. Before counting a solution was made by adding 20µl of Trypan Blue (GIBCO) to 20 µl of cell suspension. From this solution 20 µl was taken in Hemacytometer and counted. Percentage of viable cells was counted. More than 90% was considered as fine to work with.

For labeling, Calcein (Molecular Probe) was used. 50µg of calcein was dissolved in 50µl of DMSO. And for each ml of cell suspension 1µl of calcein solution was added. Cell-suspension with calcein was kept at 37 °C for at least 30 minutes for labeling.

**Addition of cell-suspension to the plate**

After one hour of incubation with solution of VN and PAI-I the plate was washed for three times with 0.5% BSA. 100 µl of cell suspension was added to each well of the plate and the plate was then incubated for 1 hour in 37 °C.

**Counting of the signal**

After incubation of the plate with cell a counting was done in Elisa Reader (Perkin Elmer) to get total signal. Then the plates was washed three times with serum free MMEM. A final count was done in the Elisa reader in presence of MMEM in each well. Each time the excitation and emission spectra used were 435nm and 535nm respectively.

**Analysis of data**

Data was analyzed by drawing a bar-graph between percentage of cells bound, subtracted from the background (cells bound to ECM alone) with respect to different concentrations of PAI-I in Kaleidagraph.

The formula of calculating percentage of cell bound is.....
Results:

These are results of the cell binding assay performed by Sumit Goswami in Dr. Peterson’s lab.

Since the majority of the experiment was not performed due to time constraints, there is not much to display as results.

CONCLUSION:

It is likely that had the cells not become contaminated, there would have been enough cells to do some RNA isolation work. However, there were many problems throughout the project’s duration that hindered its productivity. In theory, if vitronectin and PAI-1 have a serious physiological effect that is linked with tumor metastasis, then
hopefully the DNA microarray would yield specific genes that are being overexpressed as a result of the interaction of these two proteins. If results like this were reproducible, this would give someone a reason to investigate the genes that are being expressed, and hopefully they would gain insight on cancer. The clinical potential that an experiment like this has is incredible, and hopefully a successful project will lead to a more effective treatment for cancer.

WORKS CITED/CONSULTED:


