Protein Isolation and Western Blot Analysis of BCL-2 in Fibroblast Cells

Sirah Bah

Lab Partners: Devin Kuhn and Sheridan McNeill

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Abstract

This lab used the techniques of protein isolation in order to perform a western blot of fibroblast cells. The purpose of this was to study the level of cell survival in fibroblast cells by analyzing the specific protein of BCL-2. BCL-2 was used as an indicator of cell survival, because this protein is an anti-apoptotic protein. BCL-2 in this lab was isolated in the fibroblast cells in order to monitor cell survival in an array of varying cellular conditions. These cells were cultured in varying percentages of FBS and in the presence or absence of Camptothecin. The three environments created were; 5% FBS, 10% FBS, and 10% FBS + Camptothecin. After cell cultures, the samples then underwent protein isolation, and were ultimately analyzed by performing a western blot. From following these procedures and with the various cellular conditions, it was hypothesized that if the cells that were cultured in 10% FBS and in the presence of Camptothecin then they will show the lowest amount of living cells. If the cells cultured were in the 10% FBS, then they would show the highest amount of living cells in this data group.

Introduction

Protein Isolation is a technique commonly used in labs in order to extract proteins from a cell in order to complete a biochemical analysis. Protein isolation is an important technical skill in that in analyzing proteins, researchers are better adept in studying the biomarkers that are useful in diagnosing diseases (Lee, 2017). However, this procedure is not easily accomplished in that many endogenous proteins are also present in the proteins that are targeted for isolation (Lee, 2017). Due to this complexity, in order to accomplish protein isolation a series of antibodies are deployed onto the cells that have been cultured. Antibodies are used in order to target a specific protein as it can both separate and purify that protein, and by performing a western blot on the isolated it can tell a lot about the protein at hand. Protein isolation and its biochemical analysis is useful in determining the structure-function of proteins, and this is useful in not only the isolation and purification of the conventional enzymes but also the mutant forms of these proteins as well. Protein isolation is achieved by isolating the cells via centrifugation with treatments of different densities. For this given experiment the three treatments tested were; 5% FBS, 10% FBS, and 10% FBS + Camptothecin. These three different treatments were used in order to measure what treatment gave to the highest amount of living cells from fibroblast tissues.

Western blotting was another technique used to the success of this lab in order to identify the specific protein form an intricate mixture of proteins that have been extracted via protein isolation (Mahmood and Yang, 2012). This lab employed the western blot in order to investigate the presence or absence of BCL-2. BCL-2 is a protein that is a regulator for apoptosis in cells (Hardwick and Soane, 2013). This protein can either inhibit or induce cell death depending on the treatment or environment inside of the cell. This protein is also important in normal embryonic development and in preventing cancer. The three treatments that the fibroblast cells were cultured in were; 5% FBS, 10% FBS, and 10% FBS + Camptothecin. FBS or Fetal bovine serum is used in order to supplement in vitro cell cultures, and the different levels of FBS were used in order to create varying environments for the cells to culture in. Camptothecin is an alkaloid that can be derived from the Camptotheca plant. Camptothecin works by forming a stable ternary complex which inhibits normal DNA re-ligation. This effect leads to a DNA double-strand break, cytotoxicity, and apoptosis. Western blotting is performed by extracting the

proteins by cell lysis in order to prepare the samples to undergo gel electrophoresis. After gel electrophoresis occurs the samples are then electrotransferred, and undergo blocking and antibody incubation so that an x-ray image can be taken of the samples to further investigate. The samples separate in the gel according to protein size, with the largest of the protein sample at the top of the gel and the smaller protein parts separated to the bottom of the gel.

The purpose of this was to study the level of cell survival in fibroblast cells by analyzing the specific protein of BCL-2. BCL-2 was used as an indicator of cell survival, because this protein is a regulator of cell death depending on the conditions of the cell. The experimental approach for doing this was culturing cells in different treatments, protein isolation, and western blot. It was hypothesized that if the cells that were cultured in 10% FBS and in the presence of Camptothecin then they will show the lowest amount of living cells in a western blot. If the cells cultured were in the 10% FBS, then they would show the highest amount of living cells in the western blot.

Materials and Methods

Part 1: Protein Isolation

The cultured cells were examined under the microscope take note of the floaters and confluency in the three well plates; 5% FBS solution, 10% FBS solution, and 10% FBS plus Camptothecin. Once these observations were noted, three 1.5 ml centrifuge tubes were labeled to identify the three different FBS concentrated samples. The three well plates were then emptied of its liquid contents, and the now liquid free well plates were then washed with 2ml of cold PBS. 500 µl of M-PER with protease and phosphatase inhibitors were additionally added to each of the three well plates. Each plate bottom was then scraped, and the resulting samples were added to the corresponding centrifuge tubes. The tubes were mixed, and incubated on ice for 35 minutes. After incubation, the samples were spun at 4 ℃ for 5 minutes at 12,000 rpm. The supernatant was moved to newly labeled centrifuge tubes, and the ones with the pellet were thrown out. Following this, 25 µl of each sample were then added into wells A2-A10 in a 96 well microplate. 5 µl of the remaining samples were added to wells B1-B3, 20 µl of MPER protein lysis buffer was added on top of the samples in the wells, and 200 µl of the working reagent to the blank wells. Ultimately, the plate gets covered and is left to incubate at 37℃ for 30 minutes

Part 2: Western Blotting

A 4-12% gel was rinsed with deionized water, and the tape was removed from the bottom of the gel. Then, the comb was removed from the gel cassette so that the gel cassette could be placed inside a gel box so that the black writings faced the outside. The upper buffer chamber was then filled with a small amount of running buffer in order to ensure that the chamber was tightly sealed. Once the chamber was tightly sealed, 200 ml of 1 x Running buffer was added on top and left for 10 minutes. Following the making of the gel, the samples were prepared to for western blotting. The samples were heated at 70℃ for 10 minutes, and then put on ice. The samples were then loaded into lanes 1- 6 of the gel to arrange for duplicates of each sample. Lanes 1 and 2 were loaded with cells cultured in 5% FBS, 3 and 4 were loaded with cells cultured in 10% FBS, 5 and 6 were loaded with cells cultured in 10% FBS with Camptothecin, and lane 8 was loaded with the protein marker. After this 800 ml of 1 x Running buffer was added to the lower buffer chamber. Then the gel box could be connected to the electrodes, and the gel was ran at 200 Volts. The gel was left to run for 35 minutes, and then the box was shut off and the electrodes were disconnected. Then a gel knife was slipped in between the gap

between the gel cassette's two plates, and was used to carefully separate these two plates with the gel on the bottom plate. Then the top plate with the notches were discarded.

Before the gel could be transferred, a piece of nitrocellulose was labeled and soaked in transfer buffer. After the wells were removed from the gel and the foot of the gel by using the gel knife. Then a piece of presoaked filter paper was placed on top of the gel, and the trapped air bubbles were removed by the use of a glass pipette. The plate was then turned over in which the filter paper and gel were facing down over a gloved hand. Then the gel knife was used to push the gel through the aperture and onto the gloved hand. The pre-soaked nitrocellulose was then placed on the gel, and another piece of presoaked filter paper was set on top of the membrane. At this point, the gel "sandwich" was assembled as shown in Figure 1. Following this, the blot module was slid onto the guide rails on the lower buffer chamber then the Gel Tension Wedge was situated and locked in so that its vertical face was against the blot module. The blot module was then filled with transfer buffer until the gel was completely submersed, and the outer chamber was filled with deionized water. The lid was then placed on top, and the gel was ran at 30V for 1 hour 20 minutes. The sandwich was then disassembled, and placed in non-fat milk for a week. The blots were later washed, a secondary antibody was added, and incubated at 4°C overnight.

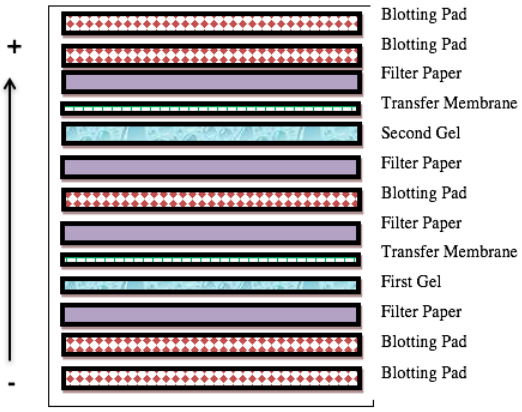

Figure 1: Shows the assembly of the gel "sandwich"

The membrane was washed in TBST three times for 10 minutes per wash. Then 10 ml of the secondary antibody solution was added to the membrane, and the membrane was left to rock for 45 minutes at room temperature. After the rocking, the membrane was washed again in TBST three more times for another 10 minutes per wash. The membrane was then transferred onto a piece of Saran Wrap with the protein side facing up. Following this, 2 ml of the developing solution was prepared, and pipetted onto the membrane to be incubated for 5 minutes. At the end of the 5-minute period, the membrane was placed on a kimwipe to remove excess developing solution. The membrane was then transferred to a sheet protector, and then taken to be analyzed.

Results

Cell Culture Treatments and their Resulting Protein Concentration

Treatment	**Protein Concentration (ug/ul)**
5% FBS (A)	1.67
10% FBS (B)	2.00
10% FBS + Camptothecin (C)	1.00

Table 1: The table above shows the protein concentration in ug/ul resulting from the three treatments types. Well plate A that had cells cultured in 5% FBS yielded a protein concentration of 1.67 ug/ul. Well Plate B that had cells cultured in 10% FBS yielded a protein concentration of 2.00 ug/ul. Well Plate C that was cultured in 10% FBS + Camptothecin yielded a protein concentration of 1.00 ug/ul.

Preparation of Samples for SDS-PAGE

Treatment	Protein Concentration (ug/ul)	Amt. Lysate (ul)	Amt Dye (uL)	Amt. MPER (uL)	Total Volume (uL)
5% FBS	1.67	12	6	8	26
5% FBS	1.67	12	6	8	26
10% FBS	2.00	10	6	10	26
10%	2.00	10	6	10	26
10% + Camptothecin	1.00	20	6	0	26
10% + Camptothecin	1.00	20	6	0	26

Table 2: The table above reflects what was loaded into the 6 wells for the SDS-PAGE. The Amt. Lysate was the protein extract, Amt. Dye was used to track movement in the gel, and Amt. MPER was used as a buffer solution to prevent annealing. The treatment that resulted in the highest protein concentration was 10% FBS, and the treatment that resulted in the lowest protein concentration was the 10% FBS + Camptothecin.

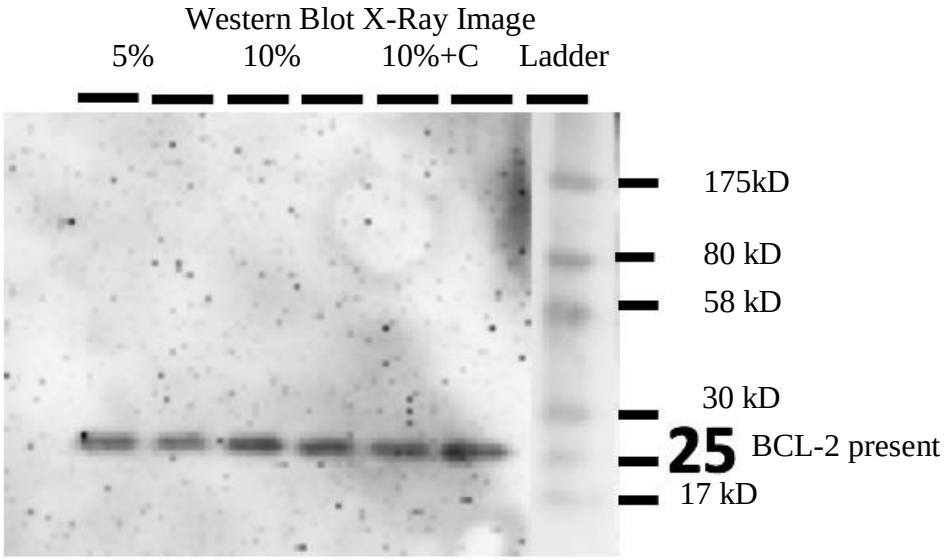

Figure 2: Image of the western blot as a result of an x-ray image. The horizontal axis shows the treatments that were tested, and the vertical shows the protein band size. The 25kD region is where the BCL-2 marker was present. There are dark bands present in the 25kD region for the 5%, 10%, and 10% + C treatments.

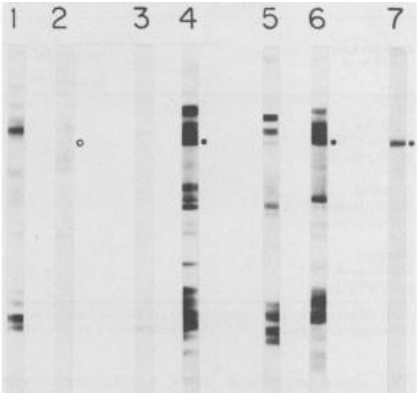

Figure 3: Example of a western blot showing HSV-1 or HSV-2 infected cell proteins. The tests were done in order to detect antibodies that were present in HSV infections. This image comes from a study done by Ashley, Militoni, Lee, Et. Al. published in the Journal of Clinical Microbiology.

Table 1 shows the protein concentrations that were a result from the protein isolations. This table shows that the treatment of 10% FBS had the highest protein concertation of 2.00 ug/ul, and the treatment with the lowest protein concentration was 10% + Camptothecin with a concentration of 1.00 ug/ul. The 10% + Camptothecin had the lowest protein concentration, because Camptothecin enhances apoptosis in cells. Table 2 shows the amounts of; Lysate, Dye, and MPER that were loaded into the SDS gels that were later imaged via x-ray. All treatments show a dark band around the 25 kD region, showing a presence of BCL-2 protein. However, BCL-2 is a regulator of cell death, and causes apoptosis or cell growth depending on the treatment of environment of the cells. The marker at 10% interpret to mean that BCL-2 was present and meant that it had the highest expression of BCL-2. The 25kD marker with the 10% + Camptothecin was interpreted to mean that BCL-2 was expressed at the lowest levels out of the three treatments.

Discussion

The results ended up validating the hypothesis in that the treatment of 10% FBS had the highest number of healthy/living cells, and the treatment with the lowest number of healthy/living cells was the 10% + Camptothecin. These results were interpreted from both table 1 and table 2. Figure 3 showed that dark bands were present at the 25kD region of the gel, which is the marker region that shows the presence of BCL-2 (Hardwick & Sloane, 2013). However, with the 10% treatment, BCL-2 induced cell growth and had the highest number of living/healthy cells and had the highest expression. BCL-2 in the presence of the 10% + Camptothecin induced apoptosis and had the lowest number of living/healthy cells and the lowest expression of this protein. The experiment was successful in addressing the question posed, as we cannot deter which treatments make for cell cultures with the greatest number of healthy/living cells and which treatments do the opposite. These findings were expected, because Camptothecin is an inducer of apoptosis, so if added to the treatment it should kill of the living cells.

This experiment is important in the world of research, because it helps explore the very much complex role of proteins and their functions in our bodies. This experiment is also useful for a future experimental design that might call to culture cells in a treatment that would produce the highest amount of living cells or the lowest amount of living cells. For example, many studies involve the use of Camptothecin and its relationship with cancer (Takimoto, 2002).

During the cell culture phase and after protein isolation the confluency of the cells and overall health of the three treatments were compared. In the bigger picture the 10% had the highest confluency, the 5 % treatment had an average confluency when compared to the other treatments, and the 10% + Camptothecin had the lowest confluency. The confluency also helped to make more sense of which treatments showed the highest and lowest expression of the BCL-2 protein. The 10% treatment had the highest expression of the BCL-2 protein, and the 10% + Camptothecin treatment had the lowest expression of the BCL-2 protein. These results show that the 10% FBS treatment is the best type of media for growing cells, because the high expression of the BCL-2 protein creates a great environment for cell growth. Another protein that could have been used in order to look for the differences between treatments is BAX. BAX is a part of the BCL-2 protein family, and an inducer of cell death (Misao, Hayakawa, et. Al, 1996). With this protein being used we would expect the treatment of 10% FBS + Camptothecin to display the highest expression of BAX protein. This would be the case, because Camptothecin is also an inducer of apoptosis. The treatment that would display the lowest expression of the BAX protein would be the 10% FBS, because it showed the highest expression of cell growth with BCL-2.

This lab had many different phases in which there could have been sources of error. One error that would be remised to not include is that the western blot used for this lab was not of my specific results. The western blot that my lab group produced did not show the striation on the gel properly, and therefore could not be interpreted. Sources of error that could have led to an error in the western blot are; high concentrations of antibody, interference from an incompatible blocking agent, insufficient washing, and contaminated buffers. To better counteract these errors more caution in technique and procedure would need to be held, and sterile technique as well. Another source of error in this lab was the loading of the dyes into the gel wells. The procedure called for 26 uL to be loaded into the wells, however, in actuality this amount was too much for the wells. This lead to the leakage and overflow of the samples into other wells. This resulted in either too little of the sample in the well or the contamination of other wells. Another error that could have resulted in loading the wells was in poking the gel, and again causing leakage and too little of a sample to run down the gel. To fix this error one should practice utmost caution in loading the wells.

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