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Lab Report: Protein Isolation and Western Blot

Abstract:

The general goal of this lab was determining the expression level of the BCL-2 protein isolated from four different cell cultures which contained different treatments. The lab focuses explicitly on a protein called BCL-2, such protein can inhibit the apoptotic pathway which means under optimal growth conditions, such protein is expressed to prevent cell death. (lab manual, 2017) Overall, the hypothesis of this lab is the cells cultured in 10% FBS serum will have the highest expression of BCL-2 while the cells cultured in 10% FBS and camptothecin will have the lowest expression of BCL-2, in other words, the camptothecin was expected to have significant effects on expression of BCL-2. To testify such hypothesis, experiment methods including protein isolation, protein quantification by BCA and western blot were applied in this lab. According to the results, samples contained 10% FBS had the most thick and dark bands indicated it had the highest expression of BCL-2 which matches the hypothesis. However, the bands of sample 4 that contained camptothecin were relatively darker and thicker than the bands of sample 1 which only contained 5% of FBS. Such results indicate sample 4 had a relatively higher expression of BCL-2 than sample 1, and it doesn't match the hypothesis.

Introduction:

The general goal of this lab was determining the expression level of the BCL-2 protein isolated from four different cell cultures, and the media of four cells cultural contained different level of FBS serum and camptothecin. Basically, the cells in culture A grew in DMEM media with 5% of FBS serum, the cells in culture B grew in DMEM media with 10% FBS serum, while the cells in culture C and D grew in DMEM media

with 10% serum and camptothecin. According to the research, ‘camptothecin is a cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase I.’ (DrugBank, 2016) By binging to the topoisomerase I, camptothecin results in a ternary complex and prevents DNA re-ligation, which causes DNA damage and induces apoptosis in cells (DrugBank, 2016). Research indicates ‘apoptosis is a process of programmed cell death that occurs in multicellular organisms.’ (Douglas. 2011) And characteristic cell changes and death caused by biochemical events, these changes including ‘blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation.’ (Douglas, 2011) According to the research, there are approximately 70 billion cells die each day due to apoptosis in the human adult. (Douglas, 2011) Another media applied in the cell cultures is Fetal bovine serum (FBS), which is ‘the blood fraction remaining after the natural coagulation of blood, followed by centrifugation to remove remaining red blood cells.’ (Carlo, 2002) The major component of FBS is the globular protein called bovine serum albumin (BSA), such proteins have a rich variety and high level of nutrients which allows FBS maintains cultured cells in media to survive, grow and divide. (Carlo, 2002) By adding a different amount of camptothecin and FBS into the cell cultures media caused the diversity of cells growth rate, which means, at the same time, the protein isolated from four different cell cultures has different concentration as well. This lab focuses explicitly on a protein called BCL-2, such protein can inhibit the apoptotic pathway which means under optimal growth conditions, the protein is expressed to prevent cell death. (lab manual, 2017). In order to determine the relative expression of BCL-2 in four different protein samples, a technique

called Western blot was used. Usually, people use Coomassie G250 stain to stain and localize protein for further observation, however, for some low-concentration-proteins, Coomassie G250 stain could not visualize them. (Lab manual, 2017) Thus, Western blot has been developed in order to identify the proteins performed at low concentrations. (Lab manual, 2017) Essentially, to achieve a western blot, native proteins were separated by the 3-D structure, and denatured protein was separated by the length of the polypeptide by using gel electrophoresis, and SDS functioned as a buffer to provide all proteins with a uniform negative charge. (Gordon J, 1979) Such type of electrophoresis uses in western blot is known as SDS-PAGE, it helps with proteins to migrate randomly around, losing the distribution of size and resolution. (Lab manual, 2017) At the same time, SDS-PAGE ensures that proteins are separated by their size and prevents enzymes break them down from degrading samples.(Gordon J, 1979) After transferring proteins in the gel onto a charged membrane, milk were applied in order to block membrane which can prevent non-specific antigens binding, and then stained with primary antibody to the specifically target protein.(Lab manual, 2017) An antibody is a large Y-shaped protein produced plasma cells and used by the immune system, it can recognize a unique molecule of the pathogen, called an antigen, and each antibody binds a specific antigen (Janeway, 2001). In this lab, by adding a primary antibody, is attached to the target protein, in this case, BCL-2. Then the secondary antibody were used to recognize the primary antibody. Lastly, chemiluminescence were used in this lab to bind to the secondary antibody as detection methods which will luminesce when exposed to the reporter on the secondary antibody. Overall, the hypothesis of this lab is the cells cultured

in DMEM media with 10% FBS serum will have the highest expression of BCL-2 while the cells cultured in DMEM media with 10% FBS and camptothecin will have the lowest expression of BCL-2, in other words, the camptothecin was expected to have significant effects on expression of BCL-2.

Materials and Methods:

Part A: isolating the protein

After passaging cells into larger Petri plate, so that they had more room to grow, the proteins got isolated from the cells.

First, four 1.5 ml centrifuge tubes got labeled to match the four dishes of cells cultured previous.

Then the media from the cell cultures got removed, and the cells left in the bottom of dishes were washed twice with 2 ml of cold PBS. Removed PBS as much as possible.

Then, 500 µl of M-PER with protease and phosphatase got added to each dish.

Scraped the cells and transferred the cells with M-PER to the labeled microcentrifuge tubes, then tubes were placed in a microcentrifuge rack.

Then the cellular contents got mixed by pipetting them down up and down for five times and incubated the tubed on ice for 35 minutes.

After incubation, the protein samples got span at 4 °C for 5 minutes at 12,000 rpm. Then the supernatant got moved to the new tubes, and pellet got discarded.

Part B: Quantifying the protein (BCA)

After isolating proteins from associated cell cultures, protein samples got quantified by using the BCA protein assay.

First, retrieved a 96 well microplate, then 25 µl of each standard were added into wells A2-A10.

Then, five µl of each of the protein samples got transferred to wells B1-B4 (protein sample 1 to B1, protein sample 2 to B2, protein sample 3 to B3, and protein sample 4 to B4). Immediately after, 20 µl of M-PER protein lysis buffer got added on top of protein samples, which provided a 1:5 dilution. Later, 200 µl of the working reagent added to wells A1-A10 and B1-B4.

Lastly, the plate got covered and incubated at 37°C for 30 minutes.

Part C: western blotting

First, around 4-12% of gel got removed from the protective pouch. The gel cassette got cleaned by deionized water and peeled off the tape from the bottom of the cassette.

The pulled out the comb from the cassette, then, the gel was placed in the gel box with the black writing faced the outside.

After setting the gel in the box, locked the gel into place using the Gel Tension Wedge.

Then, the upper butter chamber was filled with a small amount of running buffer to check for tightness of seals, and the upper buffer chamber got filled with 200 ml of 1x Running buffer once it got sealed tight.

After adding the Running buffer, set the gel for 10 minutes to equilibrate. And the protein samples got to heat up for 10 minutes at 70 degrees Celsius during the time waiting. Then put the samples back on ice immediately after.

Later, samples got loaded into lanes 2-9 of the gel (lane 2 and 3 were filled by samples from cells cultured in 5% FBS, lanes 4 and 5 were filled by samples from cells cultured in 10% FBS, and lanes 6,7,8 and 9 were filled by samples from cells cultured in 10% FBS + camptothecin).

The protein marker got loaded into lane 11, and nothing got loaded in lane 10.

After loading all samples into the gel, added 800 ml of 1x Running buffer to fill the lower buffer chamber and ran the gel at 200 Volts for 30 minutes.

Disconnected the electrodes and removed the gel from the gel box. The gel knife got inserted into the gap between the cassette's two plates with the notched 'well' side faced up. Then, pushed down on the knife handle to separate the plates, repeated the same procedure on each side of the cassettes until the plate got separated entirely. And the top plate got removed, and gel remained on the bottom plate.

Placed a labeled nitrocellulose membrane in transfer buffer, to presoak it. Then removed the wells with the gel knife after opening the gel cassette, and the foot of the gel, in the same manner, got removed at the same time.

Placed a piece of pre-soaked filter paper on the top of the gel, and used a roller to roll over the paper to remove all trapped air bubbles.

The plate got turned over, so the gel and filter paper faced downwards and pushed the gel through the slot.

The gel positioned on the pre-soaked transfer membrane, and another pre-soaked filter was used to cover the gel at the same time. Then two gels (one from the other group) were assembled as shown in Figure 1.

The blot module got slid into the guide rails on the lower buffer chamber, then placed the Gel Tension Wedge with its vertical face against the blot module.

Then the blot module was filled with transfer buffer until the gel/membrane assembly covered. Meanwhile, the outer chamber got filled with deionized water.

The samples got transferred by ruining the gel at 30 V for 80 minutes.

After transfer, the samples placed in non-fat milk, and primary antibody got added, and samples got incubated at 4 °C overnight.

Later, the membranes got washed in 10 ml of TBST, and rocked the membrane for 10 minutes, poured out TBST as much as possible, and repeated these steps two more times.

Then 10 ml of secondary antibody solution were added and rocked the membrane for 45 minutes at room temperature.

After rocking the membrane washed them with TBST for three more times (10 minutes each).

The membrane got removed onto a piece of Saran Wrap with protein side up, and 2 ml of developing solution were added onto the membrane and stayed for 5 minutes.

After 5 minutes, the membrane was picked up by forceps and placed on a Kimwipe to remove extra the developing solution. Then transferred the membrane to a sheet protector.

Finally, the banding pattern got visualized and observed.

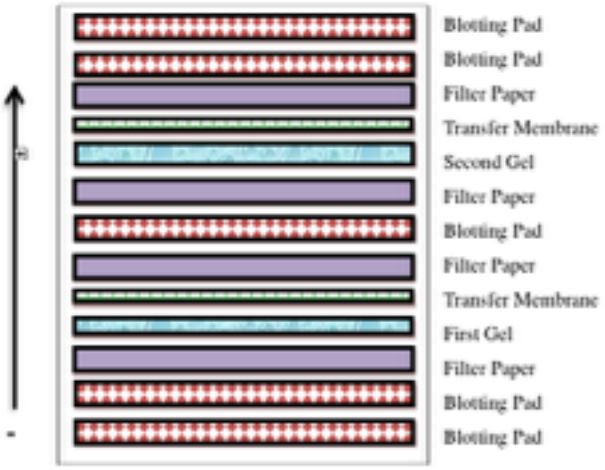

Figure 1. Orders to assemble 'gel sandwiches'.

Figure 1. Figure 1 shows the way to assemble the 'gel sandwiches' for transfer. 'The arrow represents the direction of current flow that will transfer proteins from gels onto the transfer membranes.' (Lab manual)

Results:

After the experiment, four cell cultures with different treatments and the associated protein concentrations got collected in Table. 1. In sample 1, the treatment used in cell culture was 5% FBS serum, and the associated protein concentration is 1.54 μg/μl. The treatment used in sample 2 was 10% of FBS serum, and the related protein concentration is 2.00 μg/μl. In sample 3, the treatment applied was 10% of FBS serum and camptothecin, the associated protein concentration is 1.18 μg/μl. Sample 4 had the same treatment as sample 3, and the associated protein concentration is 1.11 μg/μl.

Meanwhile, Table 2 collects the corresponding volume of each component applied in Western Blot, including the volume of protein for 20 μg, the amount of MPER and the volume of the loading dye. In general, in sample 1, the total volume that contained in 20 μg of protein was 13 μl, it received 7 μl of MPER to reach 20 μl, and 6 μl of loading dye was added to visualize the sample. In sample 2, the total volume that contained in 20 μl of protein was 10 μl, and 10 μl of MPER was added to reach 20 μl, and 6 μl of loading

dye was added in order to visualize the sample. Meanwhile, in sample 3, the total volume that contained in 20 µg of protein was 17 µl, and it received 3 µl of MPER to reach 20 µl. In sample 2, the total volume that contained in 18 µl of protein was 10 µl, and 2 µl of MPER was added to reach 20 µl. In sample 3 and 4, 6 µl of loading dye was added to visualize the sample as well.

Figure 2 shows the picture of Western blot, all the way to the left (lane 11) is the ladder of the pre-stained protein stander, and the molecular weight (in kDa) is decreasing from top to bottom. In the experiment, the same amount of protein was loaded into each lane, and the lane's order from left to right is Lane 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11. Sample 1 was loaded into lane 2 and 3; sample 2 was added into lane 4 and 5, while sample 3 and 4 were added into lane 6,7,8 and 9. Nothing got loaded into lane 1 and 10, and the one next to the line 10 is the ladder. In general, lane 5 had the thickest and darkest band, and the sample associated to lane 5 was sample 2 which contained 10% FBS. And lane 2 and lane 3 (5% FBS) had relatively lighter bands than lane 6,7, 8 and 9 (10% FBS + Camptothecin). At the same time, lane 2 was located at approximately 190 kDa of the ladder, while BCL-2 located was around 26 kDa.

Table 1. Treatments Applied in Four Cell Cultures and Associated Protein Concentration

Sample	Treatment	Protein Concentration (µg/µl)
1	DMED + 5% FBS	1.54
2	DMED + 10% FBS	2.00
3	DMED + 10% FBS+ Camptothecin	1.18
4	DMED + 10% FBS+ Camptothecin	1.11

Table 1: Table 1 includes the different treatments applied in four samples and the associated protein concentration of four samples. In sample 1, the treatment used in cell culture was 5% FBS serum, and the associated protein concentration is 1.54 µg/µl. The treatment used in sample 2 was 10% of FBS serum, and the related protein concentration is 2.00 µg/µl. In sample 3, the treatment applied was 10% of FBS serum and camptothecin, the associated protein concentration is 1.18 µg/µl. Sample 4 had the same treatment as sample 3, and the associated protein concentration is 1.11 µg/µl.

Table 2. Preparations of Four Samples for Western Blot

Sample	Volume protein for 20 µg (µl)	Volume MPER (µl)	Volume loading dye (µl)	Total volume (µl)
1(5% FBS)	13	7	6	26
2(10% FBS)	10	10	6	26
3(10% FBS+C)	17	3	6	26
4(10%FBS+C)	18	2	6	26

Table 2: Table 2 collects the corresponding volume of each component applied in Western Blot, including the volume of protein for 20 µg, the amount of MPER and the volume of the loading dye. In sample 1, the total volume that contained in 20 µg of protein was 13 µl, it received 7 µl of MPER to reach 20 µl, and 6 µl of loading dye was added to visualize the sample. In sample 2, the total volume that contained in 20 µl of protein was 10 µl, and 10 µl of MPER was added to reach 20 µl, and 6 µl of loading dye was added in order to visualize the sample. Meanwhile, in sample 3, the total volume that contained in 20 µg of protein was 17 µl, and it received 3 µl of MPER to reach 20 µl. In sample 2, the total volume that contained in 18 µl of protein was 10 µl, and 2 µl of MPER was added to reach 20 µl. In sample 3 and 4, 6 µl of loading dye was added to visualize the sample as well.

Figure 2. Picture of Western Blot

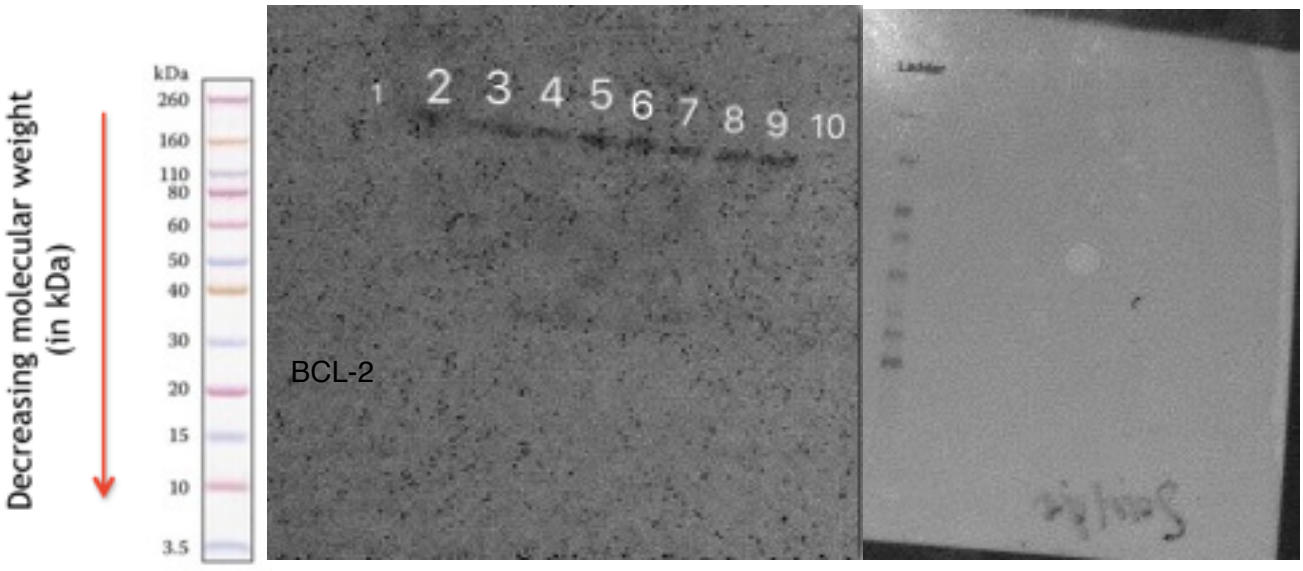

Figure2: Figure 2 includes the picture of Western blot and the pre-stained protein standard which is used as a reference. In the picture of the western plot, the lanes order from left to right is Lane 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11. Sample 1 was loaded into lane 2 and 3; sample 2 was added into lane 4 and 5, while sample 3 and 4 were added into lane 6,7,8 and 9. Nothing got loaded into lane 1 and 10, and the image next to the

lane 10 is the ladder. In general, lane 5 had the thickest and darkest band, and the sample associated to lane 5 was sample 2 which contained 10% FBS. And lane 2 and lane 3 (5% FBS) had relatively lighter bands than lane 6,7, 8 and 9 (10% FBS + Camptothecin). At the same time, lane 2 was located at approximately 190 kDa of the ladder, while BCL-2 located was around 26 kDa.

Discussion:

In this lab, cells were cultured under four different treatments, which caused varied growth conditions. In general, cells grew in 5% FBS (sample 1) had the confluence about 20% with approximately two and three dead cells. Cells grew in 10% FBS (sample 2) had 30% of confluency and four dead cells, and cells grew in 10% FBS +camptothecin (sample 3 and 4) had 40% of confluency with eight dead cells. After the observation, one can tell sample 1 had the lowest confluency and lowest number of cells killed while sample 3 and 4 which contained camptothecin had the highest confluency but contained the most amount of dead cells at the same time.

According to the research, if the cells are not undergoing apotheosis, the high expression of BCL-2 can be observed. Oppositely, if cells that are undergoing apoptosis, a high expression of BCL-2 will not be able to observed (Lab manual, 2017). In this lab, according to figure 2, one can tell the darkest lane was lane 5 which associated to sample 2. Such result indicates that treatment with 10% FBS had the highest expression of BCL-2 compared to other treatments, and it matches one of the hypothesis. Since the major component of FBS is the globular protein called bovine serum albumin (BSA), and such protein such proteins have a rich variety and high level of nutrients which allows FBS maintains cultured cells in media to survive, grow and divide(Carlo, 2002). Thus, it explains why sample 2 had the highest expression of BCL-2. Meanwhile, compared to lane 2 and lane 3, lane 6,7,8 and 9 had a relatively darker color, which means samples

contained camptothecin had a higher expression of the BCL-2 protein than samples doesn't have any camptothecin. However, due to camptothecin's unique characteristic, by adding the camptothecin to the cell culture, cells supposed to undergo apoptosis which will cause a very low expression of BCL-2 instead of having a darker band. Thus, the potential causation could be the wrong amount of protein got loaded into the gel, essentially, there is more volume of sample 3 and 4 got transferred into the lanes of the gel compared to the amount of sample 1 got transferred. According to the research, inappropriate antibody concentration might cause the inaccuracy of the results, and sometimes prolonged washing can also decrease the signal. Moreover, buffers can also contribute to the problem, thus, during the process, one should make sure that the buffers like TBST and running buffer should be new and non-contaminated. Meanwhile, if the sodium azide included in the buffer, it will inactivate HRP, and makes the results inaccurate as well (Yang, 2012).

Meanwhile, after antibodies bound to target proteins, the molecular weight of the bands are assessed by how far they traveled down the gel, and comparing their position to the ladder used in the first lane (Arold, R, 1973). Thus, since the band of BCL-2 is located approximately near 26 kDa in the ladder, which suggests the size of the BCL-2 is 26 kDa.

Besides BCL-2, another protein that can be expressed in fibroblast cells is BCLAF1, which would show variation between treatments, creating an interesting study. Bcl-2-associated transcription factor 1 (BCLAF1) belongs to BCL-2 family protein, and 'it encodes a transcriptional repressor which interacts with several members of the BCL-2

family of proteins.' (Nagase, 1996) Meanwhile, apoptosis would be induced with over-expression of such protein, but co-expression of BCL-2 proteins could suppressed apoptosis. (Entrez Gene, 2017) Thus, an experiment could be designed to detect the levels of BCLAF1 in cells that were grown under different types of growth media, and how they effects protein expression and concentration.

According to the research, 'cell culture media usually comprise an appropriate source of energy and compounds which regulate the cell cycle, and a typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose and serum as a source of growth factors, hormones and attachment factors.' (Arora, 2013) Obviously, depends on the characteristic of cells, different types of media can be used for growing different types of cells. FBS is a high-functional medium for growing cells, due to its 'high content of embryonic growth promoting factors' (Carlo, 2002) which provide cells with abundant nutrients. And it specifically contains protease inhibitors which can help regulating cells growth as well. In this lab, 10% FBS would be the best media for growing cells as cells grew in this treatment had the highest expression of BCL-2 which means compared to cells grew in other media, cells grew in 10% FBS had the least number of cells undergo apoptosis.

Lastly, in order to minimise errors in the future, during the process of transferring protein samples into the gel, one should be conscientious and make sure the volume of protein is accurate. Meanwhile, during the experiment, one should make sure the control variables like the concentration of antibody, time of cell washing, the temperature of incubation are identical all the time.

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