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Parthenolide Cytotoxic Effect on GC-2SPD Cancer Cell Line

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Abstract
The purpose of this experiment is to test the effect of the drug Parthenolide on cancer cell line GC-2SPD (2196) in vitro. This will be done by testing dose range of the drug at different two temperature; 32C, and 39C. The cells were grown at in a very similar environment as they were inside the body and they were conditioned to grow in spheroid architecture to resemble their structure as solid tumor in the tissue. Growth material was needed to maintain viable cells and routine subculture was performed as needed. Cells were drugged with different concentrations of Parthenolide and assayed, and then they were let to recover for some time and then assayed again to actually asses the ratio of viable cells to dead cells. Although there were some unintended mistakes, the experiment was successful and yielded good results. The cells showed more sensitivity at 39C as photos and curves showed.
Keywords: Parthenolide, cytotoxicity.

Purpose:
The purpose of this experiment is to test the effect of different dosages of the potential drug "Parthenolide" on cancer cell line GC-2SPD at two different temperature; 32C, and 39C, and demonstrate the results by constructing a growth curve and photos.

1. Introduction

Cancer is one of the abnormalities, deregulations or malfunctions, and may be caused by environmental and physical forces that alter the machinery of the body. Cancer develops when the cells grow out of control due to abnormal cell proliferation. It can be caused by external factors like (chemicals, radiation, smoking, etcetera), or by internal factors like (inherited mutations, hormones, or immune system malfunctioning, etcetera).

Tumors are in general two types; benign, and malignant. Benign tumor is noninvasive and poses no risk; however, malignant tumor is invasive to the circulatory system, lymphatic and surrounding tissues. Both nutrients supply and blood vessels increase the tumor size. Malignant cells are the cancerous cell and it mainly arises from mutations in somatic cells. Mutation represents the primary step in carcinogenesis. Beside the external factors that cause mutations, genomic instability can be a cause as well. Cells go through a cycle, and DNA gets copied but to ensure the complete and precise copying of DNA, cells pass G1 and G2 periods as checkpoints. The loss of this control, DNA damage accumulate and tumorogenesis results (3).

One among the tumor suppressor is gene p53 which is located on chromosome 17. P53 is a DNA binding protein that is essential for checkpoints at both G1-to-S, and G2- to M. It detects DNA damage and tells the cell to either stop growing until damage is repaired, or to kill itself by apoptosis. This gene is actually made up of 393 amino acids, and its name is derived from "p" for protein and 53 because of its molecular mass of 53,000 atomic mass units (4). P53 is found suppressed in most of cancer patients.

For this experiment purpose, GC-2SPD cell line was used. It was established by stable cotransfection of freshly isolated spermatocytes with the SV40 large antigen and a temperature sensitive mutant of the p53 tumor suppressor gene. The cells are found to be highly proliferating at 39C and slowly at 37C (1).

Scientists have studied different synthetic drugs and came up with some that showed some effect but with no 100% cure. On the other hand, one of the possible curing agents was found in naturally occurring plant that has been out there in nature for decades.

Herb Feverfew (*Tanacetum Parthenium*) was found to have a component that has an anti-inflammatory significance to it. Feverfew is also known as midsummer daisy, is a perennial herb classified in the family

Asteracea. It is found growing wild from Quebec to Ohio and South to Maryland and Missouri. It is used commercially as herbal/ homeopathic therapies as an anti-migraine remedy. The active ingredient of feverfew is the sesquiterpene Lactones (SL) Parthenolide (3). Recently, a research by Nakshatri et. Al. (2) has suggested that Parthenolide anti-inflammatory properties may be used as cancer chemotherapeutic drugs. He substantially performed some experiments to demonstrate that Parthenolide is the biological active component of feverfew, yet nevertheless, additional researches are needed to confirm the mechanism of its anti-inflammatory activity.

Parthenolide structure is composed of both exomethylene group and epoxide group, and it works by inhibiting IKKB transcription factor via NF-kB pathway. To actively test the effect of Parthenolide on cancer cells, these cells needed to be cultured outside the body in "glassware", what we call now in vitro. They have been grown in both monolayer (two- dimensional), and spheroids (three -dimensional). As a matter of fact, tumors grow naturally as in three-dimensional configuration. Spheroids in turn, consist of three distinct layers or regions; an outermost proliferating layer, intermediate quiescent layer, an innermost necrotic layer. These layers represent a measure of different sensitivity to dugs due to drug accessibility through the spheroids layers (3).

Cell culture requires the following:

- Medium, a mixture that is similar in composition to body fluid; it contains nutrients such as amino acids, glucose, hormones, trace elements, vitamins, and fatty acids. There are many kinds of media such as Eagle's, DMEM, RPMI-1640, and McCoy's.
- Serum is the most important part that is added to media, and has pH buffers and Growth factors, which promotes proliferation such as PDGF. Media contains 10% animal's serum.
- Media pH for maintaining growth for most cell lines should be between 7.0- 7.4.
- Co2 incubator, cells need to grow in 5-10% Co2 because pH must be maintained by equilibrium reaction between Co2 and Sodium bicarbonate (buffer in serum).
- Temperature, temperature should be consistent with the human or animal temperature (37°) from which cells were taken.

2. Materials

Some materials and tools were required:

- Cell line GC-2SPD (2196- cancer from ATCC).
- Media (RPMI-1640) purchased from Fisher, Trypsin from Fisher.
- Fetal Bovine Serum from Atlanta.
- Trypan blue for assay.
- Hoechst dye 33342.
- Powder Parthenolide.
- Serum from Biological.
- Hood, where air flow constantly to avoid contamination.
- Incubator to store cells and assayed cells at (37°).
- Refrigerator to keep media, trypsin (Proteolytic enzyme causes cell to detach from the growth surface), and PBS (to wash cells).
- Centrifuge.
- Sterile vessels used under the hood such as 24- well plates, 12-well plates, eppendorf tubes, etc.

3. Method

Routine subculture (passage) procedure requires stock of cell culture media provided by a source. All media preparation and other cell culture work must be preformed under the hood, which is wiped with ethanol. Before begin in subculture procedure, stock cells need to be inspected visually and preferably under the microscope.

Subculture procedure:

1- Remove media only from 3 wells out of 12, and discard
2-Wash cells with1-mL of PBS to cells to each well
3-Remove PBS and discard in liquid waste
4-Add 0.5-mL of trypsin to each well, let them set for 5 minutes, and mix well
5-Add 0.5-mL media to each well and mix well.
6- Each well will be divided to 4 new wells.

Fixed density plating was performed, in order to plate an appropriate number of cells in each well; this technique is done before dividing the cells into desired wells in the subculture procedure. This is can be done by loading the hemocytometer and count the cells under the microscope. The area of the grid can hold 10^-4 ml, and it's known the volume in the well is 1-mL. Whatever average count is obtained will be multiplied by 1 –mL and divided by 10^-4 to get the number of cells should be planted. After getting the number of cells, it should be divided by 5x10^4 cells/well to come up with the volume of each new well, and thus the split ratio was done.

Cells first were grown as monolayer on non- agaros dishes. Then transferred to agaros coated dishes to

inhibit cell- attachment. By doing so, cells will form solid bodies called spheroids resembling malignant cells in the body. To make spheroids, 2 flasks of stock were used per dish, 1-mL of media per well, and 0.2 of cells per well or less. One dish was put at 32c, and another at 39C. After couple of days, spheroids formed, and drug was applied.

Powder drug was dissolved in DM50= 50 mM. From 50 mM drug solution, a 1:100 solution was done by preparing 1-mL PBS in an eppendorf tube and adding 10 micro- liters of stock drug and mix. To prepare a 1:10 dilution, serial dilution was prepared by taking 100 µL of the 1:100 solution and adds it to 900µL PBS in eppendorf tube. Final working solution is 0.05mM or 5 µM and final volume is 1- mL. Each dish was drugged with a range of drug dosage as the following: Control (no drug), 1.25 mM, 2.5mM, 5mM, 7.5mM, and 10-mM.

Staining procedure was used to enable us to see the cells, and the dye Hoechst (33342 5mg/ml) was used following these steps:

1- Transfer spheroids and media to 1.5 mL eppendorf tube with a pasture pipette.
2- Add about 2µL of 5mg/mL Hoechst stain to each tube and mix.
3- Incubate tubes at 37C water bath for 10 minutes.
4- Transfer tubes to ice bucket; let them chill for about 5 minute until cold.
5- Spin one minute 10,000 rpm in micro- centrifuge.
6- From now and on work in ice bath bucket. Remove and discard the media.
7- Add 0.5 -mL ice cold media to eppendorf tube, mix gently.
8- Transfer 1 drop of cells (pellet) to a clean glass slide and add the cover slip.
9- Examine slides with fluorescence microscope and generate pictures.

After several days, drugged cells were ready to be assayed, and assay procedure is as follows:

1- Since this experiment was done in duplicates (2 wells of control and so on, the media of each well was taken out into an eppendorf tube.
2- Add a ½ mL of PBS to the well, discard
3- Add ½ mL trypsin to the well and let it for 5 minutes, add to media in eppendorf tube
4- Spin them in micro- centrifuge at 10,000 for about 5 minutes
5- Discard media, add ½ PBS to pellet and mix well.
6- Take about .2 mL into a new well with fresh media as recovery plate. Recovery plate was marked according to drug dosages.
7- Add.2 mL of Trypan blue to the rest of cells in eppendorf tube and leave for 15 minutes.
8- Fill hemocytometer and read 4 counts from each eppendorf tube. Clear cells indicating viability, and blue cells indicated death. The two sides will be counted by obtaining an average of only viable cells. This will be done twice to get 8 counts.

Recovery plates were assayed as well following same steps.

4. Results

By utilizing Prism computer program for scientific work, different cytotoxicity graphs were constructed, and pictures of cells on slides were taken. Graphs and pictures are attached. The most significant slides are:

- 32C control at 10x magnification.
- 32C 5mM at 10x magnification.
- 39C 5mM at 4x magnification.
- 39C 10 mM at 10x magnification.

The graphs of relative viability due to drug doses are:

- Drugged cells at 32C.
- Recovered cells at 32C.
- Drugged cells at 39C.
- Recovered cells at 39C.

5. Discussion

According to the drugged cells at 32C, the cells viability showed a dose dependant effect; where the drug inhibited cell growth but was not toxic. Maximum effect was found at 2µM; about 75% reductions in cell number. It's obvious by looking at the graph that increasing dose of drug had no effect. This data can be supported by comparing stained spheroids after drugging them. At 10x, 32C control, spheroids showed less staining, uniform surface and spheroid is more intact. At 5µM, spheroid seems to be intact with slight breakdown which suggests minor drug effect. On the other hand, recovery plate showed toxic effect at 7.5µM where cell count went down to about zero and did not recover. As suppose to recovery dish photos, at 7.5µM the cells seems to be rounded up and stressed which shows the cytotoxicity of this drug.

At 39C, drugged cells showed dose dependant effect; maximum effect was at 10µM with cell count

of .5 x10^4 viable cells, whereas recovery cells showed significant recovery at 10µM with cell count .5x10^4 as well. Obviously cells at 39C showed better response to drug than 32C cells and this can be seen at the 4x magnification of the 5µM. Spheroids are more stained, and the exterior surface started to break down significantly affected by drug. At 10µM, the spheroid is showing even more breakdown of the exterior surface. Just as the drugged cells showed response at 10µM, the recovered cells at the same concentration were stressed and apoptotic.

6. Conclusion

In conclusion, Parthenolide showed better effect at 39C than 32C that causes cell death. Although some minor experimental errors may alter the results of this experiment, prominent cytotoxic effect was observed. Further confirmation experiments may be required to determine the effect of this new agent in curing cancer patients, which may claim this feverfew agent as new remedy in the age of cancer.

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