Cell culture and proliferations | experiment

ASSIGN BUSTER

To study the cell proliferation of Mouse Y1 adrenocotical cells by using MTT (3-(4, 5-diphenyl tetrazolium bromide) and crystal violet staining methods.

INTRODUCTION

In general, Cell proliferation means multiplication/Reproduction of cells for increasing cell population in a very short span of time. The assay of cell proliferation is to measure the number of cells which are present in the dividing culture medium. Cell proliferation is controlled by using growth factor (Fetal calf serum), Which normally bind to the surface receptors present on the cell membrane generally regulate the cell signaling molecules, which pass the message to nucleus by help of receptor generally where the transcription factor gets bind to the DNA, makes turn off turn on the protein synthesis mechanism, responsible for cell division. Cell proliferation method is very important for studying various biological factors like bioassay, carcinogenic analysis and other toxicological tests. Generally two metods are used for studying cell proliferation i. e. crystal violet staining method and MTT (3-(4, 5-diphenyl tetrazolium bromide) method, here these methods are used to study cell growth in mouse Y1 adrenocotical cells.

In crystal violet staining method, the DNA of cells is going to stained by crystal violet which produces a colour intensity that is proportional to the cultured cells(including newly proliferated cells). In this method, the principle involved to calculate the cell proliferation is based on the absorbance taken up by the viable cells in culture at different concentrations after the cells are stained with crystal violet.

In MTT (3-(4, 5-diphenyl tetrazolium bromide) method, the assay depends on the amount of MTT taken up by the cells, tetrazolium salt is water soluble which produces yellow colour. The tetrazolium MTT is metabolically reduced by active cells, in presence of dehydrogenase enzymes, producing NADH and NADPH which are reducing equivalents. This results in the formation of purple formazan intracellularly, which is measured by spectrophotometer.

MATERIALS & METHODS

Cell culture:

In DMEM (Dulbecco's modification of eagle's medium), mouse Y1 adrenocotical cells which were grown on monolayer was removed by using mixture of trypsin and EDTA (0. 05% and 0. 02%). The cells are incubated by 5minutes by adding Trypsin/EDTA(7ml). After incubation the flask was removed and tapped gently to separate undetached cells. The contents of the cell are transferred to a universal container for centrifugation at 1000rpm/ 5mins. Supernatant was discarded and medium is added for resuspending the cell pellet. The cell number was estimated by using Haemocytometer counter for 15ml the cell suspension dilution was prepared containing density 1. 25X105 cells/ml of suspension. In 96 well plate, 60 wells were filled with 100µl cell suspension, in this the remaining outer wells are filled with Phosphate Buffer Solution (PBS) of 200µl. Allowed the plate overnight in a gas incubator to settle down the cells. Those cells were treated with Fetal Calf Serum (FCS) of different concentrations varying from 0% to20% in universal tubes. 12 wells of plate were filled with 200µl of different concentrations. These plates were incubated for a period of 24hours. Cells were washed with Phosphate Buffered Solution (PBS) for three

times using multichannel micropipette; later media containing various serum concentrations were added. These two 96 well plates were used for crystal violet staining and MTT (3-(4, 5-diphenyl tetrazolium bromide) assay after 72 hours of incubation.

Crystal Violet Staining Method:

The cells were removed from central 60 wells of 96 well plates and filled with 200µl of methanol in a fume cupboard. After 15minutes methanol was removed from the plate and was left to dry in the fume cupboard. Once the plates were dried the cells were stained with 200µl of crystal violet. Once the staining is complete after 20minutes the plates were washed with Distilled water for atleast three (3) times and then solubilized the cell layer by using 50µl of 10% glacial acetic acid. The plates were then kept for incubation in a gas incubator for thirty minutes after incubation Absorbance of wells was measured at 540nm.

MTT STAINING METHOD:

The cells present in the central 60 wells of 96 well plate were treated with 20µl of MTT (5mg/ml solution in PBS) and was left for 4hours in gas incubator at 370c. After incubation by using multichannel pipette medium was removed and 100µl of acid-isopropanol was added in order to dissolve the blue formazan crystals from the cell layer and then it was incubated for 30minutes at room temperature after solubilizing formazan crystals Absorbance was measured at 570nm using plate reader.

Calculation:

Total No. of cells in 5 square = 21

Average cells present in one square = 4. 2

Calculation of cell number:

The volume of each square is= 4 X 10-3

The total cell number for 5 square gives the cell = 0. 02µl

No. of cells in 1 ml = 105X104

Number of cells required = 395X104

Volume of suspension required = concentration required/ concentration got

= 395X104/105X104

= 3. 7619 ml cell suspension to be taken

Medium to be taken = 30, 000µl-3. 7619µl

= 29996. 23µl medium to be taken.

DISCUSSION:

Here the increase in the absorbance with corresponding to the fetal serum concentrations shows the sign for the cell growth. Ammonium cations bind to negatively charged DNA which in turn gave blue color to the mixture. By using the color intensity, viable cells were estimated by means of haemocytometer. No experiment will produce 100% results. So here also errors occurred due to practical errors.

Occurrence of errors might be due to:

- Washout condition of stained culture cells

- Improper solubilisation of 10% glacial acetic acid.

In MTT method the degradation of MTT gives color to the mixture. This degradation was due to the dehaydrogenases of viable cells. The color intensity is directly proportional to the cell growth. Here also the errors might be occurred due to improper solubilisation of formazan crystals (Butler. 1996), (Javoise. 1998).

DIFFERENTIATION OF K562 CELLS TO PLATELETS IN PRESENCE OF PMA:

Differentiation of K562 cells to megakaryoctes/platelets

Phorbol Myristate Acetate treated and untreated cells were spun down in a bench centrifuge and after resuspended in 1ml PBS (having 1 % Bovine serum albumin). Then by using haemocytometer the cell number was calculated, after diluting the suspension. cytospin was added to 1 ml cell volume which was adjusted to density of 106 cells per ml. In assembled cytospin 200 µl at 1000 rpm/3min. After fixing the slide in acetone /methanol(50: 50), slide was washed with 0. 15 M tris buffered saline . In humid temperature Human cd61 cells were incubated for 2 hrs & using TBS slide was washed. with rabbit anti- mouse Ig-G anti body cells incubated for for 30 mins at room temperature and washed with TBS, after washing, cells were incubated with Alkaline phosphate anti-alkaline phosphate complex, this was repeated with Ram and APAP for amplification. They were washed under running tap water after staining with red TR substrate and counter was stained with haemotoxylin. Finally the slide was viewed under microscope after washing with TBS. PMA is a diester of phorbol and a tumor promoting

agent (proc. Natl. Acad. sci. USA Vol. 82, pp, 3859-3862, june 1985Medical sciences).

PMA initiates the signal transduction by protein kinase C (PKC) enzyme which allows promoting the differentiation of K562 cells. By using CD 61 marker the K562 cells were treated . These CD61gets attached to cell network to work as primary antibodies. In addition with cells performs a seconndary antibobodies whenever exposed to Ram along with APAP and forms pink color by attaching to FC region of anti-human CD 61 antibodies. This phenomenon gives the cells under going differentiation when incubated with PMA (MSc Pharmacology & Biotechnology, cell biology laboratory manual/ January 2010)

RESULT:

The slide treated with Phorbol Myristate acetate (PMA) is in pink colour, whereas the slide which is untreated with Phorbol Myristate acetate (PMA) is in blue colour after staining. The cells when treated with PMA differentiate into Platelets/Megakaryocytes. In PMA the diseter bond promotes the tumor, which in turn activates the signal transduction of protein kinase C enzyme(PKC) inn K562 cells causes the CD61 expression.

The RAM IgG gets attached to the CD61 antibodies, these K562 cells when incubated with APAAP form a complex. Later fast red dye was added to the mixture which gets attached to the APAAP, the cells turn pink by taking the stain. The cells containing PMA expressed the CD61.

It generates the (signal transduction protein kinase C) PKC enzyme and on of K562 cells causes the expression of CD61. The rabbit antimouse IgG

antibodies attach to the antibodies of CD61 when incubated in the presence of APAAP (alkaline phosphatase anti-alkaline phosphatise) on of K562 cells complex. Then we add the fast red dye to the mixture which was attached to APAAP (alkaline phosphatase anti-alkaline phosphatise) and stains the cells pink. CD61 was expressed by only those cells which had PMA (Shelly, 2000)