Effect of growth factors fcs on cell proliferation biology essay

Introduction:

The technique in which cells normally of one type, removed from animal tissue are grown in presence of supplements and growth factors under controlled conditions is called Cell Culturing. Different cells require different conditions of temperature and gas for their appropriate growth in a cell incubator moreover growth media plays a crucial role in cell culturing, which can vary in glucose concentration, pH, growth factors like fetal calf serum (FCS).

The effect of one of the above mentioned variables, we are going to study in this experiment is growth factors. Growth factors as the name suggests enhancers the growth which supplements the media are obtained from animal blood such as calf serum.

Cell proliferation is studied by various methods, mainly used methods involve measuring the colour intensity taken up by DNA of cell during cell proliferation in presence of staining dyes e. g. crystal violet and MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide).

The aim of this experiment is to study cell proliferation by using crystal violet and MTT methods while using mouse Y1 adrenocortical cells under the effect of growth factor FCS.

Mouse Y1 adrenocortical cell are grown in Dulbecco’s modification of Eagle’s medium as monolayer cultures.

Crystal Violet:

It is biological stain used in cell proliferation method which stains DNA of the cells after permeabilisation with methanol. The positive ammonium ions of crystal violet stain DNA of cells in blue colour.

The colour intensity produced is directly proportional to number of cells, which is determined in stained cells by colorimeter. Staining with crystal violet and MTT are based on principal of absorbance.

MTT:

The MTT assay is a standard colorimetric assay in which activity of reductase enzymes are measured which convert MTT to blue insoluble formazan. The metabolic activity of viable cells is used as basic parameter in MTT assay. The tetrazolium salt, MTT is now widely used in quantitative measurement of cell proliferation. This method depends upon uptake of MTT by viable cells, which is a water soluble tetrazolium salt producing a yellowish solution. MTT is cleaved to insoluble blue formazan in the mitochondria of living cells by succinic dehydrogenases in the mitochondrial respiratory chain.

Material Used:

Mouse Y1 adrenocortical cells, Dulbecco’s medication of Eagle’s medium (DMEM) containing 10% Fetal calf serum, 2mM glutamine, 100u/ml penicillin and 100mg/ml streptomycin, crystal violet stain (0. 1% solution in 200mM boric acid), MTT (5mg/ml MTT solution in PBS), PBS methanol, 10% glacial acetic acid, distilled water, acid-isopropanol.

Apparatus used:

Laminar flow cabinet sterilized two 96 well plates, multi well pipettes, pasture pipettes, sterilized T-flasks, sterilised empty reservoirs, gas incubator, fume cupboard, spectrophotometer etc.

Method:

Cells of mouse Y1 adrenocortical were separated from their substratum with tris in EDTA as they grow in monolayer cultures. Then added same volume of medium and centrifuged after that number of cells were counted on haemocytometer and diluted to concentration of 1. 25 â…© 105 cells/ml and made it up to 30 ml.

Then cells were passaged into centre 60 wells of 96 well plate in duplicate with concentration of 0. 25 â…© 10â›µ cells/ 200Î¼l in each well while outside wells of 96 well plate were filled with same amount of phosphate buffered saline (PBS) and allowed the cells to incubate overnight at 37 °C temperature in humidified gas incubator. After that cells were washed with PBS three times and different wells of each plate were treated with different concentrations of FCS which is shown in table 1.

Hence 12 wells of each plate were treated with 0, 1, 5, 10 and 20% v/v concentration of FCS and both plates were incubated for 72 hours.

One plate was used for crystal violet staining method and other for MTT assay.

Table 1:

Dilution of FCS

% FCS

Volume of Serum

(ml)

Volume of Medium (ml)

0

0

10

1

0. 1

9. 9

5

0. 5

9. 5

10

1

9

20

2

8

Crystal violet staining method:

For this method cell media was removed first of all from incubated plate and then cells were washed with PBS. After that were fixed with 200Î¼l of methanol for 15 minutes in fume cupboard. Then methanol were removed and cells were allowed to dry in fume cupboard for few minutes.

Then cells were treated for 20 minutes with crystal violet stain 200Î¼l/well. Later cells were washed three times with distilled water and stained cell layer was allowed to solubilised in the 50Î¼l of 10% glacial acetic acid and plates were incubated for 30 minutes in gas incubator.

After that absorbance of each well was read by plate reader spectrophotometer set at 540nm.

MTT Method:

To perform MTT assay, each of centre 60 wells of 96 well plate was treated with 20Î¼l of MTT solution and plate was incubated for 4 hours at 37o C temperature in gas incubator.

After 4 hours, the medium was removed from each well and 100Î¼l of acid-isopropand was added to dissolve blue formazan crystal in the cell layer. Then plate was incubated for 30 minutes at room temperature.

When blue formazan crystal were solubilised, absorbance of each well was measured at 570nm using the plate reader.

Calculations:

Cells in five squares of Haemocytometer = 24

Volume of each square is = 4Ã-10-3Î¼l

The no. of cells in five squares multiplied with 5Ã-104 gives no. of cells in 1ml.

Hence no. of cell in 1ml = 1. 2Ã-106 cells/ml

Required cell suspension = 1. 25Ã-105 cells/ml

Dilution Factor = Concentration Required/Concentration got

Dilution Factor = 0. 104

Therefore, in order to make 30 ml of cell suspension 3. 125 ml of cell suspension was mixed with 26. 875 ml of medium.

Similarly 30 ml of cell suspension was prepared having 1. 25Ã-105 cells/ml.

Results:

Observation Table for crystal violet staining method

%FCS

Average

Standard Deviation

0

0. 205

0. 0218

1

0. 255

0. 0307

5

0. 309

0. 0450

10

0. 432

0. 0614

20

0. 581

0. 0844

Table2: Data obtain from plate reader at 540nm

Graph showing effect of FCS with Crystal Violet Method

Above graph shows that with increase in serum concentration the

absorbance increases, which is directly proportional to cell number.

Observation Table of MTT staining method:

% FCS

Average

Standard Deviation

0

0. 094

0. 0081

1

0. 115

0. 0138

5

0. 152

0. 0191

10

0. 276

0. 0724

20

0. 400

0. 0807

Table3: Data obtained from plate reader at 570nm.

Graph showing effect of FCS with MTT staining

Above graph shows that with increase in serum concentration the absorbance increases, which is directly proportional to cell number.

Discussion:

Crystal Violet Staining method and MTT Assay is based on principle of absorbance, more is colour intensity, more will be the absorbance value.

The result of Crystal violet staining method clearly indicated that absorbance value was directly proportional to cell proliferation as it was increasing with concentration of FCS.

FCS stimulated Cell Proliferation

Result in more cells and DNA

Methanol increased cell membrane permeability

Result in more stained DNA

More Colour intensity

Hence More Absorbance Value

Similar results were seen in MTT Assay but in this assay only viable cells were stained while in crystal violet method both viable and non viable cells were stained. So Crystal Violet method of staining is not specific staining technique because in this absorbance is not direct index of cell viability.

The drawback of MTT Assay is that some reducing agent may reduce MTT also which could show slight increase in absorbance, moreover this method depends on some variable like pH, presence of D-glucose and pyridine nucleotides which can affect the specificity of Assay.

In spite of above said limitations these methods are mostly followed because they are safe, simple, cheap and reproducible.

Differentiation of K562 cells to megakaryocytes/platelets

To study cell differentiation of K652 cells chronic myelogenous leukaemia, K652 cell line, indicates an early differentiation stage of granulocyte lineage. K652 cells are non-adherent, circular shaped with small microvilli.

In the presence of tumour promoters like phorbol myristate acetate (PMA) these type of cell are differentiated to megakaryocytes.

The induction of megakaryocytic differentiation of K652 cells is known to be initiated by two signalling pathways which are the nuclear factor – kappa B(NF-ÎºB) -depends pathways and other is extracellular signal – regulated kinase (ERK)/mitogen – activated kinase (MAPK) – dependent pathways.

Human chromic myelogenous leukemic cells, K652 cells have Philadelphia chromosome. Tumour promoter, PMA which is a potent mitogen for human peripheral blood lymphocyte also act as a protein kinase C (PKC) activator which differentiate K652 cells to megakaryocytes.

The various changes that occurs during differentiation of K652 cells are:

Changes in cell morphology

Cell growth arrest

Adhesive properties of cell change

Expression of markers associatated with megakaryocytes

Endomitosis

NADPH oxidase complex which is known as a primary source of ROS (Radio active oxygen species), is initiated by PMA.

PMA stimulates NADPH ROS (Signalling Molecule)

Induction of gene expression is directly related with ROS.

The Expression of CD61, a platelet cell marker helps in identifying differentiated cells. The expression of CD61 can be seen on platelets, osteoclasts, macrophages and on some tumour cells, involved in tumour metastasis and in adenovirus infections.

Results and Discussion:

It was observed that PMA treated slide was stained pink while cells devoid of PMA were stained blue as shown in Pic. 1& 2. In PMA treated slide the K562 cells were clearly differentiated to megakaryocytes which suggested that tumour promoter, PMA induced differentiation in K562 cells by signal transduction and expressed by CD61 as shown in picture below.

C: UsersmkkaushalPicturescell bio picsmail2. jpg Pic. 1

PMA Treated Cells clearly showing Differentiation to Megakaryocytes

C: UsersmkkaushalPicturescell bio picsmail. jpg Pic. 2

Cells Devoid of PMA stained blue in Colour

The expression of CD61 was recognised by addition of rabbit anti-mouse IgG antibodies that bind to CD61 antibodies when incubated in presence of alkaline phosphatise anti alkaline phosphatise (APAAP) complex.

The cells were stained pink because fast red dye get attached to APAAP so this is how CD61 was expressed in cell treated with PMA. Moreover cells treated with PMA were larger, irregular, in shape and fewer in number as compare to untreated cells.

On the contrary, Cells devoid of PMA were much smaller in size than treated cells.

Diagramatic Representations of Immunocytochemical

Reactions To Detect CD61

PMA Treated Cells PMA Untreated Cells

CD61 bound to both treated and untreated cells

Then Cells are washed to

Remove CD61 unbound &

Treated with RAM

Then RAM binds to APAAP and cells are stained pink in colour.

: Mouse Antihuman CD 61 (Primary

Antibody)

: Rabbit Antimouse IgG (RAM-

Secondary Antibody)

: Mouse Alkaline Phosphate AntiAlkaline

Phosphatase(APAAP Tertiary Antibody)