Original Article

PHORBOL MYRISTATE ACETATE (PMA) INDUCED MEGAKARYOCYTIC DIFFERENTIATION OF K562 CELLS FROM HUMAN CHRONIC MYELOGENOUS LEUKAEMIA PATIENT

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ABSTRACT

Objective: To observe the K562 cell line derived from a patient of chronic myelogenous leukemia differentiated into megakaryocytes by growing in the presence of phorbol myristate acetate (PMA).

Methodology: The differentiation process of K562 cells was monitored by the expression of a platelet cell marker, CD61 through immunocytochemistry using mouse alkaline phosphatase antialkaline phosphatase (APAAP) complex employing fast red TR as substrate, crystal violet and MTT assay used for cell growth analysis.

Results: The crystal in the presence of PMA, cells obtained were of large size and less in number as compared to cells incubated without PMA where they were of smaller size and more in number and immunochemical reaction used to detect the presence of CD61, a platelet cell marker that is expressed during differentiation of K562 cells to megakaryocytes.

Conclusion: The results showed that the addition of PMA to the growing culture of K562 cell lines induced differentiation, observed through CD61 expression and increase in cell size and cessation of proliferation.

KEY WORDS: PMA, Megakaryocytic, K562 cells, Myelogenous Leukaemia.

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INTRODUCTION

The K562 cell line was established in 1970 from a patient suffering from chronic myelogenous leukemia.[1] Human K562 cell lines isolated from patient with chronic myelogenous leukemia in blast crisis have the capacity to express characteristics of erythrocytic, monocytic, and megakaryocytic differentiation when exposed to various agents.[2]

12-O-Tetradecanoylphorbol-13-acetate (TPA), also commonly known as phorbol 12-myristate 13-acetate (PMA), is diester of phorbol and a potent tumor promoter often employed in

biomedical research to activate the signal transduction enzyme protein kinase C (PKC).

We have studied the effects of phorbol myristate acetate (PMA), a member of the family of phorbol esters. Phorbol esters are analogues of diacylglycerol (DAG), and therefore activate most protein kinase C isozymes by interaction with the DAG-binding site.[3-5] Protein kinase C, when activated, phosphorylates certain serine/ threonine residues on a large number of proteins, often inducing regulatory changes in membrane and/or cellular functions and responses.[6] Furthermore, protein kinase C pathways have been implicated in the maturation of a macrophage cell line.[7]

The effects of TPA on PKC result from its similarity to one of the natural activators of classic PKC isoforms, diacylglycerol. The K562 cells can be induced to differentiate into cells with monocytic and/or megakaryoblastic characteristics by tumor-promoting phorbol esters, e.g., phorbol myristate acetate (PMA), a protein kinase C (PKC) activator.[8-10] Previously it is also reported that some other agents used to induce differentiation in cell cultures such as, Butyric acid (BA)[11], Hemin (H)[12], 12-o-tetradecanoylphorbol 13-acetate (TPA),[13] Ionomycin, Anthracyclines[12] and Phorbol myristate acetate (PMA) to induced differentiation in different cell cultures.

The aim of present study was to observe the K562 cell line derived from a patient of chronic myelogenous leukemia differentiated into megakaryocytes by growing in the presence of phorbol myristate acetate (PMA).

METHODOLOGY

Cell Culture: K562 cells were grown as previously described with some modification in suspension in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. The cultured cells were passed twice each week, seeding at a density of about 2 × 105/ml.[14] Culture was maintained at $37C^0$ humidified atmospheres of 5% CO_2 and 95% air.

Treatment of PMA: Different concentrations (1, 10, 25, 50 and 100 nmol l^{-1}) of PMA were tried to induce differentiation of K562 cells.

Crystal violet stain: Crystal violet method of measuring cell proliferation was done as previously described with some modification (Naseer et al., 2009). Color produced was measured at 540nm using a plate reading multi-scan spectrophotometer.[15]

MTT Assay: The logarithmic growth phase of K562 cells from human chronic myelogenous leukaemia patient were taken for growth assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenly tetrazolium bromide (MTT). The MTT assay relies on the uptake of MTT, a water-soluble tetrazolium salt producing a yellowish solution. In cell cultures it is converted to an insoluble blue formazan by cleavage of the tetrazolium ring by dehydrogenases of mitochondria of living cells. The intensity of the blue colour produced is a measure of viable cell number.[5] For the MTT assay, cells were cultured with stimulatory agents for 48h and 20ml of MTT (5mg/ml solution in PBS) was added to each of the treated cultures 4h before the end of the experiment and cells were incubated at 37^0C in a humified 5% CO2 incubator. To achieve solubilization of formazan crystals formed in viable cells, DMSO was added to each well, and the absorbance was recorded on a micro titer plate reader at a test wavelength 570nm with a reference wavelength of 690 nm. The optical density (O.D) was calculated as a difference between the absorbance at the reference wavelength and that observed at the test wavelength. The effect of drugs on growth was assessed as percent of cell viability.[15]

Immunocytochemistry: After fixing the cells washed immediately in 0.15M tris buffer saline (TBS) (pH 7.6) for two hour in a humid atmosphere (sandwich box with damp paper sheets) at room temperature. Diluted antibodies dropped on a cover slip and place this on the cells in order to avoid drying so that the whole area will be covered with antibody of the same concentration. Rabbit mouse antibody IgG (RAM) (30ul of a one in 20 dilution in TBS) is

added to the cells using cover slip and incubated for 30 minutes at room temperature. Cells washed with TBS two times for one minutes each. Cells then incubated with anti-alkaline phosphates (APAAP) complex (30ul of a 1 in 20 dilution) for 30 minutes at room temperature. After washing and drying as previously, the incubations with RAM and APPAAP complex are repeated to amplify the reaction. The time for these incubations was reduced to 10 minutes each. The cells washed in TBS as before and then stained in fast red TR substrate (1mg/ml) for 15 minutes. The cells washed with running water (2-3 minutes) and counterstained with haematoxylin for 1-2 min. The haematoxylin was added directly to the cells. Finally the cells washed in TBS for one minutes and then mounted in glycerol/TBS (3:1) and viewed under the microscope. The presence of CD61 (a platelet cell marker that is expressed during differentiation) cells to megakaryocytes was indicated by a pink colouration.

RESULTS

Immunochemical reaction used to detect the presence of CD61:

The K562 cell line derived from a patient of chronic myelogenous leukemia differentiated into megakaryocytes by growing in the presence of phorbol myristate acetate (PMA). K562 cells ($2x10^5$ cells/ml) were incubated in the presence and absence of PMA (100nmole/l^{-1}) for 6 days and differentiation process was monitored by the expression of CD61, a platelet cell marker, through immunocytochemistry (Fig-1). The presence of CD61 during PMA-induced megakaryocytic differentiation of K562cells was indicated by the pink colouration that was obtained due to the immunochemical reaction carried out using mouse alkaline phosphatase antialkaline phosphatase (APAAP) complex employing fast red TR as substrate, whereas haematoxylin stained the nuclei blue in both the cases (PMA negative and positive) as shown in Figure-1. In the presence of PMA, cells obtained were of large size and less in number as compared to cells incubated without PMA, where they were of smaller size and more in number. The results showed that the addition of PMA to the growing culture of K562 induced differentiation due to the increase in CD61 expression, increase in cell size and cessation of proliferation (Fig-1).

Table-I: Effect of PMA to induced megakaryocytic differentiation of K562 cells from human chronic myelogenous leukaemia patient using MTT assay method

% CD61 (n = 5)	*Absorbance at 570 nm (Mean±S.D.)*
0	0.055±0.006
10	0.088±0.007
25	0.110±0.007
50	0.177±0.029
100	0.289±0.035

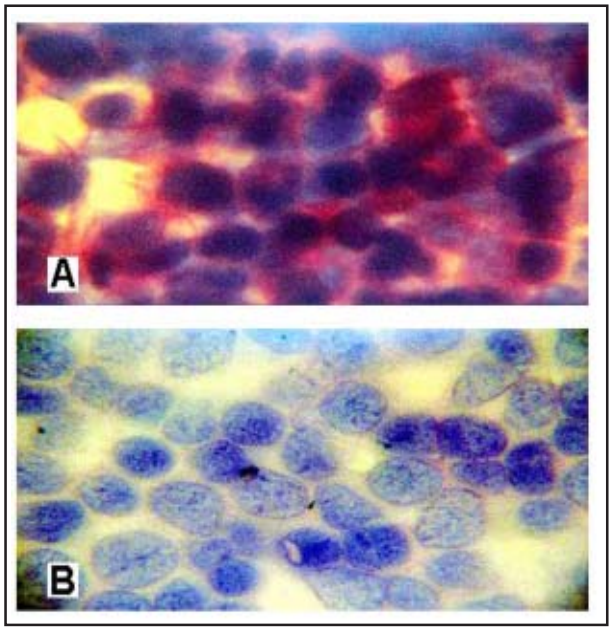

Figure-1: Differentiation of K562 cell to megakaryocytes

Diagram representing the immunochemical staining reaction used to detect the presence of CD61, a platelet cell marker, on the surface of cultured K562 cells (A) with PMA (20 x objective lens) and (B) without PMA (40 x objective lens). Presence of pink colour in PMA treated cultures showed that PMA induced differentiation in K562 cells to megakaryocytes, whereas no pink colour was observed in cells cultured without PMA. Haematoxylin stains the nuclei blue in both cases.

Determination of cell proliferation by crystal violet stain:

To asses the effect different concentrations of PMA on cellular proliferation we studied the uptake of crystal violet by K562 cells. The cells were grown as monolayer cultures and treated with different concentrations of (1, 10, 25, 50 and 100 nmol l^{-1}) of PMA to stimulate cellular proliferation. The results showed that there was significant increase in proliferation of K562 cells with the increase in amount of PMA in the DMEM medium. There was increase in absorbance value from 0.055 to 0.289 when concentration of PMA added in the medium increased from (0, 10, 25, 50 and 100 nmol l^{-1}) of PMA as shown in (Table-I).

Determination of cell proliferation by MTT assay:

To further confirm that K562 cell proliferation effected by addition of PMA, we studied MTT assay. There was a gradual increase in absorbance value up to the addition of 10, 25, 50 100 nmol l^{-1} PMA in the medium followed by a sharp increase in absorbance by the addition of 50 and 1000 nmol l^{-1} PMA (Fig-2). Moreover, the Figure-3 representing schematic diagram of the immunochemical reaction used to detect the presence of CD61, a platelet cell marker that is expressed during differentiation of K562 cells to megakaryocytes where APAAP – Alkaline phosphatase – antialkaline phosphatase complex where primary antibody – Mouse antihuman CD61 (*IgG*) and secondary antibody – Rabbit antimouse (RAM) *IgG* (raised in rabbit against mouse) (Bridging antibody) used to study the expression of K562 cells.

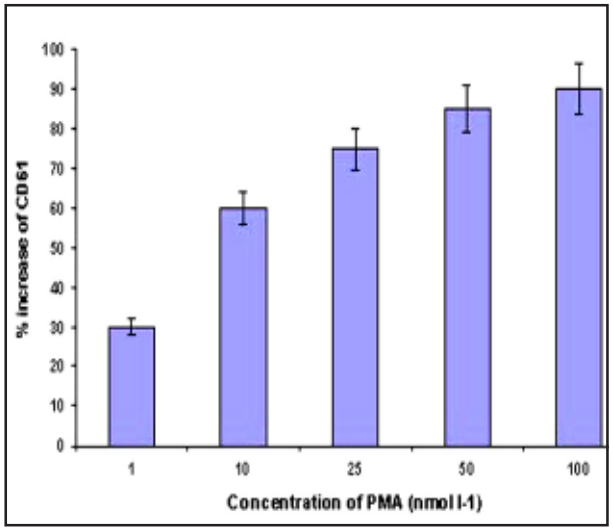

Fig-2: Graph representing the different concentration (1, 10, 25, 50 and 100 nmol l^{-1}) and percentages of K562 growth expression, showing an increase in CD61 expression with increase in concentration of PMA used. *$P < 0.05$ **$P < 0.01$

DISCUSSION

Protein kinase C has gained significant interest since it was first discovered as a major intracellular receptor for phorbol ester tumor promoters such as Phorbol myristate acetate (PMA).[6] In many cell types, including fibroblasts[16] and T lymphocytes,[17-18] PMA stimulates cellular proliferation, suggesting a role for PKC activation in this process. Paradoxically, PMA has also been shown to induce terminal differentiation in several human tumor cell lines including human chronic myelogenous leukemic cells (K562).[19]

The mechanisms of cytokine action are currently under intensive investigation, and in

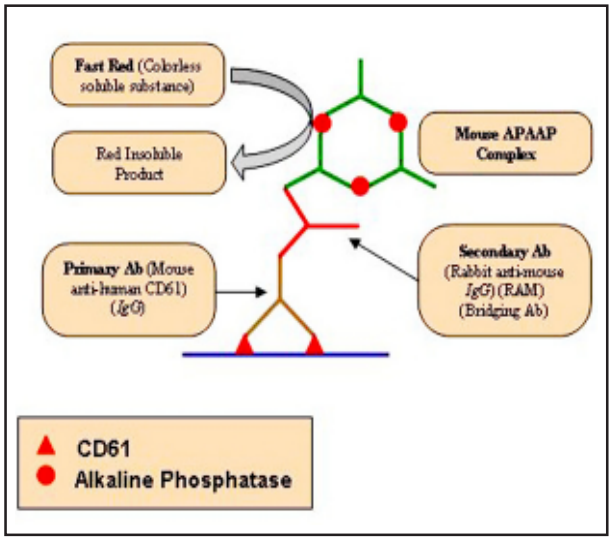

Figure-3: Schematic diagram of chemical reaction Diagram representing immunochemical reaction used to detect the presence of CD61, a platelet cell marker that is expressed during differentiation of K562 cells to megakaryocytes. APAAP – Alkaline phosphatase – antialkaline phosphatase complex Primary Antibody – Mouse antihuman CD61 (*IgG*), Secondary antibody–Rabbit antimouse (RAM) *IgG* (raised in rabbit against mouse) (Bridging antibody)

many cases, signal pathways have been identified. At least two of the proinflammatory cytokines, TNFa and IFN"y involve the protein kinase C signalling pathway,[20] and the activating effects of colony stimulating factor-1 on microglia also appear to involve protein kinase C.[21] Therefore the effects of this pathway in these phagocytic cells are of great interest. This pathway is conveniently activated by exposing the cells to PMA, which acts as an analogue of diacylglycerol, the physiological activator of protein kinase C isozymes. Another signalling pathway, that of the adenyl cyclase pathway is involved in a large number of cell functions, and this pathway is activated by forskolin.[22]

Although, the tumor-promoting PMA is a well-characterized activator of PKC, however less is known about the signaling pathways responsible for monocytic and/or megakaryocytic differentiation of K562 cells in the presence of this agent. In the present studies, the PMA-induced differentiation of K562 cells was determined by expression of a platelet cell marker, CD61. PMA induces a macrophage-like morphology with enhanced expression of proteins associated with megakaryocytes in K562 cells.[23] Although mitogen-activated protein kinase (MAPK) activity increase during the PMA-induced differentiation of K562 cells, NF-kB activation rather than MAPK activation is responsible for the PMA-induced megakaryocytic differentiation of K562 cells.[24-25]

TPA induces K562 cells to differentiate towards the magakaryocyte lineage. Treatment of K562 leukemia cells with the tumor promoter (TPA) leads to loss of their erythroid properties and to acquisition of several megakaryoblastoid characteristics. These include synthesis and surface expression of glycoprotein IIIa, an increase in platelet peroxidase activity, enhancement of thromboxane A2 receptors, and increased cell volume and DNA ploidy. TPA-treated K562 cells also synthesize and secrete platelet derived growth factor (PDGF), transforming growth factor beta 1 (TGF beta 1), urokinase-plasminogen activator (u-PA) and its specific inhibitor, type 1 plasminogen activator inhibitor (PAI-1). Induction of all these proteins, which have also been found in platelet granules (u-PA on platelet surface receptors) occur at the level of mRNA accumulation.[13]

Finally, the results indicated that the addition of PMA to the growing culture of K562 cell lines induced differentiation due to the increase in CD61 expression, and cause increase in cell size and cessation of proliferation.

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