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Sustained small and intermediate size proteins expression in phorbol 12-myristate 13-acetate/ionomycine prolonged stimulated human fibroblasts

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ABSTRACT

Objective: To compare the protein profile of culture supernatants in stimulated and unstimulated human fibroblasts to find some proteins indicating the presence of fibroblasts and their activation status.

Methods: Dermal fibroblasts were stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycine for 72 h. MTT assay was done to determine cell viability and A/E fluorescent staining was used to evaluate the cell death pattern. Protein analysis was performed by gradient SDS polyacrylamide gel electrophoresis 8%–16%.

Results: The supernatant of 24 h cultured both stimulated and unstimulated fibroblasts showed two bands in SDS-PAGE analysis with relative molecular weights of 8.59 and 78.8 kDa. These bands density was decreased during the next 48 h in unstimulated cells while their expression was continued in PMA or PMA/ionomycine stimulated cells and a new 85.3 kDa band was appeared in unstimulated and 72 h PMA stimulated cells. Moreover, we found another seven small size (10–19.5 kDa) proteins in supernatants of 48 h and 72 h unstimulated but not in PMA or PMA/Ionomycine stimulated fibroblasts. Most of these proteins expression were down regulated following fibroblast activation. This down-regulation is consistent with our finding that PMA or PMA/ionomycine stimulated cells exhibited a significant level of apoptosis cell death.

Conclusions: Human fibroblasts produce some small to intermediate sized proteins with specific SDS-PAGE profile upon cell activation. Most of these proteins can be excreted in urine and can be immunogen theoretically so this data provided a reliable clue for fibrosis biomarker screening based on designation of an appropriated immunoassay.

1. Introduction

Upon cell activation state fibroblasts have a dual role in health and disease. These cells are considered as major architect of connective tissue and secrete a dozen of growth factors and cytokine and have a close cross talk with immune system [1–4]. Similar to main cell of immune system *i.e.* lymphocyte these cells also have two facets and thus their role can be shifted to pathogenesis of some diseases. This matter is comparable to the hyperactivity or allergic reactions performed by immune system. Fibrosis is defined as an excess deposition of collagen and extracellular matrix which overproduced by activated fibroblasts [5]. Collagen fibers and other elements of extracellular matrix are main substances of lose connective tissue which wraps the cells, blood vessels and nerves fibers together to shape different types of tissues and organs constituent of our whole body [6]. Fibroblasts can be activated by different types of stimulator including histamine [7], serotonin [8], bradykinin [9], some cytokines [3] and chemokines [10], microbial products which are grouped as pathogen associated molecular pattern (PAMP) [3] and ionomycin/phorbol 12-myristate 13-acetate (PMA) [11]. PMA is a chemical compound which has a structure analogous to diacylglycerol (DAG) a natural activator of protein kinase C (PKC) [12,13]. Protein kinase C is a family of serin/theronin kinases which its

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members in presence of calcium play important roles in cell activation, proliferation, survival and death [14]. Human dermal fibroblasts express α, δ, ε, ζ isoforms of PKC. Alpha isoform is the dominant isoform of this enzyme in these cells [15] which is a Ca^{2+}-regulated isoenzymes whereas PKC-delta, -epsilon, -theta, and -zeta are not calcium dependent [16]. Activation of these enzymes by PMA mimics the effects of above mentioned various extracellular signals in intact cells which in turn results in some changes in morphology of the cells, changes in gene expression pattern and stimulation or inhibition of cells proliferation [12]. Thus activation of fibroblasts by this way can give us an overall image about the *in vivo* pattern for these cells activation. Indeed hyperactivation of these cells can be occurred in some organs like heart, lung, liver, skin and kidney which subsequently leads to serious diseases such as cardiovascular fibrosis, idiopathic pulmonary fibrosis, liver cirrhosis, systemic sclerosis and kidney post transplant fibrosis [5]. Moreover investigation of the protein secretion pattern of activated fibroblasts can provide us a strong clue for exploring of different biomarkers in urine and serum for predicting kidney rejection in clinical transplant or to predict the progress of liver cirrhosis or other tissue and organ fibrosis in above mentioned diseases. In this study the response of human skin isolated fibroblasts to PMA/ionomycin (a calcium ionophore) stimulation was investigated and protein secretion and cell survival/death patterns of activated fibroblasts were analyzed.

2. Materials and methods

2.1. Fibroblasts isolation and sub culturing

By non enzymatic method the fibroblasts were isolated from fresh foreskin prepared from 4 children aged 1.5–3 months who underwent routine circumcision from July 2014 to September 2014 at Amirkola children's Hospital, Babol, Iran. To this end, the skin samples were washed 5 times in PBS and were placed on a sterile plastic tissue culture dish (Orange scientific cat: 4450100N) and minced to small pieces using scissors. The dishes containing samples and complete growth medium [DMEM (PAA cat: E15–883) + FBS 10% (PAA cat: A 15-15) + Penicillin/streptomycin 1% (PAA, cat: p11-010)] were incubated in a humidified 37 °C, CO_2 5% incubator. The growth of fibroblasts was checked every 2–3 days under an inverted phase–contrast microscope. When the fibroblasts have been grown for 3 weeks the remaining tissue pieces were removed and the monolayer of cells were washed with Dulbecco's (D)-PBS (PAA cat: H15–002). Immediately the PBS was removed from dish and 0.25% Trypsin–EDTA (Sigma: 59418C) solution was added to dish and incubated at 37 °C for 2–3 min. Then 4 mL complete medium was added to dish and transferred the detached cells to a sterile 15 mL tube. The cells were centrifuged at 400 × *g* for 5–7 min and then the supernatant was removed carefully and the cell pellet was resuspended and transferred in a new flask for subculture. To prepare adequate cells to conduct the required experiments, the isolated fibroblasts were cultured routinely in high glucose (4.5 g/L) Dulbecco's modified eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 µg/mL penicillin-streptomycin at 37 °C in a humidified CO_2 5% environment. This research proposal was approved by ethics committee of Babol University of Medical Sciences. Based on the culture perception that considers the foreskin as a disposable tissue, the ethics committee of Babol University of medical sciences waived the requirement for obtaining written consent from children's parents in present research.

2.2. Fibroblast stimulation and MTT assay

For fibroblast stimulation 10^5 fibroblasts (n = 4) were dispensed into each well of a 24-well plate (Orange scientific cat: 5530300) and the plate was incubated at 37 °C in a humidified CO_2 5% environment. After 24 h the cell culture medium was replaced by a fresh medium contained only PMA 100 ng/mL, ionomycin 1 µmol/L plus PMA 10 100 or 1 000 ng/mL separately and ionomycin 10 µmol/L plus PMA 100 ng/mL. Cells without any stimulator were incubated for 24, 48 and 72 h as unstimulated cell control. After the indicated time the cell culture supernatants were harvested and kept at −20 °C for further analysis. To determine the cell viability following stimulation, the MTT (3-(4,5-Dimethyl thiazol-2yl)-2,5-Diphenyl tetrazolium bromide) reduction based assay was done [17]. MTT assay was carried out on the treated cells with PMA 100 ng/mL alone and Ionomycin 10 µmol/L plus PMA 100 ng/mL. After washing every well with PBS, the MTT (Sigma–Aldrich, UK) solution (5 mg/mL) added to each well and the plate was incubated for 4 h under a standard cell culture condition. The crystals of formazan were dissolved in acidified isopropanol (0.04 mol/L HCl) and the absorbance of purple solution was measured by microplate reader (Rayto, China) at a test wavelength of 570 nm and a reference wavelength of 630 nm. Each test had a blank which was complete culture medium plus MTT without cells.

2.3. Detection of apoptosis

We examined the death pattern of stimulated fibroblasts based on the method described already [18]. To this end, 2.5 × 10^5 stimulated cells with PMA/ionomycine were centrifuged at 400 × *g* for 10 min. The pellet was washed by cold PBS and was resuspended in 25 µL PBS. Then the cell suspension was mixed with 2 µL Acridin orange (AO)/ethidium bromide (EB) solution 1:1 ratio [(AO, 100 pg/mL, Sigma Chemical, St. Louis, MO); (EB, 100 pg/mL, Sigma, USA)]. Then 10 µL of cell suspension was placed onto a microscopic slide and covered with glass coverslip. Then the cells were evaluated under a fluorescence microscope and the necessary micrographs were prepared [19].

2.4. Gradient SDS polyacrylamide gel electrophoresis (SDS-PAGE)

At first the proteins of supernatant of untreated and treated cells were precipitated by acetone. To this purpose, 50 µL of supernatants were precipitated by 200 µL of ice-cold acetone (Merck) and were incubated at −20 °C overnight and then were centrifuged at 15 000 × *g*, 4 °C, for 30 min. Protein electrophoresis analysis of stimulated and unstimulated cell culture supernatants were carried out by Gradient SDS Polyacrylamide gel electrophoresis 8%–16% based on Laemmli method [20]. Briefly the samples were mixed with loading buffer containing 20% glycerol, 0.004% bromophenol blue, 4% SDS, 10% 2-mercaptoethanol and 0.125 mol/L Tris–HCl, pH 6.8. The mixture was heated in boiling water for 5 min and then was loaded in polyacrylamide gel. Electrophoresis was conducted in stacking and resolving gel,

respectively with 80 and 180 V in a tank from Bio-Rad Company. Then silver nitrate was used for gel staining.

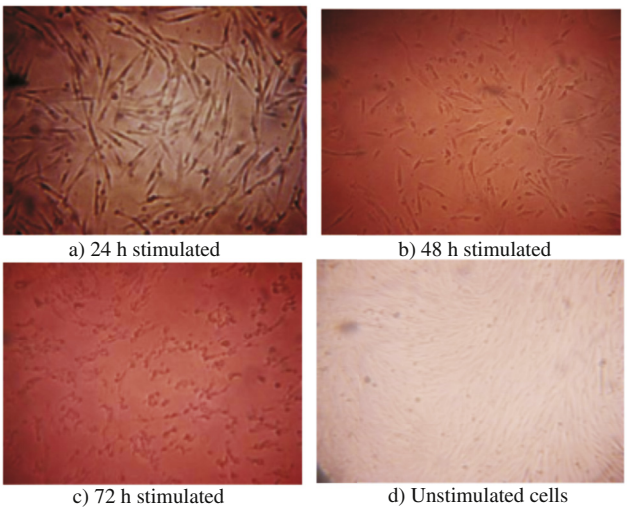
2.5. Statistical analysis

The statistical analyses were performed using SPSS 21 and One-way ANOVA was used to determine the different variables effects on fibroblast.

3. Results

3.1. PMA with ionomycine kills the fibroblasts more rapidly than PMA alone

To grasp an overall view about the effect of fibroblast stimulation on cell survival and also to explore the role of calcium ion in this phenomenon we investigated the effects of PMA or PMA with ionomycine on fibroblasts growth. As Figure 1 shows after 24 h of incubation with stimulator, the cells deformation and detachment were begun and number of round cells was increased during the next 24 h and reached its maximum level after 72 h of cells stimulation. The cells which were stimulated with PMA alone showed almost the same pattern.



a) 24 h stimulated b) 48 h stimulated

c) 72 h stimulated d) Unstimulated cells

Figure 1. Morphology of dermal fibroblasts stimulated with PMA 100 ng/mL with ionomycin 10 μmol/L after: (a) 24 h; (b) 48 h; (c) 72 h; (d) unstimulated cells (200×).
Deformation of treated cells and cell death were increased with passing of the time. Dead cells (round cells) in 72 h PMI/ionomycine stimulated group were much more than the other groups.

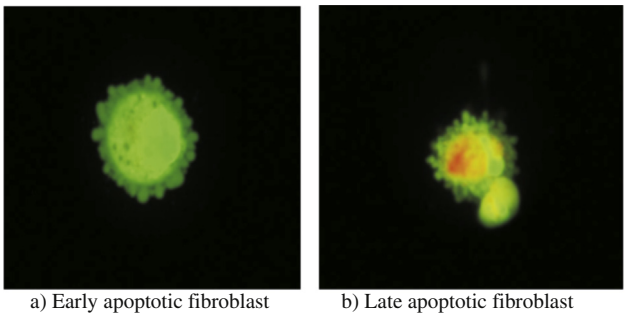
We did MTT assay to quantitize our microscopic observation. As it can be seen in Table 1 the cells which were stimulated with PMA or PMA/ionomycine after 48–72 h showed a significant decreased level of viability when compared to the cells incubated for 24 h with indicated stimulators as well as in comparison to their related unstimulated control cells whereas the PMA/ionomycine stimulated cells after 24 h exhibited a lower level of viability rate versus the cells which were stimulated only with PMA ($P < 0.05$).

Table 1
Effects of PMA 100 ng/mL alone or PMA 100 ng/mL with ionomycin 10 μmol/L ($I_{10}P_{100}$) on fibroblasts survival.

Group	% Cell viability					
	24 h	*P* value	48 h	*P* value	72 h	*P* value
Control	100	0.0130	100	0.0005	100	0.0026
PMA	90.00 ± 4.08		18.50 ± 13.30		32.80 ± 17.30	
$I_{10}P_{100}$	63.60 ± 7.90	0.0014	13.70 ± 8.65	< 0.0001	24.30 ± 13.90	0.0007

These results are expressed as mean ± SD of cell viability percentages ($n = 4$). The percentage of cell viability was calculated as OD of test for each indicated time/OD of related control × 100. Both PMA alone and PMA/ionomycine reduced the fibroblasts viability rate however with different kinetics. After 24 h of incubation the cells of PMA/ionomycine ($I_{10}P_{100}$) stimulated group exhibited a significant lower viability rate when compared with unstimulated control cells. After 48 and 72 h of cell incubation, both PMA alone and PMA/ionomycine stimulated cells exhibited a significant lower level of cell viability in comparison to the unstimulated cells. *P* value < 0.05 was considered to represent a significant difference.

To determine the fibroblast activation induced cell death pattern the AO/EB staining of 48 and 72 h PMA stimulated cells was performed. As Figure 2 shows we observed frequently the cells with bellebing membrane and green cytoplasm which we considered them as the cells showing early apoptotic cell death pattern. We also observed the cells at late apoptosis stage frequently (Figure 2) with condense nucleus and red-orange cytoplasm.



a) Early apoptotic fibroblast b) Late apoptotic fibroblast

Figure 2. Acridine orange and ethidium bromide staining of treated cells with PMA 100 ng/mL alone and ionomycin 10 μmol/L with PMA 100 ng/mL (400×).
Early and late apoptotic cells are characterized by blebbing membrane (a) and chromatin condensation and red-orange cytoplasm as well as blebbing membrane (b) respectively.

3.2. Different protein profiles in stimulated and unstimulated fibroblasts

To optimize the condition to stimulate the fibroblasts we initially examined the effect of 1 μmol/L of ionomycine with three different concentrations of PMA (10, 100, 1 000 ng/mL) on fibroblasts protein secretion and then their culture supernatant protein profiles in SDS-PAGE were compared to unstimulated fibroblasts. The protein profile of the culture supernatant prepared form the treated cells with ionomycin 1 μmol/L–PMA 10 ng/mL, ionomycin 1 μmol/L–PMA 100 ng/mL, ionomycin 1 μmol/L–PMA 1 000 ng/mL for 24 h was shown by SDS-PAGE (Figure 3). As arrows indicate two protein bands appeared in cultured fibroblasts but not in 10% FBS supplemented medium, however, no remarkable differences was observed in culture supernatants SDS-PAGE patterns among the cells which were stimulated already with 1 μmol/L of ionomycine and different concentration of PMA.

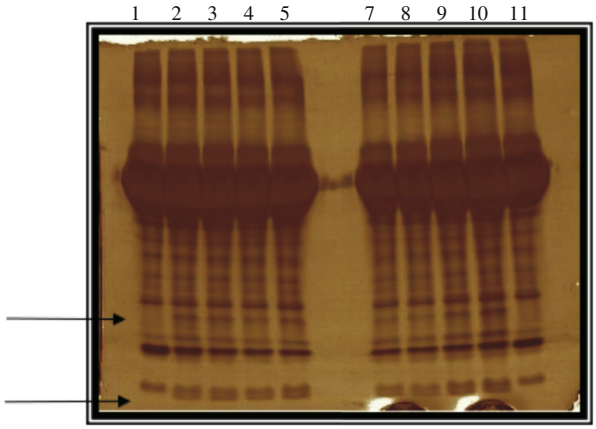


Figure 3. Protein pattern of fibroblasts culture supernatant stimulated with ionomycin and PMA.
Lanes 1 and 11: Media supplemented 10% FBS; Lanes 2 and 10: Unstimulated cells control; Lanes 3 and 9: Stimulated with ionomycin as 1 μmol/L–PMA 10 ng/mL; Lanes 4 and 8: Stimulated with ionomycin 1 μmol/L–PMA 100 ng/mL; Lanes 5 and 7: Stimulated with ionomycin 1 μmol/L–PMA 1 000 ng/mL; Lanes 1–5 are result of the test which was performed with the same volume whereas the lanes 7–11 were obtained with the same total protein concentrations of the cell culture supernatants.

Thus we can say that fibroblasts secrete some proteins which may be important to confirm just the presence of fibroblast in a special tissue. Because of no difference was observed in the protein migration pattern of the treated cells with ionomycin 1 μmol/L–PMA 10 ng/mL, ionomycin 1 μmol/L–PMA 100 ng/mL, ionomycin 1 μmol/L–PMA 1 000 ng/mL in continuation of our fibroblasts activation experiment, the fibroblasts were stimulated with PMA 100 ng/mL individually and ionomycin 10 μmol/L plus PMA 100 ng/mL for 24, 48 and 72 h (Figure 4).

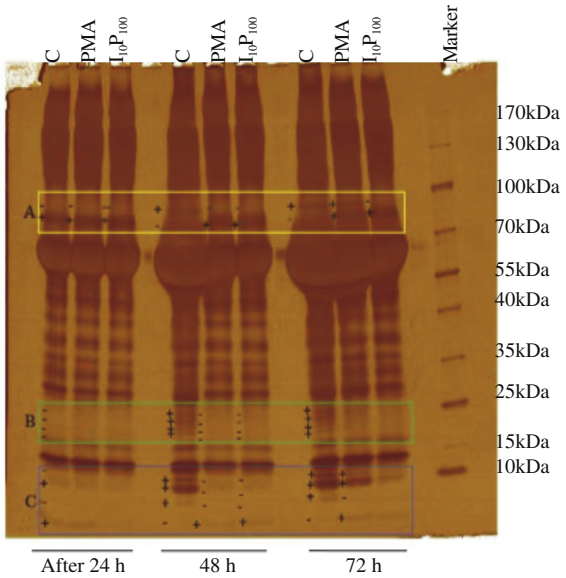


Figure 4. Protein migration profile of the fibroblasts treated ($n = 2$) with PMA 100 ng/mL and ionomycin 10 μmol/L/PMA 100 ng/mL after 24, 48 and 72 h. C: Untreated cells as control; PMA: PMA 100 ng/mL; $I_{10}P_{100}$: Ionomycin 10 μmol/L–PMA 100 ng/mL. Rectangulars A, B and C show the protein bands with relative molecular weight of 78.8 k and 85.3 kDa, 16.8–19.5 kDa and 8.5–12.3 kDa respectively. Each band in rectangular was enumerated according to its order from up to down and +, − represent the present and absent the indicated band respectively.

The supernatant of 24 h cultured both stimulated and unstimulated fibroblasts showed two bands in SDS-PAGE analysis with relative molecular weight of 8.59- and 78.8 kDa (Figure 4; C4 and A2 bands, respectively). These bands density was decreased during the next 48 h in unstimulated cells while their expression was continued in PMA or PMA/ionomycine stimulated cells and a new 85.3 kDa band was appeared in unstimulated and 72 h PMA stimulated cells (Figure 4; band A1). Moreover, we found another seven small size (10–19.5 kDa) proteins in supernatants of 48 h and 72 h unstimulated but not in PMA or PMA/ionomycine stimulated fibroblasts (Figure 4; bands B1–B4 and C1–C3). Most of these proteins expression were down regulated following fibroblast stimulation. This downregulation is consistent with our finding that PMA or PMA/ionomycine stimulated cells exhibited a significant level of apoptosis cell death.

4. Discussion

Fibrosis can be defined as a pathological condition in which activated fibroblasts secrete a large amount of collagen and other matrix substances. Fibrosis can involve our vital organs like heart, liver and kidney. Accumulating evidence show that protein kinase C plays some important roles in fibroblast migration, activation and regulation of collagen synthesis [21–23]. Different isoenzyme of protein kinase C are activated by diacylglycerol (DAG) [24]. Phorbol esters, a synthetic compound and a well known activator of protein kinase C is not metabolized readily, thus it can stimulated this enzyme for a prolonged time [24]. We took this advantage to determine the pattern of protein secretion in protein kinase C activated fibroblast aimed to find a protein with potential application to show the presence of fibroblasts in a fibrotic organ such as kidney especially to predict these cells activation status. In spite of existence of so many of protein bands due to presence of 10% fetal bovine serum in cell culture supernatants we found that almost independent to fibroblast activation status (stimulated with 1 μmol/L ionomycine and different concentration of PMA (10–1 000 ng/mL) there were some protein bands in the supernatant which were absent in 10% FBS supplemented medium only. It means fibroblast can secrete a special set of proteins which these proteins may indicate their presence in an especial tissue/organ. Interestingly with higher concentration of ionomycine (10 μmol/L) the fibroblasts predominantly showed a suppressive pattern at least for some proteins. We showed that unstimulated fibroblasts secreted at least 10 different proteins with molecular weights of 8.59–85.3 kDa. Surprisingly the secretion of one protein with molecular weight of 8.59 kDa was ceased after 24 h of the cells incubation while the expression of this protein was continued in stimulated cells after 48 and 72 h. This protein can be considered as a marker for prolonged fibroblast activation. We also showed that after 48 h of incubation unstimulated fibroblast but not PMA alone and PMA/ionomycine stimulated fibroblasts secreted an 85.3 kDa protein while the expression of a protein with molecular weight of 78.8 kDa was inversely decreased. This finding was repeated in 72 h unstimulated cells and PMA alone but not PMA/ionomycine stimulated cells. We need further study to characterize these proteins.

The importance of our finding is that these proteins mostly have a low molecular mass. Thus we suppose they can redially pass from glumerols and excreted to urine so the urine

electrophoresis may give us an important clue for predicting the fibrosis status in some serious disease like kidney, liver or heart fibrosis. Urine has a lower level of protein so determination the protein band is much easier than serum. Our viability analysis of stimulated fibroblasts was also confirmed the suppressive effect of protein kinase C activation on protein secretion. We showed that PMA/ionomycine induces apoptosis in fibroblasts. Probably decreasing or increasing in bands density of above mentioned protein in SDS-PAGE analysis is a secondary resultant of this cell death. We also suppose that the expression of new proteins during 48–72 h exposure is due that these cells gradually were activated during incubation and consequently produced these proteins. As our knowledge we are the first group to report the electrophoretic protein migration profile of PMA/ionomycin stimulated fibroblasts. However, Yang *et al.* reported that PMA/IONO promotes the apoptosis and inhibits the growth of diffuse large B-cell lymphoma (DLBCL) cells, in association with A20 upregulation [25]. Also, Traore *et al.* showed the PMA suppressed the growth of human monoblastic leukemia THP1 cells [26]. Taken together the data generated by this study showed that the human fibroblasts produce some small to intermediate sized proteins with specific SDS-PAGE profile upon cell activation. Most of these proteins can be excreted in urine and can be immunogen theoretically so this data provided a reliable clue for fibrosis biomarker screening based on designation of an appropriate immune assay.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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