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Phorbol ester phorbol-12-myristate-13-acetate induces epithelial to mesenchymal transition in human prostate cancer ARCaP_E cells

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Abstract

Background—We have reported that human prostate cancer ARCaP_E cells undertake epithelial to mesenchymal transition (EMT) when stimulated by certain soluble factors, and that EMT is regulated by surface receptor-elicited signaling pathways through protein phosphorylation. It is known that phorbol ester phorbol-12-myristate-13-acetate (PMA), a potent antagonist to both conventional and novel protein kinase C (PKC) isoenzymes, induces cancer cell scattering.

Methods—To assess the effect of PMA on EMT, ARCaP_E cells were treated with PMA and were assayed for EMT-related morphologic and behavioral changes. Specific inhibitors were used to investigate the PMA-induced EMT.

Results—PMA at 100 nM induced EMT in a time-dependent manner, resulting in a complete change from epithelial to mesenchymal stromal morphology. Concurrently, PMA inhibited expression of epithelial marker E-cadherin and increased the level of stromal marker protein vimentin, while the treated cells showed increased migratory and invasive capacities. Using specific inhibitors, we confirmed that the effect of PMA was mediated by PKC, while isoenzymes of the novel PKC subfamily were implicated as the main mediator. Finally, we determined that the EMT was dependent on newly synthesized proteins, because inhibitors for gene transcription and protein translation could both inhibit the initiation of EMT.

Conclusions—Although PMA is well known for its effects on cell migration and tumor formation, this work is the first to define PMA as an EMT inducer in prostate cancer cells. Further investigation in this experimental model may reveal important regulatory mechanisms and additional molecular changes underlying EMT.

Keywords

Epithelial to mesenchymal transition; phorbol-12-myristate-13-acetate; Protein kinase C; prostate cancer progression; metastasis

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Introduction

Epithelial to mesenchymal transition (EMT) is a fundamental process for epithelial cell remodeling during embryonic development [1–4]. EMT is accompanied by down-regulation of E-cadherin (E-cad), a key surface protein for intercellular junction between epithelial cells, and by a switch of intermediate filament protein expression from cytokeratins to vimentin [3, 4]. Following EMT, epithelial cells adopt the morphology and behavior of mesenchymal stromal cells, losing cellular polarity and becoming motile and invasive so these cells can migrate to a specified site for further development and specialization. Through sequential EMT events, epithelial cells of the primary ectoderm form endoderm and mesoderm, which reorganize into epithelial cells for further EMT to form various somatic and internal visceral organs. After completion of embryonic development and in adult stages, epithelial cells are considered to be in a stable state [5]. Additional EMT would cause migration and invasion of the epithelial cells into the mesenchymal stromal compartment.

The biological mechanism of EMT may be hijacked by prostate cancer cells, to the advantage of migration and invasion [6–8]. Although cancer cells in the transition state of an EMT are rarely seen and bona fide EMT in tumor specimens is controversial [9], prostate tumor cells are known to have the tendency to lose epithelial properties and acquire stromal characteristics. Similar to EMT in early embryogenesis, for example, prostate cancer cells are frequently seen to have lost E-cad expression [10], and to have switched expression of the intermediate filament protein expression from cytokeratins to vimentin [11], while loss of polarity and acquired motility are common features of prostate tumor cells. Moreover, the EMT-like phenotype is positively correlated to prostate cancer progression and metastasis [6], making EMT a highly relevant issue to prostate cancer progression and metastasis.

We used human prostate cancer ARCaP_E cells to study the mechanism of EMT during prostate cancer progression and metastasis [6, 8, 12]. With an epithelial morphology and tight intercellular junction, ARCaP_E cells form a cobblestone-like organization. Upon induction by soluble factors, such as epidermal growth factor (EGF), insulin-like growth factor (IGF-1), transforming growth factor (TGF β) [12, 13] and β 2-microglobulin [6], these cells undertook a series of morphologic and behavioral changes reminiscent of EMT. Upon overexpression of EMT-related genes, such as the SNAIL [12], ZEB1 [13], and LIV-1 [6], ARCaP_E cells adopted the morphology of mesenchymal stromal cells with increased tumorigenic potency. When subjected to repeated inoculation in athymic mice, ARCaP_E cells recovered from xenograft tumors showed mesenchymal stromal morphology and markedly increased tumorigenic potential [8, 14]. ARCaP_E is an ideal model for studying abnormal EMT or EMT-like changes during prostate cancer progression and metastasis [6].

The mechanism regulating EMT-like changes in prostate cancer progression and metastasis remains to be elucidated. EMT in ARCaP_E cells could be induced by soluble factors [12, 13], probably through surface receptor-mediated intracellular signaling, while intracellular signal transduction pathway critical to the EMT has yet to be identified. On the other hand, it is well known that specific chemical reagents can function as second messengers to activate specific signal transduction pathways. These reagents are useful tools for identifying critical signal transduction mediators. Employing these reagents, we examined signal transduction pathways for their role in promoting EMT in ARCaP_E cells. In this report using behavioral and expressional assays, we identified phorbol ester, phorbol-12-myristate-13-acetate (PMA), a Specific agonist of the protein kinase C (PKC) isoenzymes, as a potent inducer of EMT-like changes in ARCaP_E cells.

Materials and Methods

Reagents

PMA, Gö6983, cyclohexamide (CHX), and actinomycin D (Act.D) were purchased from Sigma-Aldrich (St. Louis, MO). Bisindolylmaleimide I was purchased from EMD Biosciences (La Jolla, CA). On receipt, all reagents were dissolved in dimethyl sulfoxide (DMSO) as stock solutions. When these agents were used in an experiment, equal volumes of DMSO were added to a parallel group as control.

Cell line

Human prostate cancer ARCaP_E cells [15, 16] were cultured in T-medium (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), penicillin (100 unites/ml) and streptomycin (100 µg/ml). Cells were cultured in a humidified incubator at 37°C with atmospheric O₂ supplemented with 5% CO₂.

Assay for cell proliferation

The protocol for assaying cell proliferation was reported previously [8]. In this study, cells were plated onto 96-well plate at a density of 2.5×10^4 /100 µl medium in each well for 24 hours. The cells were then treated in triplicate with different concentrations of PMA for 48 hours before subjected to proliferation assay.

Assay for cell migration

Two methods were used to assay for cell migration. The protocol for assaying cell migration using the Boyden chamber assay was reported previously [8]. To evaluate cell migration by the scratch wound healing method, cells on a 6-well plate were allowed to grow to full confluence. A scratch wound was made by denuding a streak of the cell monolayer with a sterile pipette tip 2.6 mm in diameter. The culture was treated with 100 nM PMA for 96 hours. Migration of the cells into the denuded space was documented by microphotography.

Assay for cell invasion

The assay method for cell invasion was reported previously [12]. In this study, cells were treated with 100 nM PMA for 48 hours in the Transwell inserts covered with 100 µl Matrigel (BD Biosciences, San Jose, CA). Cells that invaded to chamber and on the outer surface of the insert were collected and subjected to MTT assay.

Western blotting

The Western blotting protocol used was reported previously [8]. In this study, antibodies to E-cad, vimentin, cytokeratin 18 (CK-18), β-actin, and all the secondary antibodies conjugated with horseradish-peroxidase were purchased (Santa Cruz Biotechnology, Santa Cruz, CA). Specific signals were detected with the ECL plus western blotting detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ).

Microphotography

Cell images were documented with a Nikon E300 inverted microscope equipped with a MagnaFire digital camera.

Results

The phorbol ester PMA is a specific PKC activator [17] and a potent tumor promoter in experimental mice [18–20]. At the cellular level, PMA promotes migration and invasion in established cell lines [21, 22]. Because increased migration and invasion in ARCaP_E cells

are often accompanied by EMT, we assessed whether PMA could induce EMT in these cells.

1. Treatment with PMA results in EMT in ARCaP_E cells

EMT-associated morphologic changes in ARCaP_E cells have been described in detail [6, 8, 12]. When cultured *in vitro*, ARCaP_E cells display distinct epithelial morphology, in pentagonal shapes with tight intercellular junctions, forming a cobblestone-like clonal organization. After committing to EMT, ARCaP_E cells adopt the morphology of mesenchymal stromal cells, becoming dissociated from each other, losing cellular polarity, and adopting spindle-like shapes. The morphologic change is followed by increased cellular motility, as ARCaP_E cells committed to EMT displayed migratory and invasive behavior.

To assess whether PMA induces EMT, we treated ARCaP_E cells with different concentrations of PMA, and inspected cellular changes daily for signs of EMT-like morphologic changes. These experiments revealed that the phorbol ester could effectively induce EMT-like changes in ARCaP_E cells. Whereas PMA at 1 nM did not cause morphologic changes, ARCaP_E cells treated with 10 nM PMA became enlarged and flat (Figure 1). Importantly, PMA at 50 nM and 100 nM induced complete morphologic changes in 24 hours (Figure 1). Compared to a control group, the treated cells separated from each other, became spindle shaped, and lost cellular polarity. These cells rearranged in all directions and stacked on each other when cell density increased along with the treatment time. The results from these studies indicated that PMA induced EMT-like changes in ARCaP_E cells.

From four repeated experiments, we found two interesting features of the PMA-induced EMT-like changes. First, the effect of PMA is time-dependent. In every experiment performed, no morphological changes were observed in ARCaP_E cells in the first 8 hours of treatment. The morphological changes appeared gradually afterwards, with cell shape changes becoming discernible at 16 hours, and all the cells exhibited mesenchymal stromal morphology at 24 hours (Figure 1). Thereafter, the morphologic change was sustained as long as PMA was present. Second, although the effect of PMA seemed dose-dependent within the 100 nM range (Figure 1), higher concentrations of PMA (200 nM, 500 nM, and 1000 nM) did not cause additional morphologic changes, nor did these concentrations accelerate the rate of morphologic change. This observation indicated that the dose effect of PMA was limited by intracellular factors, probably by the availability of PKC proteins.

The EMT in ARCaP_E cells could be irreversible, since ARCaP_E clones retrieved from xenograft tumors displayed permanent EMT features [14]. To determine whether the PMA-induced changes were reversible, we recovered the ARCaP_E cells that were treated with 100 nM PMA for 96 hours. After being detached by trypsin and washed in PBS, the cells were cultured in the absence of PMA. Displaying spindle-shaped stromal morphology at beginning of the culture, these cells underwent cell division to form colonies that were completely epithelial in cell shape and colony organization, indistinguishable from untreated ARCaP_E cells. PMA-induced EMT in ARCaP_E cells is thus reversible. In experiments to assess the effect on cell growth and survival, PMA did not induce significant changes in cell growth at doses from 50 nM to 1000 nM. On the other hand, PMA in this dose range did not cause significant cell death within 96 hours of the treatment. It seems that EMT is a specific response of the ARCaP_E human prostate cancer cells to the PMA treatment.

2. PMA promoted EMT in ARCaP_E cells by activating PKC isoenzymes

The PKC family comprises three subfamilies of isoenzymes [17, 23, 24], while PMA is a known activator of both the conventional (α , β I, β II, and γ isoenzymes) and novel (δ , ϵ , η ,

and θ isoenzymes) PKC subfamilies in the 1 – 100 nM range. To confirm that PMA promoted EMT in ARCaP_E cells by activating PKC, we used specific PKC inhibitors to block the effect of PMA. A general PKC inhibitor, Gö6983, was used at 200 nM in order to inhibit all the PKC isoenzymes. This inhibition led to significant inhibition of EMT in ARCaP_E cells (Figure 2). A well characterized isoenzyme-specific inhibitor, bisindolylmaleimide I, was known to inhibit conventional PKC isoenzymes at 20 nM, and to inhibit novel PKC isoenzymes at much higher concentrations [25, 26]. Bisindolylmaleimide I at 50 nM did not show any inhibitory effect, whereas at 1000 nM it completely prevented PMA-induced EMT in ARCaP_E cells (Figure 2). These results supported that PMA promoted EMT in ARCaP_E cells by activating PKC, and certain isoenzymes of the novel PKC subfamily may be mediating the promoting effect. In addition, the PKC inhibitors tested in this study did not cause death in ARCaP_E cells after a 96 hour treatment, suggesting that PKC isoenzymes are not involved in maintaining the survival of ARCaP_E cells.

3. PMA inhibits expression of epithelial markers but induces mesenchymal stromal markers

Besides morphologic changes, EMT in ARCaP_E cells is accompanied by reduced expression of epithelial markers and by activated expression of stromal markers [6, 12]. Accordingly, we examined the effect of PMA on EMT-related marker protein expression (Figure 3). ARCaP_E cells treated with 100 nM PMA for different times were sampled for western blotting with specific antibodies to E-cad, CK-18, and vimentin. These analyses revealed that PMA inhibited the expression of E-cad, a critical surface protein maintaining intercellular junction between epithelial cells. The inhibition was a time-dependent process. Though there was no change in the level of E-cad within the first 8 hours, the level of E-cad decreased afterwards as treatment prolonged, and almost completely disappeared after 72 hours of PMA treatment. The decreased E-cad expression was accompanied by reduced expression of CK-18, an epithelial cell-specific intermediate filament protein. Concurrently, expression of the stromal cell-specific intermediate filament protein vimentin was activated (Figure 3). These results indicated that PMA treatment suppressed expression of epithelial cell marker proteins and stimulated expressional activity of the stromal marker.

4. PMA promotes migration and invasion of ARCaP_E cells

PMA is well known for its function of promoting cell motility and scattering [21, 22], and EMT in ARCaP_E cells was accompanied by increased migration and invasion [12]. We assessed the effect of PMA on the motility of ARCaP_E cells. Two methods, scratch wound healing and the Boyden chamber assay, were used to detect changes in cell migration. In both assays, PMA showed a marked stimulating effect on ARCaP_E cell migration (Figures 4A and 4B). A similar stimulating effect was observed in the cell invasion assay, in which PMA induced significantly accelerated invasion of ARCaP_E cells through the Matrigel (Figure 4C). In these assays, the PMA-induced cellular motility could be inhibited effectively by the PKC inhibitor bisindolylmaleimide I at 1000 nM (Figures 4B and 4C). Bisindolylmaleimide I at 50 nM did not show any inhibition. These results suggested that the increased motility of ARCaP_E cells was mediated by activation of the novel PKC isoenzymes.

5. PMA-induced EMT in ARCaP_E cells is dependent on gene transcription and translation

We noticed that in both the process of morphological changes and the process of expression alterations, PMA-induced EMT had a latent phase of more than 8 hours. The delay in EMT could be caused by the new transcription and translation of additional genes required to execute the EMT process. To explore the role of new gene transcription and translation in EMT, we added actinomycin D (Act.D), a specific inhibitor of gene transcription, together

with PMA to ARCaP_E cells. Compared to the control group, in which PMA induced marked EMT at 48 hours, cells with the combinatory treatment failed to adopt mesenchymal stromal cell morphologies (Figure 5). Similar results were obtained when cyclohexamide (CHX), a specific inhibitor of protein translation, was used in combination with PMA (Figure 5). As inhibitors for transcription and translation, both Act.D and CHX showed cytotoxicity to ARCaP_E cells after 24 hours, and beyond 48 hours many cells started to die. Nonetheless, none of the remained cells adapted stromal morphology even after 96 hours of treatment. These results suggested that that PMA-induced EMT requires transcriptional activation and EMT functions require newly synthesized proteins.

Discussion

By applying PMA treatment to the well established EMT model of human prostate cancer ARCaP_E cells, we found that PMA is a potent promoter for EMT-like phenotypes. PMA induced ARCaP_E cells to undergo marked morphologic change from epithelial to mesenchymal stromal cell shapes (Figure 1). The morphologic changes were accompanied by expressional changes, in which ARCaP_E cells lost the epithelial markers E-cad and CK-18, and switched on the expression of stromal intermediate filament protein vimentin (Figure 3). Loss of E-cad and the activated vimentin expression are the two most informative markers of EMT [27]. In addition, PMA-treated ARCaP_E cells showed significantly increased motility, both in assays for cell migration and cell invasion (Figure 4), supporting the conclusion that PMA is a potent inducer of EMT in ARCaP_E prostate cancer cells. We observed similar morphologic changes in MCF-7 breast cancer cells upon PMA treatment (data not shown). Although PMA is known to be a stimulator of cell motility and scattering, few studies have reported accompanying morphologic changes caused by PMA. This work is the first to demonstrate that PMA is an EMT inducer.

PMA, when applied *in vivo*, is a potent promoter for mouse skin tumorigenesis [18–20]. The effect of PMA is mainly through mimicking diacylglycerol, a lipid metabolite of the cytoplasmic membrane and natural second messenger activating PKC isoenzymes. As a potent agonist, PMA triggers autophosphorylation of both conventional and novel PKC isoenzymes. The activated PKC in turn functions as a serine and threonine kinase to catalyze phosphorylation of a wide panel of regulatory proteins [24]. Using a general PKC inhibitor and an isoenzyme-specific inhibitor, we demonstrated that PMA induced EMT in ARCaP_E cells by activating novel PKC isoenzymes (Figure 2). Additional studies are needed to identify which of the novel PKC isoenzymes is the principal mediator for EMT. Further investigation is warranted to elucidate the mechanism of the isoenzymes in mediating EMT.

The expression level and catalytic activity of PKC isoenzymes are correlated to cancer progression and tumor metastasis [23]. Members of the novel PKC subfamily have been known to promote cell migration and invasion in other cell lines [28–30]. Strategies targeting specific PKC isoenzymes are shown to reduce tumor growth and metastasis [28, 31–35]. It would be intriguing to investigate whether specific targeting strategies could be developed to target specific isoenzymes to prevent tumor cells from committing to EMT. On the other hand, we have reported that EMT in ARCaP_E cells is controlled by transcription factors including SNAIL, a substrate of serine and threonine kinase [12]. It is intriguing to investigate whether PMA-activated novel PKC isoenzymes causes phosphorylation and nuclear localization of these transcription factors.

We found that PMA effectively suppressed E-cad protein expression in a time-dependent manner (Figure 3). Further studies have to be conducted to determine the mechanism by which PMA suppresses E-cad expression. As a cytoplasmic membrane protein, E-cad has a dual function [36–38]. Besides functioning as a structural protein for intercellular junctions,

E-cad sequesters β -catenin to prevent it from translocation to nucleus, where β -catenin functions as a transcriptional co-regulator to activate a panel of stromal specific genes. In ARCaP_E cells, it is possible that after administration of PMA and upon loss of E-cad expression, β -catenin undergoes nuclear translocation to activate the expression of stromal specific genes, which function to cause the dramatic morphologic and behavioral changes observed in ARCaP_E cells. This study demonstrated that inhibiting either new gene transcription or new protein synthesis could inhibit EMT (Figure 5), strongly indicating that the stromal phenotype was supported by newly expressed stromal specific genes. PMA-treated ARCaP_E cells should be an ideal model for studying the expression of stromal specific genes during EMT.

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Figure 1. PMA is a potent inducer of EMT

In these experiments, human prostate cancer ARCaP_E cells were treated with different concentrations of PMA for 24 hours. ARCaP_E cells in regular culture (PMA, 0 nM) display epithelial morphology, with pentagonal cells in tight intercellular junction forming cobblestone-like cellular organization. PMA in the 100 nM range induced dose-dependent morphologic changes. Whereas lower concentrations (PMA, 1 nM and PMA, 10 nM) induced partial morphologic changes, a higher dose (PMA, 100 nM) induced complete conversion of ARCaP_E cells to mesenchymal stromal morphology, suggesting EMT. PMA at 500 nM and 1000 nM even higher concentrations (PMA, 500 nM and 1000 nM) did not cause additional morphologic changes. The result is representative of four repeated experiments. Phase contrast microphotographs are shown at 100× magnification.

Figure 2. PMA induces EMT by activating PKC

ARCaP_E cells were treated with PMA (100 nM) in the presence of PKC inhibitor Gö6983 or Bisindolylmaleimide I for 48 hours. Gö6983 at 200 nM inhibited EMT. A low concentration of Bisindolylmaleimide I (50 nM) did not show an inhibitory effect, whereas Bisindolylmaleimide I at 1000 nM completely prevented EMT, indicating that members of the novel PKC subfamily were main mediators in PMA-induced EMT. The result is representative of three repeated experiments. Microphotographs are shown at 100× magnification.

Figure 3. PMA inhibits epithelial marker E-cad expression and induces stromal marker vimentin

ARCaP_E cells treated with PMA (100 nM) were sampled at different times (hr.) for western blot analysis of EMT markers. The result is representative of two repeated experiments.

Figure 4. PMA promotes cell migration and invasion

Cell migration was assayed by two methods. **A**, in a scratch wound healing assay, ARCaP_E cells treated with 100 nM PMA were recorded for cell migration by microphotography. The result is representative of three repeated experiments. Microphotographs are shown at 40× magnification. **B**, in a Boyden Chamber migration assay, ARCaP_E cells grown on the inserts were treated for 96 hours with 100 nM PMA (PMA), 1000 nM Bisindolylmaleimide I (Bis.I), or a combination of the two (PMA+Bis.I). Cells migrating across the filters were collected and quantified by MTT conversion. Each bar represents the mean of triplicate assays from three Boyden Chambers. The result is representative of two separate experiments. **C**, to determine cell invasion, ARCaP_E cells grown on Matrigel coated inserts were subjected to the same treatment. Cells migrating through the Matrigel-coated filters were collected and quantified by MTT conversion. Each bar represents the mean of triplicate assays from three Boyden Chambers. The result is representative of two separate experiments.

Figure 5. PMA-induced EMT requires gene transcription and newly synthesized proteins
ARCaP_E cells were treated for 48 hours with PMA (100 nM) in the presence of Act.D, an inhibitor of gene transcription, or CHX, an inhibitor of protein synthesis. Compared to the control group, both Act.D and CHX inhibited ARCaP_E cells from adopting mesenchymal stromal morphology. The result is representative of three repeated experiments. Microphotographs are shown at 100× magnification.