Differentiation of Androgen-Independent Prostate Cancer PC-3 Cells Is Associated with Increased Nuclear Factor-κB Activity

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Abstract

Recently, we have reported that inosine 5′-monophosphate dehydrogenase inhibitors, such as mycophenolic acid (MPA), induce the differentiation of PC-3 cells, which are derived from a human androgen-independent prostate cancer, into cells with a phenotype resembling maturing prostate secretory cells. Here, we describe such differentiation induced by the histone deacetylase inhibitor tributyrin. The maturation was defined by cytoplasmic vacuole production and induction of CD10, CD46, CD55, GRP78, keratin 17, and zinc-α-2-glycoprotein. To identify additional genes associated with tributyrin-induced PC-3 cell differentiation and to gain some insight into the mechanism that underlies this differentiation, we have, by means of microarray analyses, compared tributyrin-induced gene expression patterns with those of MPA, which initiates PC-3 cell differentiation by a dissimilar mode of action. We suggested that genes induced by both tributyrin and MPA would be most likely associated with differentiation rather than with the unique action of each particular inducer. Our results indicated that tributyrin or MPA induced the expression of a large number of common genes, including genes known or assumed to be NF-κB dependent. The NF-κB dependency of a group of these genes, which included the PC-3 cell differentiation marker keratin 17, was confirmed by using two common NF-κB activation inhibitors, Bay11-082 and TMB-8, and p65 subunit of NF-κB complex specific small interfering RNA. Taken together, our results implicate both NF-κB–dependent and NF-κB–independent genes in the processes leading to PC-3 cell differentiation induced by tributyrin and MPA. (Cancer Res 2005; 65(24): 11588-96)

Introduction

Prostate cancer is the most commonly diagnosed malignancy in men and second only to lung cancer as a leading cause of tumor deaths in males. In 2004, ~230,000 men in the United States were diagnosed with prostate cancer and ~30,000 died from the disease (1). Unfortunately, there is no cure for locally advanced or metastatic prostate cancer. It is, therefore, imperative that new strategies for the effective management and/or treatment of this morbid disease be found.

A potential innovative treatment that may eliminate many of the consequences of the current prostate cancer chemotherapy, e.g., systemic toxicity, is differentiation therapy (2). This treatment, which has been successfully used to control promyelocytic leukemia (3), has not been as yet exploited in many other malignances. Differentiation therapy would be, in particular, valuable in the management of prostate cancer during the androgen-independent stage as this stage is usually refractory to standard chemotherapy (4).

A hindrance in the development and identification of differentiation therapy drugs was the lack of an appropriate prostate cancer cell differentiation model, in particular one that uses cells derived from androgen-independent tumors. Recently, we described such a system using androgen-independent prostate cancer PC-3 cells. By means of this model, we were able to show that inosine 5′-monophosphate dehydrogenase inhibitors, mycophenolic acid (MPA) in particular, are effective inducers of cellular differentiation of prostate cancer cells (5). The mature phenotype in the PC-3 cells was characterized by replication arrest, cytoplasmic vacuolization, and induced or increased expression of prostasomal proteins and keratins, which are most appropriate for defining maturing prostate secretory cells. Another group of inhibitors, namely histone deacetylase inhibitors, including tributyrin, is known to induce differentiation in a number of distinct cell types (6). In PC-3 cells, tributyrin was found to evoke replication arrest and to cause specific morphologic changes (7). Yet, this study failed to examine more specific markers of maturing prostate secretory cells, such as prostasomal proteins and keratins.

The present studies were initiated to further substantiate the ability of tributyrin to induce PC-3 cell maturation and to gain insight into the mechanism that underlies such a differentiation. To achieve this goal, we did microarray analyses using RNAs isolated from cells treated with either MPA or tributyrin. We assumed that at a certain stage, the signaling events initiated by these two inducers, which at the start operate by a distinct mode of action, would converge and from then on would be shared and as such be most likely specific for the differentiation process. Here, we report that both inducers lead to the increased expression of a large number of common genes, including NF-κB–dependent genes, secretory and plasma membrane proteins, and keratins. By using specific inhibitors and a small interfering RNA (siRNA) approach, we found that NF-κB activation is a key event leading to PC-3 cell differentiation.

Materials and Methods

Cell cultures and chemicals. PC-3 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured, plated, counted, and analyzed for their vacuoles content and cell cycle fractions distribution as previously described (5). MPA (Sigma, St. Louis, MO) was dissolved in DMSO (Fisher Scientific, Hampton, NH) as a stock solution of

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Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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20 mg/mL, tributyrin (3.4 mol/L, Sigma) was diluted in DMSO as a 1 mol/L stock solution, TMB-8 (Sigma) was diluted in DMSO as 300 mmol/L stock solution, and Bay 11-7082 (Biomol International, Plymouth Meeting, PA) was dissolved in DMSO as a 50 mmol/L stock solution. DMSO was also used for mock treatments.

Reverse transcription-PCR. Total RNA was isolated from cells after they were washed twice with cold PBS using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. Isolated RNA was dissolved in diethylpyrocarbonate-treated water (0.1% diethylpyrocarbonate was added to water overnight and then autoclaved for 20 minutes to destroy diethylpyrocarbonate). RNA at 4 μg was used to generate a first-strand cDNA by reverse transcription reaction according to the instructions of the manufacturer (Invitrogen).

Real-time PCR. Generated cDNAs were diluted 20 times and 1 μL was used per reaction using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) according to the protocol of the manufacturer. Primers for tested genes (Supplement 2, Table S1) were selected from the PrimerBank database (8) and synthesized by MWG-Biotech (High Point, NC). Reactions were monitored by real-time PCR system Mx4000 (Stratagene, La Jolla, CA) using settings for the SYBR Green protocol and relative changes in gene expression were calculated according the following formula: fold change = $2^{-\Delta C_t}$, $\Delta C_t = C_t$ (treated) $- C_t$ (control). Data were normalized to the expression levels of *RPS25*. The melting curve analysis was done for each pair of primers.

Microarray experiment. Total RNA was isolated as described above. RNA purification, quality evaluation, target preparation, hybridization to Affymetrix U133 2.0 chip, and basic data analyses were done at the Core Facility of University of Chicago (see Supplement 1).

Nuclei isolation and Western blot analysis. Nuclei from mock- or tributyrin-treated PC-3 cells were isolated as described in Supplement 1. Western blot analysis was done as described in Supplement 1 using a primary antibody anti-p65 (mouse anti-human, Santa Cruz Biotechnology, Santa Cruz, CA) at dilution 1:500 and a secondary antibody (goat anti-mouse labeled with horseradish peroxidase (HRP), Southern Biotechnology Group, Birmingham, AL) at dilution 1:20,000.

Chromatin immunoprecipitation. PC-3 cells (10^7 total) were either mock-treated or treated with 1 mmol/L tributyrin for 3 hours. Chromatin immunoprecipitation was done by the modifications of existing protocols (see Supplement 1; refs. 9, 10).

Transfection of small interfering RNA oligonucleotides. Previously published protocol (11) was used to perform siRNA transfection of PC-3 cells. Control nonspecific siRNA (sense 5′-AGGUAGUGUAAUCGCCUUG dTdT-3′) and p65 specific siRNA (sense 5′-GCCCUAUCCCUUUACGUCA dTdT-3′) were purchased from MWG-Biotech and diluted as a 200 μmol/L stock solution. Two days after transfection, cells were treated with 1 mmol/L tributyrin for 12 hours. At the end of treatment, cells were harvested, washed in cold PBS, and resuspended in 900 μL of PBS. Cells in 300 μL of PBS were used for Western blotting [rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Abcam, Cambridge, MA) was used at 1:2,500 dilution to monitor loading] and the rest was used for RNA isolation to perform a real-time PCR assay as described above. Western blots were quantified by the software Quantity One (Bio-Rad, Hercules, CA).

Results

Tributyrin induces replication arrest, cell cycle block, and differentiation in PC-3 cells. Tributyrin, a histone deacetylase inhibitor, was reported to induce replication arrest and cell cycle block in the human prostate PC-3 tumor cells (7). To extend these studies, we decided initially to confirm these observations under our experimental conditions. Treatment of PC-3 cells with 1 to 5 mmol/L tributyrin for 3 days resulted in replication arrest (Fig. 1*A*), which was up to 3 mmol/L dose dependent; higher doses yielded a plateau effect. This replication arrest was also validated by cell cycle analysis using flow cytometry. A 1-day mock treatment of proliferating PC-3 cells yielded 42% of cells in the G_0-G_1 phase, 26% of cells in the S-phase, and 31% of cells in the G_2-M phase. Treatment of PC-3 cells with 1 mmol/L tributyrin for only 1 day increased the percentage of cells in the G_0-G_1 phase to 82% and reduced the percentage of the cells to 4% in the S-phase and 12% in the G_2-M phase. The subdiploid population of cells accounted for ~2%. Extending tributyrin treatment for up to 3 days did not markedly alter this pattern (Fig. 1*B*).

Tributyrin induces cellular differentiation in various cell types, e.g., human myeloid leukemia, melanoma, or colon tumor cells (6, 12). It was, therefore, of interest to determine whether it can also induce the maturation of PC-3 cells, which derived from an androgen-independent human prostate cancer (13). To show this differentiation, we assayed for the production of cytoplasmic vacuoles and expression of *CD10, CD46, CD55, GRP78, zinc-α-2-glycoprotein,* and *keratin 17* by real-time PCR. These markers were chosen because they were instrumental in characterizing PC-3 cell maturation by MPA, an inhibitor of inosine 5′-monophosphate dehydrogenase (5).

Treatment of PC-3 cells with 1 mmol/L tributyrin caused an enlargement in cell volume and production of cytoplasmic vacuoles, which increased in size (Fig. 1*C*) and number in a time-dependent manner (Fig. 1*D*). After 5 days of treatment, ~60% of the cells contained these vacuoles compared with <5% of control cells (Fig. 1*D*). In addition, at 12 hours of treatment with 1 mmol/L tributyrin, there was a 2-fold increase in the expression of *CD55* and at 3 days an increase in *zinc-α-2-glycoprotein* (9×), *CD10* (6×), *CD46* (4×), *GRP78* (2×), and *keratin 17* (5×) expression as measured by real-time PCR.

Taken together, these results indicate that tributyrin induces in PC-3 cells replication arrest and a cell cycle block in the G_1 phase and triggers the expression of differentiation markers associated with a mature phenotype of prostate secretory cells (5).

Tributyrin and mycophenolic acid induce the expression of a set of similar genes. To identify additional genes whose expression is associated with PC-3 cell differentiation, we compared the patterns of tributyrin-induced gene expression with those of MPA, which initiates the differentiation process by a different mode of action—tributyrin by inhibiting histone deacetylation (6) and MPA by inhibiting inosine 5′-monophosphate dehydrogenase (14). We suggested that genes induced by both tributyrin and MPA would be most likely associated with PC-3 cell differentiation rather than with their unique actions.

The tributyrin- and MPA-induced gene expression patterns were obtained by performing microarray analyses using the Affymetrix chip. Total RNA was isolated from mock-treated control cells or cells treated for 3, 12, and 72 hours with 1 mmol/L tributyrin or for 6, 12, and 72 hours with 20 μmol/L MPA. The different initial time points were selected to reflect the stronger effect exhibited by tributyrin relative to MPA with respect to the increase in the cell size and vacuolization. Samples were labeled by reverse transcription and hybridized to the chip. Data were normalized and analyzed as described in Materials and Methods.

The result indicated an abundance of tributyrin-induced changes in gene expression, including an increase in the expression of *CD10, CD55, zinc-α-2-glycoprotein,* and *keratin 17,* thereby confirming our previous observations and serving as an internal positive control for the system. A comparison of the induced expression patterns up to 6 hours after treatment with tributyrin or MPA identified >50 transiently or continuously induced common genes that increased more than twice (Table 1). These include a number of known

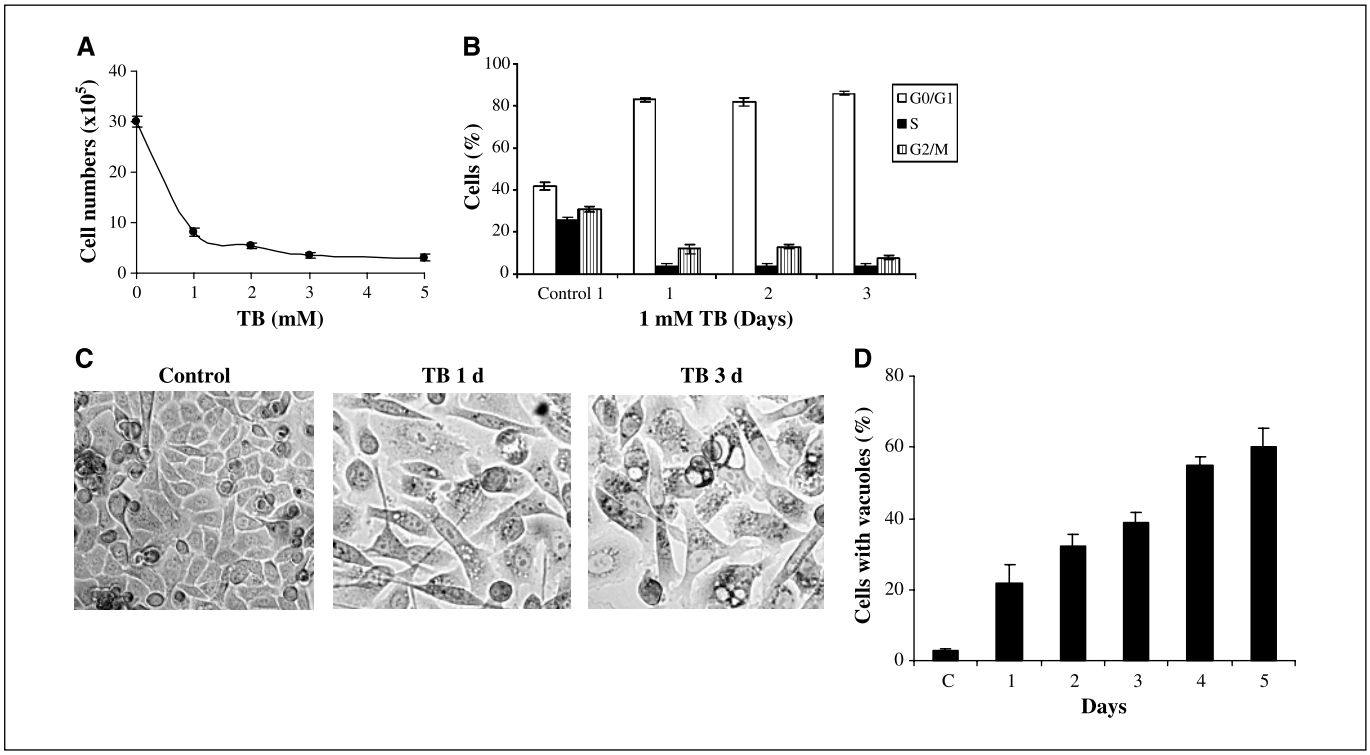


Figure 1. Tributyrin-induced cell growth arrest (*A*), cell cycle block (*B*), and cytoplasmic vacuolization (*C* and *D*). *A,* a day after cells were seeded at 3×10^5 per 60 mm plate in triplicates, they were incubated for 3 days with 1, 2, 3, and 5 mmol/L tributyrin (*TB*). *B,* for the cell cycle analysis, cells were seeded at 1×10^6 per 100 mm plate in triplicates and after 2 days incubated with 1 mmol/L tributyrin. *Columns,* percentage of cells from three independent experiments; *bars,* SD. *C,* a day after seeding PC-3 cells at 0.5×10^5 per 60 mm plate in triplicate, they were treated with 1 mmol/L tributyrin. After treatment, the cells were fixed with 3% paraformaldehyde in PBS, stained with Giemsa solution, and photographed. Magnification, ×10. *D,* percentages of cells with vacuoles were calculated. A cell with at least one visible vacuole was considered positive. *Columns,* percentages of cells with vacuoles from three independent experiments; *bars,* SD.

NF-κB-dependent genes, e.g., inflammatory cytokines and chemokines, such as *interleukin (IL)-6, IL-1β*, and related genes (*IL-1β–dependent pentaxin-related gene* or *IL-8*), *chemokine (C-C motif) ligand 20* (*CCL20*), *chemokine (C-X-C motif) ligand 3* (*CXCL3*), or *chemokine (C-X-C motif) ligand 5* (*CXCL5*) as well as *tumor necrosis factor (TNF)-α–induced protein 3* (*A20*), *antiapoptotic Bcl2-A1 protein*, and *cyclooxygenase-2* (*COX-2*). It is noteworthy that genes induced by both tributyrin and MPA also included *plasminogen activator inhibitor-2 (PAI-2),* which was shown to be associated with suppression of invasive activity in cancer TSU-Pr1 cells treated with 12-*O*-tetra-decanoylphorbol-13-acetate (15), *claudin 1,* and *occludin,* which are implicated in cell junction formation and maintenance (16), several keratins, which typically serve as epithelial cell differentiation markers (17), and the cell cycle regulator *p21/Cip1* (18).

A substantially higher number of common tributyrin- and MPA-induced genes were found at 72 hours after treatment (Supplement 2, Table S2). This group included >100 induced genes, which were not detected early after tributyrin or MPA treatment. Interestingly, tributyrin and MPA were found to induce a substantial number of genes coding for keratins and secretory or plasma membrane proteins. Changes in the expression of series of these genes were confirmed by real-time PCR using a SYBR green protocol. The tested keratins included *cytokeratin type II/K6HF* (110×, 309×), *keratin 4* (2×, 6×), *keratin 6B* (18×, 10×), and *keratin 19* (19×, 11×), whereas the secretory or plasma membrane proteins included *carcinoembryonic antigen-related cell adhesion molecule 5* (190×, 11×), *carboxypeptidase A4* (60×, 42×), *IL-13 receptor A2* (58×, 4×), *TNF ligand superfamily, member 9* (46×, 29×), *matrix Gla protein* (30×, 2×), *integrin β4* (25×, 2×), *TNF-α induced protein 2* (23×, 25×), *cathepsin B* (22×, 8×), *kallikrein 5* (19×, 69×), *Cyr61* (13×, 14×), *perlecan* (7×, 3×), *Mac-2-binding protein* (7×, 5×), *galectin* (6×, 3×), and *tryptase-ε/serine protease 22* (4×, 2×). The numbers in brackets indicate the induction fold over the control of the tributyrin- and MPA-evoked gene expression, respectively. We assume that these late induced keratins and secretory and plasma membrane proteins define the phenotype of the mature PC-3 cells and as such most likely represent bona fide differentiation markers.

MPA and tributyrin also caused a down-regulated expression of a set of common genes. Interestingly, only one of these genes (*hyaluronan synthase*) was already found to be down-regulated up to 6 hours of treatment. The number of common down-regulated genes increased with time—17 genes at 12 hours (Supplement 2, Table S3) and >500 genes at 72 hours of treatment. Gene ontology analysis revealed that MPA and tributyrin treatment for 72 hours affected mainly the expression of genes involved in cell growth and/or maintenance, cell cycle control, and DNA synthesis. Moreover, microarray data analysis revealed that tributyrin was more effective than MPA in down-regulating gene expression when compared at levels of 3-fold or higher (Supplement 2, Table S4). To confirm some of these observations, we did real-time PCR. These results confirmed that tributyrin was indeed more effective than MPA in reducing the expression of several tested genes (e.g., *hyaluronan synthase,* tributyrin 66×, MPA 3×, *farnesyltransferase β,* tributyrin 4×, MPA 2×, *cyclin B1,* tributyrin 3×, MPA 2× at 12 hours of treatment; *Cdc25C,* tributyrin 45×, MPA 2×, *thymidylate kinase,* tributyrin 3×, and MPA 2× at 72 hours of treatment). We

Table 1. Common up-regulated genes expressed after 3-hour treatment with tributyrin and 6-hour treatment with MPA

Gene	Accession no.	Tributyrin 3 h	Tributyrin 72 h	MPA 6 h	MPA 72 h
Chemokine (C-C motif) ligand 20	NM_004591	40.9	21.5	37.8	27
Chemokine (C-X-C motif) ligand 3	NM_002090	21.6		2.7	4
Interleukin 6 (IFNβ2)	NM_000600	17.1	3	5.3	15
TNF-α-induced protein 3	NM_006290	12.1	3.3	4.2	6.6
Baculoviral IAP repeat-containing 3	U37546	11.7		4.1	5.4
Pentaxin-related gene, rapidly induced by IL-1β	NM_002852	11.3	3.8	4.2	2.1
Chemokine (C-X-C motif) ligand 5	AK026546	9.6		3.9	9.2
Adrenergic, β-2-, receptor, surface	NM_000024	9.1	2.2	3	2.6
Guanylate-binding protein 1, IFN-inducible, 67 kDa	AW014593	8.1	3.9	17.8	4.6
Interleukin 1, β	NM_000576	6.7	5.3	2.8	2.5
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	NM_002575	6.6		12.9	
Activating transcription factor 3	NM_001674	6.4	2.3	2.2	11.3
Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)	NM_001945	6.3		3	2.3
Tuftelin 1	NM_020127	5		2.4	2.7
Regulator of G-protein signaling 4	AL514445	4.8	3	3.7	13
Dual specificity phosphatase 1	NM_004417	4.6		2.5	4.4
V-ets erythroblastosis virus E26 oncogene homologue 1 (avian)	BC017314	4.4		3	
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	4.2	3.5	2.2	3.4
V-ets erythroblastosis virus E26 oncogene homologue 1 (avian)	BE218980	4.2		2.1	
Endothelin 1	NM_001955	4	3.1	2.2	
Inhibitor of DNA-binding 2, dominant negative helix-loop-helix protein	D13891	3.9		3.1	
Oxidized low density lipoprotein (lectin-like) receptor 1	AF035776	3.8		3.6	
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	NM_000963	3.8	2.3	3	
Cysteine-rich, angiogenic inducer, 61	AF003114	3.7		2.3	2.6
Inhibitor of DNA-binding 2, dominant negative helix-loop-helix protein	NM_002166	3.5		2.8	
Superoxide dismutase 2, mitochondrial	X15132	3.5		2	
Interleukin 8	AF043337	3.4	2.4	2.8	2.6
Connective tissue growth factor	M92934	3.4		2.7	
A kinase (PRKA) anchor protein (gravin) 12	BF511276	3.3		3.7	
Inhibitor of DNA-binding 3, dominant negative helix-loop-helix protein	NM_002167	3.3		3.2	
Adrenomedullin	NM_001124	3.2	2.8	2.7	4.7
Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	NM_002993	3.1	2.1	2.1	2.5
Matrix metalloproteinase 13 (collagenase 3)	NM_002427	3		4	2.1
Programmed cell death 1 ligand 1	AI608902	3		3.1	2.6
Inhibin, βA (activin A, activin ABα polypeptide)	NM_002192	3		2.4	2.6
Keratin 6B	AI831452	3	8.4	2.4	3.2
Dual adaptor of phosphotyrosine and 3-phosphoinositides	AA150186	2.9		2.6	
Protein kinase STYK1	NM_018423	2.9		2.2	
Ubiquitin specific protease 53	H25097	2.9	3.1	2.1	
Claudin 1	NM_021101	2.8	2	3.1	
UDP-glucose ceramide glucosyltransferase	AI378044	2.8	2.2	2.5	
Salvador homologue 1 (Drosophila)	BF983202	2.8		2.1	
Chemokine (C-X-C motif) receptor 4	AJ224869	2.8		2	
Occludin	U53823	2.7		2.1	
Nuclear factor of κ light polypeptide gene enhancer in B-cells 2 (p49/p100)	BC002844	2.6		2.2	2.8
LIM protein (similar to rat protein kinase C-binding enigma)	AF116705	2.6		2.1	
Myosin regulatory light chain MRCL3	BU676221	2.6		2	20.7
V-myb myeloblastosis viral oncogene homologue (avian)-like 1	AW592266	2.4		2.2	2.3
Sterile α motif domain containing 4	AL117523	2.3	2.2	3	
Specifically androgen-regulated protein	NM_023938	2.3		2.9	
Small nRNA activating complex, polypeptide 1, 43 kDa	NM_003082	2.3		2.4	3
Decay accelerating factor for complement (CD55, Cromer blood group system)	NM_000574	2.3		2.1	2.9
Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA-binding protein 1, 37 kDa)	BF970044	2.3		2.1	15.8
Chondroitin sulfate GalNAcT-2	AL139812	2.3		2	
Solute carrier family 30 (zinc transporter), member 1	AI553933	2.2		2.7	
Keratin 17	NM_000422	2.1	3.9	4.1	4
Dual specificity phosphatase 14	NM_007026	2.1		2.4	

suggest that some of these down-regulated genes may serve as potential therapeutic targets to evoke cytostasis of prostate cancer cells.

Tributyrin induces nuclear factor-κB–dependent gene expression. Because the microarray data analysis revealed that both tributyrin and MPA induced the expression of known NF-κB–dependent genes, we decided to test the involvement of these types of genes in PC-3 cell differentiation. Because the level of NF-κB–dependent gene expression was more pronounced after treatment with tributyrin than MPA, we decided to concentrate on the former in the subsequent experiments.

Initially, we tested for the activation of the NF-κB pathway by examining tributyrin-induced translocation of the p65 subunit of the NF-κB complex to the nucleus by means of Western blotting and confirm p65 subunit binding to its target sites in the promoter sequence of several selected genes by chromatin immunoprecipitation assay. The results indicated that tributyrin-induced translocation of p65 was detected at 15 minutes after treatment and peaked at 45 minutes (Fig. 2*A*). A 1 hour preincubation with the specific NF-κB activation inhibitor Bay11-7082 (19) at 15 μmol/L or TMB-8, the internal calcium antagonist (20), at 300 μmol/L markedly reduced the tributyrin-induced p65 translocation, with TMB-8 being more effective (Fig. 2*B*). The inhibitors alone had little to no effect on this translocation under these experimental conditions (Fig. 2*B*).

To test for p65 binding to its target sites in the promoter sequence (gene accession numbers are in brackets) of three NF-κB–dependent genes, namely *CCL20* (AY150053), *CXCL3* (NM_002090), and *IL-8* (M28130), we used chromatin immunoprecipitation assay. After PC-3 cells were mock- or tributyrin-treated for 3 hours and subsequently protein cross-linked, the cells were collected and used to isolate chromatin. Isolated chromatin was sheared and used in chromatin immunoprecipitation experiments with an antibody against human p65. Primers encompassing the p65-binding sequence of *CCL20, CXCL3,* and *IL-8* promoters were used in the PCR reactions on the recovered chromatin immunoprecipitation DNA. The results indicated that the promoters of these three genes were amplified from the chromatin immunoprecipitation DNA and that the amount of promoter fragment recovered increased in response to tributyrin treatment (Fig. 2*C*). Moreover, this amplification correlated to the tributyrin-induced *CCL20, CXCL3*, and *IL-8* gene expression levels as assessed by the microarray analysis (Table 1).

Next, we confirmed the induced expression of a selected group of known NF-κB-dependent genes, which included *CCL20, IL-1β, Bcl2-A1, IL-6, COX-2*, and *A20*, at 12 hours after treatment with 1 mmol/L tributyrin by means of real-time PCR. The result indicated that this expression was induced from 3- to 170-fold over control values (Table 2). A 1 hour preincubation with 15 μmol/L Bay11-7082 or 300 μmol/L TMB-8 reduced the induced expression of *CCL20, IL-1β, IL-6*, and *COX-2* by 41% to 98%. The expression of the other two genes, *Bcl2-A1* and *A20*, was reduced by ~70% but only by one of the inhibitors (Table 2).

The microarray data analysis also revealed the expression of genes that have been anecdotally mentioned as NF-κB dependent. Among these were *PAI-2* (21), *IL-24* (22), and *keratin 17* (23), which were induced 2- to 10-fold over control values in PC-3 cells treated with 1 mmol/L tributyrin for 12 hours as confirmed by real-time PCR. To establish that these genes are NF-κB–dependent, we took advantage of NF-κB inhibitors Bay11-7082 and TMB-8. Using real-time PCR, we found that these inhibitors reduced the induced

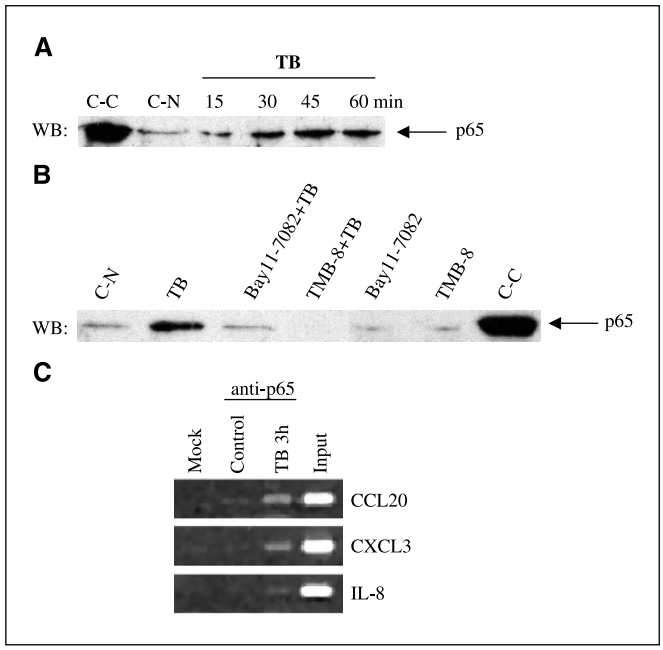


Figure 2. Translocation of the p65 subunit of the NF-κB complex into nuclei of tributyrin-treated PC-3 cells (*A*), its blockage by NF-kB activation inhibitors (*B*), and its binding to targeted promoter sequences (*C*). *A,* nuclear extracts from PC-3 cell treated with 1 mmol/L tributyrin for indicated periods of time were prepared as described in Materials and Methods. Total proteins at 30 μg were separated in a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with an anti-p65 antibody and a secondary goat anti-mouse antibody labeled with HRP. Membranes were developed as described in Materials and Methods. *C-C,* control-cytosol; *C-N,* control-nuclei; *WB,* Western blot. *B,* NF-κB inhibitors Bay11-7082 at 15 μmol/L and TMB-8 at 300 μmol/L were used to block tributyrin-induced translocation of p65. PC-3 cells were preincubated for 1 hour with the inhibitors and then treated with tributyrin for 15 minutes. Nuclear and cytosolic extracts were prepared and Western blots developed as described in (*A*). Experiments were repeated thrice and representative blots are presented. *C,* PC-3 cells were mock- or tributyrin-treated for 3 hours. Proteins were cross-linked with 1% formaldehyde and chromatin was collected and sheared by sonication. Immunoprecipitates were collected with a rabbit polyclonal antibody to p65 followed by protein A–agarose binding. For the mock precipitation, equal amount of PC-3 chromatin was used and incubated in the absence of the primary p65 antibody; all other manipulations were identical to those done on the test sample. Input chromatin (*Input*) was obtained as the DNA-containing supernatant of the mock immunoprecipitation reaction. DNA was recovered from the precipitates and analyzed by PCR using primers encompassing the NF-κB-responsive elements of the human CCL20, CXCL3, and IL-8 promoters. Chromatin immunoprecipitation assays were repeated twice with a similar outcome; the results of one of such assay are presented.

expression of *PAI-2, IL-24,* and *keratin 17* (*KRT17*) by 55% to 98% (Table 2), thus confirming their NF-κB dependency.

In untreated PC-3 cells, the expression of the NF-κB–dependent genes was in most cases reduced by the presence of Bay11-7082 or TMB-8, whereas the expression of the NF-κB–independent genes was usually not affected. Occasionally, the expression of few NF-κB genes was actually induced rather than reduced by the inhibitors (Table 2).

In addition to the chemical inhibitors of NF-κB activation, we used a p65-specific siRNA (sip65). Our initial studies confirmed that p65 protein levels were markedly reduced in the presence of 50 nmol/L sip65 2 days after transfection; p65 resumed its normal levels 4 days after transfection (data not shown). PC-3 cells were transfected with sip65 or with a nonspecific control siRNA. To confirm reduced expression of NF-κB–dependent genes in the presence of specific siRNA after tributyrin treatment, PC-3 cells were treated with 1 mmol/L tributyrin for 12 hours 2 days after

Table 2. Tributyrin-induced gene expression and the effect of Bay11-7082 and TMB-8

Gene	Tributyrin treated			Untreated		
	Control	Bay11-7082	TMB-8	Control	Bay11-7082	TMB-8
	Increase (fold)	Inhibition (%)			Inhibition (%)	
CCL20	170	93	41	1	62	0*
IL-1β	40	70	96	1	37	66
Bcl2-A1	9	68	22	1	70	0*
IL-6	3	98	62	1	70	37
COX-2	3	74	97	1	60	97
A20	3	18	73	1	44	20
PAI-2	10	75	98	1	0	95
IL-24	5	60	98	1	43	97
KRT17	2	55	86	1	57	81
RPS25	1	0	0	1	0	0

NOTE: Inhibition is defined as percentage of inhibition relative to the control level, which is considered 100%. Human ribosomal protein S25 (RPS25) served as a reference. The experiments were repeated thrice with a similar outcome; the results of one of such study are presented.
*Induction rather than inhibition, 4-fold for *CCL20* and 3-fold for *Bcl2-A1*.

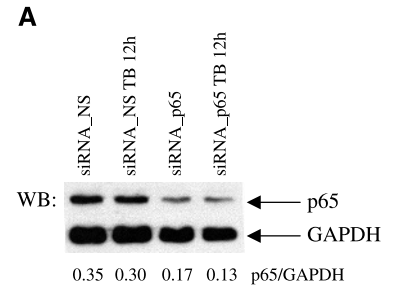
transfection. At the end of this treatment, the cells were harvested and used to determine p65 levels by means of Western blot analysis and expression of NF-κB-dependent genes by means of real-time PCR. The results indicated that p65 levels were reduced by 50% in the presence of sip65 (Fig. 3*A*) and this decrease caused a 35% to 75% reduction in the expression of the nine tested NF-κB-dependent genes (Fig. 3*B*). We speculate that some of these genes play a key role in the differentiation process.

TMB-8 inhibits vacuole production. Our previous results raised the likelihood that NF-κB–dependent genes are involved in the processes leading to PC-3 cell differentiation, because both tributyrin and MPA, which initiate differentiation by a distinct mode of action, induce their expression. The ability of Bay 11-7082 and TMB-8 to inhibit expression of *keratin 17* (Table 2), a prostate cell maturation marker (17), corroborates this notion.

To further support the above premise, we decided to test the ability of NF-κB activation inhibitors to block cytoplasmic vacuolization, a morphologic differentiation marker in PC-3 cells (5, 7). For this purpose, we preincubated PC-3 cells with 5 to 20 μmol/L Bay11-7092 and 100 to 500 μmol/L TMB-8 for 1 hour before a 2-day treatment with 1 mmol/L tributyrin or 20 μmol/L MPA. After treatment, cells were fixed with paraformaldehyde, stained with Giemsa, and photographed. The results indicated that Bay11-7092 had no effect on vacuole production (data not shown), whereas TMB-8 abolished this production (Fig. 4*A*), most likely as a result of its action as an internal calcium antagonist. As such, tributyrin- or MPA-induced vacuole production is NF-κB independent.

Taken together, our results implicate the activation of NF-κB–dependent and NF-κB–independent genes in the processes leading to PC-3 cell differentiation.

Combined treatment of differentiation inducers with nuclear factor-κB activation inhibitors increases cell death. The role of NF-κB activation in cell survival has been well documented in various cell systems (24). It was, therefore, of interest to determine whether this is also the case in tributyrin- and MPA-evoked PC-3 cell death. To test for this possibility, we preincubated the cells with 15 μmol/L Bay11-7082 or 300 μmol/L TMB-8 1 hour before a 3-day treatment with 1 mmol/L tributyrin or 20 μmol/L MPA. All attached and floating cells were collected and incubated with trypan blue to calculate percentages of dead cells. The results revealed that the combined treatments of tributyrin or MPA with Bay11-7082 was mostly the sum of the toxic effect of the individual agents whereas in the presence of TMB-8 the combined effect was more pronounced (Fig. 4*B*). In a related experiment, we double stained the treated cells with Annexin V/propidium iodide, which enables to determine percentages of apoptotic and necrotic



B

Gene	Inhibition (%)
CCL20	75
IL-1β	64
Bcl2-A1	51
IL-6	56
COX-2	35
A20	65
PAI-2	74
IL-24	45
KRT17	67
RPS25	0

Figure 3. Reduced expression of p65 subunit of NF-κB complex after specific siRNA transfection (*A*) and a consequent reduced expression of NF-κB–dependent genes (*B*). *A,* PC-3 cells were transfected with specific siRNA duplex for p65 siRNA_p65 or nonspecific control siRNA duplex siRNA_NS and 2 days later were treated with 1 mmol/L tributyrin (TB) for 12 hours. Expression levels of p65 were assessed by Western blotting and normalized to GAPDH levels. *B,* cells transfected with specific or nonspecific control siRNA duplexes were treated with 1 mmol/L tributyrin as described in Materials and Methods. Total RNA was isolated to perform real-time PCR.

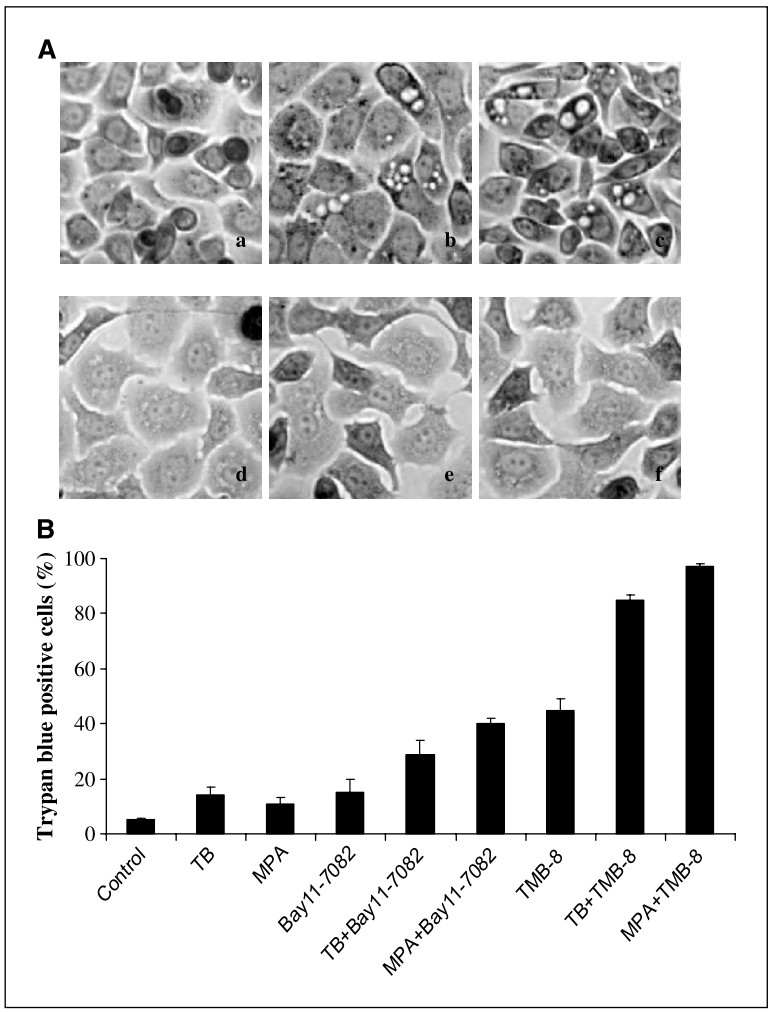


Figure 4. MPA- and tributyrin-induced PC-3 cytoplasmic vacuolization and its inhibition by TMB-8 (*A*), and PC-3 cell death evoked by MPA or tributyrin in combination with or without Bay11-7082 or TMB-8 (*B*). *A,* a day after PC-3 cells (*a*) were seeded at 0.5×10^5 per 60 mm plate in triplicate, they were preincubated with 300 μmol/L TMB-8 (*d, e*, and *f*) and treated with 20 μmol/L MPA (*b* and *e*) or 1 mmol/L tributyrin (*c* and *f*) for 2 days. After treatment, the cells were fixed with 3% paraformaldehyde in PBS, stained with Giemsa solution, and photographed. Magnification, ×10. *B,* a day after PC-3 cells were seeded at 0.5×10^5 per 60 mm plate in triplicate, they were pretreated with 15 μmol/L Bay11-7082 or 300 μmol/L TMB-8 for 1 hour and treated with 20 μmol/L MPA and 1 mmol/L tributyrin for 3 days. At the end of treatment, both unattached and attached cells were collected, stained with trypan blue solution, and manually counted. *Columns,* percentages of trypan blue–positive cells, representing a population of dead cells; *bars,* SD. Representative of three independent experiments.

cells (25). The percentages of Annexin V/propidium iodide positive cells were similar to those obtained after trypan blue staining (data not shown), suggesting that tributyrin or MPA evoke mostly necrotic rather than apoptotic cell death and suggest a role for calcium-related events in this process.

Discussion

Histone deacetylase inhibitors, such as tributyrin, have been considered as potential differentiation therapy drugs (26) and as such have been included in phase I clinical trial (27). Previously, tributyrin and a related inhibitor sodium butyrate have been found to be effective inducers of differentiation in various cell types, including promyelocytic HL-60 (28) and U937 leukemia cells (29), Y79 retinoblastoma (30), and colon tumor cells (31). It was, therefore, of interest to determine whether tributyrin could also induce differentiation in PC-3 cells, which derive from a human androgen-refractory prostate cancer, a tumor type that is usually resistant to standard chemotherapy (4) and as such is often deadly.

A previous study reported that tributyrin evokes in PC-3 cells replication arrest and morphologic changes that can be associated with cellular differentiation (7). To substantiate this differentiation, we have tested and shown that tributyrin induces PC-3 cells to display large cytoplasmic vacuoles and to express *zinc-α-2-glycoprotein, CD10, CD46, CD55, GRP78,* and *keratin 17*. These markers, which are associated with a maturing secretory cell phenotype, were chosen because they were instrumental in characterizing PC-3 cell maturation by another type of differentiation inducers, namely inosine 5′-monophosphate dehydrogenase inhibitors (5).

To identify additional genes whose expression may be associated with tributyrin-induced PC-3 cell differentiation and to gain some insight into the mechanism that underlies this differentiation, we have, by means of Affymetrix chip microarray analyses, compared the patterns of tributyrin-induced gene expression with those of MPA, which initiates PC-3 cell differentiation by a dissimilar mode of action—tributyrin by inhibiting histone deacetylase (6) and MPA by inhibiting inosine 5′ -monophosphate dehydrogenase (14). We hypothesized that at a certain stage, the signaling events initiated by these inducers would be shared and as such most likely associated with the processes that would lead to PC-3 cell differentiation rather than with the unique action of the particular inducer. To verify this hypothesis, we chose several treatment times; treatments of up to 6 hours, which we assumed would mainly involve genes associated with the induction process, or treatment for 72 hours, which would mainly identify genes that define the mature phenotype of the differentiating PC-3 cells. Our results indicated that up to 6 hours of treatment, tributyrin or MPA increased or evoked the expression of a large number of common genes and a higher number at 72 hours of treatment. Among the

latter, as we speculated, there were a substantial number of genes coding for keratins, which are traditionally used as indicators of epithelial cell differentiation (17). We also identified increased expression of series of genes coding for plasma membrane and secretory proteins; the latter can be readily detected by immunologic means in body fluids, including blood. As such, these proteins may potentially be used to monitor progression of differentiation and related chemical therapies of prostate cancer.

We also detected a large amount of commonly down-regulated genes at 72 hours of treatment. The majority of these genes are associated with cell growth and/or maintenance, cell cycle regulation, and DNA synthesis. We suggest that some of these observed down-regulated genes may serve as potential therapeutic targets to evoke cytostasis of prostate cancer cells.

A noteworthy observation was the early expression of genes known or assumed to be NF-κB–dependent and as such may be involved in the differentiation process. One of these genes was *keratin 17*, previously used as a PC-3 cell differentiation marker (5). To verify their NF-κB–dependent activation, we took advantage of two commonly used inhibitors of this activation, Bay11-082, a specific inhibitor (19), and TMB-8, an internal calcium antagonist (20). The analysis of the effect of Bay11-082 and TMB-8 on tributyrin-induced expression of a selected group of the above genes, which included *keratin 17*, confirmed their NF-κB dependency. Interestingly, one of these NF-κB–dependent genes was *PAI-2*, which has been previously found to be involved in regulating the expression of keratins, typical maturation markers of differentiating progenitors into keratinocytes (32). Unlike these results, Bay11-7092 had no effect on vacuole production, a phenotypic PC-3 cell differentiation marker (5, 7). Yet, this production was abolished by TMB-8, most likely as a result of its action as an internal calcium antagonist perhaps upstream of tributyrin-induced NF-κB activation.

In addition to these chemical inhibitors of NF-κB activation, we used a p65-specific siRNA. The result indicated that p65 reduction resulted in a reduced expression of tributyrin-induced NF-κB–dependent genes, some of which most likely play a role in the differentiation process. NF-κB seems to play a role in maturation of other cell systems, such as human myelomonocytic leukemia U937 cells induced to differentiate by phorbol 12-myristate-13-acetate (33) or sodium butyrate (34). Differentiation induction in this cell system seems to require an intact NF-κB pathway for the expression of endogenous cyclin-dependent inhibitor p21/Cip1, which consequently evokes a replication arrest at the G_1 phase of the cell cycle. In PC-3 cells, this is not always the case because MPA-induced differentiation is associated with an accumulation of the induced cells in the S phase of cell cycle (5).

The endogenous activation of the NF-κB family of transcription factors in prostate cancer cells has been associated with their increased survival (35). To confirm survival role of NF-κB in our system, we used inhibitors of NF-κB activation. Unexpectedly, the specific NF-κB inhibitor Bay11-7082 failed to markedly induce cell death, as assessed by either trypan blue or Annexin V/propidium iodide double staining. This observation suggests that PC-3 cell may have active alternative survival factors. In addition, we have investigated the mode by which tributyrin and MPA evoke cell death in the PC-3 cells by using Annexin V/propidium iodide staining in the absence and presence of the NF-κB inhibitors. Based on these results, we concluded that the majority of cells ($\sim$92-95%) died by necrotic cell death. For example, treatment with 1 mmol/L tributyrin for 3 days resulted in $\sim$17% of dead cells. Annexin V/propidium iodide staining revealed that <8% of these cells died by apoptosis. This low apoptotic rate was also confirmed during cell cycle analysis, given that the subdiploid population of cells did not exceed 2% after 3 days of treatment with 1 mmol/L tributyrin. These observations are also in agreement with our previous report, which indicated that MPA failed to induce chromatin condensation (5), caspase-3 activation, and DNA fragmentation,[1] typical apoptotic hallmarks.

Based on the present findings, we conclude that the differentiation agents MPA and tributyrin induce a substantial number of up- and down-regulated genes in PC-3 cells. Notable among these were NF-κB–dependent genes. We believe that some of the up- and down-regulated genes are key contributors to the processes leading to the acquisition of the mature phenotype of treated PC-3 cells. We also speculate that products of some of the up-regulated genes may serve as potential diagnostic biomarkers to monitor the progress of differentiation and other therapies of prostate cancer, whereas products of certain down-regulated genes may serve as potential targets for inducing cytostasis of prostate cancer.

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[1] Unpublished data.

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