

Differentiation of Human Prostate Cancer PC-3 Cells Induced by Inhibitors of Inosine 5'-Monophosphate Dehydrogenase

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ABSTRACT

To establish a system to study differentiation therapy drugs, we used the androgen-independent human prostate PC-3 tumor cell line as a target and mycophenolic acid (MPA), tiazofurin, or ribavirin, which are inhibitors of IMP dehydrogenase, as inducers. These inhibitors evoked replication arrest, caused an increase in cell size, and triggered vacuolization of the cytoplasm. By Northern and Western blotting and immunostaining, we demonstrated MPA-induced expression of 12 proteins reported to reside in prostasomes, organelles released by secretory luminal prostate cells. Additional MPA-induced proteins were identified by two-dimensional gel electrophoresis. Among these was keratin 17, a prostate cell differentiation marker. By Northern blotting, we also demonstrated the constitutive expression of keratins 8 and 18 and induced expression of keratin 19, three other prostate cell differentiation markers. In addition, we established that cells were committed to differentiate after the 2nd day of MPA treatment using guanosine, which can abrogate the effects of MPA. Based on the expression patterns of prostasomal proteins and keratins and the presence of tentative secretory vacuoles, we hypothesize that IMP dehydrogenase inhibitors induce androgen-independent PC-3 cells to mature into cells with a phenotype that resembles normal prostate luminal cells, but at their intermediate state of differentiation.

INTRODUCTION

Prostate cancer is the second-leading cause of tumor-related death in men (1). Despite the introduction of the prostate-specific antigen assay (2) and progress in androgen ablation therapy, only a limited improvement in survival benefit has been achieved. In particular, a problem persists with hormone-refractory tumors, which are resistant to an array of chemotherapeutic drugs with different modes of action (3). Although the precise reason for this resistance is unknown, it may be related to the fact that tumor formation, including prostate cancer, is caused by an accumulation of genetic alterations in a variety of genes that control malignancy (4).

A potential innovative cancer treatment that may eliminate some drawbacks of the current prostate cancer chemotherapy (*e.g.*, systemic toxicity) is differentiation therapy (5). In this treatment approach, the desired drug causes the malignant cells to undergo terminal differentiation instead of killing the tumor cells. The concept behind this therapy is based on the observations that cells of most tumors, including prostate cancers, are blocked at an early stage of cellular differentiation and that certain agents can bypass or correct this block *in vitro* (6). On the basis of these studies, a number of drugs have been identified, tested, and found to show promise in the treatment of human myeloid leukemia (7).

An obstacle in identifying drugs for prostate cancer differentiation therapy is the absence of an appropriate *in vitro* cell maturation system and useful markers that truly define the normal mature prostate

cell. A number of attempts have been made to develop such a system (8–11). These studies, however, used apoptosis indicators and/or a limited number of neuroendocrine markers rather than an array of indicators that define the particular function of normal prostate cells.

Secretory cells of the mature prostate contain highly organized organelles called prostasomes (12), which are released into the prostate fluid and semen (13). These organelles contain in their membrane a variety of proteins including complement regulators CD46 (14), CD55, and CD59 (15), an essential cofactor for coagulation, tissue factor (16), and several peptidase activities, including dipeptidyl-transpeptidase/CD26 (17), neutral endopeptidase/CD10 (18), aminopeptidase N/CD13 (19), γ -glutamyltranspeptidase (20), and aminopeptidase P (21). The prostasomes also contain neuroendocrine markers including chromogranin B, neuropeptide Y (NPY), and vasoactive intestinal polypeptide (VIP) (22) and the common secretory granule protein granulophysin/CD63 (23). Although the physiologic function of the prostasomes is still not clearly defined, they display properties that are beneficial to sperm (24–29). As such, cultured prostate tumor cells that can be induced to produce prostasomes or their building blocks coupled with replication arrest should represent a useful system to study potential differentiation therapy drugs.

IMP dehydrogenase (IMPDH) is a key enzyme in guanine nucleotide biosynthesis, and as such, it is vital for the replication of all cells (30). In addition, inhibitors of this enzyme have been found to effectively induce differentiation in a variety of distinct human tumor cell types (31). For this reason, we tested IMPDH inhibitors for their ability to induce a mature phenotype in androgen-independent prostate tumor cells. Here, we describe IMPDH inhibitor-induced differentiation of PC-3 cells into cells with a phenotype resembling that of mature prostate secretory cells.

MATERIALS AND METHODS

Cells, Giemsa Staining and Cell Cycle Analysis. PC-3 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in 8% CO₂ at 37°C in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (Invitrogen). MPA (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (Fisher Scientific, Hampton, NH) as a stock solution of 20 mg/mL, and ribavirin (Sigma) and tiazofurin (NSC 286193; National Cancer Institute Drug Synthesis and Chemistry Branch, Bethesda, MD) were dissolved in dimethyl sulfoxide as a stock solution of 50 mg/mL. Standard chemicals were purchased from Sigma. To determine cell numbers, PC-3 cells were plated in 60-mm plates at 3×10^5 cells per plate or in 100-mm plates at 1 to 2×10^6 cells per plate. Treatment with MPA, ribavirin, or tiazofurin was initiated a day after cell seeding. Treatment with guanosine was performed as indicated in the text. To demarcate vacuoles, cells were stained with a Giemsa solution (Sigma) for 1 minute and washed three times with PBS after being fixed in 3% paraformaldehyde in PBS for 10 minutes at room temperature. Images of stained cells were captured with a Zeiss inverted microscope and printed, and the cells with and without vacuoles were counted to determine their percentages. Cell cycle analysis was performed as described previously (32) using the WinMDI 2.8 software (Joe Trotter; Scripps Research Institute, La Jolla, CA).

IMP Dehydrogenase Activity. Enzyme activity was assayed as described previously (33). Specific IMPDH activity was calculated as μ mol of NADH

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formed per milligram per minute (1 unit). The results represent the mean \pm SD of three independent experiments.

Chromatin Condensation. Dissociated cells were washed twice in PBS and adhered to microscope slides by centrifugation (770 rpm, 5 minutes) using Cytospin 2 (Shandon, Pittsburgh, PA). The adhered cells were fixed with 3% paraformaldehyde in PBS for 10 minutes at room temperature, washed 3 times with PBS, permeabilized with 0.5% Triton X-100 in PBS, and stained with 100 ng/mL 4',6'-diamidino-2-phenylindole hydrochloride (Boehringer Mannheim, Indianapolis, IN) in PBS for 30 minutes at room temperature. The fraction of cells displaying chromatin condensation was determined by fluorescent microscopy of the mounted slides. Cells treated with 50 μ mol/L ZnSO₄ in serum-free medium for 1 day served as positive control.

Immunostaining. Cells were grown for 2 days on coverslips in 60-mm plates and treated with 20 μ mol/L MPA for 3 days. After treatment, cells were washed three times in PBS, fixed with 3% paraformaldehyde in PBS, washed again three times in PBS, and permeabilized with 1% Triton X-100 in PBS. To reduce the background, permeabilized cells were incubated with 5% bovine serum albumin in PBS for 10 minutes at room temperature and then stained with primary antibody mAb78 (1:2; a gift from Dr. G. Ronquist; Department of Medical Sciences, University Hospital, Uppsala, Sweden) or with a primary antibody to CD10 labeled with phycoerythrin (PharMingen, San Diego, CA) for 60 minutes at room temperature. After three washes in PBS, the cells were incubated with the secondary antibody (goat antimouse IgG Alexa Fluor 488; 1:500; Molecular Probes, Eugene, OR) for 60 minutes at room temperature. Cells were then washed again three times in PBS. Cells labeled with mAb78 were analyzed by a conventional fluorescence microscope. Images were captured at a magnification of \times 50. Cells labeled with CD10 were analyzed by flow cytometry using FACScan (Becton Dickinson, San Jose, CA) with one laser (488 nm argon laser) and three detectors. The fluorescence emission of 10,000 cells was collected in FL1 or FL2 channel. Data were analyzed with WinMDI 2.8 software (Joe Trotter; Scripps Research Institute).

Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated from cells after they were washed twice with cold PBS using the Trizol reagent according to the manufacturer's protocols (Invitrogen). RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water (0.1% DEPC was added to water overnight, and then water was autoclaved for 20 minutes to destroy DEPC). RNA (4 μ g) was used to generate a first-strand cDNA by reverse transcription reaction according to the manufacturer's instructions (Invitrogen). Complementary DNA (1 μ L) was used for polymerase chain reaction (25 to 30 cycles of 95°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute; followed by 72°C for 5 minutes) to generate DNA probes, which were excised from 1.5% agarose gel, extracted by a DNA extraction kit according to the manufacturer's instructions (Qiagen, Valencia, CA), and used for Northern blotting. The primers used in the study were synthesized by MWG-Biotech (High Point, NC) and were as follows: CD46 (NM_002389), 5'-CGCGCCATGGTGTGCTGCTGT-3' and 5'-CACATATTGGGGGCTT-ACCGT-3'; CD55 (NM_000574), 5'-GATGTACCTAATGCCAGCC-AGC-3' and 5'-CATGAGAAGGAGATGGTTGCACC-3'; clusterin (NM_001831), 5'-AGTGCCGGGAGATCTGTCTGTG-3' and 5'-GCCACGGTCTCCATAAATTAGGG-3'; 78-kDa glucose-regulated protein (GRP-78; NM_005347), 5'-GCTGAAGACAAGGGTACAGGGAAC-3' and 5'-GGAGGGCCTGCACTTCCATAGA-3'; granulophysin/CD63 (NM_001780), 5'-GTGCAAGTGGGACTGATTGCCGT-3' and 5'-GGGACTCGTCTTCGACATGG-3'; NPY (NM_000905), 5'-ATGCTAGGTAACAAGCGACTGGGG-3' and 5'-TCACCACATTGCAGGGTCTTCAAG-3'; secretory actin-binding protein/PIP (NM_002652), 5'-ATGCGCTTGTCCAGCTCCTGT-3' and 5'-CAGCATCATCAGGGCAGATGCC-3'; zinc- α -2-glycoprotein (NM_001185), 5'-ACAAAGAAATCCCAGCCTGGGTCC-3' and 5'-TGTGCTGACAGTGGCAGAGTA-3'; aminopeptidase N/CD13 (M22324), 5'-CAGCACAATCAGGCAATGAGTGGG-3' and 5'-TCATGTTGGGCAATAGACCTCGG-3'; α -glucosidase (NM_004668), 5'-GTGTCAGACCAGGTGACATGGGA-3' and 5'-TGGCACTGGTAACGGGGACT-3'; CD59 (NM_000611), 5'-AAGGAGGGTCTGTCTGTTCCG-3' and 5'-GGATGAAGGCTCCAGGCTGCT-3'; gastrin (U75272), 5'-AGCCTGTACACGGGGCAGATCTA-3' and 5'-TCTGGGAGGACAGGTAGGTG-3'; lactoferrin (NM_002343), 5'-TCTGTGATTGGCGCAGCAGG-3' and 5'-GCAAAACTTGTCCGGGCGAGTCA-3'; tissue factor (XM_001322), 5'-CCGGCGCTTACAGCACTACAAA-3' and 5'-CCCGGAGGCTTAGGAAAGTGTG-3'; VIP (NM_003381), 5'-AGGCCAGCTCCTGTGCTCCT-3' and 5'-

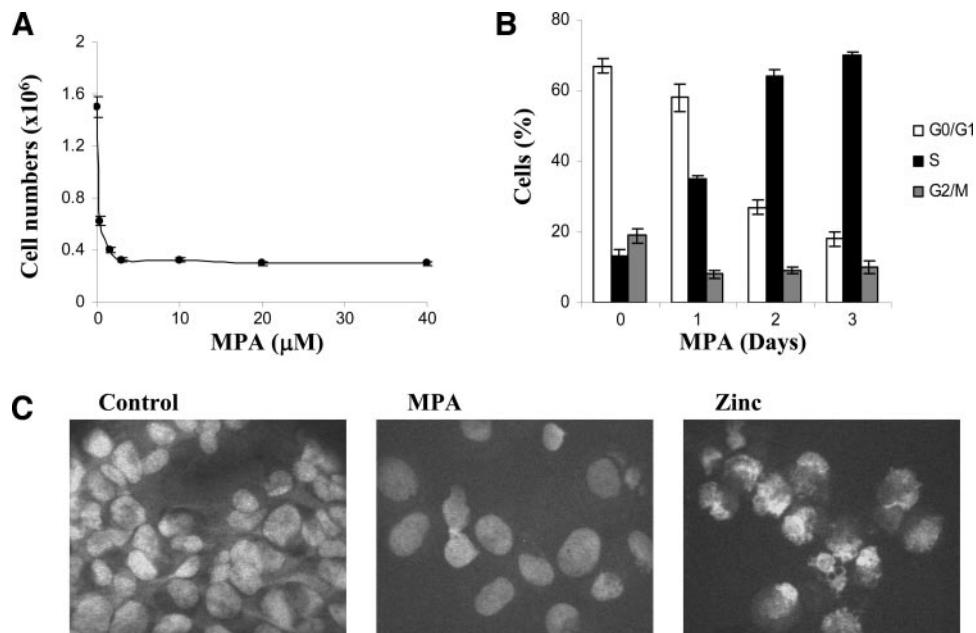
GGAAAGTCGGGAGATTCTCCCTC-3'; chromogranin B (BC000375), 5'-AACTACGGTGAGGAAGGAGCCC-3' and 5'-CCTTTTCAGGTCCAGCTTGGG-3'; fibrillin (NM_032447), 5'-CGACAATGATGAGTGCTCAGCC-3' and 5'-CCGAGGGCCTGATCAAAGTCAAAG-3'; γ -glutamyltranspeptidase (XM_209446), 5'-TGCCGGATAGAAAGGTGCTTTGG-3' and 5'-AGGGACTTGCTCTCCTGGACTGT-3'; lipoprotein lipase (BT006726), 5'-CCAAGTAAGGCCTACAGGTGCAG-3' and 5'-TTCTGAATGGCGAAGC-CGGACT-3'; PSA (X05332), 5'-GAGGTCCACACTGAAGTT-3' and 5'-CCTCCTGAAGAATCG ATTCT-3'; synaptophysin (NM_003179), 5'-CCCATGTGGACTTTCTGGCCA-3' and 5'-CCGTAGCCTTGCTGCCA-TAGT-3'; prostatic acid phosphatase (NM_001099), 5'-TCCAAGGGGGTGTCTCTGGTCAAT-3' and 5'-GTACTGTCTCAGTACCTTGATGG-3'; HMG-1 (X12597), 5'-TCCCAATGCACCCAAGAGGCCT-3' and 5'-CATCTTCTCTCTCTCATCC-3'; APG-2 (AB023420), 5'-GGATGGAGAA-GACCACCAAGC-3' and 5'-TCTGTACCCTGCTCAGCAGCT-3'; calreticulin (BC002500), 5'-AACCCCGAGTATCTCCCGATCC-3' and 5'-CCGGGGACATCTTCTCTCAT-3'; keratin 8 (NM_002273), 5'-CTGGA-GGCCGCCATTGCAGAT-3' and 5'-CAGACACCAGCTTCCCATCAG-3'; keratin 17 (NM_000422), 5'-GGTGCAGAGTGGAAGAGTGAGA-3' and 5'-TTGCCATCTGGACTTCTTCCAC-3'; keratin 18 (NM_000224), 5'-TGAGACGACGCTCACAGAGTGA-3' and 5'-TATCCCGGGTGGTGTG-TCTTTT-3'; keratin 19 (NM_002276), 5'-CTGGTTCACCCGGGACT-GAA-3' and 5'-GATCTCTCTGTCCGTCGAGCAG-3'; IMPDH II (J04208), 5'-ATTGCTGCCATCCAACACTCA-3' and 5'-CCGAGGAGGTGTGTG-TGAT-3'; and glyceraldehyde-3-phosphate dehydrogenase (NM_002046), 5'-TTAGACCCCTGGCCAAGG-3' and 5'-CTTACTCCTTGAGGCCATG-3'.

Northern Blot Analysis. Total RNA, at 5 μ g, was loaded onto a 1% agarose gel containing formaldehyde. Separated RNA was transferred to a nylon membrane (MagnaGraph; GE Osmonics Labstore, Minnetonka, MN) and hybridized with labeled DNA probes ($[\alpha$ -³²P]dCTP; Perkin-Elmer, Welle-sley, MA; 15 μ Ci per reaction). After overnight hybridization at 60°C, mem-branes were washed with 0.1% SDS in 1 \times SSPE (150 mmol/L NaCl, 162 mmol/L Na₂HPO₄, 5 mmol/L NaH₂PO₄, and 1 mmol/L EDTA) at room temperature and with 0.1% SDS in 0.1 \times SSPE at 60°C and sealed. Gene expression was determined by exposing membranes to a phosphorimager screen for 1 day. Equal RNA loading was confirmed by hybridizing the same blots with the HSP70 probe. This was done because, unlike glyceraldehyde-3-phosphate dehydrogenase, HSP70 expression was not affected by MPA treatment. Images were analyzed by a trial version of MCID analysis software (Imaging Research, St. Catharines, Ontario, Canada).

Western Blot Analysis. Dissociated cells were washed three times with cold PBS and lysed in ice-cold radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, and 150 mmol/L NaCl] containing a mixture of protease inhibitors (Invitrogen) at 4°C for 10 minutes. Cell lysates were centrifuged at 13,000 rpm for 10 minutes at 4°C. Protein concentrations were measured with a Protein Assay Kit (Bio-Rad, Hercules, CA), based on the method of Bradford (34). For electrophoresis, solubilized proteins (100 μ g per well) were separated on an 8% SDS-poly-acrylamide gel and transferred onto a Hybond-C extra membrane (Amersham, Piscataway, NJ). The membrane was blocked with freshly prepared 5% dry milk in PBS/0.05% Tween (milk/PBS/Tween) for 10 minutes at room temperature and then incubated overnight at 4°C with a primary antibody against fibronectin (1:100; Sigma). After the membrane was washed three times with milk/PBS/Tween at room temperature, it was incubated with goat antimouse secondary antibody labeled with horseradish peroxidase (1:5,000; Pierce, Rockford, IL) for 60 minutes at room temperature. Chemiluminescence was obtained by enhanced chemiluminescence detection kit (Amersham).

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electro-phoresis of lysates was performed as described previously (35). Aliquots of 200 μ g of protein were loaded onto each of four isoelectric focusing gels. Proteins in the two-dimensional electrophoresis gels were stained with 0.2% Coomassie Blue R-250 (Serva, Heidelberg, Germany) in 2.5% phosphoric acid and 50% EtOH and scanned. The resulting images were converted to a tif format and analyzed by using Progenesis software for two-dimensional gel electrophoresis data analysis (Nonlinear USA, Durham, NC) on a personal computer platform. Significance of differences between groups was evaluated using Student's two-tailed *t* test (*P* < 0.05). Proteins found to be induced or reduced after treatment by >3 \times were excised from the gels and identified by

Fig. 1. MPA-induced inhibition of PC-3 cell replication (A), cell cycle block (B), and zinc-induced chromatin condensation (C). A. A day after cells were seeded at 3×10^5 cells per 60-mm plate in triplicate, they were incubated for 3 days with MPA (0.5, 1.5, 3, 10, 20, and 40 $\mu\text{mol/L}$) and counted. B. For cell cycle analysis, cells were seeded at 2×10^6 cells per 100-mm plate in triplicate, and after 2 days, they were incubated with 20 $\mu\text{mol/L}$ MPA for the indicated periods of time. Error bars represent the SD of three independent experiments. C. After treatment with 20 $\mu\text{mol/L}$ MPA for 5 days or 50 $\mu\text{mol/L}$ zinc for 1 day, the cells were fixed, permeabilized, and stained with 4',6'-diamidino-2-phenylindole hydrochloride, a nuclear stain ($\times 50$ magnification).



peptide mass determination at the W. M. Keck Facility, Yale University (New Haven, CT).

RESULTS

Mycophenolic Acid-Induced Replication Arrest. Incubation of PC-3 cells for 3 days with 0.3 to 40 $\mu\text{mol/L}$ MPA resulted in replication arrest, which, up to 3 $\mu\text{mol/L}$ MPA, was dose dependent; higher doses yielded a plateau effect (Fig. 1A). The plateau effect was also observed after 1 day of treatment with 20 to 40 $\mu\text{mol/L}$ MPA (data not shown). Also, MPA at 1 $\mu\text{mol/L}$ reduced IMPDH activity in PC-3 cell homogenates from 0.18 ± 0.03 milliunit to $<0.02 \pm 0.01$ milliunit. Because of these observations, we used 20 $\mu\text{mol/L}$ MPA in all subsequent experiments to characterize differentiation of PC-3 cells.

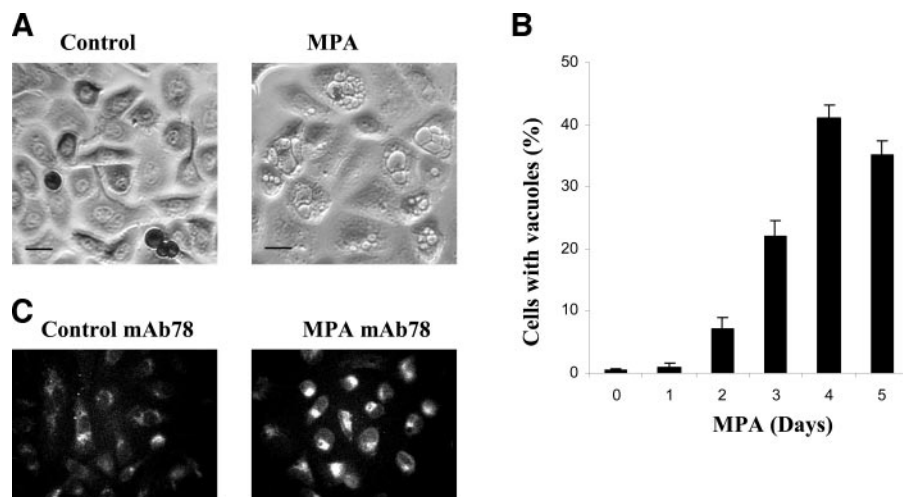
The MPA-evoked replication arrest was also validated by cell cycle analysis using flow cytometry. During a 3-day exponential growth of control PC-3 cells, the percentage of cells in the different cell cycle fractions was relatively constant, with about 70% of cells in the G₀-G₁ phase and about 15% of cells in each of the S and G₂-M phases. MPA treatment caused a major shift in this composition; by the 3rd day, the

percentage of cells in the G₀-G₁ phase was reduced to about 20%, and the fraction of S-phase cells increased to about 70%, whereas the percentage of G₂-M-phase cells was constant at about 10% (Fig. 1B). These changes were also associated with an enlargement of cell nuclei (Fig. 1C). These results demonstrate the MPA-evoked replication arrest and indicate that this arrest is due to a cell cycle block causing treated PC-3 cells to accumulate in S phase.

Despite the replication arrest, treatment of PC-3 cells with 20 $\mu\text{mol/L}$ MPA for up to 5 days failed to induce chromatin condensation, a hallmark of apoptosis. As a positive control, we used zinc, an inducer of apoptosis in these cells (36). Unlike MPA, treatment with 50 $\mu\text{mol/L}$ zinc for 1 day caused >90% of the cells to display this apoptotic marker (Fig. 1C).

Vacuole Production. In addition to replication arrest, MPA caused PC-3 cells to increase in size and to display vacuoles (Fig. 2A), which failed to stain with the lipid stain Oil Red in viable cells or with Giemsa in paraformaldehyde-fixed cells. The percentage of cells with vacuoles changed with time, reaching a maximum on the 4th day of MPA treatment. Thereafter, their fraction decreased (Fig. 2B). These results indicate that MPA induces PC-3 cells to produce vacuoles

Fig. 2. MPA-induced vacuolization (A and B) and prosomal staining (C). A. A day after cells were seeded at 2×10^6 cells per 100-mm plate in triplicate, they were incubated for 3 days with 20 $\mu\text{mol/L}$ MPA and then fixed with 3% paraformaldehyde in PBS and stained with Giemsa solution ($\times 40$ magnification; scale bar, 100 μm). B. A cell with at least one visible vacuole was considered positive. Error bars represent the SD of three independent experiments. C. Cells were treated for 3 days with 20 $\mu\text{mol/L}$ MPA and then fixed, permeabilized, and immunostained with the mAb78 antibody. Goat antimouse IgG Alexa Fluor 488 was used as the secondary antibody ($\times 50$ magnification).



containing as yet unknown substances, which most likely are released into the culture medium, at least after 4 days of MPA treatment.

Expression of Prostatosomal Proteins Induced by Mycophenolic Acid. Previous studies have reported that PC-3 cells contain prostatesome-like granules, which can be detected with the mAb78 monoclonal antibody (37). Using this antibody, we found that a 3-day treatment of PC-3 cells with MPA resulted in increased mAb78 immunostaining (Fig. 2C). To determine whether this increase was coupled with a wide-range increase in other prostatosomal proteins, we examined the expression of genes that encode these proteins using Northern blotting. Prostatosomal proteins have been reported to contain NH₂-terminal signal peptide (38), which allows their translocation into prostatesomes. Using an on-line algorithm,³ we searched for NH₂-terminal signal peptide-containing proteins, of which 21 were selected for our studies. The results indicate that MPA caused a time-dependent increase in the expression of CD46, CD55, CD59, clusterin, granulophysin/CD63, glucose-regulated protein 78 (GRP78), NPY, and zinc- α -2-glycoprotein and a transient increase in the expression of aminopeptidase N/CD13 and α -glucosidase (Fig. 3A). Unlike these results, the other prostatosomal proteins, chromogranin B, fibrillin 3, γ -glutamyltranspeptidase, lactotransferrin, lipoprotein lipase, prostate-specific antigen, and synaptophysin, were not detected in untreated or MPA-treated cells, whereas secretory actin-binding protein/PIP, gastricsin, tissue factor, and VIP were already expressed in control cells, and MPA had little to no detectable impact on their expression (Fig. 3A).

Additionally, we had readily available antibodies to two other prostatosomal proteins, namely, fibronectin, which has the previously described NH₂-terminal signal peptide, and neutral endopeptidase/CD10, which does not have such a motif. By means of Western blotting, we established that MPA induced a time-dependent increase in fibronectin expression (Fig. 3B), and by flow cytometry analysis, we demonstrated an increase in neutral endopeptidase/CD10 staining (Fig. 3B). Flow cytometry also revealed that control cells did not express CD10 (data not shown), as also reported by others (39).

These various analyses revealed that MPA induces PC-3 cells to express a considerable number of prostatosomal proteins.

Two-Dimensional Gel Electrophoresis Analysis of Mycophenolic Acid-Treated Cells. To identify additional proteins associated with MPA-induced differentiation, we performed two-dimensional gel electrophoresis of lysates from control and 5-day MPA-treated PC-3 cells. Analysis of Coomassie Blue-stained gels revealed MPA-induced changes in the proteome of PC-3 cells (Fig. 4A). Proteins displaying pronounced changes in their levels were cut out of the gels and subjected to matrix-assisted laser desorption ionization examination. The analysis identified the following five known proteins: high mobility group protein-1 (HMG-1) was down-regulated; whereas APG-2, calreticulin, keratin 17, and IMPDH II were up-regulated (Fig. 4A).

To substantiate these results, we performed Northern blot analysis with specific DNA probes. The results indicated that the changes observed by the two-dimensional gel electrophoresis approach corresponded to changes in the expression of the genes coding for these proteins. Furthermore, MPA-induced altered gene expression was detected as early as 1 day after treatment (Fig. 4A).

The identification of keratin 17, a protein associated with normal prostate cell differentiation (40), as a MPA-induced protein raised the possibility that other related keratins may also be expressed as a result of this treatment. Therefore, by means of Northern blotting, we tested the ability of MPA to induce the expression of three other keratins,

A

PROTEIN	SIGNAL PEPTIDE	NORTHERN BLOTS
CD46	MEPPGRRECPFSPWRFPGLLLAAM VLLLYSFSDA	C 1 3 5d
CD55	MTVARPSVPAALPLLDELPRLL LLLVLCLPAAVWG	
CD59	MGIQGGSVLFGLLLVAVFCH SGHS	
Clusterin	MMKTLFLVFGLLLTWESG	
Granulophysin/CD63	MAVEGGMKCVKFLLYVLLAFCA C	
Glucose-regulated protein 78 (GRP78)	MKLSLVAAMLLLSAARA	
Neuropeptide Y (NPY)	MLGNKRLGSLGLTALSLLVC LGALAEA	
Zinc- α -2-glycoprotein	MVPVLLSLLLLGPAVP	
Aminopeptidase N/CD13	MAKGFYISKSLGILGILLGVA AVCTHIALSVVYS	
α -glucosidase	MARKKLLKFFTTLEIVLSVL LLVLFISIVLIVLLA	
Secretory binding protein/PIP	MRLQLLFRASPATLLLVCLL QLGANKA	
Gastricsin	MKWMVVVLVCLQLEA	
Tissue Factor	METPAWPRVPRPETAVARTLL LGWVFAQVAGA	
Vasoactive intestinal polypeptide (VIP)	MDTRNKAQLLVLLTLLSVLFS	
HSP70		

B

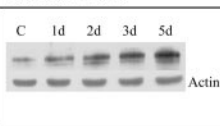
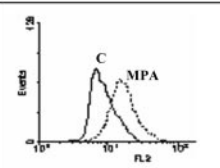
PROTEIN	SIGNAL PEPTIDE	WESTERN BLOT / HISTOGRAMS
Fibronectin	MLRGPGPGLLLAVQ CLGTA	C 1d 2d 3d 5d  Actin
Neutral endopeptidase/CD10	NO SIGNAL PEPTIDE	

Fig. 3. MPA-induced prostatosomal gene expression. A. Cells were treated with 20 μ mol/L MPA for the indicated periods of time. Total RNA was isolated, and DNA probes were prepared to analyze gene expression by Northern blotting. HSP70 was used to indicate equal RNA loading. B. Western blotting was performed to determine changes in expression of fibronectin (220 kDa) in MPA-treated cells. Actin was used to indicate equal protein loading. The fraction of CD10-immunostained cells was determined by flow cytometry using a phycoerythrin-labeled primary antibody. C, control.

namely, keratins 8, 18, and 19 (Fig. 4B). The results indicate that keratins 8 and 18 were constitutively expressed, and MPA had little to no effect on their expression. In contrast, MPA induced the expression of keratin 19; the level of keratin 19 increased by about 3-fold after 3 days of treatment. These results indicate that MPA induces the expression of two keratins that are recognized indicators of maturing normal prostate cells.

Commitment to Mycophenolic Acid-Induced Differentiation. Guanosine, a guanine derivative, which is readily metabolized into IMPDH end products, is commonly used to neutralize the impact of MPA and other IMPDH inhibitors (41). Because of this property, we

³ http://bioinformatics.leeds.ac.uk/prot_analysis/Signal.html.

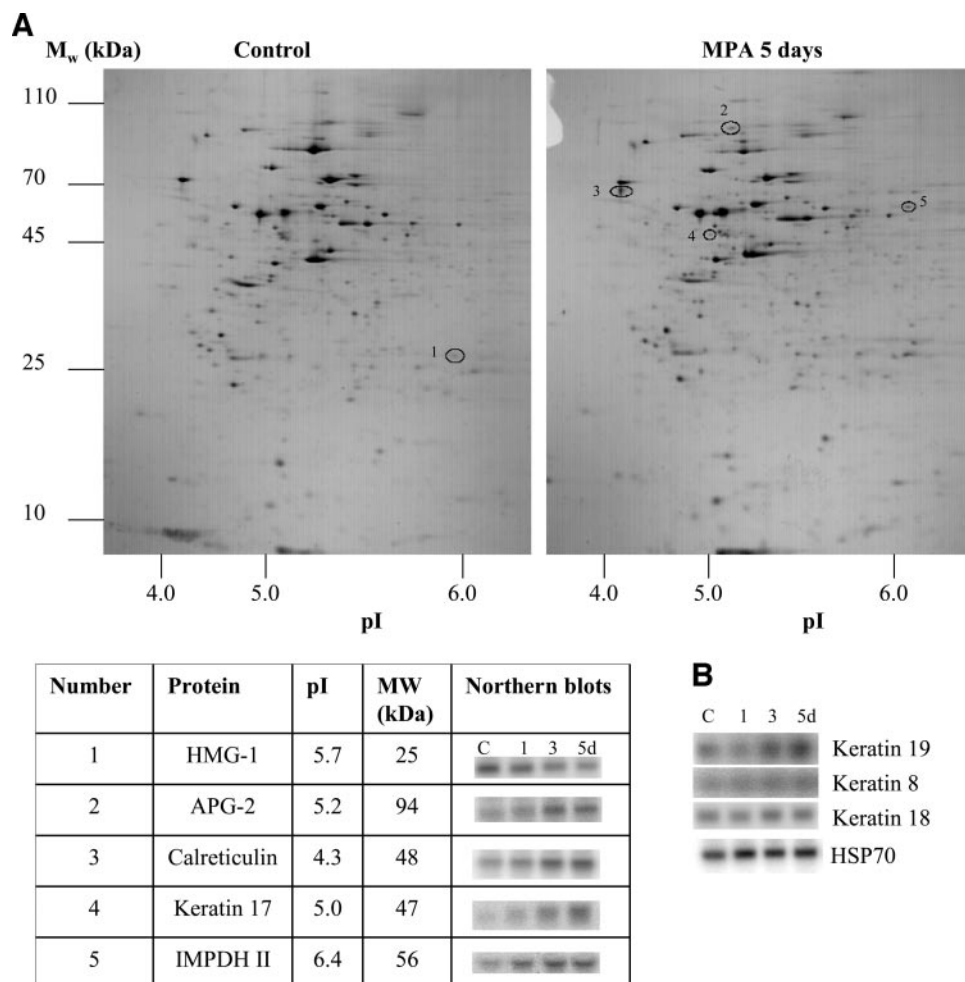


Fig. 4. MPA-induced changes in PC-3 cell proteome detected by two-dimensional gel electrophoresis analysis and confirmed by Northern blotting (A) and expression of keratins (B). A. Cells treated for 5 days with 20 $\mu\text{mol/L}$ MPA were analyzed by two-dimensional gel electrophoresis. Solubilized proteins were separated in the first dimension according to their pI and in the second dimension by a 10% to 17% polyacrylamide gradient SDS gel. The top panels depict representative Coomassie Blue-stained gels. Proteins that were identified by matrix-assisted laser desorption ionization analysis are marked and numbered. The table summarizes results of Northern blot analysis using DNA probes for genes that code for the identified proteins. B. Expression of keratins was determined by Northern blotting using specific DNA probes. HSP70 was used to indicate equal RNA loading (a representative blot is shown).

used guanosine to determine the time frame required for PC-3 cells to commit to MPA-induced differentiation.

The results indicated that 100 $\mu\text{mol/L}$ guanosine, when added together with MPA, negated the MPA-evoked replication arrest (Fig. 5A), vacuole production, and NPY gene expression (data not shown). Moreover, this neutralizing impact of guanosine was effective up to about 2 days after MPA treatment (Fig. 5B and C). After this time, the addition of guanosine had little or no effect on the manifestation of MPA-induced vacuole production (Fig. 5B) and NPY gene expression (Fig. 5C). These results demonstrate that PC-3 commitment to MPA-induced terminal differentiation occurs within the initial 2 days of MPA treatment.

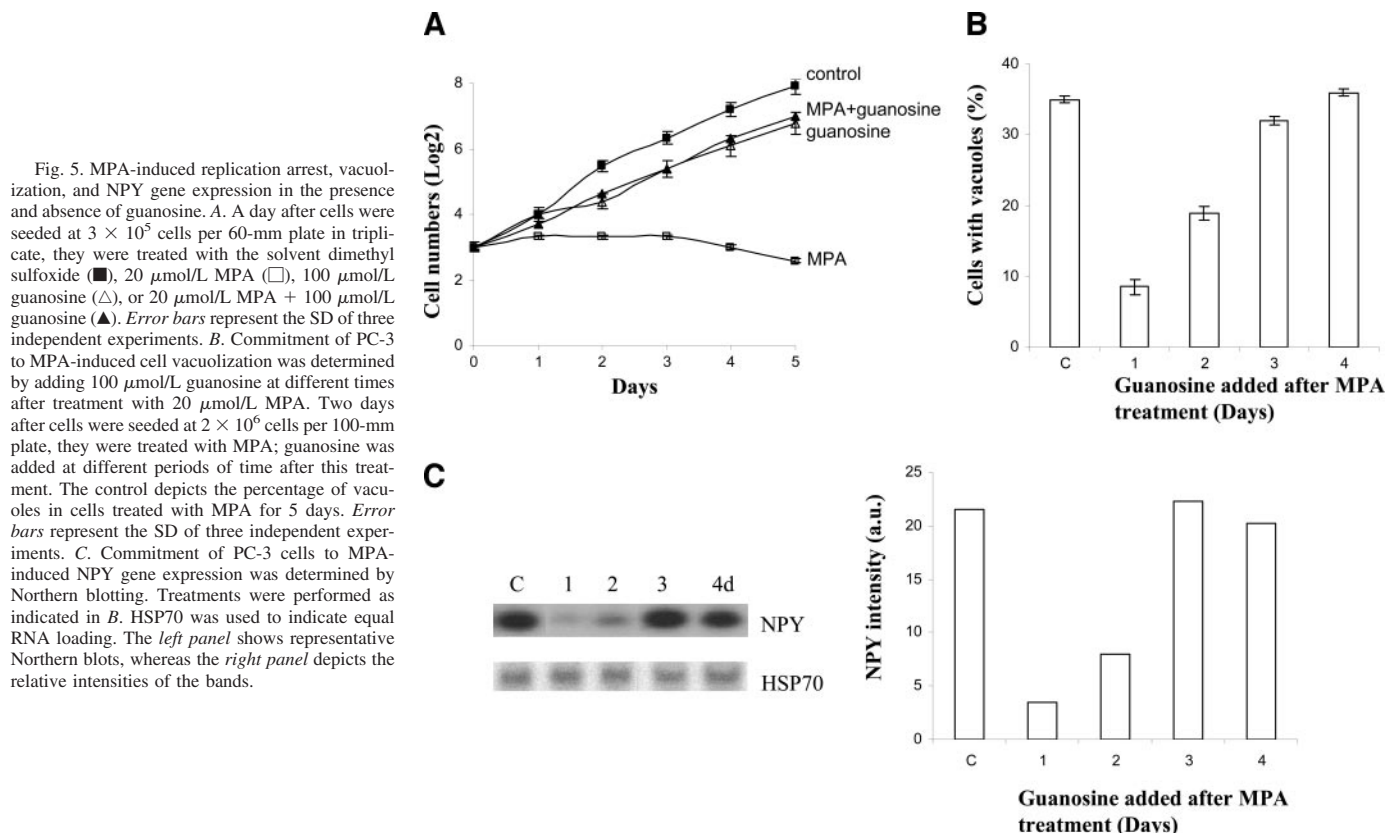
Ribavirin- and Tiazofurin-Induced Differentiation. To further confirm that MPA-induced differentiation of PC-3 cells is due to the function of MPA as an IMPDH inhibitor, we tested tiazofurin and ribavirin, IMPDH inhibitors that differ from MPA in their chemical structure (42). These inhibitors caused a dose-dependent replication arrest (Fig. 6A) and an increase in cell size (Fig. 6B). However, the doses required to achieve these changes were considerably higher than those used for MPA; a 70% to 80% reduction in cell number required a 3-day treatment with 400 $\mu\text{mol/L}$ tiazofurin or ribavirin compared with 20 $\mu\text{mol/L}$ MPA. This treatment also caused the cells to display vacuoles, with ribavirin being less effective than tiazofurin (Fig. 6B). These two IMPDH inhibitors also induced the expression of a series of prostatic proteins and keratins including APG-2, CD55, clusterin, granulophysin/CD63, GRP78, and keratin 17 (Fig. 6C). These results indicate that tiazofurin and ribavirin are inducers of PC-3 cell

differentiation; however, on a dose basis, they are considerably less effective than MPA.

DISCUSSION

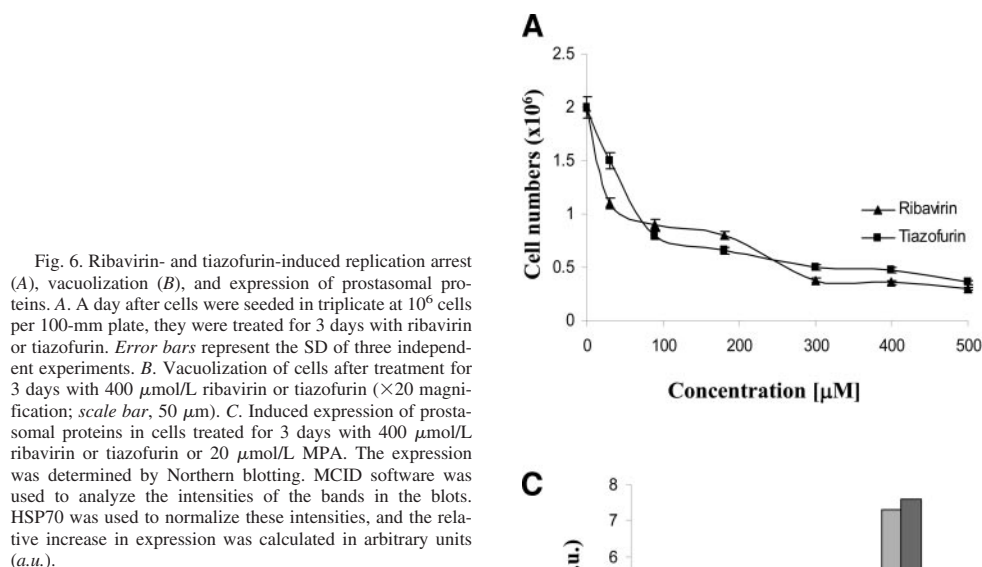
The present studies were initiated to establish a model cell system to study differentiation therapy drugs for the treatment of prostate cancers that are refractory to standard chemotherapy. Here, we describe such a system with PC-3 cells, an androgen-independent prostate cancer cell line (43). In this system, PC-3 cells were induced to differentiate into cells that display traits associated with mature prostate secretory cells. This differentiation was achieved by treating PC-3 cells with MPA, tiazofurin, or ribavirin, which are inhibitors of IMPDH, an enzyme crucial for cell replication (30). These inhibitors were chosen because they were developed as anticancer drugs (42) and have the ability to induce differentiation in human tumor cell types belonging to distinct cell lineages (41, 44). Interestingly, single oral administration of 1 g of mycophenolate mofetil, a MPA pro-drug, resulted in the appearance of 76 $\mu\text{mol/L}$ MPA in plasma of healthy volunteers (45). An oral dose of 1.5 g of mycophenolate mofetil administered to hepatic or cardiac transplant patients for longer than 6 months was well tolerated and yielded 62 $\mu\text{mol/L}$ MPA in their plasma (46). In our studies, we used MPA at doses of up to 40 $\mu\text{mol/L}$.

The central function of the mature prostate epithelium is to secrete fluids into the seminal plasma to support the viability and activity of sperm. These fluids contain, among others, small membranous vesi-



cles termed prostasomes (12). To characterize the protein composition of these organelles, Renneberg *et al.* (47) performed a two-dimensional gel electrophoresis analysis and established the presence of 80 distinct protein entities, which were not well characterized. Another

analysis of prostasomes from seminal fluid, which involved gas phase fractionation and microcapillary high-performance liquid chromatography-tandem mass spectrometry (21), identified 139 proteins, which were divided into six categories, namely, enzymes, transport/struc-



tural proteins, GTP proteins, chaperone proteins, signal transduction proteins, and unannotated proteins. This analysis revealed 128 proteins that had not been previously associated with prostasomes.

Our data, together with those of other investigators, demonstrate the presence of prostasomes in PC-3 cells (48). Interestingly, prostasomal-like granules isolated from PC-3 cells were found to enhance sperm motility (48), a function typical of normal prostasomes (26). Therefore, prostate tumor cells such as PC-3 that can be induced to express a substantial number of prostasomal proteins should represent a useful prostate differentiation cell system.

To identify such proteins in our target PC-3 cells, we used two approaches. First, we tested the expression of a selected collection of prostasomal proteins by means of Northern and Western blotting or immunostaining. Because of the large number of reported prostasomal proteins (21), we decided to concentrate on proteins with an NH₂-terminal signal peptide; these proteins would most likely be navigated into the prostasome. Our studies demonstrated that MPA induced a time-dependent or transient increase in expression of 10 proteins (CD46, CD55, CD59, clusterin, granulophysin/CD63, GRP78, NPY, zinc- α -2-glycoprotein, aminopeptidase N/CD13, and α -glucosidase), whereas another 4 proteins (secretory actin-binding protein/PIP, gastrin, tissue factor, and vasoactive intestinal polypeptide) were expressed constitutively and were not affected by MPA.

To identify additional MPA-induced proteins, we used two-dimensional gel electrophoresis technology. This approach yielded an interesting observation that among the induced proteins was keratin 17, a marker known to be involved in prostate cell differentiation (40). This led us to test for the presence of three other keratins, namely, keratins 8, 18, and 19, which are additional maturation markers of the normal prostate (40). We found that MPA induced the expression of keratin 19 but not keratin 8 or 18, which were constitutively expressed as also reported by others (49, 50).

Undifferentiated human basal prostate cells do not express keratin 8, 17, 18, or 19. These keratins are manifested as the cells progress from this state into an intermediate basal cell and turn into an intermediate differentiated and then into a well-differentiated luminal cell. The phenotype of the latter cell is characterized by the preservation of keratins 8 and 18, but not of keratin 17 or 19 (40).

Based on the fact that MPA induced the expression of about half of the tested prostasomal proteins, keratins 17 and 19, and vacuolization of the cytoplasm, we suggest that IMPDH inhibitors induce the differentiation of PC-3 cells into cells that resemble normal luminal prostate cells at their intermediate state of differentiation. Perhaps other, as yet unknown chemicals may be able to shift the PC-3 cells into the ultimate mature state. These agents may represent potential differentiation therapy drugs and, as such, should be tested in an animal model and thereafter in humans.

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