Growth Attenuation in a Human Prostate Cell Line Mediated by a Phorbol Ester (43889)

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Abstract. **Human prostatic cancer cells JCA-1 were induced with a phorbol ester 12-o-tetradecanoylphorbol-13-acetate (TPA), and cell growth differentiation were analyzed. Within hours of exposure to TPA, cell proliferation decreased and reached approximately 80% reduction after 3 days, as determined by [^{3}H]thymidine incorporation and cell counting. Cell cycle analysis revealed a blocking of cells entering into the replicating S and G_2M phases from the G_1 phase. The TPA induced cells had a reduced growth rate but remained viable. The growth-modulating activity of TPA was similarly observed with LNCaP cells. Agarose gel electrophoresis of the chromosomal DNA from induced cells exhibited a non-fragmented pattern excluding the possibility of cell apoptosis. In parallel to the growth reduction, the TPA induced cells became larger in size showing dendrite like cytoplasmic extensions. Furthermore, JCA-1 cells treated with TPA acquired the expression of cytokeratin 18 and increased the expression of actin and vimentin by 300%. These results indicate an induced growth reduction accompanied by cellular differentiation of the prostatic cancer cells.**
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It has become increasingly clear that the growth and differentiation of human tumor cells can be influenced by a variety of inducers, such as epidermal growth factor, tumor transforming growth factor, fibroblast growth factor, and other chemicals. Using these inducers and cell lines, the development of cancer cells and the induction of the mature cellular characteristics can be studied *in vitro*. The growth control of a number of human tumors have since been revealed. We have initiated studies on the differentiation induction of human prostatic cancer cells. Androgen, being the major growth and regulatory factor for the androgen dependent prostatic cells, has been well documented. The possibility of differentiation induction of the androgen-independent cells, as well as their inducing agents, remains to be fully elucidated. In order to analyze the development of androgen-independent cancer cells, we have chosen an androgen independent prostatic cancer cell line, JCA-1, as a model. This cell line was derived from a primary prostatic tumor, grows easily in culture, and is also transplantable in nude mice to form tumors (1). As a first step to elucidate the possibility of differentiation induction and the relevant mature cell markers, the JCA-1 cells were treated with 12-o-tetradecanoylphorbol-13-acetate (TPA), since it is a general differentiation inducing agent for a number of tumor cells and is a known regulator for protein kinase mediated cell growth (2, 3). The proliferation of TPA induced prostatic cancer cells was reduced, along with the detection of morphologic and phenotypic changes that are characteristic of cellular development. The potential of using differentiation induction as a control for prostatic cancer cell growth is discussed.

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Materials and Methods

Cells and Proliferation. The human JCA-1 prostatic cell line was maintained in RPMI-1640 medium supplemented with 10% FCS (1). JCA-1 cells were seeded at 1×10^5 cells/ml with or without the supplementation of 12-0-tetradecanoylphorbol-13-acetate

(TPA) (Sigma Chemical Co., St. Louis, MO) at various concentrations, in flasks or chamber slides (Lab-Tex). TPA was also supplemented to an androgen-dependent human prostatic cancer cell line LNCaP, with the same conditions. Cell culture media and TPA were changed every 3–4 days. The proliferation of cells was analyzed by the [^{3}H]thymidine incorporation procedure, by labeling cells with [^{3}H]thymidine at 0.8 μCi/ml (specific activity at 78.1 Ci/mmol, New England Nuclear, Wilmington, DE) for 4 hr before harvesting of cultures onto glass fiber filters. Incorporation of [^{3}H]thymidine into the cells was determined by scintillation counting with at least triplicate cultures (4). Separate scintillation fluid and thymidine label were used as counting controls. Cell cycle phase distribution was determined by staining cells with propidium iodide and analyzed by the flow cytometric method (4). Cell size was measured with a microscopic sizing objective after staining with hematoxylin and eosin. At least 50 cells were measured to obtain the range of cell diameters.

Cell Marker Determinations. Monolayer JCA-1 cells on the chamber slides were fixed with acetone-methanol (1:1) and stained with hematoxylin and eosine for morphology studies. Similarly fixed cells were used to react with mouse monoclonal IgG antibodies for the presence of human α-sarcomeric actin (Sigma), vimentin (Sigma), and cytokeratins 8 and 18 (Enzo Diagnostics, Farmington, NY). The immunoperoxidase procedure with a goat IgG-F(ab')$_2$ anti-mouse IgG antibody was used as a second antibody. Immunostaining was evaluated by the intensity scoring procedure of Miyamoto *et al.* (5) which quantitates both the percentage and the intensity of the stained cells. Essentially, cells were graded for the intensity of staining (0—absent, 1—weak, 2—moderate, or 3—strong; or determined as 0, +, ++, or +++), and the percentage of stained cells in each intensity level was determined. The total intensity score (TIS), from 0 to 300, was calculated as the sum of the products of each intensity score, and their corresponding percentages were determined. Cells stained with a nonspecific mouse IgG as the first antibody were used as a negative stain control.

DNA Gel Electrophoresis. DNA was extracted from TPA-treated and untreated JCA-1 cells, as well as from HL-60 cells incubated with 0.3 μm camptothecin for 3 hr (5). The cells were lysed in a solution containing 0.01 *M* Tris, 0.1 *M* NaOH, 0.5% SDS, 10 units of RNAse, and 0.001 *M* EDTA at pH 8 for 1 hr at 37°C. After the addition of proteinase K to 100 μg/ml, the incubation was continued for 2 hr at 37°C. The genomic DNA was extracted with phenol and then precipitated in 0.5 *M* NaCl and 50% isopropanol overnight at −20°C. The DNA pellet was washed in ethanol and then dissolved in 10 m*M* Tris and 1 m*M* EDTA, pH 8. Electrophoresis was performed using a 1.5% agarose gel in TBE buffer at 20 mA for 5 hr. The DNA fragments from ΦX174 Hae III digest were used as molecular weight markers.

Results

Proliferation Reduction. Incorporation of [^{3}H]thymidine into JCA-1 cells was employed to measure proliferation in response to TPA treatment. JCA-1 cells cultured with TPA had a slower proliferation than untreated cells. Figure 1 describes the TPA dose-dependent growth reduction of JCA-1 cells. TPA was used at 16 n*M* in most experiments using JCA-1 cells, since this dose caused maximum growth reduction at 3 days. Figure 2A illustrates that as early as 5 hr after TPA treatment the [^{3}H]thymidine incorporation was decreased by approximately 30% (range 20%–30%) compared with that of the untreated cells. The proliferation decreased further, until it reached approximately 80%–90% reduction after 3–4 days of TPA treatment. Figure 2B shows that the cell numbers in TPA-treated cultures were lower than the untreated cultures, with the percentage of reduction similar to that determined by the [^{3}H]thymidine procedure.

The doubling time of the JCA-1 cells grown in the presence of TPA was increased to approximately 45 hr from 17 hr in the untreated cells. With the continued presence of TPA, these slower growing cells could be maintained for further passages. Throughout the TPA treatment, the cell viability determined by trypan blue exclusion was always greater than 95%. The culture supernatant was also examined after centrifugation, and there was no shedding of dead cells from the treated cultures.

To investigate if TPA induced an apoptosis process, DNA gel electrophoresis was performed to detect the presence of the ladder pattern of the degraded chromosomal DNA. In contrast to the known apoptosis of the degraded DNA from HL-60 cells after the treatment with the topoisomerase I inhibitor camptothecin, Figure 3 shows the absence of the ladder

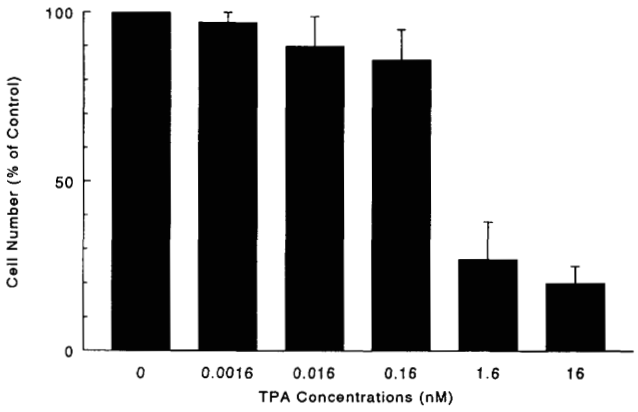

Figure 1. Graph shows TPA dose-dependent growth reduction of JCA-1 cells 3 days after the induction.

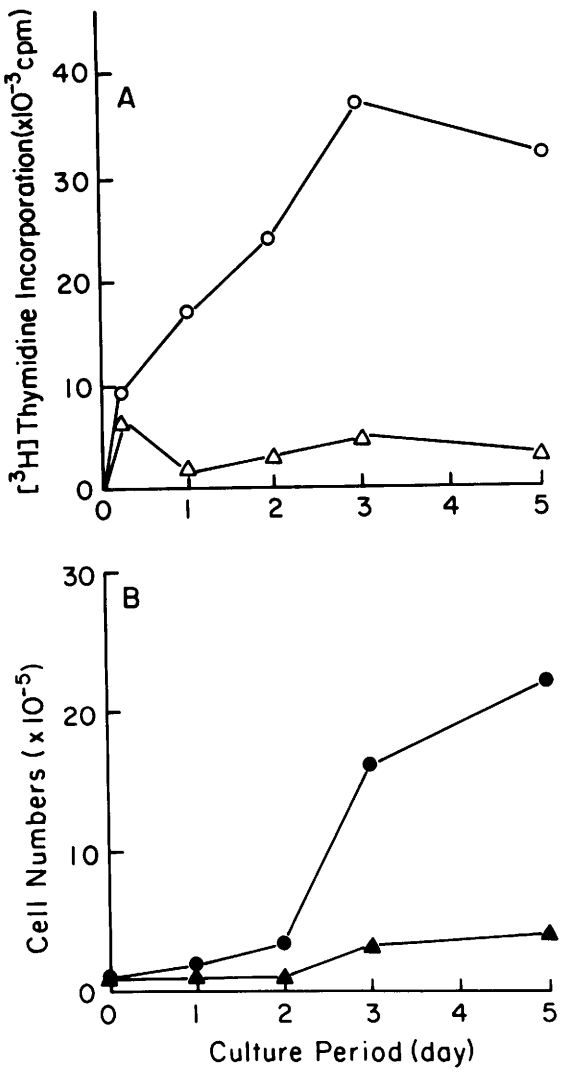

Figure 2. Graph A shows the decrease of proliferation in TPA-induced JCA-1 cells (△——△) as determined by the [^{3}H]thymidine procedure. ○——○, untreated JCA-1 cells. Graph B shows the decrease of cell number in TPA-induced culture (▲——▲). ●——●, untreated JCA-1 culture. Data points in Graph A and B were the mean of six experiments and vertical bars indicated ranges.

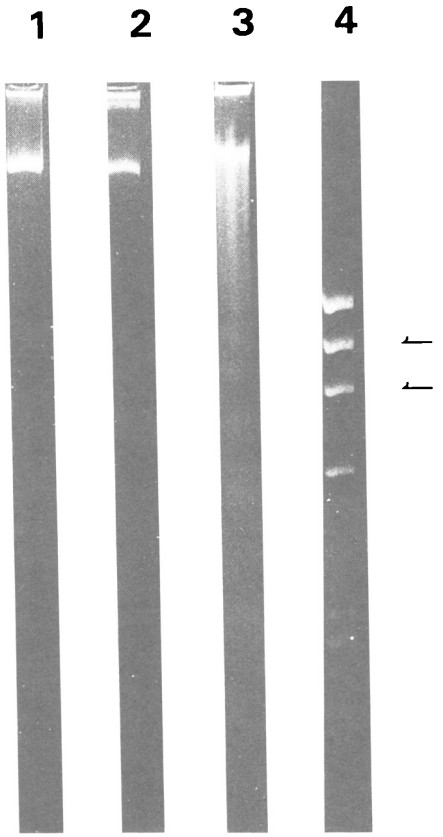

Figure 3. Gel electrophoresis of the chromosomal DNA of the TPA-treated and untreated cells. Lane 1 and 2 show the DNA obtained from the untreated and TPA induced JCA-1 cells, respectively. As a positive control for apoptosis, Lane 3 shows the fragmented DNA obtained from HL-60 cells treated with camptothecin. The molecular weight markers of ΦX174 Hae III digest are shown in the Lane 4.

pattern of chromosomal DNA in the TPA-induced JCA-1 cells. These results suggest that the proliferation reduction in response to TPA treatment was not a consequence of programed cell death.

The TPA effect on prostatic cell proliferation was also analyzed with an androgen dependent LNCaP cancer cell line. Similar to JCA-1 cells, the proliferation of LNCaP cells, as determined by [^{3}H]thymidine incorporation, had a dose-dependent reduction with TPA used. Approximately 90% reduction was observed with TPA addition at 1.6 n*M*. The earliest changes in the cell cycle phases were observed 1 day after TPA induction, revealing a decrease of cells in S and G_2M phases. The magnitude of the S and G_2M reduction became maximum after three days and remained at that level in the subsequent passages (Table I). Table I presents that approximately 30% of untreated LNCaP cells were in S and G_2M, whereas only 12% of TPA induced cells were in S and G_2M after 5 days. The reduction of the S and G_2M phases was accompanied by a concomitant increase in the G_1 phase, confirming the decrease of the replicating cell population. Table I also shows that TPA treatment of JCA-1 cells induced a similar blocking of G_1 cells entering into S and G_2M phases.

Changes in Cellular Characteristics. Cell morphology changes were observed after 2–3 days of TPA treatment. These cells became enlarged in size with prominent cytoplasm; some were pleomorphic and multinucleated. Most of the cells had spindle-like cytoplasmic extensions (Fig. 4). The diameter of the TPA-induced cells was approximately 30–40 μm compared with 10–15 μm for the untreated cells. The nucleus size also increased from approximately 8–8.5 μm to 12–15 μm in TPA-induced cells, accompanying a reduced nucleus to cytoplasm ratio.

The expressions of several extracellular matrix and cytoskeletal proteins associated with prostatic cell development were detected by antibodies with immu-

Table I. Cell Cycle Phase Distribution of Prostatic Cancer Cells After TPA Induction

Cell types	Culture period (days)	Percentage of cells in cell cycle phases in untreated culture (%)				Percentage of cells in cell cycle phase in TPA-induced culture (%)			
		G_1	S	G_2M	S + G_2M	G_1	S	G_2M	S + G_2M
JCA-1	5	61.3	24.4	13.3	37.7	81.8	3.1	15.1	18.2
	19	57.1	24.9	18.0	42.9	76.0	3.1	20.9	24.0
LNCaP	5	69.0	19.5	11.5	31.0	90.0	6.0	4.0	10.0
	9	72.5	16.1	11.4	27.5	92.0	5.5	2.5	8.0

noperoxidase staining. Figure 4, C and D, show increased content of vimentin in TPA-treated JCA-1 cells. The presence of these proteins and their staining intensity were quantitated by a total intensity score procedure (TIS). Table II represents the mean TIS values of four experiments obtained on Day 5, which is typical of these slower growing cells. TPA-induced cells acquired the intense expression of cytokeratin 18 in approximately 90% of the cells, compared with the untreated cultures which had a few cells with faint staining. The TIS scoring in JCA-1 cells changed from approximately 3 to 258 in TPA-treated cells. The staining intensity of another cytokeratin 8 was also increased in TPA-induced cells, although it was less pronounced than cytokeratin 18. Both cytokeratins 8 and 18 have been described to be associated with the more differentiated cells of the prostate tissue (7, 8). Table II also shows that TPA induced a more than 300% enhancement of actin and vimentin expression.

Discussion

TPA-induced prostatic cancer cells have been shown to be reduced in their proliferation but viable

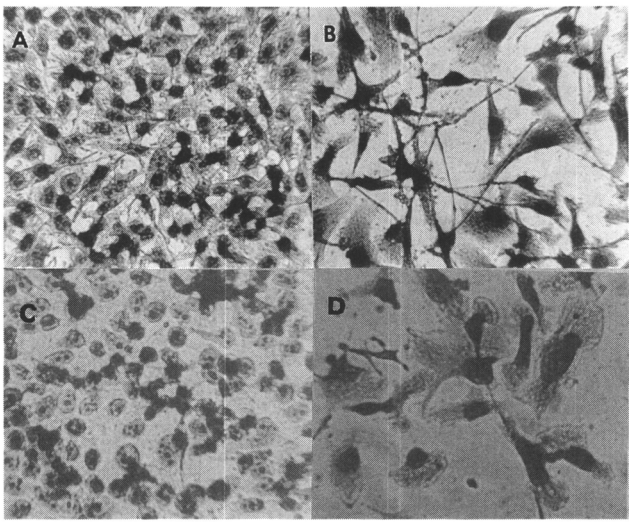

Figure 4. Morphology of untreated JCA-1 prostatic cells (Photo A) and 5-day TPA-induced JCA-1 cells (Photo B). Cells were stained with hematoxylin-eosin compared with untreated JCA-1 cells (Photo C), the TPA-treated cells showed increased cellular content of vimentin (Photo D) as assayed with an immunoperoxidase procedure (magnification ×400).

for passages in culture. These cells had an increased percentage of cells in the G_1 phase coupled with a decrease in the percentage of cells in the S and G_2M phases. The characteristics of the cell cycle phase distribution indicated that the blocking of the entry of G_1 cells into S and G_2M phases may be one of the mechanisms contributing to a slower growth.

The TPA-induced growth reduction in prostatic cells may be in tandem with their cellular development. Judging by the sequence of events detected in cultures, the proliferation reduction, which could be detected as soon as 5 hr after TPA addition, may precede other cellular changes. These changes included the development of a cell morphology with distinct characteristics. The TPA-treated cells acquired lengthened cytoplasmic extensions and had a higher content of vimentin. An increased vimentin content has been described to be related to epithelial cell shape changes and spreading (9), correlating to a new morphology. Several other important phenotype changes were detected after TPA-mediated growth attenuation. Of particular interest is the development of cytokeratin 18, which is a structural component and is associated with the differentiated luminal epithelial cells in the prostate (7, 8). Cytokeratin 8 and 18 were described as increased in differentiated human prostatic cells after retinoic acid induction (10). In line with this indication of cellular differentiation was the increased expression of actin, detected along with the cytokeratin increases. The interpretation that the differentiated cells express more actin is consistent with the observation of other cell types. Negata *et al.* (11) has described that the synthesis and content of actin were increased in myeloid leukemia cells after differentiation. The development of the cytoskeleton proteins in the prostatic cells indicated that these cells possess a better cell attachment and cytoskeleton organization, implying that they are at a different developmental stage from the original cells. Considering the sequence of events, TPA may induce a cellular development initiated with a proliferation reduction event and leading to the expression of certain phenotypes associated with the slower growing cells. The process of growth reduction and cellular development may be intertwined. At the onset of the induction period, cells were rapidly proliferating and there were more undifferentiated cells

Table II. Phenotype Expression of TPA Induced JCA-1 Cells[a] Detected by Immunoperoxidase Procedure

Markers	Cytokeratin 18		Cytokeratin 8		Vimentin		Actin	
Culture condition	−TPA	+TPA	−TPA	+TPA	−TPA	+TPA	−TPA	+TPA
% Positive cells (mean)	3	96	12.5	25	37	74	48	95
Stain intensity level (%)								
1	3	0	10	0	20	0	43	0
2	0	30	2.5	15	17	15	5	50
3	0	66	0	10	0	69	0	45
Intensity score TIS[b]	3	258	15	60	54	237	53	235
Standard deviation	0.5	42	4	12	10.8	47.3	17.5	65

[a] JCA-1 cells were treated with 16 n*M* TPA for 5 days.
[b] Procedure for quantitation of positive cells and staining intensity.

than the differentiated cells. The differentiated cells later became predominant as the rapidly proliferating cells were reduced in number.

The growth attenuation and cellular development may have also reduced the tumorigenicity of the prostatic cancer cells. Since the JCA-1 cell line is an androgen-independent cell line that can form tumors in nude mice after heterotransplantation, this tumorigenicity assay and its correlation with the cellular development is being considered.

The characteristic effect of TPA on the growth attenuation was found with both the androgen-independent JCA-1 and the androgen-dependent LNCaP cells. The LNCaP cells appeared to be more sensitive to lower dosages of TPA used. Both cell types were reduced in their growth rate but capable of continued proliferation. Induction of human promyelocytic leukemia cells with TPA resulted in their terminal proliferation and differentiating to monocytic cells, with the process paralleling our observations with JCA-1 cells (2). In contrast to a growth-terminating effect in leukemia cells, the TPA-induced prostatic cells maintained their replication. This could be caused by the different intracellular events induced by TPA in these cell types. Since the TPA effect is based on its mediation of cell proliferation with the signal transduction pathways, the prostatic cancer cells may have specific pathways and mediators unique in their interactions with TPA. Investigation in this area will further reveal the growth mechanism in prostatic cancer cells.

The TPA differentiated prostatic cells could maintain their slower replication rate and cell characteristics in continued passages. These phenomena suggest that the process of TPA-induced JCA-1 cell differentiation may be different from that of hematopoietic leukocytes in which the end-stage cells was terminal and unable to proliferate after TPA treatment (2). Whether TPA induced JCA-1 differentiation process is common to other prostatic cells remains to be investigated.

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