Patterns of Protein Kinase C Isoenzyme Expression in Transitional Cell Carcinoma of Bladder

Relation to Degree of Malignancy

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Key Words: Protein kinase C; Isoenzymes; Malignancy; Urinary bladder; Cell culture

Abstract

We determined the pattern of protein kinase C (PKC) isoform expression in human cell lines by Western blotting and immunofluorescent staining techniques. In addition, we examined PKC isoform expression in tissue samples of transitional cell carcinoma (TCC) of the bladder. PKC delta, PKC beta II, and PKC eta were found primarily in the RT4 cell line (low-grade tumor), and PKC zeta was expressed most strongly in the SUP cell line (invasive tumor). In tissue samples of urinary bladder cancer, PKC isoenzymes were expressed differentially as a function of tumor stage and grade; expression of PKC beta II and PKC delta was high in normal tissue and in low-grade tumors and decreased with increasing stage and grade of TCC. The opposite pattern was seen with PKC zeta. The differences in expression of specific isoenzymes as related to levels of malignancy of the cell lines and tissue samples suggest that the PKC family has an important role in normal and neoplastic urothelium.

The protein kinase C (PKC) family of serine-threonine kinases has an important regulatory role in a variety of biologic phenomena.1,2 The family is composed of at least 12 individual isoforms that belong to 3 distinct categories: (1) conventional isoforms (alpha, beta I, beta II, gamma) activated by Ca²⁺, phorbol esters (12-O-tetradecanoylphorbol-13-acetate [TPA]), and diacylglycerol liberated intracellularly by phospholipase C; (2) novel isoforms (delta, epsilon, eta, theta), which also are activated by phorbol esters and diacylglycerol but not by Ca²⁺; and (3) atypical (zeta, lambda, iota) members of the family, which differ in cystein-rich sequences and are not activated by Ca²⁺, phorbol esters, or diacylglycerol; PKC mu is considered to be intermediate between the novel and atypical groups. The cellular response to TPA activation can be an increase3,4 or decrease5,6 in the rate of proliferation depending on the cell type and the particular PKC isoenzymes expressed in different cells. Not all isoenzymes are activated by phorbol esters, and there is heterogeneity in response.7

It is generally considered that the enzymes when quiescent are located in the cytoplasm and on activation are translocated to the plasma membrane.8 Not all isoforms behave in this manner; indeed, not all of them are found in all tissues. Moreover, the pattern of isoform distribution varies among different tissue9 and also may change as a function of phenotype.10 Several studies have reported that the pattern of PKC isoenzyme expression and distribution in a number of cell types may change as cells are transformed from a normal to a proliferative phenotype.10-17 Thus, overexpression of PKC beta I in rat fibroblasts caused these cells to be able to form tumors in nude mice.18 It seems, however, that effects of PKC isoenzyme expression may have different influences depending on the cell type. High expression of PKC alpha
alone in MCF-7 breast cancer cells is associated with a transformed phenotype, whereas overexpression of both PKC alpha and PKC beta in these cells resulted in a less aggressive phenotype. These reports indicate that the change from the normal to the transformed state may be associated with up-regulation or down-regulation of a particular PKC isoform.

The profile of isoform expression might aid in the understanding of processes leading to cell transformation and provide information about the grade of malignancy of various types of cancer.

Tumors of the urinary bladder of transitional epithelium or urothelium exhibit the entire spectrum of biologic aggressiveness, from low-grade tumors to highly malignant carcinoma. This provides an excellent opportunity to examine the possibility that tumorigenicity of transitional cell carcinoma (TCC) may be related to the PKC isoform profile. Until now, the pattern of PKC isoform expression in urinary bladder transitional cells of varying degrees of malignancy has not been determined.

The purpose of the present study was to determine the effect of TPA on 3 TCC cell lines of different aggressiveness and to characterize the types of PKC isoenzymes in these cell lines.

**Materials and Methods**

**Cell Lines of Urinary Bladder Tumors**

The 3 different human cell lines used in the study were purchased from the American Type Culture Collection (ATCC, Rockville, MD). They were selected on the basis of degree of malignancy as determined by the depositors of the cells at ATCC according to morphologic features and ability to produce tumors in nude mice. The 3 lines were (1) TCC-RT4, originating from a TCC, grade I; (2) TCC-T24, originating from a TCC grade II, and (3) TCC-SUP, originating from a TCC, grade III that had metastasized to the bone marrow.

**Growth of Cells in Culture**

All cells were plated initially at a density of 10^5/mL. RT4 and T24 cells were grown in medium containing McCoy 5A solution (90%) and heat-inactivated fetal calf serum (10%), and SUP cells were grown in Eagle MEM with nonessential amino acids (90%) and heat-inactivated fetal calf serum (10%), to which was added a 2-mmol/L concentration of glutamine and a 1-mmol/L concentration of sodium pyruvate. All media contained 0.05 mg/mL of streptomycin and 50 U/mL of penicillin. All cell lines were incubated at 37°C in a 6% carbon dioxide atmosphere. All changes of media during the period of cell growth were done at 37°C.

**Effects of Phorbol Esters on Cell Morphologic Features**

TPA was dissolved in dimethyl sulfoxide to give a stock solution of 1 mmol/L. TPA was added to the cultures to a final concentration of 50 mmol/L and was added to culture dishes (90 mm) containing each cell line. The effect of this treatment was examined after 15 and 90 minutes of stimulation. In parallel, the same concentration of dimethyl sulfoxide was added to other dishes to control for possible effects of the diluent.

**Materials**

Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence was performed with a kit purchased from BioRad (Israel). Anti-PKC polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-PKC monoclonal antibodies from Transduction Laboratories (Lexington, KY); Horseradish peroxidase–conjugated antirabbit and antimouse IgG were obtained from BioRad. Leupeptin, aprotinin, phenylmethylsulfonyl fluoride, dithiothreitol, orthovanadate, and pepstatin were purchased from Sigma Chemicals (St Louis, MO).

**Preparation of Cell Extracts**

All cell lines were grown to equal confluency in 90-mm culture dishes. The cells were washed with Ca^2+–Mg^2+–free phosphate-buffered saline, a 2-mmol/L concentration of EDTA was added, and the cells were detached mechanically with a rubber policeman. The cells were centrifuged at 500g at 4°C for 5 minutes, and the supernatant was discarded. The cell pellet was then resuspended in sonication buffer [NaCl, 100-mmol/L concentration; tris(hydroxymethyl)aminomethane hydrochloride pH 7.5, 20-mmol/L concentration; MgCl₂, 5-mmol/L concentration; phenylmethylsulfonyl fluoride, 100-µmol/L concentration; leupeptin, 20 µg/mL; aprotinin, 10 µg/mL; dithiothreitol, 0.001-mol/L concentration] and sonicated for 1 minute (high setting, 10 pulses per second every 10-15 seconds). This preparation was then centrifuged at 500g for 10 minutes, the pellet discarded, and the supernatant stored at −70°C. An aliquot of this supernatant was retained for protein determination.

**Preparation of Tumor Extracts**

The study included 29 cases of bladder TCC obtained by transurethral resection of bladder tumors and 7 areas of normal bladder mucosa obtained during prostatectomy. After surgical excision, the specimens were divided into 2 parts. One was fixed in buffered formalin for 24 hours and then embedded in paraffin wax. All sections were stained in H&E for diagnosis. Grading and staging were performed according to the TNM system. The second part was frozen in liquid nitrogen. The frozen specimens were transferred to...
Dulbecco minimal essential medium, dissected under sterile conditions to remove blood and extraneous tissue, and cut into small (0.5-1 mm) pieces and transferred to fresh Dulbecco minimal essential medium. The samples were then centrifuged at 500 g at 4°C, and the pellet was resuspended in sonication buffer and processed as the cell extracts.

**Western Blot Analysis**

Homogenates of each cell line were electrophoresed through sodium dodecyl sulfate polyacrylamide gels (7.5%). In each case, the 3 lines were run together on the same gel and electrophoretically transferred onto Immobilon-p membranes (Millipore, Bedford, MA). The membranes were subjected to standard blocking and incubation procedures and were incubated with antibodies to specific PKC isoforms. Following 4 washes in Tris-buffered saline containing Tween 20 (0.1%), the membranes were incubated for 20 minutes at room temperature with horseradish peroxidase–labeled secondary antibody (goat antirabbit or antimouse IgG according to the primary antibody), diluted 1:10,000 in blocking buffer. After 3 washes (1 for 15 minutes and 2 for 5 minutes) in Tris-buffered saline containing Tween 20 (0.1%), the membranes were treated with enhanced chemical luminescence reagent for 1 minute and exposed on Kodak X-ray film for 10 seconds and developed.

**Densitometry**

The optical density of specific protein bands was measured from computer images of Western blots with the use of Image Pro for Windows (Media Cybernetics, Silver Spring, MD).

**Protein Determination**

The amount of protein in cellular and tumor preparations was determined from aliquots by the Bradford method. Equivalent amounts of protein were taken for subsequent sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

**Statistical Analysis**

Data were analyzed for significance using the Student *t* test or analysis of variance (InStat, Graph Pad, San Diego, CA). Data are expressed as mean ± SE, with significance taken as *P* < .05. For analyses of degree of isoform expression in tumor samples, least squares linear regression (Graph Pad, Instat) was performed.

**Results**

**Effects of Phorbol Esters on Cell Morphologic Features**

The effects of TPA (50-nmol/L concentration) after 15- and 90-minute treatments are shown in Image 1B. RT4 cells underwent morphologic changes in appearance as early as 15 minutes, when the cells became rounded with increased intercellular spaces (Image 1B). Similarly, T24 cells also began to show changes 15 minutes after addition of TPA, with slight contraction of the cell body and extension of processes. In contrast, SUP cells were unchanged at this time. By 90 minutes after TPA, RT4 cells assumed a flattened appearance with large spaces between individual cells, while T24 cells became thin with long extensions (Image 1C). At this time, the highly malignant SUP cells appeared only slightly different from control, untreated cells.

**Pattern of PKC Isoform Expression in Urinary Bladder Cell Lines**

The PKC isoenzymes beta II, delta, and eta were found to be expressed strongly in the low-grade tumor RT4 cell line but were barely detectable, if at all, in the other 2 lines (Table 1). There were also marked differences in expression of PKC zeta, which normally appeared in 2 main forms, 67-kd and 80-kd. The 67-kd band was stronger in the highly invasive SUP cell line than in either RT4 or T24 cells; densitometry measurements of Western blots from 8 separate preparations indicated that the 67-kd PKC zeta was consistently (ie, in each experiment) about 35% higher in SUP than in the other cells (Figure 1). There was no difference among the 3 cell lines with regard to the 80-kd band. PKC epsilon and PKC alpha isoforms were identified in each of the 3 cell lines without major differences among them. PKC gamma and PKC theta were not identified in any of the cell lines. PKC beta I showed decreasing expression from RT4 to T24 to SUP (not illustrated).

**Distribution of PKC Isoenzymes in Urinary Bladder Tumors**

The profile of isoenzymes in tumor samples obtained from human patients and the data regarding tumor grade and stage and 5-year follow-up are summarized in Table 2. Sixteen patients had Ta tumors, 5 had T1, 1 had T2, and 7 had T3. The tumor was grade 1 in 10 cases, grade 2 in 9 cases, and grade 3 in 10 cases. After 5 years, all patients with normal mucosa and all patients with grade G1 tumors were alive and well. In G2 cases, 3 patients were alive and well, 5 were alive with recurrent tumors, and 1 had died. In the G3 cases, 1 patient was alive and well, 2 patients were alive with recurrent tumors, and 7 patients had died of disease.

Based on the analyses of the different cell lines and tumor samples, we sought to determine whether the relative degree of specific isoenzyme expression, in particular PKC beta II, PKC delta, and PKC zeta, might be related to tumor stage. Accordingly, we determined the band intensity of each of the isoforms by densitometry measurements. In these analyses, comparisons were made among the different...
Effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on morphologic features of urothelial tumor cell lines. Left column, RT4 cells; middle column, T24 cells; right column, SUP cells. A, Control appearance before addition of TPA. B, Fifteen minutes after addition of TPA (50 nmol/L). RT4 cells became rounded with increased intercellular spaces. T24 cells showed slight contraction of the cell body and extension of processes. SUP cells were unchanged. C, Ninety minutes after addition of TPA. RT4 cells assumed a flattened appearance with large spaces between cells. T24 cells became thin with long extensions. SUP cells appeared only slightly different from control, untreated cells. (Final magnification ×1,200)
tumors by probing Western blots of all the tumors with each antibody under the same conditions. Densitometry measurements were made without knowledge of the source of the sample, ie, the degree of malignancy of the tumor as determined in the pathology reports. The data obtained on examination of 29 tumors and 7 normal tissues are summarized in Table 2. Profiles of 1 tumor of each grade and of a normal tissue sample are illustrated in Image 3. The data were analyzed by least squares linear regression, and plots of the data obtained are shown in Figure 2. As shown in Figures 2A and 2B, PKC isoenzymes beta II and delta, which were strongly expressed in the RT4 cell line (low malignancy papillary urothelial neoplasm), showed a high degree of expression in normal tissue. Expression of these isoforms decreased as a function of increasing stage and grade (PKC beta II; correlation coefficient = –0.92; \( P < .01 \); PKC delta; correlation coefficient = –0.93; \( P < .01 \)). In contrast, the 67-kd band of PKC zeta (Figure 2C) showed very low (if any) expression in normal and low-grade urothelial tumors, and its expression increased with increasing tumor stage and grade (correlation coefficient = 0.85; \( P < .05 \)). It can be seen from the plotted data that one or another isoform in certain patients does not fall on the regression line. However, in most cases when the pattern of expression for each patient is considered, as detailed in Table 2, the remaining isoforms do fit the relation. Consistent with our findings on cell lines, there did not seem to be significant differences in expression of PKC alpha among the various tumor specimens. Expression of PKC eta, however, which resembled PKC beta II and PKC delta in its distribution in the TCC cell lines, showed no consistent pattern among the various urinary bladder tumors examined.

**Discussion**

We initially compared 3 cell lines of different degrees of malignancy for their responses to stimulation by phorbol esters. We found different patterns of response indicating that there might be a relation between expression of the PKC isoenzymes and the degree of malignancy of TCC and their pattern of PKC isoform expression. Having found that there...
were clear differences, we examined the PKC profile of these cell lines and of normal urinary bladder tissue and tissue samples of urothelial cell tumors of differing stages and grades. Analyses of the blots showed clear differences among the cell lines regarding certain isoforms. As determined by Western blotting of crude homogenates, PKC delta and PKC beta II were found to be expressed primarily and PKC eta almost exclusively in the low-grade tumor RT4 cell line. As PKC delta and PKC beta II are activated by phorbol esters, the low expression of these isoenzymes in SUP cells can explain the relative insensitivity of these cells to TPA. The 67-kd band of PKC zeta, which is not stimulated by phorbol esters, was found to be most strongly expressed in the highly malignant SUP cell line. This is also consistent with the relative insensitivity of this cell line to phorbol ester stimulation. Our findings are in agreement with the findings of Bamberger et al, who reported that malignant uterine cells express higher levels of this isoenzyme than differentiated cells.

The results of this study confirm that there is a relation between PKC isoenzyme expression and cell transformation, as has been described in a number of studies on several other cell types. Our findings are compatible with studies showing high expression of PKC delta and PKC eta in differentiated cells. PKC eta has been identified in only a few epithelial tissues, and its expression level also seems to be related to the degree of differentiation. Studies suggest that PKC delta may serve an important tumor suppressor function. PKC zeta has been reported to be associated with mitogenic signal transduction in several cell types, including oocytes, melanocytes, glioblastoma, and fibroblasts.

### Table 2
Clinical Data on Relative Band Density of Protein Kinase C Isoform Expression in Transitional Cell Carcinoma and Normal Bladder Mucosa Samples

<table>
<thead>
<tr>
<th>Case No./Sex/ Age (y)</th>
<th>Grade</th>
<th>Stage</th>
<th>PKC beta II</th>
<th>PKC delta</th>
<th>PKC zeta</th>
<th>Survival (mo)</th>
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<td>2.5</td>
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N, normal.

* Intensity of band in Western blot analysis.
findings compatible with those in the present study. While we found little difference among the 3 lines in the 80-kd band, the 67-kd band was consistently most strongly expressed in the highly malignant SUP cell line. Also compatible with an expression of this isoform in cell lines with a proliferative phenotype was the pattern of expression of 67-kd PKC zeta in the tumor samples. Here, PKC zeta was found to exhibit high expression in high-grade tumors and was very low in normal and low-grade tumor cells. Similar findings were reported in a study on uterine tumor cells\(^{22}\) in which differences in phenotype—proliferative or differentiative—seemed to be related to the pattern of PKC isoenzyme expression. While slightly different in certain isoenzymes, that study provides additional evidence in support of the relation between the PKC isoenzyme profile and phenotype.

Our results on tissue samples from urinary bladder cancers are also largely consistent with those in a study of PKC isoform patterns in prostate cancer.\(^{33}\) Thus, similar to our findings, PKC beta was found to be strongly and uniformly expressed in control and benign epithelium, whereas PKC zeta was highest in cancers compared with benign and control prostate samples. On the other hand, PKC delta was not detected in either benign or malignant prostate tissue. In contrast, we found that in both urinary bladder tumor cell lines and in urinary bladder cancers PKC delta was highly expressed in low-grade malignant neoplasms, and expression decreased as the degree of malignancy increased.
One possible explanation for this difference might be related to differences between epithelial cells in the bladder and those in the prostate gland. However, it must be emphasized that PKC delta is a major PKC isoform involved in differentiation in virtually all normal tissues, and lack of expression of this isoform must be seen as the exception and not the rule. Moreover, the studies on prostate tumors were performed only with immunostaining procedures, and the findings should be confirmed by Western blot analysis.

Our analysis of PKC isoform expression in TCC and normal bladder samples showed strong correlation as predicted by the studies on the cell lines. It is important to emphasize that the total analysis takes into account the expression profile of the 3 isoforms. Exceptions in expression of one of the isoforms may provide an indication regarding tumor prognosis.

We have shown that cell lines of human urinary bladder tumors express different PKC isoenzymes that seem to be related to the degree of malignancy. The profiles are consistent with known roles of individual PKC isoenzymes in cellular proliferation, growth, and differentiation described for many transformed and normal cells and, thus, may aid in understanding the function of this family of enzymes in these processes. The clinical data for TCC and normal bladder tissues correlate well with our results in cell lines. There is no objective criterion for determination of the degree of malignancy and aggressiveness of urinary bladder tumors. The results of the present study suggest that the expression pattern of PKC beta II, PKC delta, and PKC zeta may be useful markers in the diagnosis of urinary bladder cancer and its degree of malignancy. This possibility is being studied further in our laboratory.

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Acknowledgments: This study was supported by the Gradel-Turner Research Fund, the Otto Meyerhoff Center, and the Bar-Ilan-Sharei Tzedek Joint Research Fund. Dr Sampson is Incumbent, Louis Fisher Chair in Cellular Pathology.

This work is an essential portion of the thesis submitted by Ms Langzam to the Department of Life Sciences in partial fulfillment of the requirements for the Master of Sciences degree, Bar-Ilan University.

References


