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Overexpression of Protein Kinase C in HT29 Colon Cancer Cells Causes Growth Inhibition and Tumor Suppression

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By using a retrovirus-derived vector system, we generated derivatives of the human colon cancer cell line HT29 that stably overexpress a full-length cDNA encoding the β_1 isoform of rat protein kinase C (PKC). Two of these cell lines, PKC6 and PKC7, displayed an 11- to 15-fold increase in PKC activity when compared with the C1 control cell line that carries the vector lacking the PKC cDNA insert. Both of the overexpresser cell lines exhibited striking alterations in morphology when exposed to the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Following exposure to TPA, PKC6 and PKC7 cells displayed increased doubling time, decreased saturation density, and loss of anchorage-independent growth in soft agar; but these effects were not seen with the C1 cells. Also, in contrast to the control cells, the PKC-overproducing cells failed to display evidence of differentiation, as measured by alkaline phosphatase activity, when exposed to sodium butyrate. In addition, the PKC-overexpresser cells displayed decreased tumorigenicity in nude mice, even in the absence of treatment with TPA. These results provide the first direct evidence that PKC can inhibit tumor cell growth. Thus, in some tumors, PKC might act as a growth-suppressor gene.

Protein kinase C (PKC) is a calcium- and phospholipid-dependent serine-threonine protein kinase of fundamental importance in signal transduction and growth regulation. A variety of extracellular signals have been shown to activate phospholipid turnover and the formation of diacylglycerol (DAG). DAG thus produced can activate PKC by binding to the regulatory domain of this enzyme. The tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) also binds to the regulatory domain of PKC and is a highly potent activator of the enzyme (6, 24). Once activated, PKC can modulate diverse cellular processes, presumably via phosphorylation of specific target proteins. cDNA cloning studies indicate that PKC belongs to a multigene family consisting of at least six distinct genes designated α, β, γ, δ, ε, and ξ. The β gene yields two distinct transcripts, designated β_1 and β_2. Thus, there are at least seven isoforms of the enzyme. Since these isoforms have been conserved during evolution and are differentially expressed in different tissues, it seems likely that they perform somewhat different physiologic functions, but this remains to be demonstrated.

When applied to mouse skin, TPA induces hyperplasia of the keratinocyte population, which presumably contributes to its tumor-promoting effect in this tissue. The growth of rodent fibroblasts, in both monolayer cultures and in soft agar suspension, is also stimulated when the PKC signal transduction pathway is activated by TPA (32). Recent studies on rodent fibroblasts that stably overproduce either $PKC_{\beta 1}$ (21, 29) or PKC_{α} (39) have provided direct evidence that activation of PKC can stimulate cell proliferation and perturb growth control. On the other hand, the growth of certain human cell lines is inhibited by TPA, and in some cases this is associated with induction of differentiation (9, 17, 43). Furthermore, in cultures of mouse epidermal cells, TPA induces the differentiation of some cells and stimulates the proliferation of others (48). Although the exact mechanism(s) is not known, these findings indicate that the effects of activation of PKC (or specific isoforms of PKC) on growth and differentiation depends on the particular cell type in which it is expressed. It is of interest, therefore, to study the role of PKC in growth control in cell lines in which growth is inhibited by TPA, particularly human epithelial cells.

Our laboratory has recently become interested in the possibility that PKC might play a critical role in the origin and growth of human colon cancer. Although this disease is the second major form of cancer in the United States, its precise etiology is not known. Recent studies have demonstrated point mutations in c-K-*ras* and the p53 gene and deletions at loci on chromosomes 5, 17, and 18 in some but not all malignant colon tumors (4, 12, 44). Curiously, we have found that human colon tumors generally display decreased levels of PKC enzyme activity when compared with normal colonic mucosa (18). In addition, we found that bile acid, which has been implicated as a promoter in colon carcinogenesis, can either inhibit or enhance the activity of purified PKC, depending on the Ca^{2+} concentration (10). Other investigators (30) have developed evidence that Ca^{2+}, a necessary cofactor in the activation of PKC, might have a protective role in familial colon cancer. In contrast, Craven et al. (8) have shown that topical application of TPA stimulates proliferation of the epithelium of the rectal mucosa. The physiologic significance of their study is not, however, clear since they employed a very large dose of TPA (1 to 10 μM). In a series of studies employing primary cultures of human colon specimens, it was found that normal colonic mucosa, dysplastic and villous adenomas, and carcinoma samples display increased induction of plasminogen activator by TPA (14, 16). In addition, whereas TPA does not stimulate proliferation in the normal mucosa samples, stimulation was seen with premalignant tubular adenoma cells and also with preneoplastic cells from patients with familial polyposis coli (13, 16). Specific DAG also induced mutagenesis in colonic adenoma and some carcinoma samples, but not in normal colonic epithelial cells (15). Thus, the results obtained thus far are complex, and further studies are

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needed to clarify the role of PKC in the evolution and growth of human colon cancers.

To examine the precise role that PKC plays in human colon cancer, we therefore took a direct molecular approach to address this problem. We generated derivatives of the HT29 human colon cancer cell line that stably overexpress large amounts of $PKC_{\beta 1}$. The HT29 cell line was employed, since it has been extensively characterized with respect to its growth and differentiation properties (40, 42). Our findings indicate that, in contrast to previous results obtained with rodent fibroblasts (21, 29), overexpression of $PKC_{\beta 1}$ in HT29 cells can cause a marked suppression of growth in cell culture and also suppression of tumorigenicity in nude mice.

MATERIALS AND METHODS

Cell culture procedures and isolation of PKC overexpressers. HT29 cells were grown in McCoy's 5A medium plus 10% fetal bovine serum (FBS) (Flow Laboratories, Inc.) supplemented with penicillin (50 μg/ml) and streptomycin (50 μg/ml) in a humidified atmosphere at 5% CO_2 at 37°C. The nucleotide sequence of a rat $PKC_{\beta 1}$ cDNA (clone RP58) and the insertion of this cDNA into the pMV7 expression vector have been previously described (22, 26). CsCl-banded pMV7 or pMV7-$PKC_{\beta 1}$ plasmid DNA (20 μg) was transfected into subconfluent ψ-Am cells (7, 31). After 48 h, the culture medium was collected and stored at −70°C. Recipient HT29 cells were infected with the virus-containing medium (filtered through a 0.22-μm-pore-size filter) in the presence of 2 μg of Polybrene per ml when the cultures were subconfluent (5×10^5 cells per 10-cm [diameter] plate). Forty-eight hours later, the cells were trypsinized and replated in McCoy's 5A medium plus 10% FBS with 200 μg of the neomycin derivative G418 per ml. Resistant clones were cloned by ring isolation after 4 weeks of selection.

Enzyme assays. The total PKC activity (membrane and cytosolic) present in cultured cells was determined after partial purification of cell extracts as described previously (21, 29).

The total alkaline phosphatase activity present in cultured cells was determined as follows. Confluent 10-cm (diameter) plates were treated with fresh medium alone (control), 2 mM sodium butyrate, 10 nM TPA, or 2 mM sodium butyrate–10 nM TPA and grown for 5 days. The cells were then washed twice with 10 ml of 10 mM Tris buffer (pH 7.5). The cells were scraped from the plate and homogenized in 2 ml of Tris buffer. Homogenate (40 μl) was used to assay alkaline phosphatase activity in a colorimetric assay (Sigma Chemical Co.). Total protein concentrations were determined by the method of Bradford (5).

RNA isolation and Northern blot hybridization analyses. The isolation of total cellular RNA, agar gel electrophoresis, and Northern (RNA) blot hybridization analyses with a ^{32}P-labeled probe for rat $PKC_{\beta 1}$ were performed as previously described (21), with the following modification: the ^{32}P-labeled $PKC_{\beta 1}$ probe was prepared by using a random primer generation technique (2).

Assay of growth in monolayer culture and in soft agar. Cells were seeded in a series of plates at a density of 2×10^5 per 60-mm (diameter) plate in 5 ml of McCoy's 5A medium plus 10% FBS. Two days later, the medium was changed to one containing either 10 nM TPA or 0.02% dimethyl sulfoxide (DMSO). Twice a week thereafter, the cells were fed with fresh medium. At 3-day intervals, plates were suspended with 0.5% Trypsin and cell counts per plate were performed in triplicate by using a Coulter counter (Coulter Electronics, Inc.). The results thus obtained were used to plot growth curves (see Fig. 3) and analyzed for exponential doubling times and saturation densities (Table 1).

TABLE 1. PKC activities and growth properties of derivatives of HT29[a]

Cell line	PKC activity		Growth in monolayer culture			
			Doubling time (h)		Saturation density (no. of cells [10^5/cm^2])	
	Sp act (pmol/min per mg of protein)	Fold increase	−TPA	+TPA	−TPA	+TPA
C1	1.2	1.0	37.7	37.1	6.2	5.8
PKC6	13.7	11.4	37.7	50.4	4.7	1.5
PKC7	17.9	14.9	37.4	51.8	7.2	1.2

[a] PKC fold increase is expressed relative to the increase in C1 cells. Doubling times and saturation densities were calculated from the growth curves shown in Fig. 3. TPA (10 nM) was added as indicated in Materials and Methods. For additional details, see Materials and Methods.

Growth in soft agar was assessed by suspending 2×10^4 cells in 2 ml of 0.3% Bacto-Agar (Difco Laboratories) in McCoy's 5A medium with 10% FBS plus either 10 nM TPA or 0.02% DMSO. The cells were overlaid with 2 ml of 0.3% agar in the medium every 7 to 10 days. At the end of 4 weeks, colonies were stained with the vital stain 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolinium chloride hydrate (Sigma) for 48 h at 37°C in an incubator with 5% CO_2. The resulting plates were photographed at 0.5× magnification.

Assays for tumorigenicity in nude mice. The cells in log-phase growth were trypsinized and washed twice with phosphate-buffered saline and collected by centrifugation of 1,000 rpm ($200 \times g$) for 5 min. The cells were then counted, and 4×10^5 cells were suspended in 0.1 ml of phosphate-buffered saline and injected subcutaneously in 5- or 10-week old nu/nu nude mice (Harlan Sprague Dawley Co.) under sterile conditions, by using three injection sites per mouse. Each mouse was injected with HT29 C1, PKC6, and PKC7 cells to reduce intermice variation. In addition, three different batches of each cell line were used for analyses. The growth of tumors was analyzed by measuring two dimensions at right angles to one another with a flexible ruler. The tumor volumes were calculated by the following formula: volume $= 0.4 \times A \times B^2$, where A is the larger and B is the smaller axis (38). This correlated well with the actual tumor volume and weight obtained after the tumors were enucleated. Tumor suppression was confirmed by autopsy studies. Tumor growth was monitored for a minimum of 4 weeks. Tumor histology was examined by routine methods. For statistical analyses of the tumorigenicity data (see Table 3), we used a nonparametric method, employing the Wilkinson sign rank test (20) and McNemar's test (11).

RESULTS

Generation of HT29 cell lines that stably overproduce PKC. To construct HT29 cell lines that stably overproduce PKC, we used a strategy very similar to the one previously employed to generate PKC overproducers from Rat 6 and murine C3H 10T1/2 fibroblast cell lines (21, 29), except in the present studies we employed an amphotropic-defective retrovirus packaging system, since we wished to infect human cells. In brief, a retrovirus expression vector, pMV7-$PKC_{\beta 1}$ (21, 22, 26), which contains the full-length cDNA coding

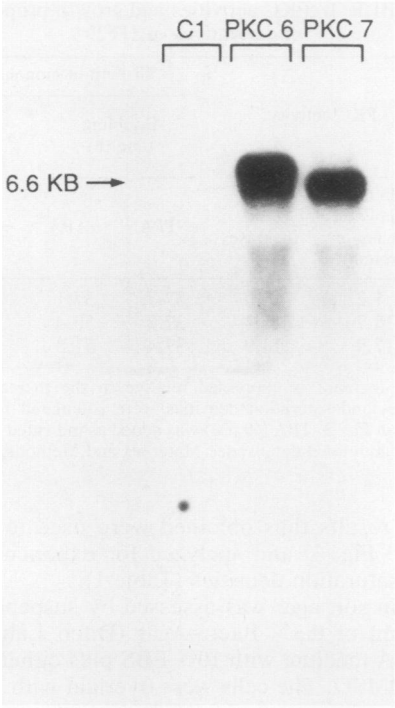

FIG. 1. Northern blot hybridization analyses. Poly(A)$^+$ RNAs were isolated from the indicated cell lines and separated by electrophoresis on a 1% agarose gel. The gel was blotted onto a nylon membrane and hybridized to a ^{32}P-labeled RP58 cDNA probe, as previously described (22). The number in the left margin indicates the size of the transcript (kilobases).

sequence for rat PKC$_{\beta 1}$ and the selectable marker gene *neo*, was used to transfect ψ-Am cells (7) by the calcium phosphate coprecipitation method. After 48 h, the medium was harvested, filtered, and used to infect subconfluent HT29 cells. Two days later, the cells were replated and grown in medium containing 200 μg of G418 per ml. Individual clones resistant to G418 were isolated and expanded. In a similar fashion, the plasmid pMV7 lacking the PKC cDNA insert was used to generate control cell lines resistant to G418.

The clonal cell lines thus derived were then analyzed for their PKC activity, as described in Materials and Methods. Several cell lines obtained with the pMV7-PKC$_{\beta 1}$ construct displayed increased levels of PKC activity when compared with the parental HT29 cells. Two of these clones, PKC6 and PKC7, which exhibited about 11- and 15-fold increases in PKC activity, respectively (Table 1), were selected for further analysis. Clone C1, a control cell line generated with the pMV7 vector lacking the PKC cDNA insert, had a very low level of PKC activity (Table 1), comparable to that of the HT29 parental cell line.

Northern blot analysis of RNA obtained from these clones (Fig. 1) with a rat PKC$_{\beta 1}$ probe revealed that the control C1 cells lacked any homologous RNA whereas PKC6 and PKC7 cells contained abundant amounts of homologous RNAs whose size (6.6 kilobases) was consistent with the length of a long terminal repeat-to-long terminal repeat transcript of the original vector. For reasons that are not apparent, the size of the transcript in the PKC7 cells was slightly smaller than that in the PKC6 cells (Fig. 1). Additional studies using Western blot (immunoblot) analyses indicated that the C1, PKC6, and PKC7 clones express very small amounts of endogenous PKC$_\alpha$ and PKC$_\beta$, but no detectable PKC$_\gamma$, protein (data not shown).

PKC overproducers display an altered morphology. In the absence of TPA, the C1 control cell line displayed morphologic characteristics that were very similar to those of the parental HT29 cell line. The addition of TPA (10 nM) to these cells induced a somewhat flatter appearance (Fig. 2). The morphologies of the two overproducer cell lines, PKC6 and PKC7, were also similar to those of the parental cells in the absence of TPA. However, both PKC-overproducer cell lines displayed marked alterations in their morphology when grown in the continuous presence of 10 nM TPA. Many of these cells became refractile, rounded up, and formed clumps. These cultures also developed large intercellular spaces (Fig. 2) and grew to a lower cell density at confluence (also see below). Higher-magnification views (data not shown) of the TPA-treated PKC6 and PKC7 cells revealed a minor population of very large flat cells with dendritic processes whose significance is not apparent at the present time.

Growth of cells that overproduce PKC is inhibited when treated with TPA. We next analyzed the growth characteristics of these cell lines in monolayer cultures in the absence and presence of TPA. Growth curves indicated that the two PKC-overproducer cell lines, PKC6 and PKC7, grew at about the same rate as the C1 control cell line when grown in the absence of TPA (Fig. 3). Continuous treatment of these cells with TPA (10 nM) resulted in marked growth inhibition of the two PKC-overproducer cell lines but had no appreciable effect on the growth of the C1 control cells (Fig. 3). Both the growth rate and the saturation density were dramatically reduced in the cultures of the PKC-overproducer cell lines treated with TPA (Table 1). Dose-response curves (data not shown) indicated that this inhibition was maximal with 10 nM TPA. These results are in contrast to those obtained in previous studies with rodent and murine fibroblast systems in which cells that overproduce PKC$_{\beta 1}$ show an increased growth rate and saturation density which are further augmented when the cultures are treated with TPA (21, 29).

The growth inhibition observed with TPA treatment of the PKC-overproducing HT29 cells was not due to a cytocidal effect, since staining with Trypan blue indicated that the treated cells were still viable. Furthermore, when the TPA-treated PKC6 and PKC7 cells were later grown in the absence of TPA, their morphologies and growth rates were identical to those of the control cells (data not shown). Therefore, the striking morphologic and growth-inhibitory effects of TPA on these cells are reversible.

Anchorage-independent growth is lost when cells that overproduce PKC are grown in the presence of TPA. Because anchorage-independent growth in soft agar is a good indicator of the in vivo tumorigenicity of several cell types including colon cancer cell lines (42), it was of interest to examine this property (Fig. 4). As expected, the control C1 cells displayed a high frequency of colony formation when grown in suspension in 0.3% agar, both in the absence and presence of TPA (10 nM). The two PKC-overproducer cell lines, PKC6 and PKC7, also showed a high frequency of colony formation when grown in soft agar in the absence of TPA. In striking contrast to the results obtained with the control C1 cells, however, growth of both PKC6 and PKC7 cells in soft agar was completely suppressed by TPA (10 nM) (Fig. 4).

PKC-overproducing HT29 cells fail to differentiate in response to sodium butyrate. It is well known that the HT29 cell

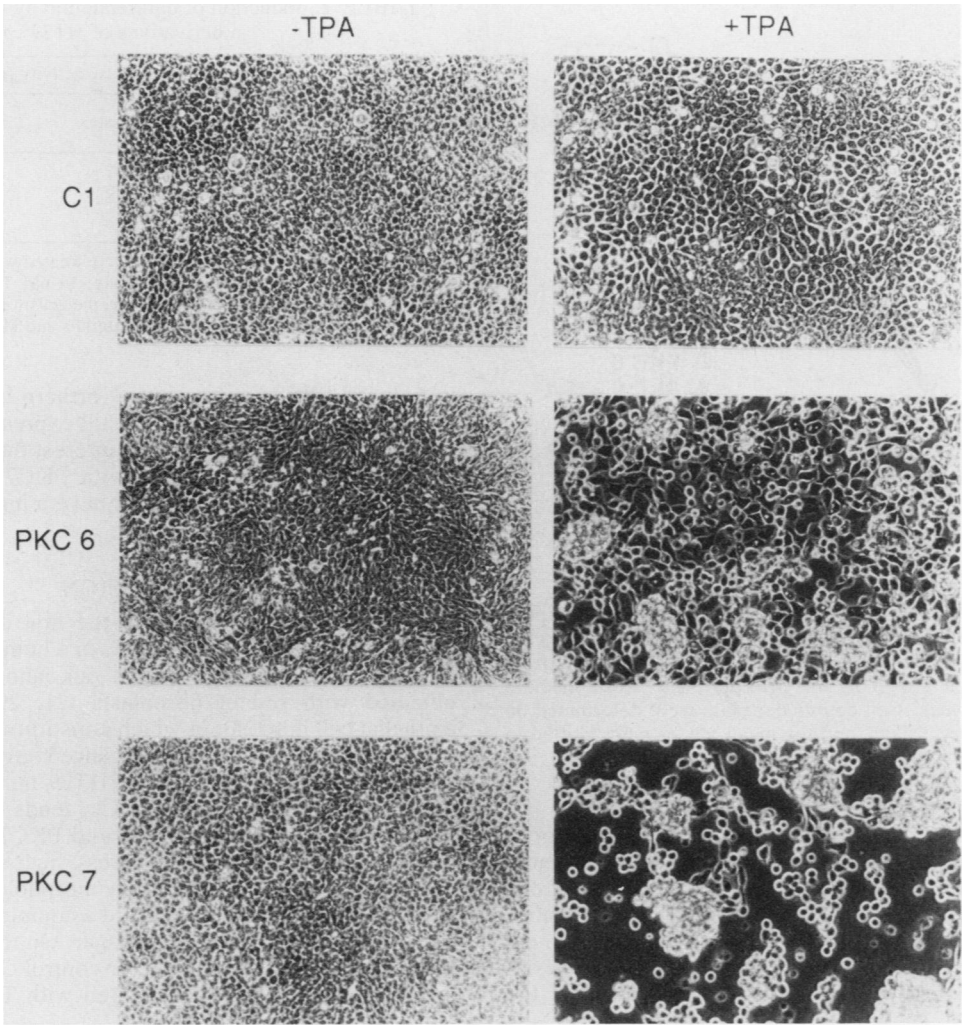

FIG. 2. Morphologic responses of the cell lines to TPA treatment. Cells in the logarithmic growth phase (2 days after seeding) were treated with 10 nM TPA in 0.02% DMSO (+ or −TPA) in McCoy's 5A medium plus 10% FBS. Photographs were taken 4 days later. Magnification, ×40.

line and certain other colon cancer cell lines undergo enterocytic differentiation when exposed to sodium butyrate (19). In fact, two permanently differentiated clones of HT29 cells have been obtained after long-term treatment with butyrate (1). The induction of alkaline phosphatase is a widely used marker for studying enterocyte differentiation (25, 47). It was of interest, therefore, to examine possible effects of PKC overproduction on the differentiation of HT29 cells, by using the induction of alkaline phosphatase as a convenient marker of differentiation (40).

In untreated cultures, the level of alkaline phosphatase was very low in the C1 control cells and in both of the overexpresser clones, PKC6 and PKC7 (Table 2). The addition of sodium butyrate (2 mM) led to a marked increase in alkaline phosphatase in the control C1 cells, but the PKC6 and PKC7 cells showed little or no response to treatment with sodium butyrate (Table 2). Treatment with TPA (10 nM) failed to produce a significant induction of alkaline phosphatase in all cells tested. The responses of these three cell types to sodium butyrate (2 mM) plus TPA (10 nM) were similar to those obtained with sodium butyrate alone (Table 2). The data shown in Table 2 were obtained after treating the cells for 5 days, since initial studies indicated that in the control cells there was marked induction of alkaline phosphatase at that time. Data from earlier and later time points confirmed the conclusion that PKC6 and PKC7 clones fail to respond to sodium butyrate as measured by the induction of alkaline phosphatase. Thus, overproduction of $PKC_{\beta 1}$ abolished the ability of HT29 cells to display the usual differentiation (as judged by alkaline phosphatase activity) in response to sodium butyrate. Furthermore, although treatment of the cells that overproduce $PKC_{\beta 1}$ with TPA inhibited growth (Fig. 3), it did not induce alkaline phosphatase (Table 2).

PKC overproducers display diminished tumorigenicity. We also characterized these cell lines with respect to tumorigenicity, since the parental HT29 cell line is known to be highly tumorigenic in nude mice (42). The C1 control cell line was highly tumorigenic when injected subcutaneously into nude mice (Table 3). Its tumorigenicity was similar to that of the parental HT29 cells (data not shown). In contrast, the two PKC-overproducer cell lines, PKC6 and PKC7, exhibited longer latency periods ($P < 0.01$ for both PKC6 and PKC7 cells), decreased tumor yields ($P < 0.03$ for PKC7 cells), and decreased mean tumor volumes on day 30 ($P < 0.01$ for both PKC6 and PKC7 cells), when compared with the C1 cells.

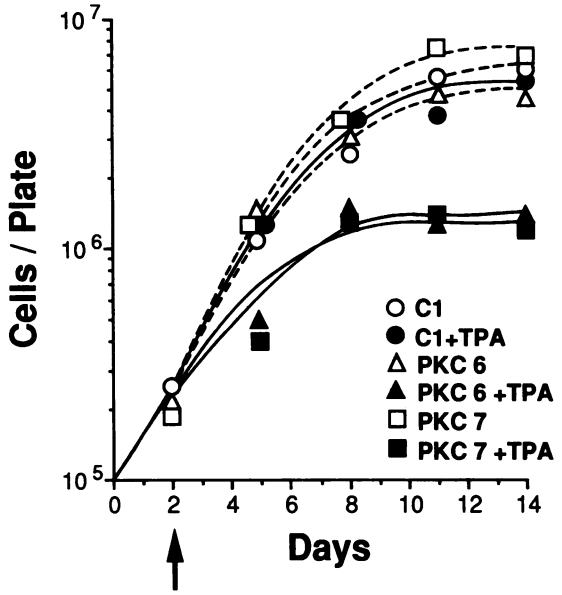

FIG. 3. Growth curves in monolayer cultures with and without TPA. Two days after seeding a series of plates, the cultures were treated with 10 nM TPA or 0.02% DMSO, as indicated by the arrow. The cells were then grown in the medium, with fresh medium changes twice per week. Cell counts per plate were determined on triplicate plates every 3 days, and the mean values were plotted to obtain growth curves.

The suppression of tumor growth was confirmed by autopsy studies. Although they were smaller in size, the tumors arising from PKC overproducers were histologically indistinguishable from the tumors obtained with the C1 control and parental HT29 cells (data not shown). The tumors obtained with the PKC6 and PKC7 cell lines did not result from the outgrowth of a cell population that had lost the introduced $PKC_{\beta 1}$ gene, since Northern blot analyses indicated that the tumors obtained still expressed high levels of the corresponding RNA. It is of interest that the suppression of tumor growth was greater with PKC7 than PKC6 cells (Table 3), since the former cells have a higher level of PKC (Table 1).

TABLE 2. Induction of differentiation by sodium butyrate in derivatives of HT29[a]

Cell line	Alkaline phosphatase activity (mU/mg of protein)			
	Control	Butyrate	TPA	Butyrate + TPA
C1	0.5	24.7	0.8	20.3
PKC6	0.4	1.8	0.8	1.2
PKC7	0.3	0.8	1.2	0.8

[a] Confluent cultures of each cell line were treated with the following: fresh medium (control), 2 mM sodium butyrate, 10 nM TPA, or 2 mM sodium butyrate plus 10 nM TPA. Five days later, the cells were assayed for alkaline phosphatase activity, as described in Materials and Methods.

DISCUSSION

The present studies provide the first description of the effects of overexpression of PKC in a human cell system. In marked contrast to the results our laboratory previously obtained with rodent fibroblasts (21, 29) or a rat liver epithelial cell line (23), in which constitutive overexpression of the β_1 isoform of PKC enhanced growth, the present study demonstrates that in the HT29 human colon cancer cell line, overexpression of $PKC_{\beta 1}$ tends to inhibit growth. When treated with TPA, PKC6 and PKC7 cells, two derivatives of HT29 cells that express high levels of $PKC_{\beta 1}$, displayed marked alterations in morphology, inhibition of growth in monolayer culture, and a complete loss of anchorage-independent growth in soft agar. On the other hand, the parental HT29 cells and a vector control cell line C1 did not display these effects when treated with TPA. Presumably,

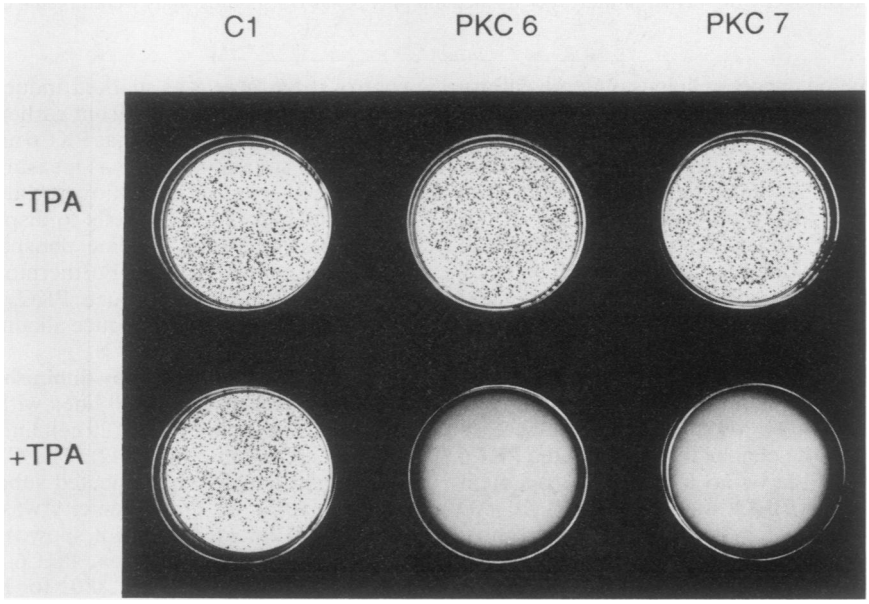

FIG. 4. Growth in soft agar. Each cell line was seeded into 60-mm (diameter) petri dishes in 0.3% soft agar in McCoy's 5A medium supplemented with 10% FBS, plus either 10 nM TPA or 0.02% DMSO. The plates were overlaid with fresh medium every 7 to 10 days. Photographs were taken after 30 days of growth. Magnification, ×0.3.

TABLE 3. Tumorigenicity of HT29 derivatives in nude mice[a]

Cell line	Tumor latency (days)[b]	No. of tumors/ no. of animals (%)[d]	Tumor vol (mm^3) (mean ± SD)[e]
C1	6.9	14/14 (100)	565 ± 323
PKC6	10.9[f]	13/14 (93)	390 ± 235[f]
PKC7	11.1[f]	8/14[g] (57)	79 ± 45[f]

[a] Each cell line was injected subcutaneously into 5-week-old nude mice (4×10^5 cells per site). For additional details, see Materials and Methods.
[b] Mean time following injection at which tumors were first palpable.
[c] Number of animals developing tumors used to calculate tumor latency and volume.
[d] Tumor yield on day 30.
[e] Determined on day 30.
[f] $P < 0.01$, compared with C1.
[g] $P < 0.03$, compared with C1.

the very low levels of endogenous PKC_α and PKC_β expressed in the latter cells (unpublished data) were insufficient to mediate these responses to TPA. Furthermore, since in the absence of treatment with TPA the PKC6 and PKC7 cells have morphologies and growth properties similar to those of the parental HT29 cells, we presume that under the usual cell culture conditions these cell lines do not contain high intracellular levels of DAG or other endogenous activators of PKC. This aspect is now being investigated.

Early studies with phorbol ester tumor promoters indicated that these compounds can have opposite effects on cells depending on the cell system studied, resulting for example in stimulation of growth and inhibition of differentiation in some cell systems or inhibition of growth and induction of differentiation in other cell systems (9, 46). The opposite effects of overexpression of PKC in HT29 cells noted in the present study when compared with the results obtained with derivatives of rodent fibroblasts that overexpress PKC (21, 29) are consistent with these earlier findings. Furthermore, since in our present studies with HT29 cells and our previous studies with rodent fibroblasts, the same isoform of PKC (β_1) was overexpressed (indeed, in the same expression vector), it is apparent that the opposite effects of TPA treatment seen in these derivatives are not due to differences between the cell systems in terms of the predominant form of PKC present in the overexpresser. Thus, the biologic effects of a specific isoform of PKC depend on the cell in which it is expressed. The same principle can apply to certain oncogenes, since an activated H-*ras* oncogene usually transforms rodent fibroblasts but inhibits proliferation and induces differentiation of the pheochromocytoma cell line, PC12 (3, 37). These findings are consistent with the general finding that the biologic effects of various components of signal transduction pathways are highly context dependent (34–36). It may be of interest that, although in both the parental HT29 and R6 cells the predominant isoform of PKC detected by Western blot analysis is PKC_α, the absolute level of PKC enzyme activity in HT29 cells is much lower than that in R6 fibroblasts (unpublished data). In a separate study, we found that primary human colon tumors also frequently display very low levels of PKC activity (18). These findings are consistent with our suggestion that PKC may be a negative growth regulator in colon cancer cells.

The mechanism by which TPA induces growth inhibition in the two HT29 derivatives that overexpress $PKC_{\beta 1}$ is not known. Studies are in progress to determine whether this effect is seen with other isoforms of PKC and other colon cancer cell lines. It seems unlikely that this growth inhibition is due to induction of differentiation, since we did not observe an increase in alkaline phosphatase in these cells, a characteristic marker of enterocyte differentiation. Indeed, it appears that the overexpression of $PKC_{\beta 1}$ blocks the usual differentiation capacity of HT29 cells, since the two PKC overproducer cell lines failed to display induction of alkaline phosphatase when treated with sodium butyrate, a potent inducer of enterocyte differentiation in the parental HT29 cells (Table 2). It is of interest, however, that sodium butyrate does induce growth inhibition of the parental, C1, PKC6, and PKC7 cells (unpublished studies). Thus, the PKC-overproducer cells are not completely resistant to the effects of this agent. It is possible, therefore, that they are blocked at an early phase of their differentiation response or that they undergo differentiation to a cell type that does not express alkaline phosphatase. Studies employing other markers of enterocyte differentiation are currently being used to examine these possibilities.

The most important finding in the present study is that overexpression of $PKC_{\beta 1}$ in HT29 cells suppresses their tumorigenicity in nude mice (Table 3). Since this effect did not require treatment with TPA, we presume that it reflects the presence in mice of a growth factor or hormone that leads to activation of the high levels of PKC present in these cells, perhaps through induction of phospholipid turnover and the generation of DAG. The failure to see complete suppression of tumorigenicity with PKC-overproducer cells might reflect limited availability of this growth factor or hormone. The data shown in Table 3 were obtained with mice that were 5 weeks old at the time of injection. In additional studies not shown here, we found that when the PKC6 and PKC7 cells were injected into 10-week-old mice, the suppression of tumorigenicity was still apparent but less striking than the results obtained with the 5-week-old mice. It is possible, therefore, that the younger mice have higher levels of this putative growth factor or hormone, but the nature of this entity remains to be identified.

At present, there is considerable interest in identifying tumor-suppressor genes and their mechanisms of action (27, 28, 45). The present study provides the first direct evidence that PKC may function as a tumor suppressor. Although these findings are thus far confined to HT29 cells, the known ability of activators of PKC to inhibit the growth of other cells (see above) makes it likely that PKC can function similarly in other cell systems. This is now being studied in our laboratory. In addition, it will be of interest to use cDNA subtraction methods to identify various genes that play a role in the PKC-mediated pathway of tumor suppression. As discussed in the Introduction, there is other evidence that PKC may play a role in certain phases of colon carcinogenesis, but this role appears to be complex. If it is found that the results of the present study have general relevance to the growth of colon tumors, then they would suggest a novel approach to colon cancer therapy. It is tempting to speculate that novel and highly potent compounds that activate PKC without exerting tumor promoting activity, such as bryostatin (41), might be used to activate the endogenous PKCs present in colon tumors, thus inducing tumor suppression.

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