Increased Expression of Protein Kinase Cα Plays a Key Role in Retinoic Acid-induced Melanoma Differentiation*

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Protein kinase C (PKC) is a calcium- and phospholipid-dependent kinase which has been implicated as a key messenger in cellular signaling (see Ref. 1 for review). There are at least seven distinct genes which constitute the PKC family. This family can be divided into two major groups (i.e. the classical and the novel group). The classical PKCs (α, βı, β2, and γ) require both calcium and phospholipid for activation, while PKCs belonging to the novel group (δ, ε, η, and θ) do not require calcium (2). PKC is also the major, if not the only, receptor for phorbol ester tumor promoters (3, 4). Besides activating PKC, prolonged treatment with phorbol esters can result in down-regulation of the enzyme (5–9). Several groups, including our own, have shown that the retinoic acid (RA)-induced differentiation of various tumor cell types is accompanied by an increase in PKC expression (9–13). In B16-F1 murine melanoma cells, RA induces a 5–8-fold increase in PKCα, message and protein (9, 10). Since this increase in PKCα levels occurs relatively early during the differentiation program, the question arises as to whether this enzyme mediates some of the phenotypic changes induced by RA (i.e. decreased monolayer growth rate, elimination of anchorage-independent growth, and increased melanin production). This question was addressed by stably transfecting and characterizing stable transfectants of B16-F1 cells that overexpress PKCα, in the absence of exogenous RA.

MATERIALS AND METHODS

Stable Transfection of B16-F1 Cells—We co-transfected early passage B16-F1 cells with a PKCα cDNA expression vector (YK504) driven by an SV40 promoter (14) and the pSV40-neo plasmid encoding the gene for neomycin resistance at a 1:10 ratio (pSV40-neo:YK504). Transfection was accomplished by the calcium phosphate/glycerol shock procedure (16). Cells transfected only with pSV40-neo (Neo) served as negative controls throughout most experiments. Neomycin-resistant clones were selected in Dulbecco’s modified Eagle’s medium (DMEM with 10% newborn calf serum) containing 1 mg/ml of the neomycin derivative G418 (Gibco). Western Blot Analysis for PKCα—Cells were seeded at 2 × 10⁴/100-mm dish. After attachment (6 h), cells were refed with DMEM containing 10% bovine calf serum with or without 10 μM RA. RA was always handled in subdued light. Forty-eight hours after addition of RA, cells were washed twice with PBS and harvested in 250 μl of lysis buffer (10 mM Tris (pH 7.5), 1 mM EDTA, 1% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride). Harvested cells were lysed on ice by three consecutive 10-s sonications with a Tekmar® sonic disruptor at power setting 60. Protein concentrations were determined by the BCA® (Pierce) protein assay. Crude samples were diluted to equal protein concentrations and electrophoretically separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. Proteins were electrophoretically transferred to Hybond-C Extra® nitrocellulose membrane (Amersham). The membrane was incubated overnight in blocking solution (5% nonfat dry milk in Tris-buffered saline (TBS), pH 8.0, containing 0.2% Tween). A 1:25 dilution of monoclonal anti-PKCα antibody (Seikagaku) was added for 1 h. This solution was removed, and the blot was washed several times in blocking solution followed by a 1-h incubation with a 1:3000 dilution of rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham), washed several times in blocking solution and then one final time in blocking solution lacking the milk. Reactive bands were visualized by the enhanced chemiluminescence method (Amersham). All washes and incubations were performed at room temperature.

Enzyme Activities of Partially Purified PKC—Culture conditions were the same as those described for Western blot. Cells were lysed on ice with 20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 5% Trion X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. Complete cell disruption was further ensured by three consecutive 10-s sonications with a Tekmar® sonic disruptor at power setting 60. The total cell lysate was centrifuged at 12,000 × g for 15 min. The supernatant was loaded onto a DEAE-cellulose anion exchange column (Cellex-D®, Bio-Rad) previously equilibrated with column buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol). The column was washed with 15 volumes of column buffer. The PKC fraction was eluted with 2 volumes of column buffer containing 100 mM NaCl and concentrated.
Overexpression of PKCa in Melanoma Cells

We transfected early passage B16-F1 cells with a PKCα cDNA expression vector as described under “Materials and Methods.” Thirty-two G418-resistant clones were screened, yielding two consistently positive clones, designated as H and K, respectively. These clones were determined to overexpress PKCα based on both Western blot analysis (Fig. 1) and enzyme activity (Fig. 2). The relative quantities of PKCα were determined by scanning the Western blot autoradiograms with a computerized densitometer. These analyses showed that clones H and K contained approximately 3.3 and 2.3 times more PKCα, respectively, than wild type (WT) cells. Western blots also showed that RA treatment induced a further increase in PKCα levels in both overexpressing clones as well as the previously described PKCα induction in control cells (WT and cells transfected only with the neomycin resistance gene (Neo)). Both positive clones were further screened for increased PKCα enzyme activity using a commercially available kit (Amersham) (Fig. 2). We found that clones H and K showed increased specific PKCα activities 3.3 and 2.0 times greater, respectively, than those of WT or Neo cells (Fig. 2). As expected, RA treatment increased PKCα activities in all cells. There was also a significant difference in PKCα activities between clones H and K (p < 0.025, Newman-Keuls).

To confirm that H and K were indeed distinct clones that had stably incorporated the foreign PKCα gene, a Southern blot restriction analysis was performed (Fig. 3). The different HindIII restriction patterns observed between DNA obtained from wild type cells and that from clones H and K illustrate that both clones have incorporated foreign PKCα DNA into their genomes. Furthermore, since clones H and K have different restriction patterns from each other, these data demonstrate that H and K are indeed separate clones.

In monolayer growth rate experiments, we found that cells from clones H and K had slower growth rates than untreated WT and Neo cells (Fig. 4). As we expected, RA treatment depressed the monolayer growth of WT and Neo cells. In comparing the growth of the two overexpressing clones, we found that by 48 h clone H had fewer cells per dish than clone K (p < 0.05, Newman-Keuls). This finding correlated with the PKC levels and activities in these clones (Fig. 1 and 2). When clones H and K were treated with RA, their respective

![Western blot analysis of PKCα overexpressing clones](image-url)
Overexpression of PKCa in Melanoma Cells

FIG. 2. In vitro enzyme activities of partially purified PKC from PKC, overexpressing clones. Cells were harvested by trypsinization and lysed. PKC-enriched fractions were obtained by ion exchange chromatography. These fractions were further concentrated by microfiltration. Equal amounts of protein were assayed using a commercially available PKC assay system (Amersham) in the presence of cofactors phosphatidylserine and phorbol ester (hatched bars) and in the absence of these cofactors (black bars). WT, wild type; H and K, PKC, overexpressing clones; Neo, cells transfected with pSV-neo only. The data are presented as the means ± S.D. where n = 2. Mean were determined to be significantly different from WT by ANOVA followed by Newman-Keuls multiple comparisons. (*p < 0.05, §p < 0.005, †p < 0.001.)

FIG. 3. Southern analysis of PKC, overexpressing clones. Fifteen µg of genomic DNA was digested overnight with HindIII restriction enzyme and separated on a 1% agarose gel. After transfer to nitrocellulose and prehybridization, the blot was probed with radiolabeled PKC, cDNA. WT, wild type; H and K, PKC, overexpressing clones.

growth rates did not significantly decrease (data not shown). At the light microscopy level, we were unable to detect obvious morphological differences between PKCα overexpressing clones and control cells.

The ability of PKCα, overexpressing clones to form viable colonies in soft agarose was significantly lower than that of untreated WT and Neo cells (Fig. 5). Cells from clone H formed significantly fewer colonies than those from clone K (p < 0.001, Newman-Keuls). As with the monolayer growth rate data, the difference in soft agarose growth between clones H and K correlated with their respective PKC levels. As expected, RA treatment of WT cells decreased their ability to form viable colonies in a concentration-dependent fashion. The colony-forming abilities of clones H and K were also reduced in a dose-dependent fashion when treated with RA (data not shown).

As an estimate of the degree of differentiation, we analyzed the cells for melanin production (intracellular as well as secreted melanin (Fig. 6). Cells from clones H and K contained and secreted more melanin than untreated control cells. RA
overexpressing PKC, exhibit phenotypes very similar to those of tumor growth rates. Due to the rapid tumor growth and lost PKC, overexpression. Due to the rapid tumor growth and had longer latency times than control cells.

It is important to note that the level of PKC, overexpression achieved in both clones was lower than that induced by RA treatment of WT or Neo cells. The reason for this finding could be explained in that PKCα is apparently associated with diminished growth (Fig. 4). Therefore, we might have been selecting cells that overexpress an apparently negative growth regulator. If a cell should overexpress very large amounts of PKCα, it might grow so slowly that no colony would be formed. Therefore, we believe that inducing higher levels of PKCα in this system would require transfection with PKCα under the control of an inducible promoter.

Other laboratories have transfected various cells with other PKC isozymes (19–21). In two of these reports, the investigators transfected nonmalignant fibroblasts and obtained clones which overexpressed PKCα and PKCδ, respectively, at very high levels. In both cases, the PKC overexpressors assumed some characteristics of transformed cells (19, 21). When malignant HT29 (human colon carcinoma) cells were transfected with PKCα, the overexpressing cells (11–15-fold) showed evidence of diminished malignancy (20). Also, when PKCα protein was introduced into erythroleukemia cells, they differentiated at a faster rate (22). In this report, we show evidence that B16 melanoma cells transfected with overexpressing and the PKC, isozyme at levels 2–4-fold higher than wild type cells, acquire a more differentiated phenotype. An important difference between the HT29 study (20) and ours is that we did not have to treat our overexpressing clones with phorbol esters in order to induce a phenotypic change. This might be explained by the presence of factors in the cell culture medium (i.e. calf serum or autocrine factors) which could stimulate a constant diacylglycerol production, thus activating the enzyme in the absence of additional cofactors such as phorbol esters. This would suggest that in B16 melanoma cells the limiting factor is the amount of PKCα enzyme and not the activators of the enzyme.

Our transfecants produced lower levels of PKCα than those in other systems, yet the phenotype of B16 melanoma cells is apparently quite sensitive to small changes in the level of this enzyme. Furthermore, the data seem to suggest that some phenotypic traits, i.e. melanin production and monolayer growth, require lower levels of PKCα, in order to saturate downstream regulators than other traits, such as colony formation in soft agarose.

The sole fact that the PKC, overexpressing clones yielded smaller tumors than their control counterparts does not necessarily indicate that their growth rates, following latency, were significantly slower. Longer latency periods by themselves could have resulted in such findings. On the other hand, one could speculate that the selective pressures within the animal might have resulted in the growth of cells that have lost PKC, overexpression. Due to the rapid tumor growth and its short duration, we were unable to accurately calculate tumor growth rates.

Although the disparate actions of PKC overexpression on the phenotypes of the host cell still seem an enigma, similar findings apply to the ras oncogene. Some cells, when transfected with v-ras, adopted a transformed phenotype (23–25), while others are induced to differentiate (26). Thus, it becomes apparent that the cellular milieu in which certain regulatory proteins function dictate the biological response.

RA has been repeatedly shown to induce differentiation in

TABLE 1

Tumorigenicity of wild type and transfected clones of B16 cells

| Cell type | Latency (median/range) | Tumor weight (median/range) | Tumor incidence |
|-----------|------------------------|----------------------------|-----------------|
| WT        | 5/3–9                  | 2.369/1.379–5.330          | 8/8             |
| H         | 9/7–13*                | 1.057/0.208–1.470*         | 8/8             |
| K         | 9/7–13*                | 0.915/0.081–1.299*         | 8/8             |
| Neo       | 5/3–7                  | 2.807/1.687–4.890          | 8/8             |

*Significantly different from WT by Dunnett's nonparametric multiple comparisons (p < 0.01).

DISCUSSION

In this report, we show that B16 cells transfected with and overexpressing PKCα exhibit phenotypes very similar to those of wild type cells treated with RA. Both of the PKCα overexpressing clones showed slower monolayer growth rates, diminished capabilities to form viable colonies in soft agarose, and increased melanin contents as well as secretion. When injected into syngeneic mice, both PKCα overexpressing clones produced smaller tumors and had longer latency times than control cells.

Although the disparate actions of PKC overexpression on the phenotypes of the host cell still seem an enigma, similar findings apply to the ras oncogene. Some cells, when transfected with v-ras, adopted a transformed phenotype (23–25), while others are induced to differentiate (26). Thus, it becomes apparent that the cellular milieu in which certain regulatory proteins function dictate the biological response.

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several different types of cancer cells. The mechanism by which RA induces differentiation is still largely unknown. Our observations, together with the findings that prolonged phorbol ester treatment of B16 cells depletes the cells of PKC, and counteracts the effects of RA treatment (10, 27), provide strong evidence that PKC, plays a key role in RA-induced B16 cell differentiation.

Acknowledgments—We thank Dr. H. Kupchik and Dr. A. Traish from the Departments of Microbiology and Biochemistry, respectively, for their critical review of this manuscript.

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