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Nonmutagenic Mechanisms in Carcinogenesis: Role of Protein Kinase C in Signal Transduction and Growth Control

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There is accumulating evidence that the multistage carcinogenic process is associated with the progressive acquisition of mutations in cellular proto-oncogenes and in growth-suppressor genes. At the same time, several types of evidence indicate that nongenotoxic agents and epigenetic events also play an important role in the evolution of tumors. One of the most intensively studied nongenotoxic agents is the phorbol ester tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and related compounds. Since TPA appears to exert its biologic effects through protein kinase C (PKC), a key enzyme in signal transduction, we have studied this enzyme in considerable detail. Our strategy has been to perturb signal transduction by developing cell lines that overexpress the β_1 isoform of PKC. Such derivatives of rat fibroblasts display alterations in morphology and growth factors, altered expression of c-*myc*, ornithine decarboxylase, and phorbin, and increased susceptibility to transformation by certain oncogenes, H-*ras*, *myc*, and *fos*. These findings provide direct genetic evidence that PKC plays a critical role in growth control and the action of certain growth factors, tumor promoters, and oncogenes. In related studies, we have characterized the β_1 isoform that is overproduced in the above cell systems in terms of its biochemical, kinetic, and immunologic properties. The enzyme has several properties characteristic of native PKCs. A surprising finding is that c-H-*ras*-transformed derivatives of the cells that overexpress $PKC_{\beta 1}$ display a several-fold increase in the expression of the endogenous α_1 isoform of PKC and a decrease in the expression of the endogenous ϵ isoform. Thus, cell transformation can lead to altered expression of specific endogenous isoforms of PKC.

Colon cancer is the second most common cause of cancer deaths in the U.S., but its precise etiology is not known. Several types of evidence will be presented suggesting that PKC plays a role in the pathogenesis of this disease. We have also found that bacteria in the human gastrointestinal flora can convert lipids to diacylglycerol (DAG), a known activator of PKC. We hypothesize that the DAG thus produced influences the growth of the colonic epithelium and thus plays a tumor-promoting role. The above findings coupled with findings from other laboratories are discussed in terms of a unifying concept of multistage carcinogenesis, which we have termed "a progressive disorder in signal transduction." This concept emphasizes the fact that signal transduction pathways are normally linked to each other via complex and overlapping networks, and the evolution of tumor cells involves both genetic and epigenetic disruptions of such networks.

Introduction

Thus far the discussions at this meeting on "Critical Target Genes in Chemical Carcinogenesis" have dealt with DNA as the critical target of chemical carcinogens and emphasized mutational changes in tumor cells. Studies in this area have revealed a remarkable number of changes in the DNA and chromosomes of tumors, both in experimental animal and human tumors. Indeed, there is accumulating evidence that the multistage carcinogenic process and the evolution of a fully malignant tumor involves the progressive acquisition of both dominant-acting mutations in cellular proto-oncogenes and recessive mutations in growth-suppressor genes (*1–3*). Since these data are rather recent and new probes for cellular proto-oncogenes and suppressor genes continue to be developed, I think one can predict that the DNA of fully malignant tumors often has a large number of changes in the structure of its DNA.

At the same time, I believe that the above changes constitute only part of the story and that nongenotoxic agents, acting on cellular targets other than DNA and through epigenetic mechanisms, also play a major role in the evolution of tumors and that epigenetic mechanisms also play an important role in the maintenance of the tumor cell phenotype. There are at least five types of evidence for this statement. *a*) The first evidence comes from studies on the phenomenon of tumor promotion, particularly studies with the phorbol ester tumor promoters (*3*). *b*) There is increasing evidence that a large number of compounds that induce tumors in

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rodents do not appear to have genotoxic effects (*4*). Although it is possible that further studies will indicate that some of these compounds do damage DNA, perhaps indirectly through oxidative damage, it is likely that many of them act through epigenetic mechanisms. *c*) The incidence of the major human tumors (other than lung cancer) in the U. S., i.e., breast, prostate, and colon cancer, appears to be related to hormonal factors or dietary lipid, which are nongenotoxic agents (*3*). *d*) There are numerous examples in which the growth and differentiation of malignant tumor cells can be modulated by nongenotoxic agents, despite the presence of activated oncogenes and/or chromosomal rearrangements. Thus, the phenotype of the cancer cell is not fully controlled by its DNA, but is also a function of epigenetic programs that control gene expression (*3*). *e*) The last type of evidence is more speculative and is based on the following logic. There is considerable evidence that the growth, development, and differentiation of normal cells both in the embryo and the adult does not involve mutational changes in DNA (except in the immune system). Therefore, epigenetic events play a profound role in determining the phenotypes of normal cells and can be quite stable at the somatic cell level, even though the mechanisms are largely unknown [for review see Holliday (*5*)]. Since the cancer cell is a gangster that uses every trick in the book to escape growth control, it is reasonable to assume that it achieves this goal by distorting both genetic and epigenetic mechanisms.

As mentioned above, tumor promotion appears to proceed through epigenetic events, i.e., nonmutational alterations in gene expression, leading to the selective outgrowth and phenotypic alteration of initiated cells. It should also be stressed that DNA-damaging carcinogens can induce not only mutations but also internal signals (yet to be defined) that apparently enter signal transduction pathways, thus leading to multiple alterations in gene expression (*3,6*). This may explain the fact that some of these agents can act as complete carcinogens (i.e., both initiators and promoters).

The most detailed information on tumor promotion comes from studies on the potent phorbol ester tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). A number of years ago our laboratory and others obtained evidence that TPA is a potent modulator of growth, differentiation, and gene expression and that its earliest effects are exerted at the level of the plasma membrane (*3*). A major advance in our understanding of mechanisms of tumor promotion came when Nishizuka and colleagues (*7*) demonstrated that TPA directly activates protein kinase C (PKC), a Ca^{2+}- and phospholipid-dependent protein serine/threonine kinase of central importance in signal transduction. Subsequent studies showed that PKC is also the major cellular receptor for TPA (*7*). Six separate genes and seven distinct subspecies of PKC have been identified, based on cDNA cloning studies (*7–10*). The first four subspecies to be identified (designated α, β_I, β_{II}, and δ have a common structure characterized by four conserved (C_1–C_4) and five variable (V_1–V_5) regions. The δ, ϵ, and ζ subspecies are related to the first four forms but lack the C_2 conserved region. Thus, like several other components of signal transduction pathways, PKC is a multigene family. The preservation of these multiple forms in higher organisms during evolution and the fact that there is differential expression of these isoforms in specific tissues (*7–10*) suggest that they differ with respect to their roles in signal transduction, but these differences remain to be elucidated.

With this background in mind, I now want to describe some of our recent studies on PKC because they illustrate certain aspects of the complex circuitry involved in signal transduction and the control of gene expression.

Effects of Overexpression of PKC on Growth Control, Oncogene-Induced Transformation, and Gene Expression

Our laboratory isolated a number of PKC cDNAs (derived from a rat brain library), including a full-length clone encoding $PKC_{\beta 1}$ (*8,9*). To gain further insight into the role of PKC in signal transduction and multistage carcinogenesis, and to better define the biochemical properties and physiologic effects of a specific isoform of PKC, we constructed a series of rodent embryo fibroblast cell lines that constitutively overproduce high levels of the β_1 isoform of PKC. We have used a retroviral expression vector (pMV7) (*11*) to transduce the $PKC_{\beta 1}$ cDNA into a variety of cell types; pMV7 lacking a cDNA insert was used to generate matched control cell lines. R6 rat embryo fibroblast cells transduced with the pMV7-$PKC_{\beta 1}$ vector express 20- to 50-fold greater PKC enzyme activity than control-vector infected cells and show several disturbances in growth control (*9*). These findings, as well as findings we obtained with derivatives of the murine fibroblast cell line C3H 10T½that also overexpress $PKC_{\beta 1}$ (*12*), provide direct evidence that this isoform of PKC can alter cell morphology and disturb growth control, particularly in the presence of TPA.

Although the R6-PKC3 cells are not fully transformed, they display a marked increase in susceptibility to transformation by an activated c-H-*ras* oncogene (*13*). We previously demonstrated that TPA strongly enhanced transformation of the parental R6 cells by the same activated c-H-*ras* oncogene (*14*). The findings with R6-PKC3 cells provide genetic evidence for a cooperative interaction between PKC and a *ras* oncogene in the process of cell transformation. This conclusion is not confined to fibroblasts since we have found that derivatives of rat liver epithelial cell lines that overexpress $PKC_{\beta 1}$ (*15*) also display increased susceptibility to transformation by the c-H-*ras* oncogene (L. L. Hsieh and I. B. Weinstein, unpublished studies).

Our studies with R6-PKC3 cells also indicated that an appropriate balance between the levels of PKC and c-H-*ras* is required for cell transformation. Thus, TPA

treatment strongly inhibited *ras* oncogene-induced transformation of R6-PKC3 cells but stimulated *ras* oncogene-induced transformation of control cells. TPA also inhibited the colony forming ability of the c-H-*ras* oncogene-transformed R6-PKC3 clones (*13*). Thus, overstimulation of a signal transduction pathway linking H-*ras* and PKC can be cytotoxic.

One can rationalize the cooperative interaction between a *ras* oncogene and PKC by the fact that *ras*-transformed cells often display increased levels of diacylglycerol (DAG) (*13*). The increased levels of DAG would provide continuous activation of the high levels of $PKC_{\beta 1}$ present in our overproducer cells. It is curious, however, that we have found that the susceptibility of R6-PKC3 cells to transformation is not confined to *ras* oncogenes, since these cells also display increased susceptibility to transformation by both the v-*myc* and v-*fos* oncogenes (W. -L. W. Hsiao and I. B. Weinstein, unpublished studies), oncogenes that act downstream from PKC. The possible significance of these findings is discussed at the end of this paper.

We recently examined the response of R6-PKC3 cells to various growth factors (S. Hoshina et al., unpublished studies). A striking finding was that several agents when tested alone (i.e., in serum-free medium), including epidermal growth factor, platelet-derived growth factor, TPA, teleocidin, and *O*-acetyl glycerol, produced a strong stimulation of DNA synthesis in quiescent R6-PKC3 cells but had a negligible effect in quiescent R6-C1 cells, a vector control cell line with normal levels of PKC. These studies provide genetic evidence that alterations in cellular levels of PKC can markedly influence the response of cells to specific growth factors. The underlying mechanisms remain to be determined, but our findings provide further evidence that PKC plays a pivotal role in growth control.

We also examined whether cells that overexpress PKC display alterations in the expression of certain mitogen-responsive genes (*15*). In these studies we used derivatives of the rat liver epithelial cell lines K16 and K22 that had been transduced with the vector pMV7-$PKC_{\beta 1}$ and express about 10-fold higher PKC activity than control cells. Despite these high levels of PKC, these cells did not exhibit gross morphologic changes, anchorage-independent growth, or tumorigenicity. When treated with 100 ng/mL of TPA, the control K16-MV7 and K22-MV7 cells displayed a slight change in morphology, whereas the PKC overexpressor cell lines K16-PKC4 and K22-PKC2 cells displayed a marked change in morphology. Northern blot analyses demonstrated that treatment with TPA induced increased levels of c-*fos*, c-*myc*, phorbin, and ODC RNAs in control K16-MV7 and K22-MV7 cells, with maximum induction occurring at about 0.5, 1, 8, and 8 hr, respectively. In K16-PKC4 and K22-PKC2 cells, TPA induction of phorbin and ODC RNAs was markedly enhanced, but this was not the case for c-*myc* and c-*fos* RNA. In addition, the levels of c-*myc* RNA were constitutively higher in both K16-PKC4 and K22-PKC2 cells than in the control cells. Taken together, these results provide direct evidence that PKC plays a role in modulating the expression of c-*myc*, phorbin, and ODC RNAs. It seems likely that PKC also plays a role in the TPA induction of c-*fos*, but this was not enhanced in the cells that overproduce $PKC_{\beta 1}$, perhaps because the endogenous PKC is sufficient to saturate this response.

Biochemical and Immunologic Properties of PKCs in Overproducer Cells

In addition to mechanistic studies on the role of PKC in growth control and transformation, the cell lines we have developed that overproduce PKC are a convenient source for the purification of relatively large amounts of a specific isoform of the enzyme. This offers a considerable advantage since at the present time it is still difficult to isolate homogeneous preparations of specific isoforms of PKC from natural sources. An additional advantage of this approach for studying biochemical properties of specific PKC isoforms is that the enzyme is synthesized and posttranslationally modified in the same cell type in which its biological properties are determined. We have used 10T1/2-PKC4 cells, a derivative of C3H 10T1/2 cells that expresses high levels of $PKC_{\beta 1}$ (*12*), for this purpose (*16*). The $PKC_{\beta 1}$ purified from these cells had the following properties: Activity was dependent on phospholipid and Ca^{2+} (optimum 500 μM), but arachidonic acid could be substituted for the phospholipid, yielding about one-third the activity; TPA or teleocidin stimulated the activity and shifted the Ca^{2+} optimum to 10 μM; activity was inhibited by H-7, tamoxifen, and staurosporine (*16*). In general, these properties of $PKC_{\beta 1}$ are similar to those of preparations of PKC isolated from rat brain.

We have also characterized the PKCs present in our overproducer cell lines by immunologic methods (C. M. B. Borner, et al. unpublished studies). Immunocytochemical studies conducted with Susan Jaken indicate that in the R6-PKC3 cells the overexpressed $PKC_{\beta 1}$ enzyme is rather diffusely distributed in the cytoplasm, with little or no detectable material in the nucleus. After treatment of the cells with phorbol dibutyrate, there is a dramatic translocation of the enzyme to the periphery of the cell. Cell fractionation studies also indicate that phorbol esters induce translocation from the cytosol to the membrane fraction and downregulation of the $PKC_{\beta 1}$ in these cells. Thus, the overexpressed $PKC_{\beta 1}$ behaves similar to that of endogenous PKC. Since, however, the initial level of enzyme is much higher in the overproducer cells, the biologic responses to TPA are exaggerated and more prolonged.

Western blot analyses using isoform-specific antibodies indicate that the overexpressed $PKC_{\beta 1}$ has a molecular weight of 78 kDa, which is slightly smaller than that of PKC_{α} (80 kDa). We have also characterized the PKCs present in the H-*ras* transformed R6-PKC3 and K16-PKC4 cells. In both cell systems, transformation was associated with an increase in membrane-associated

$PKC_{\beta 1}$, suggesting that the *ras* oncogene leads to persistent activation of the enzyme. Surprisingly, we found that the level of the endogenous PKC_{α} isoform was increased about 6- to 10-fold, and the expression of the endogenous PKCε isoform was decreased several-fold, in both the H-*ras* transformed R6-PKC3 and K16-PKC4 cells. Although it is well established that different isoforms of PKC are expressed in different normal tissues (*7–10*), to our knowledge this is the first evidence that cell transformation can lead to altered expression of specific endogenous forms of PKC.

In previous studies we have found that the total level of PKC enzyme activity is usually decreased in human colon tumors (*17*), and we are currently examining the isoform profiles. Increased levels of specific isoforms of PKC are seen during induction of granulocyte differentiation of HL60 cells by vitamin A or dimethylsulfoxide (*18*) and during macrophage differentiation induced by 1,25-dihydroxy D3 (S. Solomon et al., unpublished studies). Increased levels of PKC have also been seen in some cell lines that display the multidrug resistance (MDR) phenotype (*19,20*). It seems likely, therefore, that the DNA sequences that encode different isoforms of PKC are flanked by different promoter-enhancer sequences that respond to specific transcription factors during the course of differentiation and tumor formation. Differential regulation at the levels of RNA turnover, translation, and posttranslation could also play a role in cellular profiles of specific isoforms of PKC.

Role of PKC in Colon Cancer

Despite the fact that colon cancer is the second most common cause of cancer deaths in the U. S., the etiology of this disease is not known. Factors that have been implicated include a high fat and low fiber diet; mutagens (i.e., fecapentaene) produced by the intestinal microflora; bile acids, which in experimental systems have a tumor-promoting effect; and genetic predisposition (*21–24*). We have obtained the following evidence that PKC plays a critical role in colon carcinogenesis: *a*) the bile acid deoxycholic acid (DOC) stimulates PKC activity *in vitro* (*22*), and in intact cells it induces PKC translocation and the expression of PKC-responsive genes (*24*); *b*) human colon tumors have decreased levels of PKC, suggesting downregulation of the enzyme (*17*); and *c*) human colon tumors display increased expression of phorbin, a PKC-responsive gene (*25*).

In recent studies we have examined the hypothesis that specific bacteria in the intestinal lumen might convert lipids to DAG, which could then enter the colonic epithelium and activate PKC, thus modulating cell proliferation (*26*). This would cause a chronic state of increased proliferation of the colonic epithelium, a situation that is seen in populations at high risk of colon cancer (*21–25*).

Indeed, we have found that when bacteria obtained from normal human feces are incubated with ^{14}C-labeled phosphatidylcholine, there is appreciable production of DAG. This reaction is strictly dependent on addition of specific bile acids to the incubation system. Assays of fecal specimens from 10 normal individuals demonstrated a 27-fold interindividual variation in the production of DAG in the *in vitro* assay system and also in the absolute levels of DAG present in the same fecal samples. On the other hand, both parameters of DAG are quite constant in repeated fecal samples obtained from the same individual.

Other investigators (*23*) have also found that DAGs are present in human feces, but they did not identify their source. Studies are in progress to isolate the specific intestinal bacteria that produce DAG and to determine whether DAG present in the intestinal lumen can actually enter colonic epithelial cells, stimulate PKC, and thereby modulate the growth of these cells. Should this prove to be the case, then we would have a mechanism to explain how an interaction between dietary lipids, bile acids, and specific bacteria in the intestinal lumen could contribute to the risk of colon cancer in humans.

Holistic View of Signal Transduction

The above findings indicate that overexpression of the β_1 isoform of PKC can produce highly pleiotropic effects on cellular morphology, growth, growth control, and susceptibility to malignant transformation by diverse types of oncogenes. Furthermore, some of these effects depend upon the specific cell type in which this enzyme is expressed and the environment in which the cells are grown. Studies are in progress to determine whether other isoforms of PKC produce similar effects.

It is difficult to rationalize the present findings in terms of simple linear pathways of signal transduction. Results obtained with various hormones, neurotransmitters, growth factors, receptors, ion channels, protein kinases, protein phosphatases, second messengers, and transcription factors lead to similar conclusions. It is apparent, therefore, that the signal transduction pathways of mammalian cells constitute a dynamic and highly interactive network that includes considerable "cross-talk" and both positive and negative feedback circuitry. In a sense, all components of the system have the potential of influencing each other. A crude analogy would be a mechanical clock in which all of the gears are connected to each other, and a perturbation in one gear is perceived by the entire system. The complex maps of intermediary metabolism in which diverse biochemical pathways can influence each other provide another analogy. This complexity in signal transduction probably explains why multiple types of exogenous agents, multiple steps, and multiple mechanisms are involved in the carcinogenic process and why tumor phenotypes are highly heterogeneous. It is for these reasons that I have defined multistage carcinogenesis as a "progressive disorder in signal transduction."

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