Disorders in Cell Circuitry Associated with Multistage Carcinogenesis: Exploitable Targets for Cancer Prevention and Therapy

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Abstract

The development of a malignant tumor involves the progressive acquisition of mutations and epigenetic abnormalities in multiple genes that have highly diverse functions. Some of these genes code for pathways of signal transduction that mediate the action of growth factors. The enzyme protein kinase C plays an important role in these events and in the process of tumor promotion. Therefore, we examined the effects of three inhibitors of protein kinase C, CGP 41251, RO 31-8220, and calphostin C, on human glioblastoma cells. These compounds inhibited growth and induced apoptosis; these activities were associated with a decrease in the level of CDC2 and cyclin B1/CDC2-associated kinase activity. This may explain why the treated cells accumulated in G2-M. In a separate series of studies, we examined abnormalities in cell cycle control genes in human cancer. We have found that cyclin D1 is frequently overexpressed in a variety of human cancers. Mechanistic studies indicate that cyclin D1 can play a critical role in carcinogenesis because: overexpression enhances cell transformation and tumorigenesis; introduction of an antisense cyclin D1 cDNA into either human esophageal or colon cancer cells reverts their malignant phenotype; and overexpression of cyclin D1 can enhance the amplification of other genes. The latter finding suggests that cyclin D1 can enhance genomic instability and, thereby, the process of tumor progression. Therefore, inhibitors of the function of cyclin D1 may be useful in both cancer chemoprevention and therapy. We obtained evidence for the existence of homeostatic feedback loops between cyclins D1 or E and the cell cycle inhibitory protein p27Kip1.

On the basis of these and other findings, we hypothesize that, because of their disordered circuitry, cancer cells suffer from “gene addiction” and “gene hypersensitivity,” disorders that might be exploited in both cancer prevention and therapy.

Introduction

It is a thrilling experience to honor J Freireich by recalling the important advances that have been made over the past few decades in the care of cancer patients. I (I.B.W.) first met J in 1957 when I was a Clinical Associate on the Metabolism Service of the National Cancer Institute and he was just beginning his pioneering studies on platelet transfusions and the chemotherapy of leukemia. I tremendously appreciate the subsequent advances in these and other areas that he spearheaded, his leadership role in clinical research, and his stimulating friendship.

This is the era of molecular genetics. To discover the molecular basis of Freireich’s many talents, I wanted to analyze his genome, but I didn’t have a sample of his DNA. So, I took the sequence of the letters in his name and entered it into the GenBank database on nucleic acid sequences to search for homologous sequences that might be informative. The readout I obtained is displayed in Fig. 1. It is amazing and prophetic! There is over 62% homology between the sequences Emil Freireich, M.D., and Emil Frei, M.D. In an evolutionary sense, Emil Freireich, M.D., is an extended (one might say jumbo) version of Emil Frei, M.D. We are now trying to transfer the consensus sequence into our oncology fellows so that they will acquire some of the marvelous skills shared by these two leaders in cancer therapy.

My career has emphasized research on cancer causation, with a view toward prevention. Until recently, this field seemed separate from that of cancer therapy, but in recent years, this has changed dramatically. Indeed, the fields of cancer causation, prevention, and treatment are rapidly merging, with the ultimate goals of reducing both cancer incidence and mortality. I will discuss how recent studies on disorders in signal transduction and cell cycle control, which develop during the multistage process of carcinogenesis, provide new targets for both cancer prevention and treatment. I will also discuss our hypothesis that, because of their disordered circuitry, cancer cells suffer from “gene addiction” and “gene hypersensitivity,” disorders that might be exploited in cancer prevention and therapy.

It is now apparent that the development of a fully malignant tumor involves the progressive acquisition of mutations and epigenetic abnormalities in multiple genes (1, 2). Because of the large number and diverse functions of these genes, we believe that the two categories “oncogenes” and “tumor suppressor genes” are not adequate because they do not indicate the specific biochemical functions of the individual genes or con-
There is cross-talk between components in each category (tumor promoters and related compounds) and its role in mediating the action of the phorbol ester because of its central role in the action of the phorbol ester (i.e., PKC). Indeed, recent studies on PKC have rapidly expanded this subcategory. This subject is discussed in greater detail below. The second category (b) includes genes that influence how cells interact with the extracellular matrix and/or neighboring cells. This category includes various cell surface proteins, cell adhesion molecules, extracellular proteases, and angiogenesis factors. Obviously, alterations in these genes are especially relevant to tumor cell invasion and metastasis. I should emphasize several caveats related to this classification scheme: some of the above-mentioned genes perform multiple functions that extend across these categories (i.e., p53); there is cross-talk between components in each category and between categories; and the biological effects of some of these genes are dependent upon the context of the specific cell type in which it is expressed. Therefore, the classification scheme shown in Table 1 is an oversimplification. Nevertheless, it is more informative than simply using the terms oncogenes and tumor suppressor genes, and it may be useful in conceptualizing novel approaches to cancer prevention and therapy. With this theme in mind, I now want to discuss recent studies from our laboratory related to a family of genes in category a.1 of Table 1, namely, PKC, and then I will turn to recent studies on genes in category a.3, namely, cyclin D1 and related genes.

**Inhibitors of PKC**

For several years our laboratory has been interested in PKC because of its central role in the action of the phorbol ester tumor promoters and related compounds and its role in mediating the action of several growth factors and oncogenes (3). Indeed, in recent studies, we demonstrated that a specific isoform, PKCε, plays a role in oncogenesis by activating the Ras/Raf/mitogen-activated protein kinase pathway of signal transduction (4). For these reasons, inhibitors of PKC might be useful in cancer prevention and treatment. Therefore, we examined, in detail, the effects of a potent inhibitor of PKC, the staurosporine derivative CGP 41251, on a series of nine human glioblastoma cell lines (5). This compound caused irreversible inhibition of the proliferation of these cell lines, with an IC_{50} of about 0.4 μM. This was associated with an increase of cells in the G2-M phase of the cell cycle and the induction of apoptosis. These effects occurred even in cell lines carrying mutations in the p53 gene. The treated cells displayed a decrease in the level of the CDC2 protein (also termed CDK1) and a decrease in cyclin B/CDC2-associated kinase activity. The latter effects may explain why the cells accumulated in G2-M, but the precise mechanism remains to be determined. We obtained similar effects with another staurosporine derivative, Ro 31-8220, and with the PKC inhibitor calphostin C, which acts on the regulatory rather than the catalytic domain of PKC. Taken together, these findings suggest that, although these compounds are not generally thought of as cytotoxic agents, they may be effective in cancer chemotherapy because they can induce irreversible growth inhibition and apoptosis in a p53-independent manner. Indeed, CGP 41251 inhibited the growth of a human glioma cell line in nude mice (5). Therefore, we are optimistic that PKC inhibitors and other compounds that target functions in category a.1 in Table 1, for example, inhibitors of tyrosine kinase receptors and inhibitors of farnesylation of the Ras protein, might preferentially inhibit the growth of tumor cells.

**Disturbances in Cell Cycle Control in Human Cancer**

We will now discuss recent studies on disturbances in cell cycle control genes (category a.3 in Table 1) in human cancer and their relevance to cancer prevention and therapy. As shown in Fig. 2A, the orderly progression of dividing mammalian cells through G1, S, G2, and M is governed by a series of proteins called cyclins, which exert their effects by binding to and activating a series of specific CDKs. This process is further modulated by the phosphorylation and dephosphorylation of CDK proteins by specific protein kinases and phosphatases and

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**Table 1** Categories of genes targeted during multistage carcinogenesis

<table>
<thead>
<tr>
<th>Subcategory</th>
<th>Description</th>
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<tbody>
<tr>
<td>a. Intracellular circuitry</td>
<td>1. Agonist-induced signal transduction 2. DNA replication and repair</td>
</tr>
<tr>
<td></td>
<td>Adhesion molecules; proteases; angiogenic factors, and so on</td>
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3 The abbreviations used are: PKC, protein kinase C; CDK, cyclin-dependent serine and threonine protein kinase.

by a series of specific CDK inhibitor proteins called CDIs (Fig. 2B; Refs. 6–8).

There is accumulating evidence for mutations or abnormalities in the expression of various cyclins, CDKs, and CDIs in several types of human cancers (for review, see Refs. 6–16). The most frequent abnormalities that have been found are in the G1, cyclin, cyclin D1. Cyclin D1 plays a key role in the G1-S progression of the cell cycle. It binds to and activates CDK4 and CDK6. This leads to phosphorylation and inactivation of Rb, thus resulting in activation of the transcription factor E2F, which enhances S-phase progression. cyclin D1, also termed prad1 or bel-1, is located at chromosome 11q13. Chromosomal rearrangements at this locus in parathyroid tumors or centrocytic B-cell lymphomas cause increased and constitutive expression of this gene. The cyclin D1 gene is amplified and overexpressed, at both the mRNA and protein levels, in a significant fraction of primary human breast carcinomas, esophageal carcinomas, squamous cell carcinomas of the head and neck, non-small cell lung carcinomas, hepatocellular carcinomas, and bladder carcinomas. Cytogenetic and molecular studies indicate that the amplified cyclin D1 gene is part of a much larger amplicon located at chromosome 11q13. Overexpression of cyclin D1 in the absence of gene amplification is also seen in about 45% of human breast carcinomas (10) and about 40% of colon carcinomas (11, 12), but the mechanisms responsible for this overexpression are not known.

The increased expression of cyclin D1 could be useful in identifying preneoplastic lesions because we have found that increased expression of cyclin D1 can be detected in adenomas of the colon, i.e., at a relatively early stage in the process of colon carcinogenesis (12), and in Barrett’s esophagus, a disease associated with an increased risk of esophageal cancer (13). Other investigators recently reported that cyclin D1 is also overexpressed in the small polyps of patients with familial polyposis coli and in a mouse model of this disease (14). Increased expression of cyclin D1 is a marker of poor prognosis in squamous cell carcinomas of the esophagus, squamous cell carcinomas of the head and neck (8, 15, 16), and carcinomas of the pancreas (17).

In studies on human esophageal carcinomas, we noted that the subset of tumors that had amplification and increased expression of cyclin D1 always displayed expression of the Rb protein, whereas the subset of tumors that did not express the Rb protein (presumably due to inactivating mutations or deletions) did not show amplification and increased expression of cyclin D1 (9). A similar relationship between cyclin D1 and Rb was subsequently seen in human breast (18) and non-small cell lung cancers (19). Thus, it would appear that, during the clonal evolution of tumors, the inhibitory effect of the Rb gene on cell cycle progression can be abrogated, either by increased expression of cyclin D1, which would increase phosphorylation of the Rb protein, thereby inactivating its inhibitory function, or actual loss of the Rb protein (9). Alternative mechanisms include inactivation of the CDI p16INK4a, which acts on cyclin D1/CDK4 and cyclin D1/CDK6. These alternative mechanisms provide a paradigm for explaining why individual tumors of the same histological type can differ with respect to the spectrum of genes that are mutated because the same regulatory pathway can be perturbed by mutations in different genes in the pathway. Therefore, in the design and clinical use of new gene-specific anticancer agents, it may be necessary to score individual tumors for the specific mutation involved or design agents that are pathway specific rather than gene specific.

Several types of mechanistic studies specifically implicate the cyclin D1 gene in tumorigenesis. Thus, using gene transfer studies, we found that stable overexpression of cyclin D1 in
rodent fibroblasts enhanced their growth in cell culture and tumorigenicity in nude mice (20). Cotransfection studies indicated that cyclin D1 cooperates with a defective adenovirus E1A gene (21) or an activated ras oncogene (22) in the transformation of rodent cell lines. Overexpression of a cyclin D1 sequence under the control of a mouse mammary tumor virus promoter in transgenic mice resulted in mammary hyperplasia and tumors of the mammary epithelium (23), and cyclin D1 cooperated with a myc oncogene in producing B-cell lymphomas in transgenic mice (24, 25). On the other hand, cyclin D1-deficient mice have reduced proliferation of the mammary epithelium (26).

**Antisense to Cyclin D1 Inhibits Growth and Reverses the Transformed Phenotype of Human Esophageal and Colon Cancer Cells**

Although in early studies it was known that the cyclin D1 gene is amplified and overexpressed in a significant fraction of human esophageal tumors and several other types of human cancer, the functional significance of this overexpression had not been established. This was an important issue because, as mentioned above, the amplicon on chromosome 11q13, in which the cyclin D1 gene resides, contains other genes, and it is possible that they, rather than cyclin D1, might be critical to tumorigenesis. To address the specific role of cyclin D1, an antisense cyclin D1 cDNA construct was expressed, either constitutively or inducibly, in the HCE7 human esophageal cancer cell line, in which the endogenous cyclin D1 is amplified and expressed at high levels (27). The expression of antisense cyclin D1 led to decreased expression of cyclin D1 at both the mRNA and protein levels, and this was associated with a marked inhibition of cell proliferation. Antisense cyclin D1-expressing cells displayed a decreased plating efficiency, increased doubling time, decreased saturation density, increased cell size, decreased cyclin D1-associated in vitro Rb kinase activity, decreased anchorage-independent growth, and a loss of tumorigenicity in nude mice (27). We recently obtained similar results when an antisense cyclin D1 cDNA was stably expressed in the SW480E8 human colon carcinoma cell line that expresses high levels of cyclin D1 in the absence of gene amplification (28). These derivatives also reverted toward normal phenotypes and lost their tumorigenicity in nude mice.

These findings provide direct evidence that the overexpression of cyclin D1 in certain tumor cells contributes to their abnormal growth and tumorigenicity. The ability to revert the transformed phenotype of these cells with antisense cyclin D1 suggests that cyclin D1 may be a useful target in cancer therapy. This could be achieved by designing antisense oligonucleotide or gene therapy approaches that would inhibit the expression of cyclin D1 in these human tumors, or, as a more feasible approach, designing drugs that inhibit the action of cyclin D1 by inhibiting the kinase function of CDK4 and CDK6.

**Overexpression of Cyclin D1 Enhances Gene Amplification**

A critical aspect of the multistage process of carcinogenesis is the apparent ability of tumor cells to develop genetic variants with an abnormally high frequency. Normal mammalian cells have checkpoints at the G1-S and G2-M stages of the cell cycle, at which cells can delay progress through the cell cycle to permit repair of damaged DNA and, thereby, prevent various types of mutations. Therefore, defects in cell cycle control and the normal function of these checkpoints might enhance genomic instability. A frequent example of genomic instability in tumors is the occurrence of gene amplification, which is often seen in cellular oncogenes and genes that play a role in drug resistance. Previous studies by other investigators demonstrated that homozygous loss of function of the p53 gene was sufficient to increase the susceptibility of cells to gene amplification (29, 30). However, other factors can also play a role because some tumor cells with wild-type p53 genes can still display a high frequency of gene amplification. Because cyclin D1 plays a pivotal role in the G1 phase of the cell cycle and this gene is frequently overexpressed in several types of human tumor, we postulated that this overexpression might contribute to genomic instability during tumor progression. Indeed, we demonstrated that ectopic overexpression of cyclin D1 in a rat liver epithelial cell line markedly increased amplification of the CAD gene (31). This effect was associated with impairment of G1-S checkpoint control, although the cyclin D1-overexpressing cells had a normal p53 gene. Overexpression of cyclin D1 also enhanced acquisition of resistance to methotrexate. The capacity of cyclin D1 to enhance gene amplification could contribute to the process of genomic instability during tumor development (31). Therefore, inhibition of the action of cyclin D1 might provide a strategy for inhibiting tumor progression and the acquisition of drug resistance.

**Paradoxical Expression of p27KIP1 and Rb in Cancer Cells**

During the course of our studies on the expression of various cell cycle control proteins, we were surprised to find relatively high levels of expression of p27KIP1 and, sometimes, high levels of the Rb protein in some cancer cell lines because both of these proteins are growth inhibitors. In a series of human esophageal cancer cell lines, there was a positive correlation between the level of cyclin D1 (which would be expected to enhance growth) and levels of the p27KIP1 and Rb proteins (32). Several human colon and breast cancer cell lines also expressed high levels of the p27KIP1 protein, but this protein was expressed at low levels in three normal mammary epithelial cell lines (33–36). Furthermore, ectopic overexpression of cyclin D1 in an esophageal cancer cell line that expressed a low level of cyclin D1 was associated with increased expression of both p27KIP1 and Rb (32). Ectopic overexpression of cyclin D1 or cyclin E in mammary epithelial cell lines that express low levels of both of these cyclins was also associated with increased expression of p27KIP1 (33–35). The reciprocal effect was also seen because, when we used an antisense cyclin D1 cDNA to reduce the expression of cyclin D1 in an esophageal or colon cancer cell
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findings, we postulated the existence, in some cell types, of a human breast (36) and colon tumors (37). On the basis of these findings, we postulated the existence, in some cell types, of a feedback loop between cyclins D1 or E and p27KIP1, the function of which might be to maintain a homeostatic balance between positive and negative regulators of G1-S progression of the cell cycle (32–36). Recent studies suggest that breast and colon tumors that display low expression of p27KIP1 are associated with a poor prognosis (38–40); perhaps they have lost this homeostatic mechanism.

High levels of expression of another CDI, p21WAF1, have also been seen in some human tumors, including glial tumors (41), non-small cell lung carcinomas (42), leiomyosarcomas (43), breast carcinomas (44), and pancreatic carcinomas (16). In addition, cyclin D1 can induce increased expression of p21WAF1 through an E2F mechanism (45). These apparent paradoxes may provide another example of a homeostatic feedback mechanism that is retained in many tumors.

**Gene Addiction and Hypersensitivity as Exploitable Targets**

A remarkable finding in our studies using an antisense cyclin cDNA vector was that simply inhibiting the expression of cyclin D1 in either an esophageal or colon cancer cell line caused marked growth inhibition and loss of tumorigenicity, despite the fact that both cell lines are highly aneuploid and carry mutations in additional genes. Furthermore, the reverted cells still expressed appreciable levels of the cyclin D1 protein (27, 28); therefore, the growth inhibition we obtained was not simply because we blocked the expression of an essential gene. Indeed, the residual level of expression of cyclin D1 in the reverted esophageal cancer cell line was much higher than that of a tumorigenic esophageal cancer cell line in which the endogenous cyclin D1 gene was never overexpressed (27). These findings led us to suggest that the intracellular circuitry of cancer cells that overexpress cyclin D1 requires a higher level of this protein than do cells that developed through alternative circuitry, in which this gene is not overexpressed (2, 27, 28). In this sense, the former cells are addicted to cyclin D1, which may explain the profound growth-inhibitory effects we obtained with antisense cyclin D1 in the cyclin D1-overexpressing cell lines. The above-described homeostatic feedback loop between cyclin D1 and p27KIP1 or similar homeostatic mechanisms could be the basis for this addiction, as illustrated schematically in Fig. 3. According to this scheme, tumor cells that express high levels of cyclin D1 might also have increased levels of the inhibitory protein p27KIP1, but cyclin D1 would be in relative excess and, thus, would cause growth stimulation. A decrease in the level of cyclin D1 in these cells could then cause marked growth inhibition because there would now be a relative excess of p27KIP1, especially if the normal homeostatic balance between cyclin D1 and p27KIP1 is impaired. Similar mechanisms might confer addiction to other dominant acting oncogenes in tumor cells, for example, tumor cells carrying an activated ras oncogene or tumor cells that express increased levels of growth factors or growth factor receptors.

Our ability to revert the malignant phenotype of cancer cells by altering the expression of a single gene resembles the findings by other investigators in which restoration of a single wild-type tumor suppressor gene to a cancer cell can markedly inhibit its growth and/or tumorigenicity, despite the presence of several other genetic abnormalities in the recipient cells (for examples, see Refs. 46 and 47). We postulate that the latter results also reflect abnormalities in cell circuitry in these cancer cells, such that, if they lack the expression of a tumor suppressor gene, they may be hypersensitive to the inhibitory function of that gene if it is restored. A hypothetical model based on the tumor suppressor gene Rb is shown in Fig. 4. In normal cells, the inhibitory functions of Rb are opposed by cyclin D1 and favored by a low level of expression of the cyclin D1/CDK4 inhibitory protein p16INK4, thus providing a homeostatic control mechanism. Inactivation of Rb in a tumor cell is often associated with decreased expression of cyclin D1 (9, 18, 19, 48) and increased expression of p16INK4 (49). Therefore, if a wild-type Rb gene is introduced into these cells, its growth-inhibitory effects could be greater than its effects in normal cells because the low level of cyclin D1 and the high level of p16INK4 will prevent inactivation of the Rb protein by phosphorylation. The tumor suppressor gene p53 induces the protein Mdm2, which antagonizes the action of p53 (50, 51). Disturbances in this homeostatic feedback loop in tumor cells in which the p53 gene is inactivated might confer hypersensitivity to the restoration of wild-type p53, thus explaining the subsequent loss of tumorigenicity.

The above models related to gene addiction and gene hypersensitivity are hypothetical and probably oversimplified. Nevertheless, they may provide a rationale for new approaches to cancer prevention and therapy that exploit the bizarre circuitries of cancer cells.

**Conclusion**

The multistage process of carcinogenesis is associated with numerous mutations and epigenetic abnormalities in genes that
carry out highly diverse intracellular and extracellular functions. The biological consequences and clinical significance of any single genetic abnormality in cancer cells can, therefore, only be understood within the complex network of signal transduction pathways and mechanisms of gene expression that control cellular homeostasis, cell proliferation, differentiation, and apoptosis. Recent studies on abnormalities in cyclin D1 and related genes in human cancers illustrate these principles. This emphasis on cell circuitry and cell context has important implications in terms of targeted approaches to cancer prevention and therapy.

References


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Disorders in cell circuitry associated with multistage carcinogenesis: exploitable targets for cancer prevention and therapy.

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