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I. Bernard Weinstein, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, New York 10032

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This project examines the role of cyclin D1, and related genes, in multistage breast carcinogenesis. It may provide new biomarkers and strategies for the prevention and treatment of breast cancer. During the past year two studies were completed and published. The first provides further evidence that increased expression of cyclin D1 can inhibit the growth of mammary epithelial cells. It can also increase the sensitivity of these cells to the induction of apoptosis by various agents. These effects appear to be due to increased levels of the cell cycle inhibitory protein $p27^{kip1}$. The second study demonstrates that increased expression of cyclin E in mammary epithelial cells can also inhibit growth and that this is also due to increased expression of $p27^{kip1}$. Breast cancer cell lines with increased expression of cyclins D1 and/or E also have high levels of $p27^{kip1}$. Thus, mammary epithelial cells have a homeostatic feed-back loop that regulates the G1 to S progression of the cell cycle. Studies are in progress to evaluate the clinical relevance of these findings.

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PRINCIPAL INVESTIGATOR: I. Bernard Weinstein, M.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

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I. Bernard Weinstein — 8/9/96

Principal Investigator's Signature — Date

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THE ROLE OF CYCLIN D1 OVEREXPRESSION IN BREAST CANCER PROGRESSION

(5) INTRODUCTION

Nature of the problem. Although there is considerable evidence that the majority of human breast cancers are caused by environmental factors (including dietary factors) and reproductive factors, rather than hereditary factors, the specific causes of human breast cancer are not known with certainty. Furthermore, although exciting progress has been made in identifying mutations and or aberrant expression of cellular oncogenes and tumor suppressor genes in human breast cancers, the precise mechanisms responsible for the uncontrolled proliferation of breast cancer cells, the apparent genomic instability of malignant breast tumors, and the often relentless course of tumor progression, are poorly understood at the present time.

Background. Cyclins are a recently identified family of proteins that regulate the passage through the G1, S, G2 and M phases of the cell cycle (for review see 1-3). These proteins complex with specific cyclin-dependent serine-and threonine-protein kinases (CDKs), thereby regulating the activity of these kinases. This process is further modulated by the phosphorylation and dephosphorylation of CDK proteins by specific protein kinases and phosphatases, and by specific inhibitory proteins called CDKIs. At least 8 distinct cyclin genes have been identified in the human genome and at least six CDKs (CDK 1-6) form complexes with these cyclins. Based on their conserved sequence motifs with cyclins in other species and their patterns of expression and apparent functional roles during the cell cycle, they are grouped into three categories: G1 cyclins, including cyclins D1-3, C and E; the S-phase cyclin, cyclin A; and G2/M phase cyclins, cyclins B1 and B2. Cyclin B is the best characterized mammalian cyclin. It complexes with (CDC2) to regulate both mitotic entry and exit. It is not known whether cyclin B1 and B2 are interchangeable. The precise functions of the other cyclins in the cell cycle is not as well defined in mammalian cells as in lower organisms. After stimulating quiescent cells by growth factors, cyclin D1 is expressed maximally during mid to late G1, although it appears to be expressed at a constant level in continuously dividing cells. Cyclin D1, and cyclins D2 and D3, associate with CDK kinases thereby activating their activities. The Rb protein is a target for phosphorylation by these complexes in vitro (12). There is conflicting evidence, however, on the ability of cyclin D1 to complex directly with the Rb protein. It is not known whether all of the biologic effects of cyclin D1 are mediated via the Rb protein.

Several lines of evidence implicate the role of cyclins D1 and E in human breast cancer (see 1-4). 1) The cyclin D1 gene (originally termed Prad1), is located at chromosome 11q13. This locus is frequently amplified in human breast cancers. We and other investigators have demonstrated amplification and increased expression of cyclin D1 in a subset of primary human and breast cancer cell lines. 2) The cyclin E gene was found to be amplified and expressed at a high level in a human breast cancer cell line (1-4). 3) Increased expression, and abnormalities during the cell cycle, in the expression of cyclins D1 and E have been described in human breast tumors and cell lines (1-4). It is of interest that the increased expression of cyclin D1 did not always correlate with amplification (7,8).

Our laboratory has previously described amplification and increased expression of cyclin D1 in human tumors of the liver and esophagus (3-6). We have also demonstrated that stable overexpression of cyclin D1 shortens the G1 phase of the cell cycle and enhances malignant cell transformation (4). These studies from our laboratory, coupled with the other evidence (cited above) of abnormalities in cyclins in human breast cancers, provide the basis for this grant.

Purpose of the present work. The overall purpose of this work is to examine the hypothesis that amplification and/or increased expression of the cyclin D1 gene plays an important role in multistage breast carcinogenesis by enhancing the process of tumor progression. These studies might also provide new biomarkers and diagnostic tools to more precisely detect and stage breast tumors. This approach could also lead to the development of agents that inhibit the action of specific cyclins or cyclin-dependent protein kinases in human tumors, and thus lead to new strategies for breast cancer chemoprevention and therapy. If abnormalities in the expression of cyclin genes enhance genomic instability, as suggested by our recent studies (11), then such inhibitors might specifically block the process of tumor progression and the emergence of hormone independent and drug resistant variants of breast tumors.

Methods of approach. As discussed above, the cyclin D1 gene is frequently amplified and overexpressed in primary human breast cancers and breast cancer cell lines, but the functional and prognostic significance of this finding is not known. We are using well defined normal human mammary epithelial and human breast cancer cell culture systems to analyze the role of cyclin D1 in cell cycle control, gene expression and amplification, cell transformation and tumorigenicity. Similar studies are also being done with cyclin E. A major strategy employed in our studies is to utilize gene transfer methods to develop derivatives of normal mammary epithelial cells that stably overexpress either cyclin D1 or cyclin E and then examine possible effects on growth control, differentiation and various cell cycle parameters (6).

(6) BODY

(a) Increased Expression of Cyclin D1 in a murine Mammary Epithelial Cell Line Induces $p27^{kip1}$, Inhibits Growth, and Enhances Apoptosis

There is considerable evidence that cyclin D1 plays a critical role in cell cycle progression during the early to mid-G1 phase of the cell cycle. The study of cyclin D1 is directly relevant to human breast cancer, because we and other investigators have found that the cyclin D1 gene frequently amplified and/or overexpressed in both primary human breast carcinomas and breast carcinomas cell lines. Last year we reported that cyclin D1 is also overexpressed in carcinogen-induced rat mammary carcinomas.

In studies in which an exogenous cyclin D1 cDNA was overexpressed either stably or inducibly, in murine, rat or human fibroblasts, this led to shortening of the G1 phase and in some cases enhanced growth and tumorigenicity. To further understand the role of cyclin D1 in breast tumorigenesis, in studies reported last year we overexpressed cyclin D1 in the human mammary

epithelial cell line HBL-100 (6). In contrast to the previous results obtained with fibroblasts, we found that cyclin D1 overexpression markedly inhibited the growth of the HBL-100 cells, and this was associated with lengthening of the S-phase. Although the HBL-100 cells were originally isolated from normal human mammary epithelial cells, they express the SV40 large T-antigen and exhibit characteristics of transformation. Therefore, in studies carried out during the past year (9) we sought to overexpress cyclin D1 in a more normal mammary epithelial cell line. We chose the mouse mammary epithelial cell line HC11 since, although it is immortalized, it is highly epithelial, has normal growth properties, is non-tumorigenic and can be induced to express the milk protein casein, upon stimulation with specific hormones. The same human cDNA construct used in our previous studies with rat fibroblasts and HBL-100 cells was introduced into the HC11 cells via retrovirus mediated infection and neo+ clones were selected, as previously described (6). Several neo+ clones expressed about an 8-fold increase in cyclin D1 protein, as determined by western blot analysis using a cyclin D1 antibody. When compared to vector control clones the cyclin D1 overexpressors had a similar exponential doubling time but they had a lower saturation density and a lower cloning efficiency in soft-agar assay, and there was an increased fraction of cells in the G1 phase of the cell cycle. These results are in contrast to our previous findings in which stable overexpression of the same cyclin D1 construct in R6 rat fibroblasts stimulated growth and decreased the proportion of cells in the G1 phase (4). In the human mammary epithelial cell line HBL-100 overexpression of cyclin D1 inhibited growth and caused an increase in the proportion of cells in the S-phase (6). The growth inhibitory effects of cyclin D1 are not confined to HBL-100 and HC11 cells since in recent studies we found that when cyclin D1 was overexpressed in the normal human mammary epithelial MCF-10F or 184B5 cells, this also caused growth inhibition (recent unpublished studies).

An unexpected finding was that the HC11-cyclin D1 overexpressor cells expressed about a 3-fold increase in the level of expression of the cell cycle inhibitory protein $p27k^{kip1}$. We have also seen increased expression of the latter protein in derivatives of MCF-10F cells that overexpress cyclin D1. The increased levels of this inhibitory protein could account for the above described growth inhibition and also the increase of cells in the G1 phase, since $p27^{kip1}$ can inhibit cyclin D1-cdk4, cyclin E-cdk2 and cyclin A-cdk2 kinase activities, to varying degrees. We suggest that in certain cell types overexpression of cyclin D1 can induce the expression of $p27^{kip1}$, either directly or indirectly, perhaps because of a homeostatic feedback regulatory loop involved in cell cycle control.

Overexpression of cyclin D1 in HC11 cells was also associated with increased levels of expression of the cyclin E and cyclin A proteins. In addition to the characteristic 52 kDa cyclin E protein band these cells also expressed two lower-molecular-mass (42 and 50 kDa) cyclin E-related proteins. Similar lower-molecular-mass cyclin E proteins have been noted in a variety of human tumors. In addition to the increased levels of the major 58 kDa cyclin A protein, the HC11 cyclin D overexpressor cells also displayed a 56 Kd cyclin A related protein. To our knowledge this form of cyclin A has not been described previously and its precise nature is not known. Despite the increased levels of cyclins E and A, the HC11 cyclin D1 overexpressor cells did not display increased cdk2-associated <u>in vitro</u> kinase activity, perhaps because of the above mentioned increased expression of $p27^{kip1}$.

A novel finding in this study is that stable overexpression of human cyclin D1 in the HC11 cell line induces apoptosis, which was demonstrated by characteristic morphological criteria. We also observed the same phenomenon in a cyclin D1 overexpressor derivative of the human mammary epithelial cell line HBL-100. Thus, increased levels of a human cyclin D1 can induce apoptosis in both mouse and human mammary epithelial cells. It is of interest that increased expression of endogenous cyclin D1 occurs in postmitotic neurons undergoing apoptosis, and that senescent fibroblasts also express increased levels of cyclin D1. The difference in apoptosis indices between the HC11 and HBL-100 cyclin D1 overproducer cell lines and the corresponding vector control cell lines was particularly striking after serum starvation. Thus when transferred from medium containing 10% FCS, to medium containing 0.1% FCS the cyclin D1 overexpressor cells displayed about a 8- to 24-fold increase in apoptosis when compared to similarly treated control cells. These results are reminiscent of previous findings indicating that when fibroblasts that overexpress c-myc or rho-p21 are starved of serum they also display increased apoptosis. Thus, the overexpression of genes involved in signal transduction and/or cell cycle control may, paradoxically, make these cells more dependent on growth factors for survival. We found that the cyclin D1 overexpressor cells were also much more sensitive to the induction of apoptosis by certain cytotoxic agents including the PKC inhibitors CGP 41251, Ro31-8220 and calphostin C, and the inhibitor of DNA synthesis hydroxyurea. Our findings may be relevant to the role of cyclin D1 in the in vivo physiology of the mammary gland since cell death has been shown to play a pivotal role during the involution of the lactating mammary gland and also in breast cancer. We speculate that cyclin D1, in conjunction with other factors, regulates apoptosis in both normal and malignantly transformed mammary epithelium. In fact, apoptosis might in unusual cases account for the published cases of spontaneous regression of advanced breast cancer and other tumors. Further studies are required to determine the mechanism by which increased expression of cyclin D1 can enhance apoptosis and the clinical significance of this effect.

Regardless of the underlying mechanism, the results obtained with HC11 cells in the present study, taken together with our previous findings with HBL-100 cells, indicate that in both a murine and human mammary epithelial cell lines, stable overexpression of cyclin D1 can inhibit growth and enhance apoptosis. It is paradoxical that increased expression of cyclin D1 in mammary epithelial cell cultures inhibits growth, since this gene is often amplified and/or overexpressed in both human and rat mammary carcinomas. Presumably during the development of breast cancer there are other events that compensate for these potential growth inhibitory effects. Studies are in progress to identify these factors.

(b) Overexpression of Cyclin E in the HC11 Mouse Mammary Epithelial Cell Line Is Associated with Growth Inhibition and Increased Expression of $p27^{kip1}$

In addition to the increased expression of cyclin D1, described in the above study, there is evidence that human breast cancers often display increased expression and dysregulation in the expression of cyclin E. Cyclin E binds to and activates CDK2 and this complex plays a critical role in cell cycle progression, acting in late G1 after cyclin D1.

To further address the role of cyclin E in mammary tumorigenesis, in this study (10) we developed derivatives of the normal mouse mammary epithelial cell line HC11, which stably

overexpress a human cyclin E cDNA (HU4). We found that this causes the expression of two different forms of the cyclin E protein, a major band at about 50 kDa and a minor band at about 42 kDa. With the use of a specific antihuman cyclin E antibody we were able to demonstrate that the 42 kDa cyclin E was consistently expressed in parallel with the 50 kDa band in the derivatives of the HC11 cells. We suggest that the 42 kDa bank observed in the HC11/cyclin E-overexpressing derivatives is due to further splicing of the overexpressed cyclin E mRNA, since this mRNA contains both potential splice-donor and splice-acceptor sites. The existence of multiple cyclin E-related proteins that range in size from kDa 52 to about kDa 35 has been described previously in human breast cancer cells. The precise origin of these multiple forms of the cyclin E protein and their functional significance is not, however, apparent at the present time.

The present study demonstrates that overexpression of the same cyclin E cDNA can exert distinct biological effects in different cell types. Thus, whereas previous studies found that stable overexpression of the UH4 cyclin E cDNA in rat or human fibroblasts shortened G_1, decreased cell size, and enhanced growth, we found that stable overexpression of the same cDNA in the HC11 cells lengthened G_1, increased cell size, and inhibited growth. Our findings are reproducible since they were seen with several clonal derivatives of HC11 cells that overexpress this cyclin E cDNA and were not seen in vector control clones. Furthermore, sequencing studies indicated that the present results are not due to mutations in this cDNA that might have occurred during construction of our PMV12-cycE plasmid. In addition, when we introduced the same construct into rat fibroblasts, we reproduced the stimulatory effects obtained previously by other investigators.

We believe that the most likely explanation for why the HC11-cyclin E derivatives have a prolonged G_1 phase and display growth inhibition is that stable overexpression in these cells of this human cyclin E cDNA induces, either directly or indirectly, the expression of the inhibitory protein $p27^{kip1}$. The increased expression of this inhibitory protein was seen in the HC11-cyclin E cells when they were in continuous exponential growth and also in cultures synchronized by serum starvation and refeeding. It was also a reproducible finding in several cyclin E-overexpressing clones and was not seen in the vector control clones. This interpretation is consistent with the fact that although these derivatives displayed a marked increase in the levels of the exogenous cyclin E proteins, extracts of these cells did not display an increase in cyclin E-associated kinase activity when compared to extracts from control cells. Extracts of the overexpressor cells also displayed an increase in inhibitory activity in <u>in vitro</u> assays for cyclin E-associated kinase activity. In addition, both the exogenous cyclin E protein and the $p27^{kip1}$ protein could be detected in cyclin E/Cdk2 immunoprecipitates obtained from the overexpressor cells.

We hypothesize that the increased expression of $p27^{kip1}$ in the HC11/cyclin E-overexpressing cells is a manifestation of a homeostatic feedback loop between cyclin E and $p27^{kip1}$, which is present in HC11 cells but may be absent or have a different set point in fibroblasts. Consistent with this hypothesis is our finding that rat fibroblasts that stably overexpress the same human cyclin E cDNA do not display an increase in the $p27^{kip1}$ protein. The increase in $p27^{kip1}$ in the HC11-cyclin E overexpressor cells might protect these cells against potentially toxic effects of cyclin E overexpression, thus enhancing their viability although they grow more slowly. The

present model system may be instructive for revealing regulatory loops that play a role in mammary carcinogenesis. Thus, as discussed above, certain tumor cells and some normal cell lines often display dysregulation in the expression of their endogenous cyclin E gene, and these cells also accumulate lower molecular weight cyclin E proteins. Some of these proteins might be defective in binding to and activating Cdk2, like the 42 kDa protein, but they could also play a role in feedback regulation of cell cycle progression. It is of interest that the HC11 derivatives that express the exogenous cyclin E cDNA display not only increased expression of the endogenous $p27^{kip1}$ but also decreased expression of their endogenous cyclin E protein. Additional studies are required to determine if this represents an independent negative feedback loop in the control of cyclin E expression or is a secondary effect due to changes in cell cycle control

The findings in the present study are relevant to human breast cancer since we found that a series of tumorigenic human breast cancer cell lines express a high level of $p27^{kip1}$. The high level of expression of $p27^{kip1}$ is surprising since this protein is thought to act as a tumor suppressor. Thus, our hypothesis of a feedback loop between cyclin E and $p27^{kip1}$ may apply to these breast cancer cell lines. As discussed in Section (a) above, we have also found that overexpression of cyclin D1 in mammary epithelial cells can also induce the expression of $p27^{kip1}$ (9). Thus it appears that in these cells increased expression of either cyclin D1 or cyclin E can induce increased levels of $p27^{kip1}$, presumably reflecting feed-back loops that normally maintain a homeostatic balance between positive and negative cell cycle control factors. Studies are now in progress to evaluate the clinical relevance of these findings in a series of primary human breast cancers.

(7) CONCLUSIONS

During the second year of this research project we have completed and published (9, 10) two specific studies that are directly related to the original goals of this research proposal.

Taken together these studies indicate that increased expression of either cyclin D1 or cyclin E in mammary epithelial cells can inhibit rather than enhance growth. In the case of cyclin D1, the increased expression markedly sensitizes the cells to the induction of apoptosis. A striking and novel finding is that the increased expression of either cyclin D1 or E in these cells leads to increased expression of the cell cycle inhibitory protein $p27^{kip1}$. These findings reveal for the first time the existence of a homeostatic feed-back regulatory loop that regulates G1 to S progression of the cell cycle in mammary epithelial cells. Studies are now in progress to evaluate the clinical relevance of these findings in human breast cancers.

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