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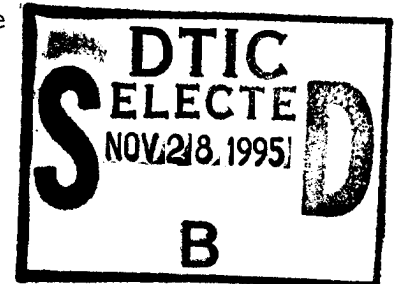
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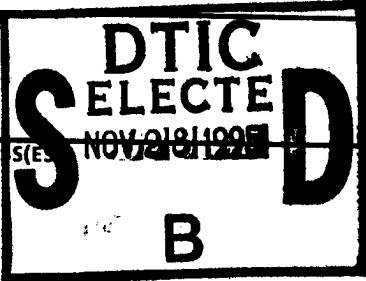
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Introduction

The overall objective of the work proposed in this grant is to provide a detailed understanding of the molecular mechanism by which G2/M regulation is achieved in human breast epithelial cells. In particular we are focusing on how initiation of M-phase is delayed in cells that have been treated with agents that induce DNA damage or that prevent synthesis of DNA. By providing a more detailed explanation of how cytotoxic therapies brings about cell death we hope to provide clinicians with better tools for the treatment of breast cancer.

Methodology and Results

Biochemical and cell biological techniques are being employed to address these problems. The three technical objectives that were defined in the proposed work are summarized below:

Technical Objective 1

Examine the pattern of expression and activity of mitotic checkpoint control proteins in transformed and non-transformed breast cell lines. (Equivalent to Task 1 in SOW 1-15 months)

Technical Objective 2

Examine the response of transformed and non-transformed breast cell lines to cytotoxic treatments in particular to correlate the operation of normal checkpoint controls with cell survival or death. (Equivalent to Task 2 in SOW 12-30 months)

Technical Objective 3

Determine the importance of checkpoint control proteins in cytotoxic sensitivity by genetic manipulation of WEE1, CDC25, Cyclin B and CDC2 *in vivo*. (Equivalent to Task 3 in SOW 30-48 months)

The first tasks we defined in this grant was the characterization of the CDC2 and Cyclin B proteins in normal and transformed breast epithelial cell lines. Previously published data suggested that CDC2 and Cyclin B proteins are significantly over-expressed in transformed breast epithelial cells compared to normal controls (1). We reasoned that the high levels of CDC2 and Cyclin B proteins in these cells might be expected to lead to deranged regulation of the G2/M transition and hence to some form of mitotic catastrophe. However the fact that these cell lines are viable when cultured *in vitro* suggests that the proteins that regulate CDC2/Cyclin B are able to maintain control of CDC2/Cyclin B despite the increased level of expression and despite alterations in the timing of expression. Our initial experiments therefore centered on examining the abundance of CDC2 and cyclin B protein in a number of

transformed breast epithelial cells compared to normal breast cells grown under identical conditions. We examined the abundance of CDC2, Cyclin B and Cyclin A in 5 different transformed breast cell lines and normal breast cells. (Normal breast epithelial cells (184) were obtained from Dr. Martha Stamer (UCSF) through Dr. Steve Reed (TSRI).) Samples were harvested when plates were ~50% confluent. The samples were normalized before loading 75µg of protein. After probing for cell-cycle related proteins the blot was re-probed with an antibody that recognizes Hsp70, a control protein that would not be expected to vary significantly on transformation. The normal cell extract was loaded at 75µg and 250µg in order to assess the extent of increased abundance in the transformed cells. The blots (shown in Figure 1) were developed by enhanced chemiluminescence and films were subjected to quantitation by densitometric scanning. This analysis showed that in one of the transformed cell lines (Zr75T) which grows very slowly in our hands had significantly less Cyclin A and Cyclin B than the control cells. The lower levels of Cyclin A and B in this cell line is consistent with its slow rate of proliferation. The remaining cell lines were doubling at approximately the same rate as the normal control population. CDC2, Cyclin A and B was found at increased levels in one cell line (MDA-MB-231). All three proteins were judged to be present at 3-4 times the levels found in normal cells. A modest (2-fold) increase in cell-cycle related proteins was also observed in two other cell lines (MDA-MB157 and T47).

Our analysis agreed in principle with previously published results, however the extent to which cell-cycle related proteins are over-expressed was not as great as previously reported (1). Despite the over-abundance of G2/M controlling proteins in some of the breast cell lines we have examined, we were concerned that these alterations would not be sufficient to yield a clear difference between normal and transformed breast cells when treated with cytotoxic agents and that any differences we might observe could not be directly correlated with a change in CDC2/Cyclin B activity profile. Furthermore, there is some variation in the reported abundance of cyclins between different workers using the same cell-lines (1, 2). Therefore, we were concerned that slight differences in growth properties might give rise to significant differences in the apparent abundance of cyclins in asynchronous cultures. We reasoned that it would be advantageous to create a situation in which the proteins that control G2/M are over-expressed (by transfection) in a controlled manner. Furthermore, by using test transfected and control transfected cells we would be able to correlate alteration in cell physiology directly to the gene product being manipulated. Once we have achieved a system that allows controlled expression of the gene products we are interested in, we will return to Technical objective 2. This will allow us to simultaneously examine the response of transfected, transformed and normal breast cells to cytotoxic treatments and thereby correlate the operation of normal checkpoint controls with cell survival or death

We set about producing stable human cell-lines which over-express CDC2, CDC2AF, Cyclin B, CDC25 or WEE1. Initial experiments concentrated on the use of the tet repressible system developed by Gossen and Bujard (3). Using previously established conditions (3, 4) we were unable to establish cell-lines which expressed

the cDNAs of interest. Control experiments in which CD8 or bacterial B-galactosidase was introduced into the cells showed that the transfection and selection procedures were working. Although we cannot be certain why these experiments failed, it is possible that over-expression of these proteins is detrimental to the cells and that low level leaky expression under repressed conditions prevented the isolation of stable cell lines. Therefore, we have switched to two different systems. We are currently working on the use of a sheep metallothionein promoter based vector (pMT which was obtained from Dr. F Rauscher III, The Wistar Institute). We have finished making the necessary constructs and will be testing them by transfection shortly. We are also using a transient transfection system in which cells are co-transfected with, CD8, a cell-surface reporter protein that can be stained for cell-cycle analysis. Preliminary experiments in HeLa cells show that the constructs direct the over-expression of CDC2, Cyclin A, B and WEE1. Cells that have been transfected with the gene of interest and the marker protein CD8 were irradiated, harvested, processed and the cell-cycle distribution of CD8 positive cells scored relative to the CD8 negative cells. Any difference in the response of cells to irradiation will therefore be directly attributable to the over-expression of the given protein. We are currently working on the exact conditions that allow analysis of co-stained. This work is being carried out in HeLa cells. As soon as the technical details have been defined we will begin transfection of breast cell-lines.

Conclusions.

The results obtained from our analysis of the expression patterns of CDC2, Cyclin A and Cyclin B led us to alter the order in which Technical objectives 2 and 3 will be approached. They do not detract from our hypothesis that a more complete understanding of G2/M regulation is essential as the basis for improved treatment of breast and other cancers. We have had some technical difficulty in establishing cell-lines which over-express the cDNAs of interest. However, it is worth noting that these technical problems were anticipated in the original proposal and although they have slowed our progress we have no reason to expect that the alternative strategies that we outlined in the proposal will not succeed. We therefore plan to continue with the experiments described in the proposal.

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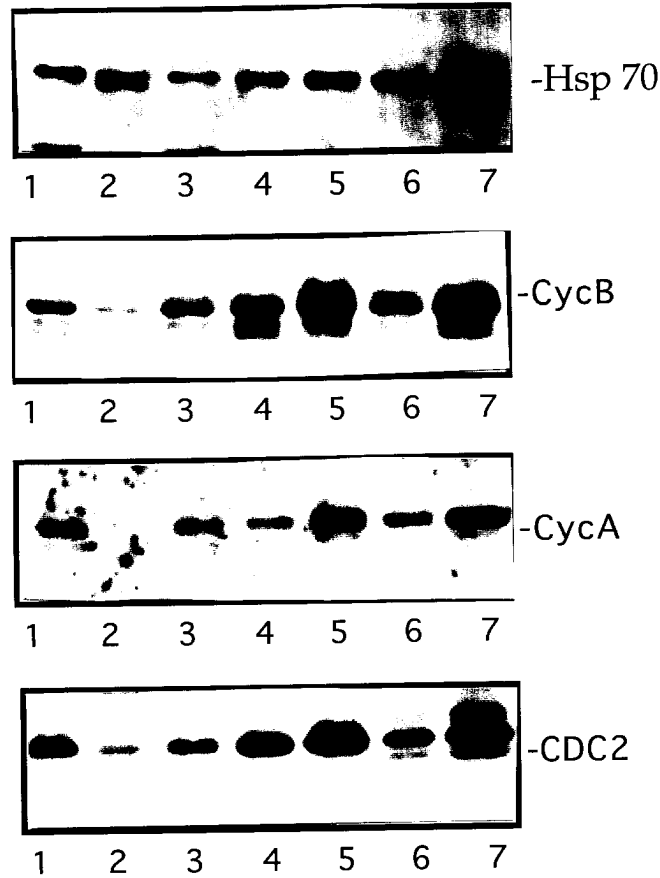
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APPENDIX

Figure 1



Expression of cell-cycle related proteins in normal and transformed human breast cell lines. Breast cells were grown to ~50% confluence the cells were harvested by trypsinization. Cell pellets were lysed by boiling in 4% SDS. The protein concentration was determined by Bradford reagent (Bio-rad). 75 μ g of each extract was loaded onto a 6-15% gradient acrylamide SDS gel. Proteins were transferred to nitrocellulose membrane using standard conditions. The nitrocellulose was probed with affinity purified antibody to human cyclin B, human CDC2, serum against human cyclin A and with monoclonal antibody to human Hsp70 (a gift from Dr. L. Karlson. TSRI). 75 μ g of transformed breast cell line MDA-MB-157 (lane 1), 75 μ g of transformed breast cell line Zr-75-30 (lane 2), 75 μ g of transformed breast cell line HS578T (lane 3), 75 μ g of transformed breast cell line T-47D (lane 4), 75 μ g of transformed breast cell line MDA-MB-231 (lane 5), 75 μ g of normal breast cells (184) (lane 6), and 250 μ g of normal breast cells (184) (lane 7).