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13. ABSTRACT (Maximum 200 Words)

The overall hypothesis that drives this project is that the involvement of particular tumor suppressor genes, such as BRCA1 and BRCA2, in familial breast cancer (BCa) provides important clues regarding the etiology of the far more common, sporadic form of this disease. These two breast cancer susceptibility genes play central roles in genome surveillance during the process of DNA replication in proliferating cells. We hypothesize that BCa progression is fueled by the accumulation of cancer-promoting mutations due to errors in genome duplication during S phase. According to our model, hypoxia in developing tumors leads to cell growth arrest during S-phase, and selects for cells that have bypassed the this S-phase checkpoint due to mutations in the ATR-hChk1 pathway. The specific aims of this project are: 91) to define the role of the ART checkpoint pathway in hypoxia-induced cytostasis, and (2) to determine whether defects in this checkpoint pathway promotes BCa progression, and confers sensitivity to killing by certain anticancer agents.

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INTRODUCTION

The identification of genome surveillance proteins, such as *BRCA1*, *BRCA2, ATM*, *hCHK1*, and *hCHK2*, as BCa susceptibility genes leads us to hypothesize that defects in S phase checkpoint function are major contributors to breast cancer development and/or progression. In a report that served as the background for this proposal, we demonstrated that deep hypoxia (0.1% O_2) caused cancer cells to arrest in S phase of the cell cycle, and that this arrest was accompanied by the activation of the ATR-hChk1 checkpoint pathway (*1*). These results led to the hypothesis that escape from the ATR-hChk1 –induced growth arrest will confer a proliferative advantage on malignant clones in the developing tumor. At the same time, these clones will gain an increased level of genomic instability due to the disruption of the ATR-dependent S phase checkpoint. Thus, hypoxia-induced S phase arrest may fuel BCa tumorigenesis in part by increasing the rate of gene mutation in the affected BCa cells.

This project is intended to further understand the interplay between tumor hypoxia-reoxygenation, the ATR-Chk1 checkpoint pathway, and malignant progression in human BCa. Our goal is to establish relevant in vitro culture systems involving human breast epithelial cells (hBrEC) that are either precancerous or fully malignant. These model systems will be used to dissect the roles of surveillance proteins such as ATR, hChk1 and BRCA1, as suppressors of tumor progression, and the impact of defective ATR-mediated checkpoint activation on genetic instability and the emergence of fully tumorigenic BCa clones. Finally, using the knowledge gained from these studies, we will explore rational therapeutic strategies targeted against cells bearing defective ATR-dependent S phase checkpoints.

BODY

Task 1: To define the role of the ATR checkpoint pathway in hypoxia-induced cytostasis, and to determine whether hypoxic adaptation promotes genetic instability in human BCa cells (Months 1-18).

During the first year of the proposal, we have, as proposed in the original grant application, invested considerable effort in generating the model systems needed to explore the interplay between hypoxia and genetic instability in developing BCa cells. We now have normal hBrEC cells, "premaligant" hBrEC-TAg cells, and fully transformed hBrEC-TAg/*Ras cells as described in the experimental methods. During this timeframe, we have established the apparatus needed to culture hBrEC cells and their transformed derivatives under conditions of "deep" hypoxia (0.1% O_2). In pilot studies, we used the MCF-7 BCa cell line as a preliminary model system to determine the effects of deep hypoxia on the viability and cell cycle distribution of BCa cells. As shown in **Figure 1**, hypoxia-reoxygenation caused a substantial decrease in the viability of MCF-7 cells. This drop in viability was exacerbated by treatment of the cells with siRNA directed against ATR, consistent with our hypothesis that ATR is important for the recovery of hypoxic cells from S-phase arrest. We are currently testing the idea that the cell death observed in these studies is due to unrepaired DNA damage leading either a general loss of cell viability, or to a more specific mechanism involving a catastrophic

mitosis (due to incompletely or erroneously replicated DNA) and consequent apoptotic cell death.

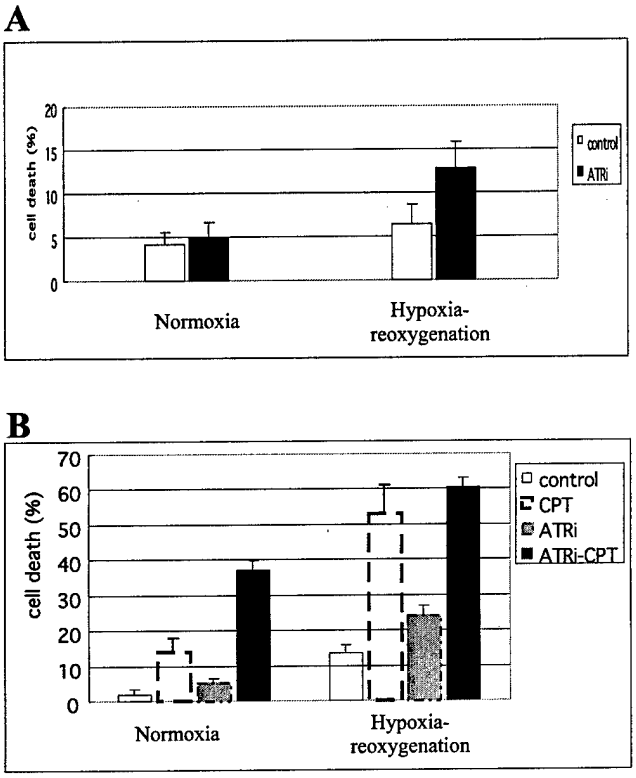

Fig. 1. A, Effects of severe hypoxia on camptothecin (CPT)-induced BCa cell killing. MCF-7 cells were seeded into 6-well plates 20 h prior to transfection with luciferase or ATR siRNA. After transfection, the cells were cultured for an additional 24 h, and then transferred into a 0.1% O_2 chamber at 37°C. Cells were cultured for 8 h under hypoxia, and were then shifted to normoxic conditions for 48 h. Cell viability was measured by trypan blue dye exclusion assay. **B,** siRNA transfection was the same as in A. Cells were treated with CPT (500 nM) for 15-20 min before transfer into the 0.1% O_2 culture chamber. After 8 h, the cells were cultured for 12 h under normoxic (20% O_2) conditions in the presence of CPT. The medium was removed, and the cells were cultured in fresh, drug-free medium for 48 h. Cell viability was assessed by trypan blue dye exclusion.

The results in **Figure 1** also show the effect of the anticancer drug camptothecin (CPT) on MCF-7 cell killing under normoxic versus hypoxic conditions. The original grant application stated that CPT, a topoisomerase I poison, was a potentially valuable therapeutic agent for killing of BCa cells that had adapted to hypoxia-induced S phase arrest through acquisition of mutations in the S phase checkpoint machinery. Consistent with this model, we found that the cytotoxic activity of CPT was enhanced when cells were exposed to hypoxia and subsequently allowed to recover under normoxic conditions. In the coming year, we will intensively examine the effects of CPT on the hBrEC lines that have just become available in the laboratory. Our studies to date indicate that topoisomerase I poisons and other S-phase specific cytotoxic agents merit further investigation as therapeutic agents for BCa cells bearing mutations in ATR, BRCA1, or other component of the S-phase checkpoint machinery in human cells.

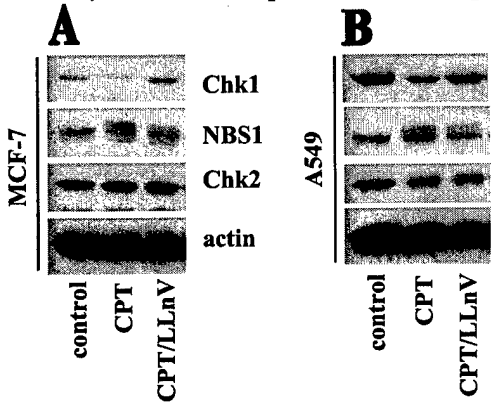

Fig. 2. Induction of hChk1 degradation by CPT. MCF-7 breast cancer (A) or A549 lung cancer (B) cells were treated for 8 h with 500 nM CPT. During the last 4 h, the indicated samples were exposed to the proteasome inhibitor (LLnV). The cells were harvested, and detergent-soluble proteins were separated by SDS-PAGE. Proteins were transferred to PVDF membranes and immunoblotted with the indicated antibodies.

During these studies with CPT, we made the unexpected finding that CPT exposure induces the downregulation of the checkpoint kinase, hChk1, in human BCa and lung cancer cell lines (**Figure 2**). Follow-up studies showed that the decrease in hChk1 was due to drug-induced ubiquitination and proteolysis of the hChk1 protein (**Figure 3**). To our studies, these findings are entirely novel, and merit intensive investigation, in that they may well explain, at least in part, the therapeutic selectivity of CPT in human cancer patients. In principle, CPT and related topoisomerase I poisons should show indiscriminate cytotoxic activities against all proliferating cells, regardless of whether they exhibit a normal or malignant phenotype. However, the drug clearly does show a useful therapeutic index in humans, and more than a two-log variation in cellular sensitivity to CPT is found in the NCI's 60-cell line screen (see the Developmental Therapeutics Program website at http://dtp.nci.nih.gov). These studies have direct implications for the present project, in that the loss of Chk1 induced by CPT may well interfere with the recovery of stalled replication forks in BCa cells from hypoxia-induced S phase arrest. This model will be pursued vigorously during the coming year, and could provide major insights into the therapeutic activity of CPT in BCa and other solid tumors.

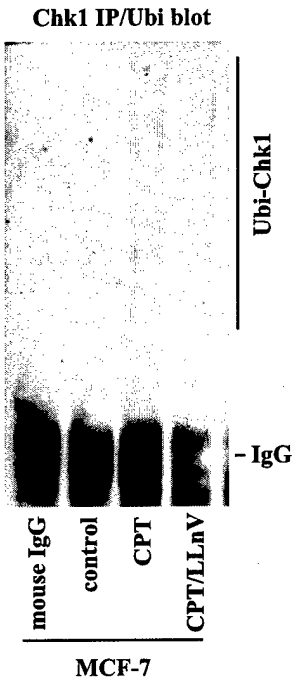

Fig. 3. CPT treatment induces the ubiquitinoylation of hChk1 in MCF-7 BCa cells. MCF-7 cells were treated with 500 nM CPT and 10 uM LLnV as described in Fig. 2. Cellular extracts were immunoprecipitated with anti Chk1 antibodies at 4°C. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-ubiquitin antibodies.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of hBrEC cell lines in the laboratory.
- Establishment of the hardware that will allow us to culture the hBrEC cell lines under hypoxia and nutrient-deprived conditions.
- Demonstration that loss of ATR function impairs the recovery of BCa cells from hypoxia-induced S phase arrest.
- The novel finding that CPT (and not other anticancer agents tested to date) triggers the downregulation of hChk1 in human breast and lung cancer cell lines.

REPORTABLE OUTCOMES

No reportable outcomes from project year 1.

CONCLUSIONS

During project year 1, we have laid the groundwork needed to proceed to the next phase of this project. With the hBrEC cell lines in hand, we are now positioned to explore the role of hypoxia-reoxygenation in genetic instability during BCa development, which is the major goal outlined in Task1 of the original application. We have made the highly novel finding that CPT causes the proteolytic degradation of hChk1 in human BCa cells, and have preliminary evidence that the loss of hChk1 enhances the cytotoxic effects of CPT by preventing cellular recovery from S-phase arrest induced by hypoxia or other environmental stresses. The latter studies will provide critical insights into the mechanism of action of an important class of anticancer agents, and may well define a mechanism whereby tumor cells acquire resistance to these drugs. Year 2 of this project will yield some major reportable outcomes related both to the hypoxia-genetic instability model and the mechanism of BCa cell killing by CPT.

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1. E. M. Hammond, N. C. Denko, M. J. Dorie, R. T. Abraham, A. J. Giaccia, *Molecular and Cellular Biology* **22**, 1834.