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13. ABSTRACT ***(Maximum 200 Words)***

During this three year IDEA Award we have made progress towards all three Technical Objectives. Despite significant efforts we encountered scientific problems isolating novel cDNAs encoding human homologs of yeast DNA damage response genes *RAD9* and *DUN1* during year 1 and year 2. In contrast, two hybrid screens resulted in the isolation of human homologs of *RAD18* and *RAD21*. Thus, the focus over year 2 and 3 has been the characterization of the human Rad21 protein in mammalian cells and breast cancer samples.

Alterations in expression of human Rad21 mRNA and protein in human breast cancer cell lines was detected. Using antibodies produced by this grant we are now determining expression of RAD21 protein in human cancers stratefied for aneuploidy. We also demonstrated that Rad21 is cleaved upon induction of the apoptotic pathway (as opposed to DNA damage itself). The cleavage site has been biochemically identified and the characteristics of the cleavage enzyme determined. Cleavage also results in mislocalization of the protein. This cleavage product may play a role in signalling subsequent events in apoptosis or result in aneuploidy which is associated with a poorer prognosis in breast cancer patients.

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A. Introduction

The goal of this project is to further define at a molecular level the human gene products required for the normal cell cycle response after DNA damage. The checkpoint response is a fundamental mechanism by which cells control their cell division cycle after experiencing DNA damage from radiation. This response results in an arrest in the G1, S and G2 phases of the cycle until damage is repaired. This checkpoint response is conserved among eukaryotes including the budding yeast *Saccharomyces cerevisiae.* Human cells have an additional response which results in apoptosis after DNA damage. In our application, we proposed to exploit the conservation between yeasts and humans to isolate human checkpoint genes by large scale complementation screens and homology searches isolating novel human cDNAs which can complement yeast G2 checkpoint mutant strains. Subsequent Technical Objectives were directed towards understanding the structure and expression of these genes in both normal and malignant mammary cells. The final Technical Objective was to assay changes in expression of these genes after DNA damage. In this final report we detail progress during the three years of this award towards all three objectives. This grant was co-funded with a companion CDA Award for the PI, Dr. Sharon Plon (grant #DAMD17-97-1-7284).

B. Progress toward completing the proposed Technical Objectives.

RESULTS

Technical Objective 1 - Isolation of additional human G2 checkpoint genes.

a. **Complementation Assay:** As previously described, this work comprised the first year of the IDEA award. Human cDNA libraries were screened by expression in yeast *rad9, cdc9-8* and *mec1, cdc9-8* strains as described in the application. Despite extensive screening no human cDNAs which could reproducibly rescue the checkpoint defect of these strains was identified

b. As described in the year 1 progress report, a second approach to isolating human checkpoint genes utilized homologous regions between evolutionarily distant species (*S. cerevisiae and S. pombe)* to develop degenerate PCR based primers. For example, a fission yeast homolog of *RAD9* named *rhp9* was published. Alignment of those sequences revealed areas of homology that may suggest conserved regions of the protein. One such area is in the carboxy terminus consistent with the known BRCT domain. During year 1 we made a major effort to develop a series of degenerate PCR primers to these regions but the amplified sequences obtained did not demonstrate additional regions of homology to the *S. cerevisiase RAD9* or *DUN1* genes. We proposed to try direct amplification from human mammary cDNA and mammary carcinoma cDNA in order to prevent any bias against long messages or "unclonable" sequences that might not be represented in a cDNA library.

During year 2, amplification was performed on multiple human cDNA samples including normal mammary cells, breast carcinoma cell lines and ovarian cDNA. The resulting PCR products were cloned and sequenced. No sequences obtained provided evidence for additional regions of homology between the isolated sequence and the yeast *RAD9* or *DUN1* genes respectively. Thus, the use of direct cDNA sources from either benign or

malignant mammary cells did not result in isolation of novel human cDNAs for these checkpoint genes. During year three of the grant the human genome project has neared completion, searches of these resources using a variety of techniques has not identified human genes or cDNAs with significant homology (outside the BRCT domain) for *RAD9* and *DUN1*. Thus, these two genes may not have a direct homolog in the human genome, but their functions may be carried out by other types of proteins, e.g., p53 in the case of Dun1 and BRCA1 for Rad9.

c. **Isolation of human *RAD21* cDNA.**
We used a different form of genetic screen including two-hybrid screens in yeast for human cDNAs in the DNA damage checkpoint and repair response have been accomplished. As part of those screens cDNAs encoding the human homolog of *S. pombe RAD21* and *S. cerevisiae RAD18* were isolated. The *RAD21* sequence has been previously reported in the literature although the human gene and protein has not been thoroughly characterized previously.

Technical Objective 2A – Checkpoint gene structure and expression in human breast cancer cell lines.

As accomplished during year 1 of this grant, RNA, DNA and protein were derived from eight human mammary derived cell lines, MCF10A, MCF7, MDA-MB-157, MDA-MD-231, MDA-MB-136, BT-20, HBL100 and SKBR-3 grown in culture under controlled conditions. Analysis of expression of *RAD21* reveals increased expression at the RNA level in breast cancer cell lines, specifically MDA-MB-436 and SK-BR-3 in comparison to the HMEC controls.

We then pursued the development of reagents in order to study the expression of human Rad21 in mammary cell lines and breast cancer samples. A polyclonal antibody was obtained towards the end of year 1. During year 2, we obtained a large stock of this anti-sera and obtained affinity purified antibody that is being used in immunohistochemistry and immunoflourescence experiments. We also isolated a monoclonal antisera for additional studies (eg, immunoprecipitation with polyclonal antisera followed by Western blotting with monoclonal antibody). Monoclonal antibody which recognizes the human Rad21 protein was obtained by immunization with a GST-Rad21 fusion protein. The monoclonal is functional in both Western blot and immunoprecipitation assays.

During year 2, we performed Western blots analysis of the cell lines listed above with the anti-Rad21 polyclonal antibody. Expression of Rad21 is variable in human breast cancer cell lines. In particular, the level of Rad21 protein is elevated in MCF-7 and SK-BR-3 cell lines in comparison to MCF10F cells. In contrast Rad21 protein is absent in the BT-20 cell line suggesting a possible mutational mechanism. These results provided a rationale for further analysis of Rad21 protein expression in human breast cancers with known levels of aneuploidy.

In order to accomplish this last goal, during year 3 we have collaborated with the Baylor Breast Cancer Center (Kent Osborne, Director). They have now tested the anti-Rad21 antibody on a pilot sample of breast cancer samples and optimized the protocol to provide excellent detection by immunohistochemistry after antigen retrieval. This

protocol is now being used to systematically determine the expression of Rad21 in breast cancer samples as a function of chromosome number or aneuploidy. These studies are continuing after the end of year 3.

Technical Objective 3 - Determination of Changes in Response to Radiation of a Human Breast Cancer Cell Line upon Expression of Human Checkpoint Genes.

We used cDNA probes and antisera to study Rad21 expression in normal and checkpoint deficient cell lines after DNA damage. *RAD21* mRNA is not up or down regulated in response to ionizing radiation. Western analysis revealed that after ionizing radiation there was no change in the major bands representing Rad21 in several cell types including the totally checkpoint deficient A-T cells. Thus, we did not see evidence for a change in phosphorylation or expression after ionizing radiation. However, some cell types demonstrated the production of a lower molecular weight band consistent with cleavage.

Further analysis revealed that cells in the early stages of apoptosis demonstrated cleavage of the endogenous Rad21 protein. Induction of apoptosis in multiple human cell lines results in the early (4 hours post insult) generation of a 64 kD cleavage Rad21 product. Identity of this product is confirmed by recognition by affinity purified polyclonal and monoclonal antibody to human Rad21. This product is detected after induction of apoptosis by both DNA damaging agents (ionizing radiation and topoisomerase inhibitors) as well as non-DNA damaging agents (cycloheximide treatment and cytokine withdrawal). Induction of apoptosis is assayed by cleavage of the endogenous PARP protein and morphological changes consistent with apoptosis. In addition, equivalent doses of ionizing radiation in cells which are resistant to apoptosis do not generate this band; thus, it is not a simple byproduct of DNA damage. Addition of caspase inhibitors to the cells blocks the cleavage of Rad21 after an apoptotic signal. Given the role of rad21 in chromosome cohesion, the cleavage product may signal subsequent events of apoptosis including DNA degradation. Further evidence for signalling is provided by the finding that the Rad21 cleavage product is translocated to the cytoplasm after cleavage.

The finding that Rad21 is regulated during apoptosis (as opposed to DNA damage itself) is a novel finding. This has lead to a new hypothesis that there may be a direct link between the development of aneuploidy (given Rad21's role in chromosome cohesion) and apoptosis. During year 3 we used our biochemical reagents to purify the cleaved this fragment. The isolated peptides were then sequenced in order to determine the specific cleavage site within the protein. This site is unique from the previously identified cleavage that oocurs at the end of mitosis. We have also identified that the cleavage is sensitive to the presence of a particular set of phosphatase inhibitors. Current experiments are underway to determine whether expression of either the full length or cleaved product in the cytoplasm will induce apoptosis.

Personnel Supported by this Award over the Term of the Grant

Debananda Pati - Assistant Professor
Anu Gannavarapu - Research Assistant II
Allison Franz - Research Technician II
Amy Barbour - Lab Assistant
Luke Engelking - Lab Assistant
Margot Perez - Research Associate
Shirong Luo - Research Tech III
Rita Wilkerson - Lab Tech II

C. Key Research Accomplishments

- Extensive degenerate PCR cloning to obtain human homologs of *RAD9* and *DUN1* completed utilizing cDNA sources from both normal mammary cells and breast carcinoma cells.
- Polyclonal antibody to human Rad21 protein produced and affinity purified.
- Monoclonal antibody to human Rad21 protein produced.
- Determined expression of Rad21 protein assayed in human mammary and breast cancer cell lines.
- Development and optimization of technique to assay Rad21 expression in human breast cancers accomplished.
- Identification of Rad21 cleavage as an early step in apoptosis.
- Identification of Rad21 cleavage site after induction of apoptosis.

D. Reportable Outcomes:

- Results of this project presented at three meetings:
  - International Meeting on Forkhead/Winged Helix Proteins" Analysis of *CHES1*, A Human Checkpoint Suppressor," Scripps Institute, La Jolla, CA, November, 1998
  - Cold Spring Harbor Cell Cycle Meeting, May, 2000, Cold Spring Harbor New York (abstract attached).
  - DOD Era of Hope Meeting, June 2000, Atlanta GA.
- Monoclonal antibody to human Rad21 protein produced.
- Manuscript describing regulation of Rad21 during apoptosis is in preparation.
- DOD Concept, IDEA award and CDA award grant applications were selected for award to co-Investigator, Debananda Pati, to explore the newly identified link between apoptosis and chromosome cohesion.

E. Conclusions

The most challenging aspect of this project was the isolation of novel cDNAs encoding human homologs of yeast DNA damage response genes. Complementation of the yeast mutant *rad9* did not yield human cDNAs with significant homology. Major efforts to isolate cDNAs by degenerate PCR strategies for *RAD9* and *DUN1* during year 1 and year 2 were also not successful and analysis of the human genome sequence suggests that these homologs may not exist. In contrast, two hybrid screens using known human DNA damage response/cell cycle genes did result in the isolation of human homologs of *RAD18* and *RAD21*. Thus, the focus over year 2 and 3 has been the characterization of the human Rad21 protein in mammalian cells and breast cancer cells.

The subsequent objectives focused on determination of whether cDNAs isolated in genetic screens are altered in expression or structure in breast cancers. As described above we do see alterations in expression of human Rad21 mRNA and protein in human breast cancer cell lines. This has lead to development of immunohistochemistry techniques and ongoing experiments on primary human breast cancer samples with the

Baylor Breast Care Center investigators. These studies will in particular look for a correlation between Rad21 expression and aneuploidy.

The results of Technical Objective 3 have been most surprising to date. We did not see alteration in *RAD21* mRNA or Rad21 phosphorylation in human cells exposed to DNA damage. However, we did detect specific cleavage of the protein. This has lead to determination that induction of the apoptotic pathway (as opposed to DNA damage itself) induces specific cleavage of the human Rad21 cohesin protein. The biochemical characteristics of the cleavage have been identified and the cleavage site in the protein determined. This cleavage product may play a role in signalling subsequent events in apoptosis or result in aneuploidy in cells that survive the apoptotic response.

Appendix 1 – Abstract from Cold Spring Harbor Laboratories – Cell Cycle Meeting

CLEAVAGE OF HUMAN Rad21 COHESIN PROTEIN: POTENTIAL ROLE IN EARLY APOPTOSIS

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Sister chromatid cohesion during DNA replication plays a pivotal role for accurate chromosome segregation in eukaryotic cell cycle. Analysis of Rad21 function in fission yeast and *SCC1/MCD1* in budding yeast have demonstrated that it is required for appropriate chromosome segregation during normal mitotic cell cycles and double strand break repair after DNA damage. In budding yeast sister chromatid separation is promoted by the cleavage of the cohesin sub-unit Scc1 and may involve ubiquitin-mediated proteolysis of regulatory molecules. In a two-hybrid screen for potential targets of human Cdc34 (hCdc34) ubiquitin-conjugating enzyme, we have isolated human Rad21 (hRad21) as an hCdc34 interactor. Transfection studies in mammalian cells have indicated physical association of hCdc34 and hRad21 using co-immunoprecipitation experiments. Level of hRad21 was significantly enhanced in the presence of proteasome inhibitors, indicating the involvement of ubiquitin-mediated proteolysis. In a parallel set of studies to analyze the role of Rad21 in mammalian cells after DNA damage, we have identified a novel regulation of hRad21 protein in apoptosis. Induction of apoptosis in multiple human cell lines results in the early (4 hours post insult) generation of a 64kDa cleavage hRad21 product. Although Rad21 is a nuclear protein the cleaved 64 kDa product is found in both nuclear and cytoplasmic fractions. Identity of this product is confirmed by recognition by affinity purified polyclonal and monoclonal antibody to hRad21. This product is detected after induction of apoptosis by both DNA damaging agents (ionizing radiation and topoisomerase inhibitors) as well as non-DNA damaging agents (cycloheximide treatment and cytokine withdrawal). In addition, equivalent doses of ionizing radiation in cells which are resistant to apoptosis do not generate this band; thus, it is not a simple byproduct of DNA damage. Given the role of Rad21 in chromosome cohesion, this cleavage product may signal subsequent events of apoptosis including DNA degradation. A role for Rad21 in apoptosis has been further strengthened by identification of a number of genes involved in apoptosis as interactors of hRad21 in a two-hybrid assay. In summary, ubiquitin-mediated proteolysis may play a role in the cleavage of hRad21 during metaphase-anaphase transition. In addition to previously described functions of Rad21 in chromosome segregation and DNA repair, cleavage of the protein is an early event in the apoptotic pathway. These results provide the framework to identify the physiologic role of hRad21 function in the apoptotic response of normal and malignant cells.

Appendix 2 – Abstract from DOD Era of Hope Meeting – 2000.

CHARACTERIZATION OF THE HUMAN RAD21 COHESIN PROTEIN AND DETECTION OF SPECIFIC CLEAVAGE EARLY IN APOPTOSIS

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The goal of this project is to identify human homologs of yeast genes proven to play a role in the response to DNA damage. Isolation of these genes will then allow characterization of their expression and activity in both normal and malignant cells exposed to DNA damage. Our laboratory has employed a number of techniques to identify human homologs of these genes including yeast two hybrid screens with human cell cycle genes. We have identified the human homolog of the yeasts Rad21/*SCC1/MCD1* genes. Prior analysis of Rad21 function in fission yeast and *SCC1/MCD1* in budding yeast have demonstrated that it is required for double strand break repair after DNA damage and appropriate chromosome segregation during normal mitotic cell cycles.

We have now characterized the expression of human Rad21 RNA and protein in mammalian cells in response to DNA damage and in human breast cancer cell lines. Expression of Rad21 is variable in human breast cancer cell lines. In particular, the level of Rad21 protein is elevated in MCF-7 and SK-BR-3 cell lines in comparison to MCF10F cells. In contrast Rad21 protein is absent in the BT-20 cell line suggesting a possible mutational mechanism. These results provide a rationale for further analysis of Rad21 protein expression in human breast cancers with known levels of aneuploidy. Analysis of mammalian cells after DNA damage has identified a novel regulation of Rad21 protein in apoptosis. Induction of apoptosis in multiple human cell lines results in the early (4 hours post insult) generation of a 64 kD cleavage Rad21 product. Identity of this product is confirmed by recognition by affinity purified polyclonal and monoclonal antibody to human Rad21. This product is detected after induction of apoptosis by both DNA damaging agents (ionizing radiation and topoisomerase inhibitors) as well as non-DNA damaging agents (cycloheximide treatment and cytokine withdrawal). In addition, equivalent doses of ionizing radiation in cells which are resistant to apoptosis do not generate this band; thus, it is not a simple byproduct of DNA damage. Given the role of rad21 in chromosome cohesion, this cleavage product may signal subsequent events of apoptosis including DNA degradation. In summary, expression of the human cohesin protein Rad21 is altered in human breast cancer cell lines and in addition to previously described functions in chromosome segration and DNA repair, cleavage of the protein is an early event in the apoptotic pathway. These results provide the framework to identify the importance of Rad21 function in the apoptotic response of breast cancer cells to treatment.

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