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13. ABSTRACT *(Maximum 200 Words)*

The HER-2/neu extracellular domain (ECD) is shed from breast carcinoma cells in culture and is found at elevated levels in sera of patients with metastatic breast cancer where it may predict poor prognosis. Our studies show that an N-terminally truncated HER-2/neu product, p95, is produced when the ECD is shed, that p95 has kinase activity, and is expressed to a greater extent in breast cancer patients with lymph node metastasis. **Purpose.** The objective of this proposal is to directly test the hypothesis that shedding of the extracellular domain of HER-2/neu, which creates the truncated p95 kinase, promotes oncogenesis. **Scope.** The effect of shedding to oncogenesis will be examined by further characterizing the control of shedding and genetically altering shedding activity to test the impact on tumorigenesis and oncogenesis. **Results:** We have developed and characterized strategies for generation of mutants of p185HER-2 to alter shedding. Moreover, to identify the shedding enzyme, we expressed p185HER-2 in cells that are genetically deficient in the disintegrin metalloprotease, TACE. Our results suggest that TACE does not function in shedding of p185HER-2.

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INTRODUCTION

Subject: The HER-2/neu extracellular domain (ECD) is shed from breast carcinoma cells in culture and is found at elevated levels in sera of patients with metastatic breast cancer where it may predict poor prognosis, response to adjuvant endocrine and chemotherapy, and allow tumor cells to escape immune surveillance. Our studies show that an N-terminally truncated HER-2/neu product, p95, is produced when the ECD is shed, has kinase activity, and is expressed to a greater extent in breast cancer patients with lymph node metastasis (1). **Purpose:** The objective of this proposal is to directly test the hypothesis that shedding of the extracellular domain of HER-2/neu and creating of the truncated p95 kinase promotes oncogenesis. **Scope:** To examine the impact of shedding on oncogenesis: (a) We will genetically alter shedding of HER-2/neu. To alter shedding, deletion and domain replacement mutants will be constructed within the HER-2/neu juxtamembrane cleavage domain. The mutations to be made will be based on known structural determinants of shedding defined through studies of diverse transmembrane proteins. A second approach to genetically alter shedding will be pursued by expressing HER-2/neu in cells that are null for shedding enzyme. (b) The impact of altered shedding to oncogenesis will be examined in cell culture and animal models. The transforming activity of HER-2/neu with genetically altered levels of shedding will be examined by well-established cell culture models of transformation, by tumorigenesis assays in nude mice, and by metastatic potential in immune compromised mice.

BODY

STATEMENT OF WORK

The following outlines the approved statement of work to be conducted in the first year and the progress we have made in this direction.

Task 1. Genetically alter the proteolytic shedding of p185HER-2/neu (months 1-24).

A major strategy to be used to examine whether shedding of the extracellular domain of p185HER-2 affects tumorigenesis was to genetically alter the structure of the juxtamembrane domain of p185HER-2 to either enhance of block shedding. The mutants were then to be characterized for their shedding activity and for their effects on p185HER-2 kinase activity. Once they were characterized, the next plan was to stably transfect the shedding mutants into different cells and to eventually examine whether shedding correlates with tumorigenesis. To construct mutants with altered shedding activity, we proposed to delete the juxtamembrane stalk of p185HER-2 to inhibit the shedding activity and to swap the juxtamembrane domain with that of other receptors with the goal of enhancing shedding. We have spent the past year trying to genetically alter the juxtamembrane stalk of p185 HER-2. One plan was to use PCR mutagenesis to delete the 12 residue stalk of HER-2/neu, which is bordered by the conserved Cys at position 642 and the transmembrane domain beginning at Ile 654. The approach we used was to PCR amplify the sequence 5' to the desired deletion site between Cys 642 and Pro 643 using a forward primer that spans the start codon, and a reverse primer that is complementary to the desired deletion site and that also incoporates an AatII restriction site. This PCR product was then digested with AatII and then ligated with the gel purified fragment of p185HER-2 cDNA that was

digested with the same enzyme. This enzyme cuts at a unique site in the p185HER-2 cDNA, AatII, that is immediately adjacent to the transmembrane coding sequence. The ligated product, cloned into the pcDNA 3.1 mammalian expression plasmid, was then sequenced. Initial analysis of the sequence suggested the correct product. The expression plasmid with the p185HER-2 juxtamembrane deletion mutant was examined for expression by transient transfection into Cos-7 cells. Production of the product was monitored by Western blot analysis using an antibody specific for the C-terminus of p185HER-2. Initial attempts at expression failed to reveal a product detectable by Western analysis whereas expression of the wildtype p185HER-2 in pcDNA 3.1 was easily detected. We initially suspected that the mutant protein was unstable because of the juxtamembrane deletion. We next reexamined the nucleotide sequence of the deletion mutant. After repeated sequencing attempts we detected a single point mutation that introduced a stop codon in the region that encodes the extracellular domain of p185HER-2 presumably creating a truncated protein. This mutation was found to be introduced as a PCR artifact. A second attempt at generating the juxtamembrane deletion mutant using the same PCR strategy and the Pfu turbo polymerase, which has proof reading activity, resulted in creation of the identical point mutation. We concluded that the PCR cycling conditions were somehow enhancing the probability of creating this particular mutant. We are now in the process of altering cycling conditions in order to avoid creating this point mutation.

Another plan for generating a mutant with altered shedding activity was to swap the juxtamembrane domain of tumor necrosis factor-α precursor (preTNFα) with that of p185HER-2. The shedding of TNFα is well characterized and is efficiently induced by addition of tumor promoters (2). In contrast p185HER-2 shedding is very slow and is not inducible by tumor promoters (1,3). Replacement of the p185 juxtamembrane domain with that of TNFα should create a p185HER-2 mutant with greatly enhanced inducible shedding. The strategy we used was to digest the p185HER-2 cDNA with AatII, which cuts at a unique site immediately adjacent to the p185HER-2 transmembrane coding sequence. The next step was to have an oligonucleotide synthesized that is identical to the 36 nt sequence encoding the TNF-α stem sequence that also contains an AatII restriction site at each end. The oligonucleotide was then ligated into the AatII digested p185HER-2 cDNA. This has been accomplished and we are now in the process of sequencing different clones to identify one with the correct orientation. In the process of constructing this mutant, we became aware of a manuscript recently published by Codony-Servat et al., (3) in which they constructed a swap of the juxtamembrane domain of p185HER-2 for that of proTGFα, with the similar goal of modulating the shedding of p185HER-2. They showed that the p185HER-2 mutant indeed had tumor promoter induced shedding at a much greater rate than the wildtype p185HER-2. Their findings validate this strategy. We are in the process of contacting them to try and obtain their mutant. If we cannot obtain this mutant, we will follow their strategy to create the proTGFα juxtamembrane swap since it's enhanced shedding capacity has been established. Although Codony-Servat et al., (1999) showed that tumor promoter induced shedding of this mutant, they did not characterize its kinase activity or other properties. This will need to be accomplished to sort out whether properties of this mutant protein other than shedding are affected by domain substitution.

In the last year of funding, we also became aware of a report by Siegel et al., (4) describing an alternative splicing event that results in deletion of an exon. The removal of the exon caused a deletion of 16 amino acid residues adjacent to the transmembrane domain. This deletion effectively removed the stem sequence. The authors of this paper reported that this deletion corresponded to enhanced transforming activity but did not conduct a biochemical characterization. Interestingly, Elenius et al., (5) reported a similar alternative splicing event in which 10 amino acid residues were spliced out of the juxtamembrane region of the HER-4 receptor tyrosine kinase. This alteration affected the shedding activity of the HER-4 receptor suggesting the possibility that the comparable alternatively spliced form of p185 HER-2 may also have altered shedding activity. Based on this finding, we sought to identify cells that express the alternatively spliced form of p185 HER-2 and to clone the alternatively spliced form to characterize its shedding activity. Two carcinoma cell lines that overexpress HER-2, but have vastly different levels of shedding (1) were investigated for

the presence of the alternatively spliced form. To accomplish this we conducted reverse transcriptase PCR (RT-PCR) on SKOV-3 cells that overexpress HER-2 but do not shed and on SKBR-3 cells that overexpress and have shedding activity, using primers that flank the juxtamembrane region. The results of this analysis revealed a single PCR product of identical size from each cell line. Nucleotide sequencing demonstrated identical sequences. The conclusion is that the defect in shedding in SKOV-3 cells is not caused by altered structure of the HER-2 product either due to alternative splicing or to mutation. Therefore, the shedding enzyme may be deficient in these cells. Because the alternatively spliced form discovered by Siegel et al (4) may provide important information regarding the control of shedding and may be used to exam the role of shedding in oncogenesis, we will investigate additional cells for the presence of the spliced variant. In addition, in the upcoming year, we will try and construct a mutant that mimics the alternatively spliced form to exam its shedding activity and to investigate its oncogenic potency. To summarize, we have made advances in further understanding and characterization of shedding of p185HER-2. We have encountered problems in the construction of shedding mutants of p185HER-2 as described above. We now have strategies in place to surmount these obstacles. The construction of the mutants and their expression in mammalian cells should be forthcoming.

Another task outlined in the statement of work was to genetically alter shedding through the use of cells that are null for shedding enzyme. Our preliminary studies revealed that shedding of p185HER-2 was blocked by TAPI (1) a metalloprotease inhibitor that was originally designed to inhibit TACE, the metalloprotease that causes shedding of proTNF-α (5). We obtained the TACE null cell line from Dr. R. Black (6). The first strategy was to transfect TACE null and wildtype mouse fibroblasts with the wildtype HER-2/neu expression plasmid, and select stably transfected colonies.

Further, the plan was to analyze stably transfected cells for HER-2/neu expression and compare stably transfected TACE null and wildtype cells lines for shedding, for synthesis, transport, and kinase activity (months 6-12). We were able to obtain stably transfected cells, however p185HER-2 expression was somewhat low. This made it difficult to compare the extent of p185HER-2 shedding from the TACE null versus wild type cells. Most of the data indicate that there is no difference in the extent of shedding from the TACE null cells, however shedding from both cell lines was very low. The conclusion is that TACE may not be the enzyme involved in p185HER-2 shedding, but it is difficult to get a definitive answer from this system. We have no plans at this point to pursue the goal of establishing shedding enzyme negative cells. This will require additional work, outside the scope of this grant, to identify the enzyme or enzymes responsible for shedding of p185HER-2.

KEY RESEARCH ACCOMPLISHMENTS

- Successfully constructed a juxtamembrane deletion mutant. (We are in the process of eliminating the artifactual mutation introduced.)
- Identified a recently discovered mutant p185HER-2 with the juxtamembrane domain swapped. This mutant has enhanced shedding activity. (We have requested this mutant. If we cannot obtain it, we will constructing the mutant in the lab.)
- Have determined that TACE is not likely the proteolytic enzyme involved in shedding of p185HER-2. Similar p185HER-2 shedding activity was observed in TACE null versus wildtype cells stably transfected with HER-2.
- Have determined that the defect in shedding from the SKOV-3 cells is not due to altered p185HER-2 structure and therefore may be caused by reduced levels of shedding enzyme.
- Have not been able to detect the alternative spliced product of p185HER-2 with the altered juxtamembrane domain in two carcinoma cells lines.

REPORTABLE OUTCOMES

- Manuscripts, abstracts, presentations:
  None.

- Patents and licenses applied for and/or issued:
  None
- Degrees obtained that are supported by this award:
  None
- Development of cell lines, tissue, or serum repositories:
  None
- Informatics such as databases and animal models:
  None
- Funding applied for based on work supported by this award:
  None
- Employment or research opportunities applied for and/or received :
  None

CONCLUSIONS

To summarize, we have made advances in further understanding and characterizing the process of shedding of the extracellular domain ofp185HER-2. We have developed strategies for constructing deletions mutants and domain substitution mutants to alter the extent of shedding. Because all the background work has been accomplished to construct these mutants, we should be in a position to characterize their activity and to begin testing their oncogenic potency in the next year. We have encountered problems in the construction of shedding mutants of p185HER-2, but we now have strategies in place to surmount these obstacles. The hypothesis that TACE may be the enzyme involved in shedding of HER-2 and the plans to use TACE null cells to control shedding have not worked. Our studies suggest that a shedding enzyme, rather than TACE may be involved in shedding of p185HER-2.

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APPENDICES

None