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

The HER-2/new extracellular domain (ECD) is shed from breast carcinoma cells in culture and is found at elevated levels in era of patients with metastatic breast cancer where it may predict recurrence. Our studies show that an N-terminally truncated HER-2/new 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 several strategies for generation of mutants of p185HER-2 to alter shedding and have investigated approaches to modulate shedding by treatment with exogenous effectors. We have been unsuccessful in development of mutants that specifically alter shedding, but that do not affect other receptor activities. These mutant proteins either are unstable or have altered kinase activity. We conclude that juxtamembrane mutants are problematic for examining the function of shedding on receptor-mediated tumorigenesis. Efforts to use approaches that employ chemical inhibitors and activators of shedding to examine the impact on transformation and tumorigenesis have not yielded interpretable results that distinguish the effects on receptor activity versus receptor shedding. Moreover, these modulators, alone, appeared to affect cell behavior. We conclude that modulation of shedding cannot be clearly separated from direct effects on the activity of the receptor itself of from secondary effects on the cells. We conclude that future studies need to be conducted on the p95 in human breast cancer samples to determine whether it predicts outcome in patients. While the epidemiological study may not yield proof that p95 causes metastasis and poor outcome, ultimately this line of study may be more beneficial to breast cancer patients than in vitro analyses that are subject to numerous problems with interpretation as determined in this project.

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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 statement of work to be conducted and the progress we have made in this direction.

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

To examine the impact of shedding of the ectodomain of p185HER-2 on tumorigenesis, we proposed to alter the extent of shedding by engineering altered sequence in the juxtamembrane domain of p185HER-2. The juxtamembrane region of the ectodomain is known to be the site of proteolytic cleavage of transmembrane proteins during shedding (Arribas et al., 1996). The mutants were then to be characterized first for their proper location at the cell surface and for kinase activity by examining autophosphorylation of the mutant receptor and by analyzing the kinase enzymatic activity of the receptor by immunoprecipitation and in vitro kinase activity. The characterization of the mutant receptor was an important first step since alterations in biosynthesis, processing, or kinase activity would be expected to affect tumorigenic potency of the receptor obscuring effects of shedding.
In the first and second years, a deletion of the juxtamembrane stalk of p185HER-2 was constructed and secondly an altered sequence of the juxtamembrane stalk was created by swapping with the juxtamembrane of tumor necrosis factor-α precursor (preTNFα), which undergoes potent tumor promoter inducible shedding. From this study it was concluded that deletion of the juxtamembrane of p185HER-2 protein resulted in folding defects leading to deficient protein production. We next designed a strategy to delete the 16 amino acids adjacent to the transmembrane domain, to exactly mimic an alternative splicing event described by Siegel et al., (1999), which results in deletion of an exon. Further biochemical characterization of this variant product of HER-2 has revealed that the protein has enhanced in vitro kinase activity. Altered kinase activity and enhanced transforming activity of this mutant protein precludes efforts to examine effects of altered shedding on tumorigenesis, since any effect of shedding would be secondary to altered kinase activity. 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 efficiently induced by addition of tumor promoters (Blobel, 1997). In contrast p185HER-2 shedding is very slow and is not inducible by tumor promoters (Christianson et al., 1998, Codony-Servat et al., 1999). To examine the properties of this mutant, we performed transient transfection into Cos-7 cells. The protein was produced and detected by immunoblotting. To further characterize this mutant, we examine the kinase activity by examining the tyrosine phosphorylation level by conducting anti-phosphotyrosine blots. In this system, overexpression of the wildtype p185HER-2 results in dimerization and constitutive tyrosine phosphorylation. Comparisons of the tyrosine phosphorylation level of the mutant and wildtype have initially revealed that the mutant has decreased levels suggested that the kinase activity is altered. Further characterizations of this mutant

conducted in the last year of funding further determined that effects on altered shedding could not be distinguished from direct effects of the mutation on the receptor activity. Moreover, the effects on shedding on cell proliferation and transformation were further complicated by the cellular effects of the tumor promoters that are required to induce shedding.

In summary, the juxtamembrane region of p185HER-2, which is the site of proteolytic cleavage to achieve shedding is also a region of the receptor that affects receptor activity. Consequently, it is difficult, if not impossible to distinguish effects on shedding versus effects on receptor activity. The problems involved in interpretation of results obtained using juxtamembrane mutations are further emphasized by recent studies (Burke and Stern, 1998) showing the importance of the juxtamembrane domain sequence of p185HER-2 in dimerization and receptor activation. This study suggests that any attempts to disrupt the juxtamembrane region will have the primary consequence of affecting receptor activation.

In the final year of funding we tested alternative methods to alter shedding. though the use of cleavage inhibitors and activators that used alone and in combination. For inhibitors, we tested TAPI (Christianson et al., 1998) and BB-94 (Codony-Servat et al., 1999) both metalloprotease inhibitors shown to inhibit HER-2 shedding. To stimulate shedding, we used the 4-aminophenylmercuric acetate (APMA), a matrix metalloprotease activator known to cause potent stimulation of HER-2 shedding (Molina et al., 2001). HER-2 transfected 3T3 cells and control 3T3 cells were treated with 10 μM TAPI, and as previously observed, shedding of the ectodomain and production of p95 were inhibited. We then tested the effects of TAPI on the growth properties of the cells. For the focus forming assay, the 3T3/HER-2 cells were plated at low density in 12 well plates. For the anchorage independent growth assays, the cells were plated in soft agar. Triplicate wells were treated with 10 μM TAPI or with the control vehicle. As a futher control, the parental 3T3 cells were also treated in parallel experiments with 10 μM TAPI. While TAPI appeared to have a slight (~30%) inhibitory effect in both assays, the parental 3T3 cells were similarly affected in the focus forming assay. Therefore, the effects of TAPI appeared to be nonspecific for HER-2 shedding. Next we investigated the effects of the metalloprotease activator APMA on shedding, focus formation, and anchorage independent growth of parental and 3T3/HER-2 cells. As previously reported by Molina et al., 2001), APMA strongly stimulated shedding. However, as observed when TAPI was employed, the effects of APMA on the parental and HER-2 cells were comparable. There appeared to be no specificity for the 3T3/HER-2 cells. For the final year of study, we also proposed to conduct a similar set of studies employing MCF-7 breast carcinoma cells. The MCF7 cells were stably transfected with HER-2 and several colonies were characterized for HER-2 expression. After analysis of ~ 15 clones of stably transfected cells, the maximum level of HER-2 overexpression achieved was 3 fold. Despite this small increase, we observed a striking 15 fold increase in Heregulin mediated tyrosine phosphorylation of HER-2. We next measured the shedding of HER-2 by the amount of ECD released in the culture media determined by ELISA, and by the level of p95 in the cell extract. Initial analyses revealed little or no shedding in these cells. Next, we tried to enhance shedding by treatment with the APMA metalloprotease activator. Still, there was no detectable shedding from these cells. A recent publication further indicated that the MCF7 cells are defective in shedding activity. Consequently,

we were unable to determine the effects of altered shedding on the behavior of these cells.

KEY RESEARCH ACCOMPLISHMENTS

In the final year of funding:

Further analyzed juxtamembrane domain swapping mutants of HER-2 for kinase activity and for intracellular signaling

Characterized the effects of TAPI and APMA metalloprotease activator on shedding from 3T3/HER-2 cells

Tested the effects of TAPI and APMA on the focus forming activity of parental versus 3T3/HER-2 cells

Tested the effects of TAPI and APMA on the anchorage independent growth of 3T3/HER-2 cells

Generated stable HER-2 overexpressing MCF7 breast carcinoma cell lines

Characterized the MCF7/HER-2 cells for HER-2 levels and for HER-2 mediated signal transduction

Tested the MCF7/HER-2 cells lines for shedding by measuring the ECD and p95

Treated MCF7/HER-2 cells with APMA and tested shedding and p95 production.

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
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  None
- Employment or research opportunities applied for and/or received :
  None

CONCLUSIONS

To summarize, we have employed several different strategies for experimental modulation of shedding in order to test effects on tumorigenic activity of HER-2. First we used a genetic approach and constructed deletions mutants and domain substitution mutants to alter the extent of shedding. Because of altered receptor processing and kinase activity caused by these mutations and recent information suggesting that the juxtamembrane region of p185HER-2 is critical for receptor dimerization and kinase activity, we concluded that juxtamembrane mutants will not be an effective approach for testing the role of shedding of the ectodomain in receptor mediated tumorigenesis. Next, we used additional approaches involving the use of chemical inhibitors and stimulators of shedding for testing the effects of shedding. While we determined that metalloprotease inhibitors and activators were effective in modulating the extent of shedding, these effectors also had an effect on cell growth that was independent of HER-2 shedding. Therefore, the effect of these effectors on shedding versus other secondary effects on the treated cells could not be distinguished.

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APPENDICES

None