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We are investigating the need for cooperating genes in the development of ErbB2-induced mammary tumors using the mouse as a model system. In one set of experiments, we are investigating the role of ligands for the ErbB family of tyrosine kinase receptors, specifically focusing on NDF. Using a transgenic approach, we are investigating the effect of different NDF isoforms and mutant NDFs on ErbB2-induced tumors in the mouse. In a second set of experiments, hypomorphic alleles are being created at NDF, to determine the contribution of this gene in ErbB2-induced tumors. Finally, in a wider screen to identify genes that can cooperate in ErbB2-induced mammary carcinogenesis, we are performing MMTV-mutagenesis in ErbB2 transgenics to activate and tag cooperating oncogenes, with the goal of isolating and characterizing these genes.

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FOREWORD

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Archibald S. Perkins 7/19/96

PI - Signature Date

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INTRODUCTION

Like most human cancer, breast cancer is the result of cumulative genetic alterations resulting in loss of growth control. The genes involved in this multistep process have not been elucidated, but include p53, which is altered in more than 50% of cases, the Rb tumor suppressor gene, BRCA1 (5% of cases, mostly inherited, early onset), and the *neu/erbB-2/HER-2* gene in 20-30% of cases [1]. It is this last gene, *neu*, which is the focus of our research. *neu* is a member of a family of genes that encode receptor tyrosine kinases. Other family members include the epidermal growth factor receptor (*EGFR*), *erbB-3*, and *erbB-4*. Activated *neu* oncogenes are potent in transforming cells in culture and transgenic mice overexpressing either mutationally activated *or* normal *neu* in the mammary gland succumb to adenocarcinomas. The oncogenic effect of both activated and normal neu alleles was evident from whole animal studies [2]. When normal c-*neu* gene was driven by MMTV in transgenic animals, the tumors were focal adenocarcinomas surrounded by hyperplasia, and were not pregnancy dependent [3]. Since the mode of c-*neu* participation in oncogenesis in humans is amplification rather than activating mutations at c-*neu*, this transgenic model more closely resembles the situation in humans, and the long latency and stochastic nature of the tumors emphasizes the need for other events in carcinogenesis.

The $p185^{neu}$ receptor encoded by *neu* is stimulated by two families of ligands: the EGF family, and the NDF family, which includes heregulin [4], also known as neu differentiation factor (NDF) [5]. None of the EGF family members appears to bind directly to $p185^{neu}$, yet several can activate the receptor via transmodulation. This is believed to occur by binding of the ligand to a high affinity receptor (e.g., EGFR) that then physically associates with $p185^{neu}$ and heterodimerizes. The result of this physical association is phosphorylation and activation of $p185^{neu}$. Thus a variety of ligands can channel their signal through $p185^{neu}$, and the partners created depend on what other receptors are expressed in a given cell, and what ligands the cell is exposed to.

NDF is synthesized initially as a transmembrane glycoprotein with a 242 amino acid ectodomain that has an IgG-type motif and an EGF homology domian. The latter, contained in all members of the ErbB-binding ligand family, most likely functions in receptor binding. The transmembrane form, via proteolysisis at a site near the ecto-/transmembrane domain junction, is likely to be the precursor for the released form, as is the case for other membrane-bound growth factors. There are two possible EGF homology domains of NDF: α and β, determined by alternative splicing. The β isoforms bind with higher affinity, and appear to be expressed at higher levels in neural tissue. The α isoforms are expressed at higher levels in mesenchymal cells and in the breast, including breast carcinomas; however, β isoforms are also expressed in some mammary tumors.

While data is now emerging concerning the role of NDF in mammary carcinogenesis, considerably more is known concering the role played other ligands that act through ErbB family members. Transgenic mice overexpressing TGFα, either with promoters targeting mammary epithelium, or generalized promoters, display mammary epithelial hyperplasia and neoplasia that is often malignant, and often involves the terminal ducts and secretory alveoli [6]. Recent studies show a potent interaction between TGFα and c-*neu* overexpression in transgenic mice: By crossing the MMTV-c-*neu* transgenics with MMTV-TGFα mice, a strong cooperativity was found, resulting in rapid hyperplasia and milk production (Muller, pers. comm). Clearly TGFα has a mitogenic, growth-stimulatory role in breast development and in mammary carcinogenesis. The role of NDFs is unclear, but given the finding that it can promote differentiation and growth cessation in cultured mammary epithelial cells, it may act antagonistically to TGFα. It is the goal of these studies to explore the role of NDFs in mammary carcinogenesis in whole animals using genetic approaches.

BODY

Task 1. Overexpression of NDF: Transgenics *neu* is one of the few genes clearly implicated in the development of human mammary tumors. In addition, TGFα, a ligand for a related growth factor receptor, EGFR, can stimulate $p185^{neu}$ activity via transmodulation, and can also play a stimulatory role in mammary carcinogenesis. We hypothesize that NDF, a putative ligand for $p185^{neu}$ that can stimulate its activity, but which induces the differentiation of mammary cell lines, plays an important role in mammary tumors, especially those in which *neu* also has a causative role. To address this hypothesis, we proposed to direct overexpression of NDF to the mammary gland of transgenic mice, and to see what effect this has on mammary growth and development, as well as susceptibility to c-*neu*-induced or MMTV-induced mammary tumors in mice. To this end, we have so far made transgenic mice with NDF under transcriptional control of MMTV (14 founders) and under control of the whey acidic promoter (18 founders). For this experiment, we utilized the β2A isoform of NDF. As discussed above, this isoform has the β-type EGF homology domain that binds with higher affinity to the ErbB4 receptor [7], and the A isoforms contain the longest cytoplasmic domain. We chose this domain since it stimulates higher tyrosine kinase activity than the α isoforms, and because it was thought that the longer cytoplasmic tail may have some important role. We have analyzed these mice for expression, and were disappointed to find that the level of stable transcripts in lactating or late pregnancy mammary glands was very low, especially when compared with that level obtained with the WAP promoter driving other genes, or the MMTV promoter driving TGFα [6].. Nonetheless, we have expanded three of the highest expressing lines, and have crossed them to the MMTV-c*neu* mice for two reasons: one is to see if there is synergy between the two genes; and also to see if transgene expression increases in the tumor (as is often the case).

We have also made three other types of transgenics. As discussed above, NDF is produced as a transmembrane protein that is later cleaved at a specific site on the ectodomain to release a soluble ligand molecule. This is similar to the situation with stem cell factor (SCF, also known as Steel factor or mast cell growth factor). With this factor, it has been shown that the membrane-bound and soluble forms have different activities, and are probably both essential in mouse development. We wondered if a form of NDF that could not be cleaved would have higher activity than one that can be cleaved. To test this, we created transgenic mice that contain MMTV-NDF with a point mutation in the proteolytic cleavage site that is used to generate the soluble form. Unfortunately, expression of the transgene could only be detected by reverse transcription-PCR, followed by Southern blotting. Nonetheless, the founders (no offspring) are being maintained in mating, to see if tumors develop.

We were concerned about the problem of low expression of the transgene, and have approached this in several ways. One is that we obtained MMTV

expression vectors from two other investigators (Phil Leder and Paul Jolicoeur), and constructed MMTV-NDF plasmids with these. We have transfected these into tissue culture cells and induced with dexamethasone (the MMTV LTR is responsive to dexamethasone). We compared the level of expression of these constructs to that seen with our transgenic construct, which was made with a vector from Robert Coffey. There was no significant difference in the level of expression between the different plasmids: all expressed at a fairly low level. These results are consistent with the notion that high level expression of NDF may be detrimental to the cell or the mouse, and is selected against. To test this, we have done the following. We know that the MMTV-TGFα plasmid from R. Coffey worked well in transgenics and in cell culture, so it is likely that this expression is not selected against. Since the receptor specificity of the ligand is determined by the EGF homology domain, we thought we could change the specificity of the MMTV-TGFα ligand by replacing its EGF homology domain with that from NDF (the β-type EGF homology domain). This chimeric protein may express better than NDF, yet have the same biologic activity of NDF. A converse swap was also made: replacing the EGF homology domain of NDF with that from TGFα. In cell culture, TGFα-derived constructs expressed at significantly higher levels, indicating that the inclusion of other parts of the NDF gene has a negative effect on expression. We have used these to create transgenic mice, and will be testing these soon for expression.

Another approach that we have taken is to utilize a different NDF isoform for making the MMTV-NDF transgenic animals. As discussed above, the α isoforms bind to ErbB4 with lower affinity, yet appear to be more breast-specific, in that they are expressed at higher levels in breast tissue and in breast carcinomas, and in a mouse mammary implant study, the α isoform showed a more potent effect on inducing lobuloalveolar development [8]. This suggests that in a transgenic mouse, the α isoforms may have a more potent effect than the β isoforms. Given their pattern of expression, the α isoforms may be more appropriate. To test this, we have made an MMTV-NDFα2A plasmid, and will be making transgenics from this construct soon.

In the course of these studies, a report has been published describing the phenotype of transgenic mice having a MMTV-NDFβ2C transgene. (This NDF cDNA was cloned from a mouse mammary tumor, indicating that this isoform can be expressed in mammary tumors) [9]. The report describes a persistence of the terminal end buds in the mammary glands of virgin transgenic mice, and the presence of mammary gland adenocarcinomas in older mice. While this report has diminished the novelty of our transgenic experiments, it suggests that NDF does play an important role in mammary carcinomas in the mouse, and that further experiments are worth performing. The isoform that we used was different from the one that they used, which may give us a different result. And as described above, we are also pursuing the testing of transgenic mice with other isoforms, which lends novelty to our experiments.

Task 2. Targeted deletion of NDF via homologous recombination. In these experiments, we take another approach to testing the same hypothesis that NDF plays an important role in mammary development and neoplasia. If our hypothesis is true, then deletion of the gene encoding NDF should have effects on either mammary gland development or neoplasia, or both. It turns out that the creation of a null allele at the NDF locus is lethal in the homozygous state [10, 11], and thus a null allele is uninformative in terms of the effect of NDF on mammary carcinoma. Thus, we are embarking on an exciting new set of experiments to address this issue. Dr. Yilong Sun, a postdoctoral fellow who just joined my lab, will be creating more discrete mutations in the NDF gene via homologous recombination in embryonic stem cells, with the goal of creating "hypomorphic" alleles of NDF. These mutations will be designed so that they are partially functional rather than null, and thus may give viability and an informative phenotype. These mutations will be based on in vitro studies that examine the effect of the mutations on ErbB4 signaling (to be done in collaboration with Dr. David Stern here at Yale) and on classical mouse mutations in genes such as *Steel*, which encode molecules structurally analogous to NDF. We are particularly enthusiastic about this approach, and should be able to report some significant progress in next year's report.

Task 3 Identification of protooncogenes that can cooperate with *neu*. It is clear from the studies of Muller and coworkers that *neu* does not act alone in the generation of mammary carcinomas in transgenic mice. The long latency (5-8 months) and the solitary, stochastic nature of the tumors argues that other factors are necessary in the disease process. We thus hypothesize that while *neu* is an important oncogene in mammary tumorigenesis, other genes are involved, and we propose to identify what these other genetic factors are by retroviral mutagenesis and proviral tagging. This is being accomplished by infection of transgenic MMTV-c*neu* mice with mouse mammary tumor virus (MMTV). We expect that infection of transgene with the virus will cause an acceleration of tumorigenesis: a shortening of tumor latency, due to the activation of cellular genes that can cooperate with c*neu* in the development of tumors. The presence of the proviral tag in *cis* to the implicated oncogene will enable us to molecularly clone and characterize them. This approach will allow us to find out what other genes need to be altered, and if the function of these genes can be discerned, what other aspects of cellular growth control must be deregulated, in order to arrive at a fully malignant cell. To perform the experiment correctly, we have backcrossed the MMTV-c*neu* transgene onto the C3H background for five generations, so that the genetic background will be essentially identical to C3H, the high mammary carcinoma strain that carries MMTV. We have completed with this phase of the project, and have begun to age mice that have both the transgene and MMTV, to look for acceleration of tumorigenesis. We now have over twenty mice that have both MMTV and the MMTV-c*neu* transgene, and will continue to produce these until 50 are obtained. We expect tumors to begin to arise in the next few months.

In relation to the statement of work, we are on schedule with Task 1, but behind on Tasks 2 and 3. However, we are making progress on Task 3 and should attain our goals by the end of the grant period. Task 2 is being initiated now, but given that our proposed approach would have failed, that this was reported by others allowed us to pursue other parts of the project and now allows us to design and implement hopefully better approaches

CONCLUSIONS

The projects are progressing. The technical approaches have shifted slightly based on data from our lab and those of others. The NDF transgenics that we are now developing promise to address the role of an α isoform in mammary tumorigenesis, and will extend those of others as to the effect of β isoform overexpression. We intend to expend considerable effort in the coming year on the creation of hypomorphic NDF alleles, an approach that promises to be very interesting. We expect that tumors will be arising relatively soon in the mice carrying both MMTV and the MMTV-*cneu* transgene, and can begin to analyze these for proviral insertions.

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