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13. ABSTRACT *(Maximum 200 Words)*

Approximately 50% of human cancers have accumulated missense mutations in the gatekeeping tumor suppressor protein p53, usually resulting in genomic instability and a very poor prognosis. The wild-type p53 protein is required for assessing DNA damage in cells and making the decision to either induce cell cycle arrest to facilitate DNA repair, or to induce a suicide response in those cells with irreparable damage. In human tumors, many hot-spot mutations are found within the DNA-binding domain of p53, rendering it incapable of sequence-specific transactivation of target genes such as p21, bax, and mdm2. Some of these mutants, in addition to having dominant-negative functions, also gain novel functions by interacting with proteins differently from the wild-type p53 protein. One such gain-of-function p53 mutant possesses an Arg to His substitution at codon 175 (172 in mice) and has been shown to be involved in the dysregulation of centrosome duplication leading to abnormal mitoses and subsequent aneuploidy. Because centrosome abnormalities and aneuploidy are often seen in high-grade breast tumors, unraveling the mechanism behind the involvement of p53172 R-H in centrosome dysregulation will help us to understand the progression of mammary carcinogenesis. In order to identify potential indirect target genes regulated by this mutant, we employed a suppressive subtractive hybridization technique to generate a cDNA library specific to p53 null mammary epithelial cells (MECs) expressing the 172 R-H mutant. cDNA made from p53 null mammary epithelial cells transiently transfected with wild-type p53 was subtracted from cDNA made from mutant p53 transfected cells. The subtraction procedure generated a pool of cDNAs differentially expressed in the presence of the mutant protein; many interesting genes were revealed to be candidates for regulation by mutant p53. Some of these include developmental, metabolic, transcriptional, translational, and structural genes, as well as genes involved in transformation, signal transduction, chromatin remodeling, DNA repair and apoptosis. Characterization of a subset of these genes is underway using both Northern and Western blots and functional studies will be performed with candidate genes to determine their relationship

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Introduction

Approximately 50% of human cancers have p53 alterations, and patients with these cancers have poorer prognoses. When functional, the p53 protein blocks proliferation of cells that have sustained DNA damage and induces apoptosis in cells too badly damaged to undergo repair. Mutant forms of p53 are often no longer protective, and in many cases have acquired additional functions which make them more deleterious than the simple absence of wild-type p53. The p53 175R-H mutant is one such gain-of-function mutant. It is a confomational mutant and can no longer bind DNA, although it retains its ability to bind other proteins. It is hypothesized that mutant p53 proteins may gain novel functions by interacting with other proteins that supply a DNA-binding domain. The resulting complex could then use the p53 transactivation domain to modulate a novel set of genes.

p53 has also been implicated in the regulation of the G2/M spindle checkpoint and mitosis, with particularly striking effects upon centrosome duplication. Centrosomal hypertrophy is implicated in at least two processes that adversely affect prognosis in cancer patients: 1) loss of cell polarity and tissue organization, and 2) an increased occurance of multipolar mitoses, which predisposes to the development of aneuploidy. In addition, centrosomes have been shown to be larger and more numerous in high-grade breast adenocarcinomas. Unraveling the mechanism behind the involvement of the 172R-H mutant in the dysregulation of centrosome duplication leading to aneuploidy is of great interest to this project and could help us to better understand tumor progression.

A p53 172R-H transgenic mouse model was generated in the laboratory, and studies of this mouse indicated that the presence of this p53 mutant did not decrease apoptosis or increase proliferation, but did promote the development of aneuploid tumors following carcinogen treatment. Similar results were seen when these mice were crossed with mice carrying other mammary-targeted oncogenes; tumors arising in the p53 mutant-carrying bitransgenic mice were frequently aneuploid. The current studies were initiated in order to address the mechanistic issues: specifically, how the p53 175R-H mutant contributes to mammary tumorigenesis and the development of genomic instability.

Body

Specific Aims:

In order to understand the role of the p53 172R-H mutant in mammary tumorigenesis, the scope of the grant proposal is outlined by the following specific aims :

1) To study the effect of the WAP-p53 172R-H transgene on genomic instability in early stages of mammary tumorigenesis

2) To investigate p53 172R-H as a gain-of-function mutant by identification of potential indirect target genes and/or novel protein-protein interactions

Progress toward these aims:

Specific Aim 1: To study the effect of the WAP-p53 172R-H transgene on genomic instability in early stages of mammary tumorigenesis.

Initial characterization of mammary tumors arising spontaneously (following pituitary isograft) in mice bitransgenic for WAP-p53 172R-H and WAP-TGF-a indicated that 57% of tumors arising in the mutant p53-expressing bitransgenic mice were aneuploid, whereas no aneuploid tumors were observed in the WAP-TGF-a control mice. Both sets of tumors arise with a latency of 100-110 days post-isograft. Loss of wild-type p53 is known to influence aneuploidy through dysregulation of centrosome duplication. Furthermore, centrosomes from high-grade human breast cancers are abnormal in many respects, indicating that there may be a relationship between centrosome abnormality and aneuploidy, which is a marker of poor prognosis. It has been shown that aberrant centrosome duplication can be detected in early hyperplasias and even in phenotypically normal, but transformed, tissue. We initially hypothesized that the aneuploidy we had observed in bitransgenic tumors might be accompanied by centrosome dysregulation. Furthermore, given the short timeframe in which both of these sets of tumors arose, we could examine mammary glands of mice at defined timepoints within the 110 day window for aberrant centrosome numbers occurring in phenotypically normal tissue and preceding the development of aneuploid tumors.

Although preliminary results indicated that there might be more centrosomes in bitransgenic tumors, the complete study did not bear this out. Neither group of tumors appeared to contain aberrant centrosome numbers, despite the ploidy variance. Furthermore, the pretumor

samples from both groups appeared to have normal numbers of centrosomes at the timepoints examined, which were 15, 30, 45, 60, and 90 days post-isograft.

However, this did not discount the possiblity that some transient centrosome abnormality resulting in later ploidy-related consequences might be induced very soon (*i.e.*, less than 15 days) after expression of the p53 172R-H mutant in the mouse mammary gland. In order to address this possibility, we extended this specific aim to include *in vitro* studies. p53-null mammary epithelial cell [MEC] cultures were transfected with either wild-type p53 or p53 172R-H using an adenoviral method, and subsequently immunostained for both p53 and gamma-tubulin, a component of centrosomes. Transfected (*i.e.*, p53-positive) cell centrosome numbers were analyzed and compared to those of untransfected cells. These experiments indicated that cells transfected with the p53 mutant did indeed demonstrate aberrant centrosome numbers by 3 days post-transfection, whereas this was not seen in mock-transfected cells or cells transfected with wild-type p53. However, very few MECs stably-transfected with the mutant p53 had aberrant centrosome numbers, suggesting that the majority of the abnormal cells seen in the transient transfection experiments underwent apoptosis due to failure in mitosis. Additional experiments indicated that MECs stably transfected with the p53 mutant displayed reduced apoptosis, both basal and DNA-damage induced, suggesting a second mechanism by which this p53 mutant might also contribute to mammary tumorigenesis. We hypothesize that the early centrosome abnormalities create a cellular environment in which genomic instability is more frequent than usual, compounded by diminished apoptotic capabilities in the same cells, and that this forms the basis of the tumor predisposition seen in the mouse models.

Specific Aim 2: p53 172R-H as a gain-of-function mutant: the identification of potential indirect target genes and/or novel protein-protein interactions

Previous Observations

It is known that the p53 172R-H mutant cannot bind to p53 consensus binding sites, but there are several papers suggesting that p53 may have indirect transcriptional effects. Furthermore, it is possible that this p53 mutant may mediate its gain-of-function effects partially through aberrant protein-protein interactions. Any genes up- or down-regulated preferentially in the presence of the mutant could be directly involved in the dysregulation of normal centrosome number and maintenance of chromosomal stability. The potential transcriptional effects of the p53 172R-H mutant are currently being addressed. Initially, we proposed to use cDNA obtained from p53-null MECs transiently transfected with mutant p53, wild-type p53, or mock-transfected cells to screen Atlas™ Arrays (Clontech) for differentially expressed genes. However, due to difficulties with data analysis, we were not confident regarding the validity of data obtained from these screens, and so took an alternative approach. We employed CLONTECH's PCR-Select™ cDNA Subtraction Kit to perform a suppressive subtractive hybridization in which cDNA made from p53 null cells transfected with wildtype p53 was subtracted from cDNA made from mutant p53 transfected cells. The subtraction procedure generated a pool of cDNAs differentially expressed in the presence of the mutant protein two days after transfection. These cDNAs were then cloned into pGEM vectors; 900 plasmids were differentially screened with forward and

reverse subtracted probes, and 120 clones were chosen to be sequenced following the screening. Of the102 clones identified in sequence homology databases, many interesting genes were revealed to be candidates for transcriptional regulation by mutant p53. A wide variety of genes were identified – some of these include developmental (EED), metabolic, transcriptional (TRAP100, SRA), translational (Trt, Naca), and structural genes (γ-actin), as well as genes involved in transofmation (MAT1), signal transduction (SDF1, $G\alpha_s$, Pitpn, MRK, calcyclin), chromatin remodeling (Psma1, Hmg1, H2A), DNA repair (Ku70) and apoptosis (Naip1). In addition, 11 ESTs were identified and are currently being investigated. We are especially interested in the genes involved in transcription, chromatin remodeling, and DNA repair and plan to pursue the involvement of mutant p53 in these processes in an attempt to discover its role in mammary carcinogenesis. Following the verification of up- or down-regulation of these genes by p53 172 R-H, the interaction between mutant p53 and the known transcriptional regulators of these genes will be investigated. It is expected that novel protein-protein interactions between the mutant and other proteins directly or indirectly involved in transcription of key genes involved in tumor progression will be revealed.

Current Observations

As of the date of the last status report, many technical difficulties have arisen on this project. Much time has been spent trying to recover from the disasterous flood that Baylor and the rest of Houston experienced in June of 2001. In addition, experimental techniques critical for the verification of up- or down-regulation of the genes identified in the subtractive hybridization screen have presented problems with reliability and duplication. There were difficulties with using the same adenovirus/lysine-mediated approach that was originally used to express the mutant p53 protein. Expression of the mutant p53 protein in a p53 null mammary epithelial cell line is necessary in order to obtain the representative sample of RNA needed to identify the relative expression levels of candidate clones in the presence of the mutant protein. For this reason we have switched to a retrovirally mediated system in which a retroviral construct containing p53 R172H is transfected into a 293T packaging cell line. Subsequently, the p53-virus-containing media from the 293T cells is transferred to the p53 null mammary epithelial cells, thereby infecting these cells with the virus and ultimately inducing mutant p53 expression. Although this technique has recently been successfully established with this mammary cell line using B-gal controls, the experimental infections with the actual mutant p53 virus is still being worked out. This technique is expected to be successfully employed for this purpose very soon.

An additional method for looking at gene expression in the presence of p53 R172H is to make use of our mutant p53 transgenic mouse model, WAP-p53R172H. WAP (whey acidic protein) is a milk protein gene expressed at high levels during pregnancy; therefore, the WAP promoter drives strong expression of the mutant p53 during pregnancy. We were forced to re-establish this line of mice from cryopreservation from another institution, and this took many months. The colony is now plentiful, and we are currently in the process of using mammary tissue from pregnant WAP-p53R172H mice to observe gene expression and how it is affected by this particular mutant p53.

One of the most interesting genes identified in the SSH screen is the developmentally regulated polycomb gene, EED. EED is the mouse homolog of the Drosophila extra sex combs gene, and is turned on very early in development to repress a wide variety of genes involved in

differentiation and other critical processes. In addition, EED has recently been shown by another group to be required for X-inactivation in females. Our preliminary data suggest that EED could possibly be down-regulated in the presence of the R172H mutant. This begs the question of whether or not mutant p53 could be directly or indirectly regulating EED expression. Due to the fact that differentiation is often repressed in order for proliferation to occur, the up-regulation of a repressor of differentiation (EED) would not be unreasonable in a tumor pre-neoplastic environment. Due to our interest in this genes involvement in mammary tumorigenesis, we decided to look at it's expression in the mammary gland. Using both semi-quantitative RT-PCR and In situ hybridization techniques, we have shown that EED is indeed expressed in this tissue. In addition, preliminary data suggest that EED is expressed at higher levels in both pregnant and cancerous glands, as compared to the glands of virgin or lactating mice. To date, we are unaware of any other reports of EED being expressed in the mammary gland. In the very near future we will be using methods currently under development to look more closely at EED with respect to it's involvement with either wild-type or mutant p53.

Previous Research Accomplishments

1. Determined that centrosome amplification is not necessarily coincident with aneuploidy in a bitransgenic mouse mammary tumor model

2. Demonstrated transient centrosome abnormalities in p53 175R-H-transfected MECs

3. Demonstrated that stably-transfected cell populations mimic tumor populations in that they lack significant percentages of cells with centrosome abnormalities

4. Demonstrated that MECs stably transfected with p53 175R-H display diminished basal and DNA damage-induced apoptotic responses

5. Generated a subtractive cDNA library containing cDNAs differentially expressed in p53 null MECs transiently transfected with p53 172 R-H

6. Revealed important and significant candidate genes possibly regulated by mutant p53, such as EED, Hmg1, Ku70, SRA, and Nm23

Key Research Accomplishments since the last Status Report

1. Generation of a retroviral construct containing the p53 R172H mutant cDNA
2. Development of a retrovirally-mediated gene transfer technique using a p53-null mammary epithelial cell line
3. Propagation and re-establishment of the WAP-p53R172H transgenic mouse colony
4. Characterization of EED expression in the mouse mammary gland

Reportable Outcomes

Data from this project was presented at two national meetings:

Molecular Biology and Pathology of Neoplasia Workshop, Keystone, CO, July 1998.

Mouse Models of Mammary Tumorigenesis Meeting, Bar Harbor, ME, October, 1999.

Data from this project has been published in two peer-reviewed journal articles:

Murphy, K.L., and Rosen, J.M. (2000) Mutant p53 and genomic instability in a transgenic mouse model of breast cancer. *Oncogene*, **19**:1045.

Murphy, K.L., Dennis, A.P, and Rosen, J.M. (2000) A gain-of-function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model. *FASEB J.*, in press.

Conclusions

Discovering the role played by mutant p53 in the development of breast cancer is the primary goal of this study. We believe that the p53 172 R-H mutant contribution to cancer progression is primarily due to different protein-protein interactions conferred by the mutant with proteins that would otherwise interact differently, or not at all, with wild-type p53. These new or aberrant interactions could then disrupt the cells' ability to successfully maintain the integrity of the genome either directly by disrupting proper mitotic processes (including centrosome duplication) or indirectly, by altering gene expression via another protein's DNA-binding domain. We are anticipating the identification and further characterization of some of these interactions. Specifically, we expect to have verified the up- or down-regulation of several candidate genes identified in the SSH screen, and furthermore, to be able to further characterize genes that are indeed differentially regulated in the presence of the p53 mutant.

References

Murphy, K.L., and Rosen, J.M. (2000) Mutant p53 and genomic instability in a transgenic mouse model of breast cancer. *Oncogene*, **19**:1045.

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