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**ABSTRACT (Maximum 200 Words)**

This proposal tests a novel concept that H$_2$O$_2$ generating oxidase-mediated production of reactive oxygen species in breast epithelium contributes to the development of breast cancer. Two tasks are currently being pursued. The first deals with the overexpression of xanthine oxidase (XOX) or urate oxidase (UOX) in a non-tumorigenic human mammary epithelial cell line to ascertain whether oxidase overexpressing cells undergo transformation when exposed to substrate xanthine for XOX and uric acid for UOX. We have made constructs to express these oxidases under the direction of cytomegalovirus or mouse mammary tumor virus (MMTV) promoter. Transfections are being performed to generate cell lines stably expressing these enzymes. The second task deals with the generation and characterization transgenic mice expressing UOX under the transcriptional control of MMTV promoter, an in vivo model system to explore the role of oxidative stress in mammary carcinogenesis. The choice for urate oxidase is based on the fact that we have used this enzyme in previous studies showed that cells stably expressing this enzyme reveal the characteristic crystals of this enzyme in peroxisomes. We have injected the construct in fertilized ova and have identified five founder mice. These are being bred to ascertain germ line transmission and expression.
FOREWORD

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INTRODUCTION

Carcinoma of the breast is overwhelmingly a disease of females. In the United States, the age-standardized incidence of breast cancer has doubled during the past four decades. The established risk factors are both non-hormone mediated and hormone-mediated. Oxygen free radicals are a well-established risk factor for cancer and aging. Evidence for the role of free radical-induced DNA damage in aging, and cancer comes from the correlations between high consumption of fruit and vegetables, or of specific dietary antioxidants and a relatively low incidence of several types of cancers. Recent literature suggests a role for free radical induced injury in the development of breast cancer. The idea of free radical induced injury having a role in breast cancer development is intriguing since it opens up the possibility of antioxidants being able to prevent its development.

Our idea or hypothesis is that the increased incidence of breast cancer in the United States is due to increased generation of reactive oxygen species (ROS) in the breast epithelium during the reproductive period and antioxidant activity will be beneficial in preventing breast cancer. Accordingly, our idea is based on the notion that xanthine oxidase (XOX), which is present in milk for possible antimicrobial activity, to keep the milk sterile, plays havoc with the breast epithelium of women at risk. We will test the idea that XOX or a different hydrogen peroxide-generating oxidase such as urate oxidase (UOX) over-expression in breast epithelium leads to neoplastic transformation using in vitro and in vivo transgenic approaches. For this purpose stably transfected mammary epithelial cells will be generated and exposed to xanthine for XOX or uric acid for UOX cells to produce $H_2O_2$/ROS and transformation potential will be assessed. Likewise, we will generate transgenic mice over-expressing UOX under the control of mouse mammary tumor virus (MMTV) promoter. We have considerable past experience in vitro transformation work using peroxisomal fatty acyl-CoA oxidase and peroxisomal urate oxidase in African green monkey kidney cells and we also have the expertise in our laboratories to generate transgenic mice[1-3].
We are testing the idea that XOX or UOX overexpression in breast epithelium leads to neoplastic transformation using *in vitro* and *in vivo* transgenic approaches. Our proposal will test the idea that XOX mediated generation of ROS in breast epithelium contributes to the development of breast cancer and that XOX levels are hormonally regulated, with highest enzymatic activity in breast epithelium during the reproductive phase of female biology. The hypothesis that breast cancer is due to ROS generated by XOX, or other \( \text{H}_2\text{O}_2 \)-generating oxidases such as UOX, will be tested using molecular genetic approaches. The specific aims of the proposed study will address the fundamental issues related to our idea/hypothesis regarding the role of ROS in breast cancer pathogenesis. Work related to the following specific aims were initiated during the first year.

**Specific Aim 1:** Overexpress XOX in non-tumorigenic human mammary epithelial cells (MCF-10A) under the control of MMTV-LTR promoter and ascertain the development of neoplastic transformation when exposed to the substrate xanthine. For this purpose, stably transfected cell lines will be isolated, the levels of XOX expression and \( \text{H}_2\text{O}_2 \) generation analyzed, and the ability to transform in response to xanthine will be assessed.

**Development of construct -mouse mammary tumor viral (MMTV) promoter-XOX.** Human xanthine oxidase (hXOX) full-length cDNA is inserted into the pMAMneo mammalian expression vector designed for high level expression of eukaryotic genes. pMAMneo contains the RSV-LTR enhancer linked to the dexamethasone-inducible MMTV-LTR promoter. This chimeric construct was used to transflect MCF-10A cells. MCF-10A cells represent a spontaneously immortalized, untransformed human mammary epithelial cell line. These cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12. These cells were plated at \( 5 \times 10^5 \) cells per dish on 100-mm tissue culture dishes and transfected with 10 \( \mu \)g of pMAMneo-Hxo DNA by the standard calcium phosphate precipitation method. After incubation for 16 hr with the DNA, the dishes were washed with phosphate-buffered saline and reincubated with fresh medium containing 200 \( \mu \)g of neomycin per ml. The medium was replaced once every 4 days.

A number of G418 resistant colonies were isolated and are currently being analyzed for integration of the exogenous XOX cDNA by Southern blot analysis. Initial work showed minimal or no detectable expression of transgene. After establishing the additional stably transfected cell lines, we will continue to analyze these for expression of XOX mRNA by Northern blotting and XOX protein by western blotting. We will then analyze the enzymatic activity and delineate low and high XOX expressors. We hope to accomplish this work during the second year.

**Specific Aim 2:** Generate transgenic mouse lineages that overexpress urate oxidase under the control of MMTV-LTR promoter and utilize this *in vivo* model to explore the role of urate oxidase-generated ROS in the development of breast cancer. The goal here is to develop transgenic mouse models to study the role of ROS in breast carcinogenesis.
Construction of construct for generating transgenic mice: The construct used for microinjection is shown below (Fig. 1).

Rabbit beta globin genomic DNA

Figure 1: Transgene construct of MMTV-UOX

Characterization of transgenic mice expressing urate oxidase: We have microinjected the MMTV-UOX transgene into fertilized ova and generated mice by implanting these ova in pseudopregnant mice. Analysis of 2-week-old mouse-tail DNA was performed by Southern blotting and by PCR to ascertain the integration of transgene into the genomic DNA. We have identified 5 founder mice (3 males and 2 female, Fig. 2) so far.

Figure 2: MMTV-UOX

These are currently being bred to ascertain the germ-line transmission and expression. This work is progressing well. We will continue to inject the ova to generate additional founders. Our goal is to generate mice that express UOX in mammary epithelium. After ascertaining germ-line transmission, expression of transgene in breast epithelium will be assessed by northern analysis of RNA isolated from breast tissue, western blotting with antibodies raised against urate oxidase and by immunoperoxidase staining of the breast tissue for urate oxidase expression. Finally, we will examine the breast epithelium by transmission electron microscopy and by using protein A gold immunocytochemistry to visualize the presence of urate oxidase containing crystalloid cores with in peroxisomes.
KEY RESEARCH ACCOMPLISHMENTS

- Construction of plasmids for in vitro expression
- Construction of MMTV-UOX transgene for the generation of transgenic mice
- Microinjection of transgene into fertilized ova and generation of 5 founder mice
- Breeding founders and ongoing analysis of offspring for germ-line transmission
REPORTABLE OUTCOMES

CONCLUSIONS

During the first year, we have been successful in the construction of plasmids for in vitro work and for microinjection to generate transgenic mice. In vitro work is in progress to generate stable cell lines. Our attempts to generate transgenic mice expressing hydrogen peroxide generating oxidase has resulted in 5 founder mice. The offspring of these animals are being analyzed to ascertain germ line transmission and expression. This line of investigation will continue during the second and third year.
References:

