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GRANT NUMBER: DAMD17-94-J-4287

TITLE: Analysis of the Regulation of Expression of Transforming Growth Factor-Beta in Human Breast Cancer Cells

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REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Annual 1 Oct 94 - 30 Sep 95

4. TITLE AND SUBTITLE
Analysis of the Regulation of Expression of Transforming Growth Factor-Beta in Human Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-94-J-4287

6. AUTHOR(S)
Bradley A. Arrick, M.D., Ph.D.,

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dartmouth College
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This grant entails two objectives. These objectives derived from prior work by ourselves and others which have identified TGF-β as an important cytokine in the biology of breast cancer. For established tumors, overexpression of TGF-β may result in increased in vivo tumor growth and metastatic spread. The first objective involves the analysis of resected breast cancer specimens, with the ultimate goal of determining the mechanism(s) by which TGF-β could be overexpressed by some tumors. There are as yet no definitive data or conclusions from this aspect of the work. The second objective of the grant was to identify the molecular determinants of promoter usage for TGF-β3 in breast cancer cells. These studies stem from our prior work in which we have characterized an alternative TGF-β3 promoter uniquely functional in breast cancer cells. We have made some progress in designing promoter constructs which yield authentic promoter function upon transfection into breast cancer cells. The Statement of Work did not call for any conclusions by this point in the grant, and none are available.

14. SUBJECT TERMS	15. NUMBER OF PAGES
gene expression, promoter analysis, TGF-β , breast cancer	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

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298-102

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*U.S.GPO:1993-0-358-779

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Bradley Dow 10/20/95

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REVISED ANNUAL REPORT
Grant Number DAMD17-J-4287
PI: Bradley A. Arrick, M.D., Ph.D.
Institution: Dartmouth College
Reporting Period: 10/1/94-9/30/95

Title: Analysis of the Regulation of Expression of Transforming Growth Factor-Beta in Human Breast Cancer Cells.

Introduction

This grant entails two objectives. These objectives derived from prior work by ourselves and others which have identified TGF-β as an important cytokine in the biology of breast cancer. For established tumors, overexpression of TGF-β may result in increased in vivo tumor growth and metastatic spread (Gorsch et al. 1992). The first objective involves the analysis of resected breast cancer specimens, with the ultimate goal of determining the mechanism(s) by which TGF-β could be overexpressed by some tumors. The second objective of the grant was to identify the molecular determinants of promoter usage for TGF-β3 in breast cancer cells. These studies stem from our prior work in which we have characterized an alternative TGF-β3 promoter uniquely functional in breast cancer cells (Arrick et al. 1994).

Body of First Yearly Report

First Objective (Objective A)

We had previously reported that intensity of anti-TGF-β1 immunoreactivity within breast cancer specimens correlated with poor clinical outcome (Gorsch et al., 1992). That study involved 57 heterogeneous patients. As an initial effort, we felt it important to confirm that observation by analysis of a larger number of tumor specimens from a homogeneous set of patients. We have recently completed the analysis of anti-TGF-β1 immunoreactivity in approximately 140 patients who had node-positive breast cancer and were treated as part of a multi-institutional clinical trial (by the CALGB cooperative group). Statistical analysis of these data, with correlation with markers of angiogenesis performed by collaborators, will begin shortly. Overall, among this group roughly 20% of patients were found to have tumors which overexpressed TGF-β1, 20% were determined to be underexpressors of TGF-β1, and the remainder (approximately 60%) were graded as expressing normal amounts of TGF-β1 protein.

Awaiting the analysis of the immunohistochemical data, we have conducted preliminary experiments to determine the optimal method of processing frozen tissue so as to obtain protein, DNA, and RNA of sufficient quality for subsequent quantitative analysis. We have compared grinding frozen tissue into a powder under liquid nitrogen using mortar and pestle with the use of a "tissue pulverizer" which essentially

allows one to whack at it with a hammer. The optimal method for tissue handling turned out to be a combination of the two, although we have yet to compare with a motor-driven homogenizer. We have had difficulty in obtaining RNA of high quality, that is to say without evident degradation, and so are exploring alternative freezing and processing procedures.

The *Statement of Work* in the grant application did not call for the completion of studies within this objective within the first year -- we aim to determine whether those tumors which overexpress TGF-β1 protein do so as a result of gene amplification of the TGF-β1 gene locus by the conclusion of the 18th month of the grant. Since the determination of the frequency of gene amplification is essentially a function of comparative PCR, this analysis of the genomic DNA from the frozen specimens must await the collection of a sufficient number of quality specimens. It would be inappropriate to compare PCR results from reactions conducted over a span of many months. We anticipate being able to report these results in the second yearly report.

Second Objective

The entirety of this objective is to understand the molecular basis by which breast cancer cells, unlike all other cell types examined by ourselves and others, utilize a different promoter for transcription of the TGF-β3 gene (Arrick et al., 1994). Our initial intention was to devote the first 18 months of the grant to the analysis of DNaseI hypersensitivity and genomic methylation in the vicinity of the TGF-β3 promoter. We had planned to not pursue experiments involving transfection of TGF-β3 promoter constructs until after the first 1.5 years of the grant. As we will describe below, we have since decided to work on both of these fronts during the first year.

Experiments for Objectives B-1 and B-2

DNase hypersensitivity assays were conducted as outlined in the grant proposal. In summary, cells (both breast cancer cell lines which express both transcripts of TGF-β3 and non breast cancer cell lines which express only the 3.5 kb transcript of TGF-β3) were grown to near confluence in 6 cm dishes in standard serum-containing medium. They were, while still adherent in the culture dishes, treated with DNase I at doses of 0, 2, 20, and 200 units/ml in the permeabilizing solution of Stewart et al (Nucleic Acid Research 19: 3157) for 4 min at RT, at which time the genomic DNA was isolated. This was then digested with BglII overnight and applied to a 1% agarose gel. Ethidium bromide staining revealed the expected "smear" of genomic DNA. This was transfered by capillary transfer to nylon membrane and probed with radiolabeled probe which was the BsU36I-BglII fragment from the TGF-β3 genomic clone. No differences in hybridizing band patterns was evident between cells which utilize the downstream TGF-β3 promoter (we tested T47-D and SK-BR-3) compared with cells which only utilized the upstream TGF-β3 promoter (HT-1080 and A-673 cells were tested). We also isolated nuclei from these cells by hypotonic lysis and treated the intact nuclei with the

same range of DNase I concentrations, prepared genomic DNA and analyzed the DNA exactly as outlined above. Again, we did not detect any differences among the cells.

Using the genomic DNA preparations from the cells not treated with DNase I we also analyzed for differences in methylation at HpaII vs MspI restriction sites by digesting overnight with either of these two enzymes, and including these digests in southern analysis as outlined above. This is a limited analysis of methylation status, but there were no differences between cells noted. The more robust methods outlined in the proposal involving sequencing require some prior knowledge of the approximate area of genomic DNA involved in the regulation of expression. It has thus become evident that efficient analysis of genome structure (methylation, DNase hypersensitivity) would be optimal if we could narrow our focus by comparison of a panel of expression constructs containing varying amounts of 5′ flanking sequence.

Experiments for Objective B-3

Although the Statement of Work did not call for any experimental efforts towards the completion of this objective, as discussed above, we have since decided that information that might be obtained by these experiments could facilitate the successful completion of objectives B-1 and B-2, and so have begun work on objective B-3.

The preliminary data regarding promoter activity that were presented in the grant application were obtained with a chimeric promoter-CAT construct which contained an enhancer element from the SV40 virus. In addition, that plasmid did not contain any sequences upstream of commonly-utilized TGF-β3 promoter (P1). We first prepared by standard subcloning techniques the two additional constructs outlined in Fig 9 of the proposal, ones which contain sequences which will also activate P1. To avoid confounding effects of the SV40 enhancer, this was removed. Transient transfection of these constructs into cells (HT-1080, SK-BR-3, T47-D, and A673) did not result in highlevels of CAT activity, and RNA analysis of the transfected cells failed to reveal detectable levels of CAT-specific RNA. These experiments were conducted many times, using a range of transfection techniques (Ca-phosphate precipitation, lipofectin, lipofectamine), and RNA analysis was done by standard Northern blotting as well as RNase protection assays.

To try to optimize expression, we prepared the following construct: the putative TGF-β3 promoter sequence was placed upstream of the neomycin resistance gene in an otherwise-promoterless plasmid. We have transfected with this construct T47-D and SK-BR-3 cells and have selected for stable transfectants by incubation of the cells in the presence of G418. We have recently done our first Northern with RNA from a few such clones, probing for RNA containing the neo resistance gene cDNA sequence, and believe that this will serve as a somewhat cumbersome but effective approach to this problem.

References Cited

Arrick, Bradley A., Richard L. Grendell, and Loree A. Griffin. 1994. Enhanced translational efficiency of a novel transforming growth factor-β3 mRNA in human breast cancer cells. Molecular and Cellular Biology 14:619-628.

Gorsch, Stefan M.Vincent A. Memoli, Thérèse A. Stukel, Leslie I. Gold, and Bradley A. Arrick. 1992. Immunohistochemical staining for transforming growth factor-β1 associates with disease progression in human breast cancer. Cancer Research 52: 6949-6952.