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This is a career development award. The primary focus of the research is the expression and function of TGF-β by breast cancer cells. 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 goal of confirming our prior work correlating clinical outcome with TGF-β expression levels in tumors. There are as yet no definitive data or conclusions from this aspect of the work. The second objective of the grant was to evaluate the impact of TGF-β on the tumorigenic and metastatic potential of human breast cancer cell lines in nude mice. We have made progress on generating the transfected cell clones, but do not yet have data regarding animal studies. The third research objective is the analysis of the regulation of TGF-β expression in breast cancer cells. We have made some progress in designing required promoter constructs for this work, but do not have data as yet.

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Bradley A. ...
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10/20/95
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ANNUAL REPORT

Grant Number DAMD17-J-4130

PI: Bradley A. Arrick, M.D., Ph.D.

Institution: Dartmouth College

Reporting Period: 10/1/94-9/30/95

Title: Studies on Human Breast Cancer and Transforming Growth Factor-beta --
Application for a Career Development Award.

Introduction

This is a career development award. The primary focus of the research is the expression and function of TGF- β by breast cancer cells. The specific grant objectives derived from prior work by ourselves and others which have identified TGF- β as an important cytokine in the biology of breast cancer (Gorsch et al. 1992). 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 goal of confirming our prior work correlating clinical outcome with TGF- β expression levels in tumors. The second objective of the grant was to evaluate the impact of TGF- β on the tumorigenic and metastatic potential of human breast cancer cell lines in nude mice. The third research objective is the analysis of the regulation of TGF- β expression in breast cancer cells. This primarily deals with identification of the promoter elements responsible for the function of a breast cancer-specific TGF- β promoter (Arrick et al. 1994).

Body of First Yearly Report

Objective #1

We had previously reported that intensity of anti-TGF- β 1 immunoreactivity within breast cancer specimens correlated with poor clinical outcome (Gorsch et al., 1992). That study involved 57 heterogeneous patients. Confirmation of this correlation can most convincingly be done by analysis of tissue specimens from a large cohort of patients that have participated in one of the adjuvant chemotherapy trials conducted by a multi-institutional cooperative group. Before we were able to do this immunohistochemical analysis, we had to establish the staining conditions for a different anti-TGF- β 1 antibody since the polyclonal antisera we had been using ran out. We settled on a polyclonal antibody available from Promega, Inc., which is also specific for TGF- β 1. Antigen retrieval with an 8 min treatment of sections with Pronase E turned out to be optimal for formalin-fixed sections. We have recently completed the analysis of anti-TGF- β 1 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- β 1, 20% were determined to be underexpressors of TGF- β 1, and the remainder (approximately 60%) were graded as expressing normal amounts of TGF- β 1 protein.

As outlined in the *Statement of Work* in the grant application, these studies will be ongoing over the course of the entire grant. We will report results yearly as they are obtained.

Objectives #2A and 2B

In order to evaluate the in vivo impact of a breast cancer cell which overexpresses TGF- β , we are preparing stable transfectants of the MDA-MB-231 cell line which contain TGF- β 1 expression constructs. We had considerable difficulty in obtaining the desired clones. Starting off by co-transfecting the TGF- β plasmid with a neomycin-resistance plasmid, we isolated many clones and found that nearly all did not express the TGF- β plasmid. This was not surprising since TGF- β can slow the growth of these cells. To maximize transfection efficiency, we began two parallel experiments. First we prepared retroviral vectors containing the TGF- β cDNA sequence. We also prepared bicistronic expression plasmids in which both TGF- β and neo would be translated off of the same transcript. To accomplish this, we obtained a plasmid from Dr. John Majors which contains the internal ribosome entry site from the encephalomyocarditis virus (Ghattas et al., 1991). This plasmid, called pCEN, allows for insertion of the coding sequence of interest upstream of the internal ribosome entry site via a unique EcoRI site, with the neomycin resistance gene situated downstream of the internal ribosome entry site as the second cistron. By situating the two cistrons in this manner, we felt that selection for G418 resistance would ensure for the expression of mRNA encoding the desired cDNA.

Recently-completed analysis of clones transfected with the bicistronic vectors has indicated that the vast majority of neomycin (G418)-resistant clones also produced high levels of TGF- β as a result of expression of the transfected cDNA. RNA analysis by Northern blot documented that transfected cells expressed RNA species which included the coding sequences of both the neo resistance gene and the TGF- β 1 cDNA cassette. We measured expression at the protein level by collection of conditioned medium and assay with the standard mink lung assay. Conditioned medium from clones transfected with pCEN alone and cells transfected with the pCEN-C2S2 plasmid was collected, half was treated with HCl to activate all TGF- β , and then the condition medium was placed in a dilution series on mink lung cells. These cells are exquisitely sensitive to the growth inhibitory effects of TGF- β , and serve as the read-out in the TGF- β bioassay which measures the amount of BrdU incorporated by cells over a 4 hr time period as a measure of DNA synthesis. In each experiment, a standard curve with recombinant TGF- β was generated. By comparison of the amount of active TGF- β in the conditioned medium after acid activation to the amount present without activation, we could calculate the percentage of secreted TGF- β that is in the active state de novo. We pursued two of our pCEN-C2S2 clones. Both were found to secrete over 10-times more TGF- β than the pCEN clones. Furthermore, as expected, the pCEN-C2S2 clones

produced TGF- β that was partially active. From both clones approximately 35-50% of the TGF- β was active without acid activation.

In a similar manner, we transfected cells with a construct which would have yielded a dominant negative mutant of TGF- β . Despite the isolation of successfully transfected clones expressing the expected RNA, we were unable to document any reduction in the amount of TGF- β secreted by these cells. Discussions with the scientist who gave us this cDNA confirmed that they have had similar difficulty in other cell types. We elected to not pursue the preparation of cells which underexpress TGF- β . Rather, with increasing attention to the importance of the predominant TGF- β receptor (termed the type II receptor) as a target of mutation in malignant progression, we have decided to expand our analysis to include breast cancer cells in which we have down-regulated expression of this receptor by transfection of a dominant negative receptor expression plasmid. Since we will want to prepare cell clones which not only have diminished receptor expression but also overexpress the ligand TGF-b1 (which may represent the most malignant phenotype), we will need to prepare expression plasmids which rely on a different selectable marker. We will use a zeocin-resistance cDNA for this purpose.

According to the *Statement of Work* in the grant application we would be beginning to test these stable cell clones in nude mice (Objective 2B). With our failure to prepare underexpressors, and with our decision to prepare clones with reduced receptor expression, we have delayed the beginning of the animal experiments until those cells are ready. We cannot begin the animal experiments until all of the clones are in hand. Therefore, we anticipate being able to report results of the first animal experiments in the second yearly report.

Objective #3

#3A

During this first year of the grant 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.

#3C

A major component 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- β 3 gene (Arrick et al., 1994). In the *Statement of Work* in the grant application we had indicated that we would not pursue these studies until after the first year. We have since decided to work on some of these

experiments during the first year while we optimize our methodology for frozen tissue processing and the collection of specimens.

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- β 3 and non breast cancer cell lines which express only the 3.5 kb transcript of TGF- β 3) 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 BglIII overnight and applied to a 1% agarose gel. Ethidium bromide staining revealed the expected "smear" of genomic DNA. This was transferred by capillary transfer to nylon membrane and probed with radiolabeled probe which was the BsU36I-BglIII fragment from the TGF- β 3 genomic clone. No differences in hybridizing band patterns was evident between cells which utilize the downstream TGF- β 3 promoter (we tested T47-D and SK-BR-3) compared with cells which only utilized the upstream TGF- β 3 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.

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- β 3 promoter (P1). We first prepared by standard subcloning techniques the two additional constructs 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 high levels 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 (Calcium 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- β 3 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.

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