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GRANT NUMBER DAMD17-96-1-6146

TITLE: Characterization of Breast Cancer Cell Death Induced by Interferons and Retinoids

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REPORT DATE: July 1997

DTIC QUALITY INSPECTED 2

TYPE OF REPORT: Annual

19971210 049

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

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1997	Annual (1 Jul 96 - 30 Jun 97)

4. TITLE AND SUBTITLE
Characterization of Breast Cancer Cell Death Induced by Interferons and Retinoids

5. FUNDING NUMBERS
DAMD17-96-1-6146

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland at Baltimore
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 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*

We have previously observed that a combination of human Interferon-β (IFN) and all-trans retinoic acid (RA) induces superior growth inhibitory responses in several human tumor cells. In particular this combination induces cell death similar to apoptosis *in vitro*, which could not be observed with individual agents. Preliminary studies identified no changes in the levels of known regulators of cell death such as p53, cyclin D and Bcl2. Thus it is important to understand the cell growth suppressive mechanisms and the genes that mediate these effects. We hypothesized that the observed cell death might be mediated by either novel or hither to unimplicated gene products. Therefore, the major aim of this project is to identify the gene products that mediate the growth inhibitory/ cell death inducing activities of the combination of IFN and RA in human tumor cells. To directly identify these gene products we have proposed to employ a genetic technique that utilizes anti-sense knock-out as the strategy. In this technique, cells are transfected with cDNA libraries cloned in antisense orientation in an episomal vector and then are challenged to survive the effects induced by human IFN-β and RA. The cDNA libraries were prepared from BT-20 human breast carcinoma cell line that was treated with the IFN/RA combination for various lengths of time. The surviving cells will carry an antisense product that inhibits the function of an endogenous gene thereby blocking its growth inhibitory functions. By preparing the Hirt DNAs, the episome can be isolated from the cells and can be used to transform bacteria. Sequencing of the episome identified in bacterial screening would permit the identification of the gene products.

14. SUBJECT TERMS Breast Cancer	15. NUMBER OF PAGES 11
	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

FOREWORD

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Edward R. Hamm 7/25/97

PI - Signature Date

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Introduction:

This training fellowship was awarded to a pre-doctoral student, Edward R. Hofmann, at the University of Maryland School of Medicine. Research data generated from this study will be submitted as a Ph. D. thesis in the future.

Interferons (IFN) are a group of multi functional cytokines which regulate cellular antiviral, antitumor and immunological responses (1,2). Products of IFN-stimulated genes (ISGs) mediate the biological actions of IFNs (2). IFN-α/β treatment of cells leads to the tyrosine phosphorylation of cytoplasmic STAT proteins by JAK family protein kinases JAK1 and Tyk2 (3,4). Upon phosphorylation, two such proteins, STAT1 and STAT2, then migrate to the nucleus. They then associate with another DNA binding protein, p48, and subsequently bind to IFN stimulated response element (ISRE) to activate gene expression. IFN-γ, on the other hand, activates STAT1 through protein kinases JAK1 and JAK 2, which then migrates to the nucleus and binds to the γ-IFN activated site (GAS) to stimulate transcription (3,4). Despite the description of multiple cellular ISGs, only two major mechanisms have been implicated in their growth regulatory actions (1,5,6). These are: i) the 2-5A pathway and ii) the PKR (protein kinase R) pathway. Both these pathways are operated by double stranded RNA-dependent enzymes. We have not found evidence for the involvement of these pathways in IFN + RA mediated growth inhibition. Although IFNs as single agents have strong anti-tumor activities in a variety cancer cells (7), they have limited effects on breast tumor cell growth (8). Therefore, we explored whether a combination of IFNs with retinoids would cause strong growth inhibition in breast cancer cells.

Retinoids encompass vitamin A related compounds which have profound influences on cell growth and differentiation (9). A prototype of these is all-trans retinoic acid (RA), a natural metabolite. Upon entry into the cells, retinoids bind to specific nuclear retinoic acid receptors (RAR) which then associate with cognate retinoic acid response elements (RARE) to stimulate gene expression (9,10). Retinoids suppress the growth of several tumor cells *in vitro* and *in vivo* (11). Despite their strong cell growth regulatory action, the candidate genes for retinoid mediated growth inhibition have been unknown. Although many studies have suggested a role for RARs in growth inhibition of RA (12,13), these data so far have not identified the ultimate gene product(s) that mediate the growth inhibition. Furthermore, our laboratory and others have found that RA by itself is not sufficient for breast tumor growth inhibition *in vitro* and *in vivo* (14).

Previous studies in our lab have demonstrated that IFN-β and RA alone are unable to inhibit cell growth in several breast tumor cell lines. However, IFN-β + RA combination induces growth arrest of several breast carcinoma cells both ***in vitro*** and ***in vivo*** (15). Furthermore, high concentrations or extended exposure to IFN-β + RA induces a cell death consistent with programmed cell death. Preliminary studies identified no changes in the levels of known regulators of cell death such as p53, cyclin D and Bcl2 in IFN+RA treated cells (16-18). Thus it is important to understand the cell growth suppressive mechanisms and the genes that mediate these effects. We hypothesized that the observed cell death might be mediated by either novel or hither to unimplicated gene products. Therefore, the major aim of this project is to identify the gene products that mediate the growth inhibitory/ cell death inducing activities of the combination of IFN and RA in human tumor cells.

Body:

Experimental Approach

To directly identify these gene products, we have proposed to employ a genetic technique that utilizes anti-sense knock-out as the strategy (19, 20). In this technique, cells are transfected with cDNA libraries cloned in antisense orientation in an episomal vector and then are challenged to survive the effects induced by human IFN-β and RA. The cDNA libraries were prepared from BT-20 human breast carcinoma cell line that was treated with the IFN/RA combination for various lengths of time. The surviving cells will carry an antisense product that inhibits the function of an endogenous gene thereby blocking its growth inhibitory functions. By preparing the Hirt DNAs (21), the episome can be isolated from the cells and can be used to transform bacteria. Sequencing of the episome identified in bacterial screening would permit the identification of the gene products.

Methods

Cell growth: All estrogen dependent cells were cultured in phenol red free EMEM supplemented with 5% charcoal stripped fetal bovine serum (CSFBS) and 10^{-11}M estradiol. All other cell lines will be cultured in the media with phenol red but supplemented with 5%CSFBS (15). Drug selections were performed with empirically determined concentrations for 2-3 weeks. Surviving cell clones were expanded and total DNA was extracted by Hirt method (21).
RNA isolation and cDNA synthesis: Total RNA will be isolated using RNAZol B reagent (Tel-Test Inc, TX). PolyA+ RNA was isolated using polyAtract system (Promega Inc.) cDNA libraries will be constructed with commercially available kits (Stratagene Inc.) per the recommendations of the manufacturer.
Drugs: IFN-β_{ser} (Berlex Inc. CA) and all trans retinoic acid (Sigma chemical co, MO) and Hygromycin B (Boehringer-Mannheim Inc.) will be employed through out these studies.
Electroporation of mammalian cells: Cells (10^6) were trypsinized and collected into 2mm gap BTX electroporation cuvettes. 20μg of antisense library cDNA was used in each cuvette along with 30μg of salmon sperm DNA as carrier. Electroporation conditions were as follows: 250V, 900μF, 13ohms.
Transformation of bacteria: Hirt extracts were done as described before. DH10B strain *E. coli* were electroporated with 30-50μg Hirt extract DNA. Conditions were as follows: 2.5mV, 129ohms.

Results:

We have prepared antisense cDNA library using polyA+ mRNA from BT-20 cells treated with IFN-β (100U/ml) + RA (1μM) for various lengths of time . This cDNA was then ligated with a bifunctional linker which upon attachment to 3' end of the cDNA created a HindIII restriction site (22). Attachment to the 5' end created a BamHI site. This cDNA was then digested with HindIII and BamHI enzymes for further cloning into expression vector, pTKO1. Since the cDNA was

created using a methylated dCTP, it protected the internal digestion of cDNAs. These cDNAs were then cloned in to HindIII and BglII digested episomal eukaryotic expression vector, pTKO1. This vector contained a HindIII close to an IFN stimulated promoter and the BglII site at the close to polyadenylation site. Cloning of cDNA into this vector thus allowed the cDNA to be inserted in an antisense orientation. This vector also carried a Hygromycin resistance marker for selection in eukaryotic cells. Further, it also contained Ori-P (origin of DNA replication) and EBNA1 (Epstein-Barr viral nuclear antigen 1) of Epstein-Barr virus for its autonomous replication in mammalian cells. It also carried a ampicillin resistance marker for selection in bacteria. In order to identify the genes that are responsive to IFN/RA combination, we aimed cloning the genes that would express in a variety tumors. For this purpose, these libraries were transfected into a HeLa cell line in which IFN (1000U/ml)+RA (1μM) combination induces cell death. After 1st round transfection of the library, we have identified several surviving clones. Hirt preparations from the first round were transformed into bacteria. This resulted in the isolation of 22 possible episomes that carried cDNA inserts of variable length. Subsequently, individual episomes were transfected into MCF-7 breast carcinoma cells and the selected with IFN/RA combination (200U/ml+1μM) and Hygromycin (100μg/ml) for 27 days. Figure 1 represents the data obtained with some of the rescued clones. These candidate clones were further analyzed by restriction digests and southern analyzes to confirm they were pTKO1 vectors with cDNA inserts. Of the 22 rescued clones, 7 were false positives. Second and third round transfections were performed to further screen for true clones. Of the 15 that were tested all were found to protect the cells against the growth inhibitory effects of IFN/RA combination in human breast carcinoma cell lines, MCF-7, BT-20 and T47D. We have named them as Death associated genes (DAGs). Figure 2 represents the cell protective effect of one of the rescued episomes, DAG1. These experiments were long term time because each time the experiment took nearly a month to obtain results. Initially, southern blot analyzes were performed to detect whether these independent isolates were related to each other. These studies suggested that these clones are independent of each other. A representative southern blot is shown in Figure 3. In this case, DAG1 cDNA insert was employed as a probe against other rescued inserts. As can be seen in the figure it has no relationship to other DAG inserts (faint signals in other lanes are not specific to DAG1 cDNA because the probe encompasses primer sequences from the vector). We have partially sequenced six of the isolates. Partial sequences from these genes did not match to known genes in the Genebank. Therefore, they appear to be novel products. Northern blot analyzes identified specific mRNAs expressed in the cells. One of these clones DAG1 has been chosen for further study. A partial sequence (1 Kb) of DAG1 has been established. It has no homology to any known gene sequences found in GeneBank as of today. Since these isolates did not contain full length inserts, we have prepared new libraries, in sense orientation, to isolate full length clones. We have just begun the cloning of these inserts into bacterial expression vectors to identify their gene products.

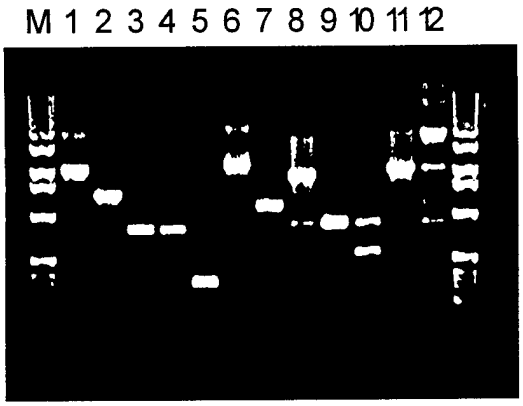

Fig.1: Identification of multiple inserts from individual clonal isolates from 1st round transfection BT-20 anitsense library and selection with IFN-β (200U/ml), RA(1μM) and Hygromycin-B (100μg/ml). M= Molecular weight markers. Representative samples from different episomes rescued were subject to PCR using primers specific for the pTKO1 vector. Note the differences in the sizes of inserts.

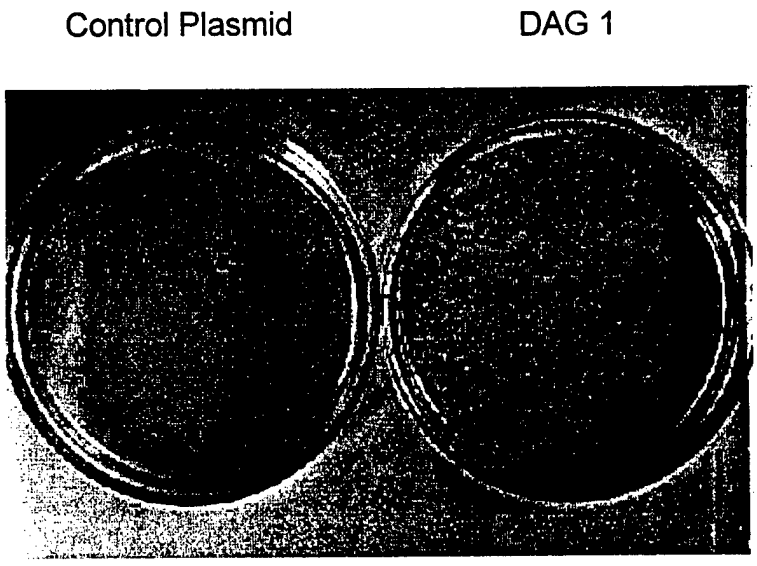

Fig. 2: Protection of MCF-7 breast carcinoma cell line from against the cytocidal effects of IFN-β and RA the DAG clones. Cells were transfected with DAG 1 episome and selected for 3 weeks in the presence of Hygromycin-B (100μg/ml), IFN-β (200U/ml) and 1μM RA. Control plasmid is parent vector (pTKO1) carrying chloramphenicol acetyl transferase gene. Similar studies were also performed in other breast carcinoma cells.

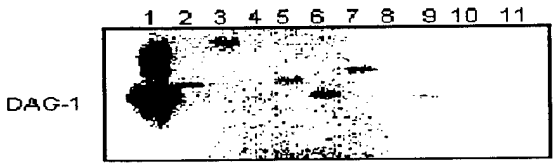

Fig.3: Inter-relationships of the isolated death associated genes. PCR products of each clonal isolate were separated on a 1% agarose gel. They were southern blotted and probed with DAG 1 PCR product labeled with ^{32}P. Each lane had comparable amount of DNA as visualized by ethidium bromide staining (data not presented). Specific bands with intense signal correspond to the probe.

Conclusions:

Our studies have established the validity of using antisense knockout technology for identifying genes that cause cell growth arrest or death. Consistent with our hypothesis that IFN-β + RA combination employs novel gene products, we have identified several potential independent cDNAs. The role of these genes in the cell death process will be examined in future studies. As a step towards this, we have begun the characterization of DAG 1.

Studies planned for the next budget year:

1. We will complete the sequence of DAG1 and establish its complete map. We will transcribe the gene products in vitro. We will predict the structural domains using GCG program. These data would aid further in the mutational analysis.

2. We will express the gene products in PET bacterial vectors to isolate the pure proteins and raise antibodies against the expressed proteins. If these proteins could not be expressed in bacterial cells, we will explore their expression in baculoviral vectors.

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