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13. ABSTRACT *(Maximum 200 words)*

The purpose of the work in year 01 of this study was to generate stable lines of human breast carcinoma cells which expressed varying levels of plasminogen activator inhibitor type-1 (PAI-1) as a consequence of transfection with a vector bearing a PAI-1 cDNA insert under control of MYC-responsive E box sequences. A panel of 14 such genetically-engineered cells was developed which varied significantly in the level of vector-driven PAI-1 mRNA and protein expression. Two of these cell lines were assessed as to their ability to adhere to the extracellular matrix protein vitronectin. High level PAI-1 synthesizing transfectants were significantly impaired compared to parental control breast carcinoma cells with regard to their adhesive ability. A comprehensive characterization of the growth traits of these cells, as well as the continuing generation of new estrogen-dependent transfectants will be completed in year 02.

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Table of Contents

Introduction

Amplification or genomic rearrangements involving the c-myc locus, resulting in increased MYC protein expression, is a frequent event in breast tumor development occurring in 20 to 57% of human breast carcinomas (reviewed in Hesketh, 1994; Nass and Dickson, 1997). Indeed, all human breast tumors appear to have some basic defect in MYC expression control as evidenced by the frequent overexpression of MYC protein in this tumor type (Hesketh, 1994). c-myc amplification has important biologic consequences for the breast cancer patient and is predicitive of shortened relapse-free time and decreased overall survival. In general, c-myc amplification correlates with high tumor proliferative rate, lymph node involvement, tumor size, extracellular proteolytic activity, aneuploidy, and aggressive behavior. Recent attempts to control growth of c-myc overexpressing breast carcinomas have included targeted suppression of MYC synthesis using antisense deoxyoligonucleotides and genotoxic drugs (topoisomerase II inhibitors). These efforts have met with limited success and certain unexpected toxicities.

The MYC protein is a member of the helix-loop-zipper family of transcription factors which function as dimers to regulate expression of particular genes that possess a specific hexanucleotide sequence (CACGTG; termed an "E"-box) in their "promoter" regions or intronic regions. E-boxes occur in certain growth or cell cycle-regulated genes and it is presumed that MYC functions to transcriptionally activate this set of growth genes. Work in our laboratory has revealed that one growth-regulated gene which is consistently overexpressed in aggressive breast carcinomas and encodes the type-1 inhibitor of plasminogen activator (PAI-I) also contains an E box in its promoter.

Cancer invasion and metastasis are complex processes in which degradation of the extracellular matrix (ECM) plays a critically important role. Such matrix degradation, which facilitates cell migratory activity, is accomplished by the concerted action of several cascading proteolytic systems including the generation of the serine protease plasmin by the urokinase (uPA)-dependent pathway of plasminogen activation **(figure appended)** (Dano et al., 1994; Laiho and Keski-Oja, 1989) and subsequent activation of metalloproteinases (Liotta et al., 1991). Indeed, ECM degradation is regulated largely by the plasmin-based proteolytic cascade (Vaheri et al., 1990; Pollanen et al., 1991; Laiho and Keski-Oja, 1989). Plasmin, in turn, degrades the ECM directly as well as indirectly by activating latent metalloproteinases (Dano et nal., 1987). Within this context PAI-1 plays a primary role as a negative regulator of this pericellular proteolytic cascade by complexing to and inhibiting the catalytic activity of uPA (Blasi et al., 1987; Laiho and Keski-Oja, 1989) **(figure appended)** By virtue of the specific subcellular localization of elements of the plasmin-based pericellular proteolytic cascade to regions of cell-to-substrate adhesion **(figure appended)**, modulation of the expression of any or each of these factors would be expected to have significant ramifications with regard to the control of cellular growth and migration abilities.

Clinical studies have demonstrated that, in general, elevated tumor levels of uPA, the uPA receptor, and/or PAI-1 are consistently associated with poor disease outcome and appear conducive to tumor spread and metastasis (Schmidt et al., 1992; Duffy, 1992, 1996; Pedersen et al., 1994). The role of PAI-1 as a determinant in aggressive growth behavior is particularly relevant in the case of breast cancer. Elevation of uPA, uPA receptor and, more consistently, PAI-1 in the primary breast carcinoma signaled an elevated risk for metastasis and a poor prognosis (Costantini et al., 1996; Gandolfo et al., 1996; Mayerhofer et al., 1996; Fersis et al., 1996 Torre and Fulco, 1996; Foekens et al., 1995). Because of the increasing appreciation of the contribution of these elements of the plasmin regulatory cascade to growth and invasiveness of human cancers, various approaches to block expression of uPA, the uPA receptor, and PAI-1 at the gene level (with antisense deoxyoligonucleotides) or neutralizing antibodies have met with varying success in mouse model systems (reviewed in Schmitt et al., 1997) We proposed to utilize a metabolic anomaly of many breast carcinomas (i.e., elevated MYC expression) to specifically drive expression of an introduced therapeutic construct in tumor cells. This would be expected to greatly enhance the specific effects of the introduced vector and eliminate the often non-specific and potentially toxic aspects of large dose deoxyoligonucleotides and antibodies.

The current general consensus is that expression of PAI-1 protein by breast tumor cells is either **(1)** insufficient to effectively block the excessive production of uPA characteristic of highly aggressive tumor cell types or **(2)** permissive for the formation of new ECM by the invading tumor cells thus providing the ECM scaffold on which to propagate and migrate. Our experiments are designed to address both potentialities (using sense vectors to further increase PAI-1 expression and antisense constructs to suppress endogenous PAI-1 synthesis). Indeed, the Principal Investigator's laboratory has considerable experience in the field of PAI-1 gene expression and its relevance to cell growth control. We were the first to demonstrate that PAI-1 transcription is growth state-regulated (Ryan et at., 1996; Kutz et al., 1997), that such expression is modulated by cell-to-substrate adhesion (Slack and Higgins, 1996; Ryan et al., 1996) and that, at least in *ras* oncogene-transformed cells, PAI-1 has the characteristics of a tumor suppressor gene (Higgins et al., 1997).

Our laboratory has taken the novel approach of targeting expression of myc-responsive, cell cycle-dependent, genes using engineered expression vectors designed to respond to the endogenous breast cancer MYC protein as a means to regulate cell growth behavior. The gene we have selected to genetically manipulate encodes PAI-1. We have constructed vectors bearing a full-length PAI-1 cDNA; expression of this cDNA insert is under control of specific myc-responsive E-box promoter sequences which differ in copy number to regulate level of induced PAI-1 expression **(figure appended)**. The insert encodes a PAI-1 species distinguishable from the endogenous breast PAI-1 to insure that the observed biological effects are due to the vector-driven PAI-1 "gene". Such utilization of tumor-associated anomalies in gene (i.e. c-myc) expression to direct genetic-based intervention therapies is a new approach to cancer treatment. This strategy, moreover, addresses the more aggressive breast tumor cell type, the highly proteolytically active potentially metastatic cell, for specific therapy. These studies will provide information critical to the eventual design of tumor type-appropriate targetable delivery systems for genetic therapy of breast cancer. This approach, moreover, takes advantage of the breast carcinomas own amplified MYC expression to transactivate the transfected PAI-1 vector E-box resulting in high level PAI-1 transcript production. Transfectant targets include both estrogen-responsive and independent human breast cell lines; levels of PAI-1 expression attained are monitored by Northern blotting (for mRNA) and ^{35}S-methioning labeling/immunoprecipitation (for protein). Once expression status has been confirmed, morphology, growth characteristics and invasive potential of the derived lines will be established. This study constitutes the first assessment of the potential usefulness of the PAI-1 gene in genetic therapy of human breast cancer.

Body of Report

Experimental results and methods for year 01 studies.

Results:

In accord with the goals in ***Task 1*** in the originally proposed Statement of Work which was as follows:

***Task 1*:** To assess the effects of vector-directed PAI-1 expression on *in vitro* growth traits of human breast carcinoma cells.

(a) develop a panel of transfectant MCF-7 (estrogen responsive) and MDA-MB-231 (estrogen receptor negative) breast carcinoma cells which express differing levels of vector-driven PAI-1 mRNA and protein and protein (months 1-8)

(b) perform assays to assess the *in vitro* growth characteristics of the individual transfectant cell lines (months 9-14)

we have successfully generated a total of 14 different stable transfectant breast carcinoma cell lines which vary in constitutive levels of PAI-1 protein and in RNA expression as a consequence of expressing our positive sense E-box-driven Rc/CMVPAI vector **(figure appended).** These lines have been designated MDA-Rc/CMVPAI-A through N. Levels of PAI-1 synthesis obtained closely correlated with the number of E box sequences ligated supporting our original hypothesis that the CACGTG motif was an important regulatory element in PAI-1 expression in human breast carcinomas. These resource cell lines will be utilized in work carried out in years 02 and 03 which is devoted to a detailed characterization of the growth traits of these stable transfectants. We have initiated an analysis of the growth characteristics of two of these transfectant lines using the appropriate parental strain as a control. These results are also described in the appended table. It is apparent that cells induced to express high levels of PAI-1 are significantly compromised with regard to their ability to adhere to the extracellular matrix protein vitronectin. It will be important to evaluate (in year 02) each of the generated cell lines as to their adhesive and migratory abilities, including their matrix degrading capability, as proposed originally. The specific methodology utilized in this phase of the study is described below. In addition, we are currently generating additional transfectant lines in the MCF-7 cellular background which provide the unique quality of estrogen-inducibility of MYC expression thereby allowing for controlled vector-driven PAI-1 gene expression for use in growth trait assessments.

Methods:

Preparation of transfectant human breast tumor cells. The estrogen receptor negative MDA-MB-231 cells have a constitutively high level of MYC synthesis (Nass and

Dickson, 1997) and were the host cell selected for the initial preparation of stable transfectants for long-term growth studies. The transfection protocols used, selection of individual clones, and assessment of extent of perturbation of PAI-1 expression at both the mRNA and protein levels were described in detail previously (Higgins et al., 1997). A panel of transfectants was established which vary significantly in levels of PAI-1 protein expression over transfectant controls (cells transfected with vector minus PAI-1 cDNA insert) and parental cells **(described in appended table).**

Assessment of in vitro growth traits of transfectant cells. Initial studies were designed to evaluate if cells which vary in ability to synthesize sense PAI-1 have distinctly different adhesive traits. Control and genetically-engineered cells were evaluated with respect to potential differential cell adhesion to protein-coated surfaces. Adhesion was assessed using the ECM protein vitronectin pre-coated onto quadruplicate wells of Immulon 1 (Dynatek) microliter plates. Mid to late log phase cells previously labeled with ^{3}H-thymidine(3 μCi/ml) for 24 hours were harvested and resuspended in DMEM/15 mM HEPES.0.2% BSA. Cells were added to wells for 30-120 minutes at 37^{0}C. After removal of nonadherent cells and extensive washing, adherent cells were lysed and radioactive counts determined per well compared to total radioactivity added per well (these data are presented in the appended table). Optimal assay conditions are currently being established with regard to cell number, protein coating concentration, length of incubations.

Conclusions

Several important conclusions were reached as a result of work in year 01 which were consistent with the tasks outlined in the original Statement of Work proposed.

(1) Various transfectant breast carcinoma cell lines were developed from a defined parental stock which express different levels of vector-driven PAI-1 mRNA and protein. The PAI-1 expressing status of these genetically-engineered cells correlated with the number of MYC-responsive E box modules ligated to the basal PAI-1 promoter supporting our contention that the E box is an important regulator of PAI-1 expression in breast carcinoma cells. The constitutively-produced MYC protein produced by the transfectant lines likely transactivated the expression vector construct although this will be confirmed in year 02 by means of mobility shift experiments to demonstrate MYC binding to the myc-responsive sequences in the plasmid.

(2) Preliminary assessments of the growth and adhesive traits of genetically-engineered cells compared to parental controls indicated that PAI-1 over-expressing lines were significantly less adhesive to the matrix protein vitronectin. These findings are preliminary and restricted to only several of the transfectant lines derived but are consistent, nevertheless, with clinical observations relating high PAI-1 expression to increased motility and metastasis in breast cancer.

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APPENDIX

TABLE

Transfectant Cell Line	PAI-1 Expression Level [a] (fold increase to MDA parental)	Vitronectin Adherence [b] (% of MDA parental)
MDA-Rc/CMVPAI-A	1.6 ± 0.7	
MDA-Rc/CMVPAI-B	23.6 ± 4.4	
MDA-Rc/CMVPAI-C	7.8 ± 1.9	
MDA-Rc/CMVPAI-D	2.2 ± 0.8	
MDA-Rc/CMVPAI-E	11.9 ± 3.4	
MDA-Rc/CMVPAI-F	0.9 ± 0.1	
MDA-Rc/CMVPAI-G	1.5 + 1.1	
MDA-Rc/CMVPAI-H	47.3 + 7.0	12.1 ± 4.2
MDA-Rc/CMVPAI-I	9.1 ± 2.7	
MDA-Rc/CMVPAI-J	15.5 ± 3.0	31.4 ± 9.1
MDA-Rc/CMVPAI-K	4.7 ± 0.8	
MDA-Rc/CMVPAI-L	1.0 ± 0.6	
MDA-Rc/CMVPAI-M	19.7 ± 5.8	
MDA-Rc/CMVPAI-N	33.3 ± 8.0	

[a] assessed by gel electrophoresis of metabolically – labeled cultures (Higgins et. al., 1997)

[b] Relative vitronectrin adherence of ^{3}H-thymidine-labeled cells (MDA parental adherence = 100%)

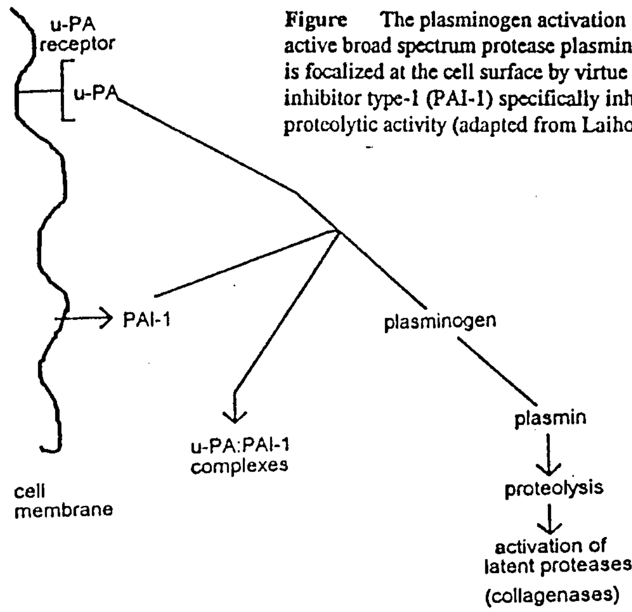

Figure The plasminogen activation cascade. Conversion of the inactive zymogen plasminogen to the active broad spectrum protease plasmin is catalyzed by urokinase type plasminogen activator (u-PA) which is focalized at the cell surface by virtue of interaction with its specific receptor. Plasminogen activator inhibitor type-1 (PAI-1) specifically inhibits this reaction resulting in a net decrease in pericellular proteolytic activity (adapted from Laiho and Keski-Oja, 1989).

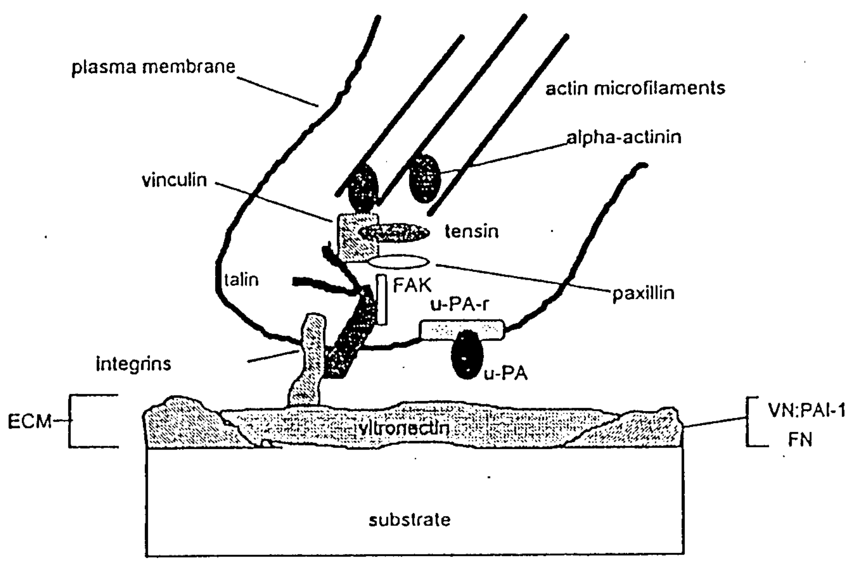

Figure Idealized schematic illustrating localization of u-PA and PAI-1 at focal adhesion sites. The relationship between constituents of the focal contact and components of the plasmin-mediated proteolytic system demonstrate the potential importance of the plasmin-mediated proteolytic cascade as a regulator of cell-to-substrate adhesion. u-PA (associated with its receptor) and PAI-1 are localized to the extracellular face of the cell within and surrounding the focal contact, respectively (derived from Pollanen et al., 1991 and Ezzell, 1993).

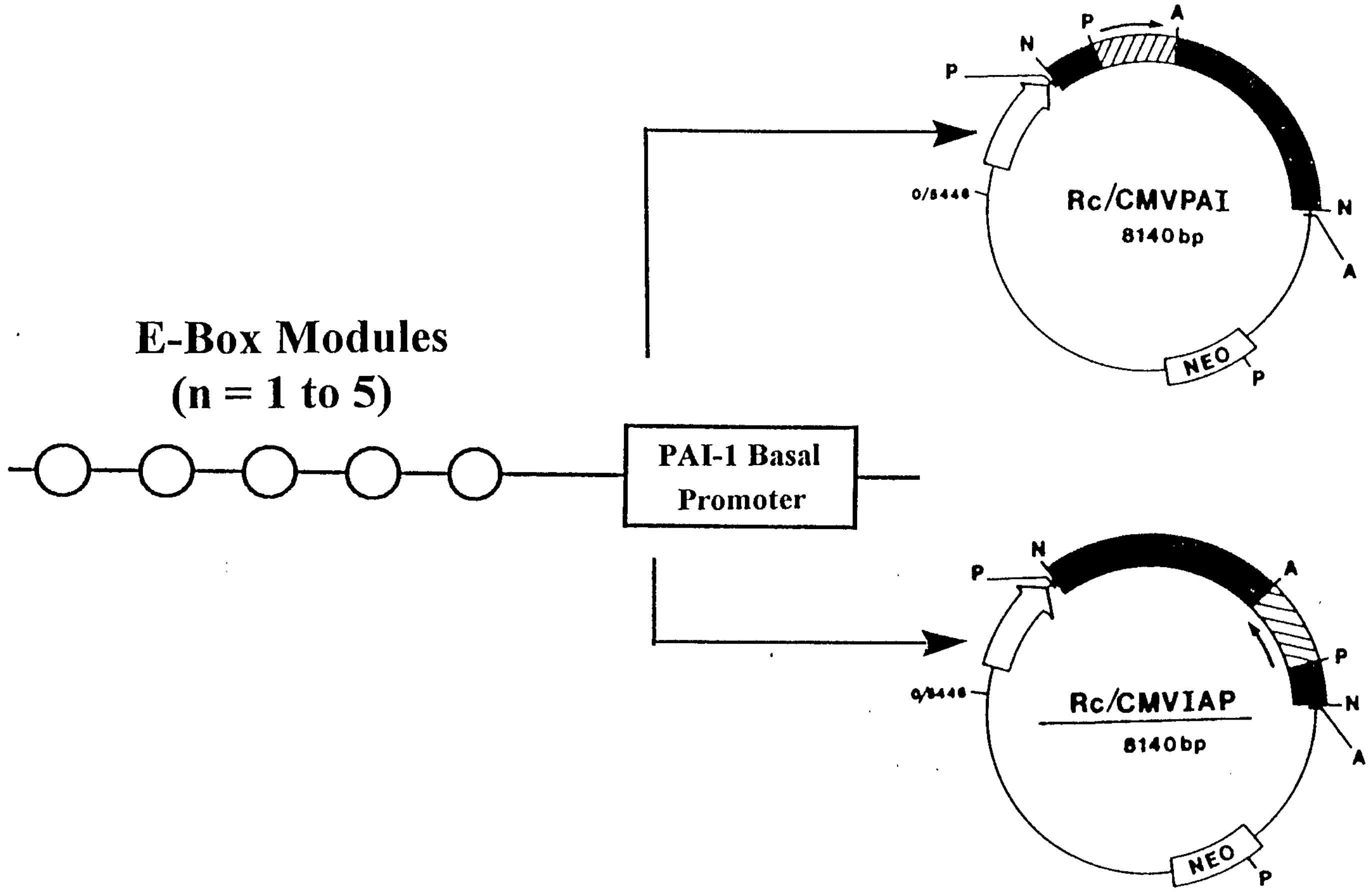

Figure Stratagy for construction of PAI-1 sense and antisense expression vectors under the control of E-box-driven transcription. The CMV promoter was restricted out of our previously designed Rc/CMV-based PAI-1 sense (PAI) and antisense (IAP) vectors (Higgins et al., 1997) and replaced with a cassette comprised of *myc*-responsive hexanucleotide E-box elements (varying in copy number from 1 to 5) and the basal PAI-1 promoter fragment (consisting of nucleotides -162 to -1 and containing the PAI-1 TATA box). This basal promoter fragment is necessary for transcriptional activation of the PAI-1 gene when ligated to various heterologous and PAI-1-specific enhancer elements but is itself a poor activating sequence.