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We have determined by western blot analysis the level of expression of a number of isoforms of Protein Kinase C in a panel of breast cancer cell lines of differing invasive and metastatic potential. The cells were found to express no PKC-β, similar levels of PKC-ι/λ and various levels of PKCs δ, ε and α. Of these, PKC-α expression levels, particularly those of the small, active form of the enzyme, were found to correlate with invasive and metastatic behavior. These findings strengthen the hypothesis that the expression of particular isoforms is associated with invasive and metastatic behavior.

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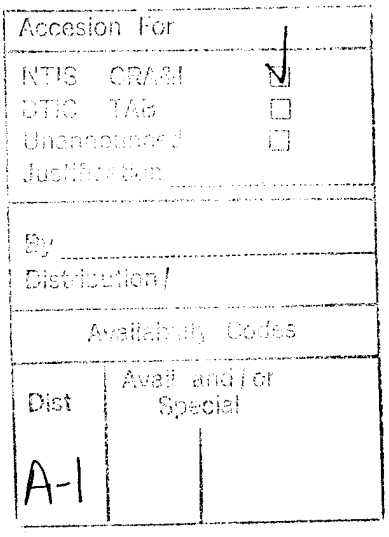
Table of Contents:



Introduction:

The establishment of a metastasis is the result of complex series of processes which seem to require that the tumor cell possess a number of specific abilities. Metastasizing cells must first change their adhesion properties to allow them to break away from the primary tumor. They must become more motile, and elaborate proteases to allow them to degrade and pass through the extra-cellular matrix and basement membranes and thereby gain entry to a lymphatic or blood vessel. Once in the circulatory system they must avoid destruction by immune surveillance or by the significant physical strains imposed by turbulence and sheer forces in the circulatory system. Next, tumor cells arriving in the blood vessels of a target organ must attach to the endothelial cells that line the blood vessel, and somehow pass between them. After crossing another basement membrane, the cells must start to proliferate in the new site and recruit blood vessels into the growing secondary tumor (1).

Much remains to be discovered about the regulation of all of these processes, but one thing is clear, all of them are regulated by Protein Kinase C (PKC) at some level: The expression and activity of many of the cellular adhesion molecules implicated in these processes are regulated by PKC, including E-cadherin, CD44 (Clusters of Differentiation 44), and some integrin subunits (2, 3). The adhesion of tumor cells to basement membrane components is inhibited by PKC inhibitors (4). The motility of cancer cells is stimulated by molecules that use PKC signaling pathways, and direct activation of PKC can stimulate motility (5). The expression of matrix degrading enzymes is stimulated by PKC activation (6). The adhesion of tumor cells to endothelial cells is enhanced by PKC activation (7). The retraction of endothelial cells in response to tumor cell binding is blocked by PKC inhibitors (8). Finally, protein kinase C is intimately involved in the control of the proliferation of all cell types including tumor cells and hence the growth of any metastasis.

There is an increasing body of evidence that different isoforms of PKC (a family of at least 11), are responsible for the regulation of different processes, and in light of this we hypothesize that metastatic behavior may in part result from: 1) the overexpression of specific isoforms of protein kinase C leading to increased activation of PKC responsive metastatic processes, and/or 2) an increase in the sensitivity of the cell to the signals that activate protein kinase C, resulting in increased kinase activity and stimulation of metastatic processes. In the grant DAMD17-94-4166, we proposed to test this hypothesis and: 1) determine if specific patterns of PKC isoform expression are associated with a more invasive and metastatic phenotype. 2) investigate whether increased invasive and metastatic behavior may be promoted by an increase in the sensitivity of a cancer cell to signals in its environment that activate PKC. 3) directly test whether the overexpression of particular PKC isoforms can lead to increased invasive and metastatic potential. 4) determine whether compounds that reduce PKC activity in vivo can inhibit the metastasis of tumor cells in the nude mouse. As we describe below, we have made significant progress on these studies and have generated data that are consistent with our hypotheses.

Body:

In the first year of the grant, our major objective was to determine the pattern of expression of the different isoforms of PKC in a panel of breast cancer cell lines of various invasive and metastatic potential. The level of both protein and mRNA (messenger ribonucleic acid) was to be examined. A significant portion of this work has been completed, and the remainder of these studies should be finished relatively soon.

Task 1. Determination of Pattern of PKC isoform expression in breast cancer cell lines.

A) Determination of PKC isoform expression by Western Blot Analysis.

The cells used in these studies are listed in table 1. As can be seen from the table, these cells vary in their metastatic and invasive potential from cells that are both non-metastatic and poorly invasive to cells that are highly invasive and metastatic. Whole cell lysates were prepared by solubilizing the cells in a sub-confluent 75 square centimeter (Sq cm) flask with 5 milliliters (ml) of boiling lysis buffer (10 millimolar (mM) Tris pH 7.4, 1% Sodium Dodecyl Sulphate (SDS)). The lysates were boiled for 5 minutes and then sonicated for 20 seconds. The concentration of protein was determined and the lysates mixed with an equal volume of double concentration (2 X) Lammeli buffer. The samples were stored at -70 Celsius (°C) until assayed.

Table 1. The invasiveness, tumorigenicity, Estrogen Receptor status, and PKC expression of human breast cancer cell lines.

Cell Line	Invasion[a]	Tumor formation	ER	PKCα 82K	PKC α 46K	PKC β	PKC δ	PKC ϵ	PKC λ/ι
T47D	+	P	+	+/-	+	-	++	+	+++
ZR-75-1	+	P	+	+/-	+	-	-	++	++
Hs578T	+++	M	-	++++	++++	-	+/-	++	+
MDA-MD-231	+++	LI	-	+++++	++++	-	+	++++	++
MDA-MB-435	++	M	-	++	+++++	-	+++	+++++	++
MDA-MB-468	+	P	-	+	++	-	+/-	++	++
MDA-MB-453	+		-	+	+	-	+++	+	++
SKBR3	+		-	+++	+/-	-	++++	++++	+++
MDA-MB-436	++	LI	-	++++	++++				
MCF-7-ADR	++	P	-	++++	++++	-	+/-	++	++
MCF-7	+	P	+	+	+	-	++++	+++++	+++

[a]Data from ref 9. P, Primary tumor formation only, no local invasion or metastasis; LI, Local invasion through peritoneum, colonization of visceral organs; M, Metastasis to lungs and other organs. Level of PKC immuno reactivity expressed as an estimate of intensity relative to the signal for PKC α in MDA-MB-231 cells.

20 microgram (μg) samples of the lysates were run on 7% polyacrylamide gels and transferred to nitrocellulose. The filters were blocked with 3% Bovine Serum Albumin (BSA) in Phosphate Buffered Saline containing 0.1 % tween-20 (PBST) overnight followed by incubation with anti -PKC antibodies for 12 hours diluted in 3% BSA/PBST. After washing with 5 changes of PBST for five minutes each, the filters were incubated for 2 hours with anti-mouse antibody conjugated to horseradish peroxidase diluted in 3% BSA/PBST. After further washing in PBST the filters were rinsed in Enhanced Chemi-Luminescence (ECL) reagent and exposed to x-ray film.

The results from representative experiments are shown in Figures 1-4 (Fig 1-4.), and these data are summarized in Table 1. The levels of PKC ι/λ do not differ much between the cell lines, and PKC β expression was not detected in any of the lines. The levels of both PKC δ and PKC ϵ vary significantly between the various cell lines but do bot seem to be correlated particularly well with invasive or metastatic behavior.

As can be seen in Fig 1, two specific bands were seen for PKC α. One is at the size expected for the full length protein (82 Kilo Daltons, KDa), and the other is at about 46KDa. This size corresponds to the reported size of the catalytic subunit of the protein that is believed to be generated by proteolytic cleavage upon activation of the enzyme (10). These two forms are listed separately in the summary table (table 1) and as can be seen, there is a significant correlation between the level of both of the forms and the invasive and metastatic potential of the cells. There are, however, two lines where there is a much better correlation between aggressive phenotype and the level of the short form of PKC α, than the full length form. This is an interesting finding because, as stated above, the short form is believed to represent the active form of the enzyme. This suggests that although the level of full length PKC α seems to be associated with a more aggressive phenotype, PKC activity

Fig.1 PKC - α

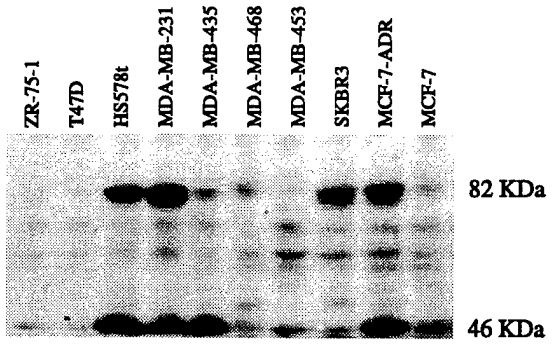


Fig. 2 PKC - δ

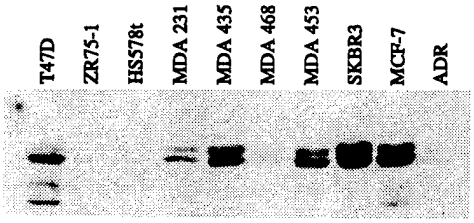


Fig. 3 PKC - ε

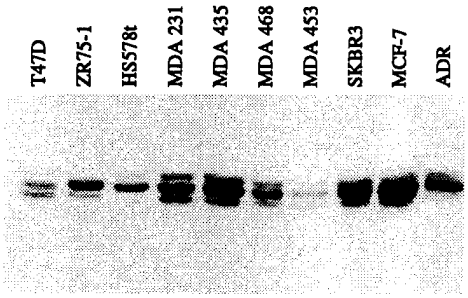
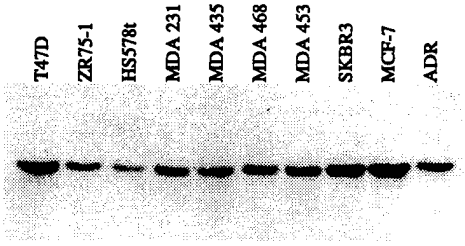


Fig. 4 PKC - ι/λ



may be a more precise indicator of invasive and metastatic potential. Measurement of PKC activity in the cell lines in various extracellular contexts is to be done in Task 2. Experiments are ongoing to conduct western blot analysis for the other isoforms of PKC. This work should be completed in approximately one month.

B) Determination of PKC isoform expression by Northern Blot Analysis.

This work involves the same panel of cells examined by western blot. Whole cell RNA was prepared from each of the cell lines listed in Table 1. The cells in a sub-confluent 75 Sq cm flask were lysed by incubation with 5 ml of lysis solution (4M Guanidine thiocyanate, 2.5mM sodium citrate,). Subsequently, 0.5 ml of 2M sodium acetate pH 4, 5 ml of phenol and 2 ml of chloroform/isoamyl alcohol (49:1) were added and the solution mixed, and allowed to sit on ice for 15 minutes. After centrifugation for 20 minutes at 12 thousand (K) revolutions per minute (RPM), the upper phase was removed and the RNA precipitated with 2 volumes of ethanol. The RNA was pelleted by centrifugation at 12k RPM for 20 minutes and then redissolved in 0.5 ml of lysis solution and re-precipitated with ethanol. The RNA was dissolved in water, the concentration measured spectrophotometrically, and the samples stored at -70°C until required.

We are still in the process of constructing the riboprobe constructs that will be used to generate probes with which to analyze the expression of the PKC isoforms at the level of the mRNA. This work should be completed in approximately two months and at that time the RNA prepared from the cell lines will be fractionated through 1.2% agarose gels containing 2.2M formaldehyde and transferred to nylon membranes. These membranes will be hybridized with RNA probes transcribed from the linearised riboprobe constructs. After washing the filters will be exposed to x-ray film and the signals quantitated by densitometry. This work should be completed in a few months, and should allow us to determine whether the cells that express high levels of the protein do so because they have elevated RNA levels of because of other mechanisms.

Task 2: Determination of PKC activity in breast cancer cells *in vitro* and *in vivo* in the nude mouse.

A) Construction of reporter construct.

Another task that was to be initiated in the latter half of the first year of this proposal was the construction of a reporter construct to allow PKC activity to be measured in cancer cells growing in *vivo in* the nude mouse. This approach was to be used because such measurements could not be made using existing technology. We have made significant progress with this work which is nearing completion. We have demonstrated that the

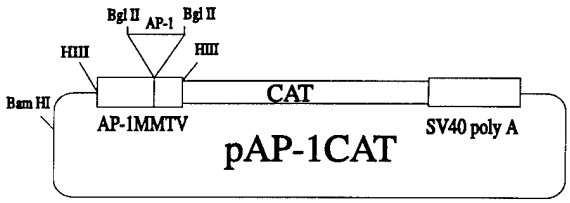


Fig. 5. Map of the PKC responsive reporter construct pAP-1CAT.

reporter construct, as envisioned, is going to work as we had hoped and that our overall strategy is, therefore, likely to succeed.

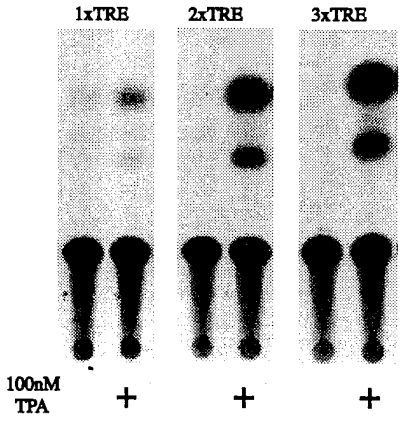


Fig. 6 Induction of CAT activity in cells transfected with pAP-1CAT.

The estrogen responsive reporter construct pERECAT (estrogen responsive chloramphenicol acyl transferase) was used to produce the PKC responsive reporter construct pAP1CAT which is shown in Fig. 5. Oligonucleotides containing the PKC responsive sequence (AP-1 site) from the human collagenase gene was synthesized and ligated into the enhancer site of pERECAT in place of the estrogen responsive sequences. Three versions of this plasmid have been prepared thus far, containing one, two, or three on these AP-1 elements, all in the same direction as in the natural gene (confirmed by sequencing). The correct function of these constructs has been tested by transient transfection into MCF-7 (Michigan Cancer Foundation-7) cells. The transfected cells were grown for 24 hours in media that contained no drug or 100 nanomolar (nM) 12-O-tetradecanoylphorbol-13-acetate (TPA). Cell lysates were prepared by freezing and then thawing a cell pellet 3 times in 100 microliters (μl) of 100 mM Tris pH7.8. CAT assays were conducted using standard methods, in which radioactive chloramphenicol is acetylated by the cell extract, and the reaction products run on thin layer chromatography. Fig 6. shows the results of such an experiment. In this assay CAT activity is demonstrated by the appearance of more mobile, acetylated forms of the chloramphenicol. As can be seen, treatment with TPA resulted in an increase in CAT activity from all three constructs but that the plasmid with three AP-1 sites worked the best in these cells. As can be seen for table 1, MCF-7 cells express relatively low levels of PKC, which probably explains why the construct with three AP-1 sites worked so much better than the others. 100 nM TPA produces maximal stimulation of PKC activity in these cells, and so to get good signal strength from cells in which PKC has not been activated to that extent we should probably use constructs with 4 or 6 AP-1 sites. Work is continuing to generate these constructs, and when that is complete we will determine the optimal construct for each cell line. We will finally replace the CAT gene in the constructs with the β-galactosidase gene as stated in the proposal.

Task 3: Determination of the effect of over-expression of PKC on the metastatic phenotype.

We were not intending to start work on Task three until the beginning of the second year of the proposal. However, the progress achieved in Task 1 made it clear that PKC α would be a good target for this study. We have constructed the PKC α expression construct using the strategy described in the grant and are in the process of testing it in transient transfection studies before proceeding to generate stable transfectants. We transiently transfected MCF-7 cells with the expression construct and after 72 hours made a whole cell lysate from these cells. We will analyze this lysate by western blot to determine if a protein recognized by the anti-PKC α antibody is being produced by these cells and that it is of the correct molecular weight. This will be done in the next few weeks at which time we will start to make stable transfectants with which to conduct the next

phases of our studies.

Task 4:

Work on Task 4 was not scheduled to start until the beginning of the third year, however, we will start on this work as soon as the necessary parts of Task 2 are completed.

Conclusions:

We have demonstrated that the level of expression of at least one isoform of PKC is closely correlated with invasive and metastatic potential. It is possible that over the corse of the next few weeks when the evaluation of the levels of the other isoforms is completed that other associations will become apparent. We have established that the AP-1 site from the human collagenase gene works well in the context of the Mouse Mammary Tumor Virus (MMTV) derived promotor to be used in these studies, and that the expression of a reporter gene driven from this promotor is indeed responsive to agents that modulate PKC activity.

In conclusion, significant progress has been made on the work that we had anticipated that we would complete in the first year of this project, and although we have not completed it we have made progress on work that it was not envisioned that we would do in the first year. We, therefore, are confident that we will be able to complete the studies within the remaining period of the grant. Furthermore, the data generated thus far, strengthen the hypotheses upon which the study is based and clearly demonstrate that the approach that we initially chose to answer the questions posed are highly likely to provide the answers that we require.

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APPENDIX

Abreviations used:

BSA	Bovine Serum Albumin
CAT	Chloramphenicol Acyl Transferase
cd	Clusters of Differentiation
°C	Celsius
cm	Centimeters
ECL	Enhanced Chemi-Luminescence
K	Thousand
KDa	KiloDaltons
MCF	Michigan Cancer Foundation
µg	Microgram
ml	Milliliter
mM	Millimolar
M	Molar
MMTV	Mouse Mammary Tumor Virus
mRNA	Messenger Ribonucleic Acid
nM	Nanomolar
PBST	Phosphate Buffered Saline
PKC	Protein Kinase C
RPM	Revolutions Per Minute
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
Sq	Square
TPA	12-O-tetradecanoylphorbol-13-acetate