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Role of protein kinase C isoforms in human breast tumor cell survival

Meredith Ann McCracken
West Virginia University

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**Role of Protein Kinase C Isoforms in Human
Breast Tumor Cell Survival**

Meredith A. McCracken

**Dissertation submitted to the
Davis College of Agriculture, Forestry, and Consumer Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Genetics and Developmental Biology**

**Jeannine Strobl, Ph.D., Chair
Michael Mawhinney, Ph.D.
Daniel Panaccione, Ph.D.
Yon Rojanasakul, Ph.D.
Knox Van Dyke, Ph.D.**

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ABSTRACT

Role of Protein Kinase C Isoforms in Human Breast Tumor Cell Survival

Meredith A. McCracken

Protein kinase C (PKC) is a family of serine/threonine kinases composed of 12 unique isoforms that function in a variety of cellular processes including proliferation, apoptosis, differentiation and carcinogenesis. The contribution of individual PKC isoforms to these processes is largely unexplored. The purpose of this study is to identify specific PKC isoforms that promote breast tumor cell survival following radiation treatment. This work demonstrated that depletion of PKC δ and PKC ζ isoforms from MCF-7 and MDA-MB-231 human breast tumor cells by antisense oligonucleotides significantly impaired survival following radiation insult. Antisense oligonucleotides were shown to be effective and selective inhibitors of specific PKC isoforms. The PKC δ inhibitor, rottlerin reduced cell survival and confirmed that PKC δ inhibition attenuates breast tumor cell survival, both in the presence and absence of radiation insult. Inhibition of PKC ζ significantly reduced the survival of radiation treated breast tumor cells. DNA damage, assessed using the comet assay provided evidence that depletion of PKC δ from MDA-MB-231 cells results in loss of DNA integrity. In contrast, PKC ζ antisense oligonucleotide treatment did not result in DNA damage. Consistent with participation in cell survival, PKC δ protein levels were elevated in response to radiation treatment. These studies have identified two PKC isoforms that function in breast tumor cell survival and support the application of isoform specific PKC inhibitors to the treatment of human breast tumors.

DEDICATION

To my grandparents, ‘Lil’ and ‘Doc’ McCracken.

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LIST OF ABBREVIATIONS

AT	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia Mutated
BSA	Bovine Serum Albumin
CDC	Cell Division Cycle Protein
CDK	Cyclin Dependent Kinase
CHK	Checkpoint
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
HBSS	Hank's Balanced Saline Solution
IKK	I κ B Kinase
IP ₃	Inositol 1,4,5-triphosphate
IR	Ionizing Radiation
JNK	Jun N-terminal Kinase
MAPK	Mitogen Activated Protein Kinase
MARCKS	Myristoylated Alanine-Rich C Kinase
MCF	Michigan Cancer Foundation
MDA-MB	M.D. Anderson Metastatic Breast
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium)
NF κ B	Nuclear Factor κ B
ODC	Ornithine Decarboxylase
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PDK-1	3-Phosphoinositide-Dependent Kinase
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PIP ₃	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Phenylmethylsulfonyl Fluoride
PR	Progesterone Receptor
PVDF	Polyvinylidene Difluoride
SAPK	Stress Activated Protein Kinase
SM	Sphingomyelin
SMase	Sphingomyelinase
STD	Standard
T ₃	3,3',5-triiodo-L-thyronine
TNF- α	Tumor Necrosis Factor- α
TPA	12-O-tetradecanoylphorbol 13-acetate

I. INTRODUCTION

1. Breast Cancer

Women in the United States have a one in eight (12%) relative lifetime risk for the development of breast cancer, the most frequent form of cancer in women. Breast cancer incidence in the U.S. has steadily risen since the 1930's, by an average increase of 1.2% per year. In the year 2001, breast cancer is predicted to affect 192,000 women, resulting in death for 40,000 women in the U.S. alone. Additionally, 1500 men will be diagnosed with breast cancer in the U.S. The first significant reduction in breast cancer mortality rates was observed in data from the 1990-1997 period and is probably due to improved therapies and earlier detection (Cancer Facts and Figures 2001, American Cancer Society).

The therapeutic benefits of single agents are often improved upon by utilizing combinations of treatment modalities. The purpose of this dissertation was to improve breast cancer therapy by increasing the effectiveness of radiation therapy through a novel approach. Protein kinase C (PKC) has been a relatively understudied target for radiosensitization. Our approach to enhance radiation therapy was through the inhibition of PKC. The intent of this introduction is to provide a brief overview of the underlying genetic factors in breast cancer and current breast cancer treatment strategies as well provide an understanding of radiation biology and PKC.

2. Genetic Susceptibility

Breast cancer susceptibility is a culmination of several different risk factors including age, family history, obesity, menstrual history, pregnancy, oral contraceptive use, diet and alcohol consumption. The single most important factor in breast cancer risk assessment is family history. A positive family history may be a consequence of inherited germ-line mutations in genes which

result in a predisposition to breast cancer. Table 1 provides a summary of inherited mutations that produce breast cancer susceptible phenotypes.

Early studies sought to identify a 'breast cancer susceptibility gene' and suggested that the autosomal dominant inheritance of a gene on chromosome 17 was partly responsible for the predisposition to breast cancer (Lynch et al., 1972). The location of the gene was narrowed to the q12 region on chromosome 17 and identified as *BRCA1* (Miki et al., 1994). Linkage analysis studies conducted on families with early-onset breast cancer revealed linkage between *BRCA1* and early-onset breast cancer in 45% of the families (Easton et al., 1993). To determine the penetrance of the *BRCA1* mutation, breast cancer incidences in families carrying the *BRCA1* mutation were observed. The penetrance of the *BRCA1* mutation increases with age and by age 70 years is estimated to be 85% (Easton et al., 1995).

Since mutations in *BRCA1* account for only half of familial breast cancers and do not seem to be linked to male breast cancer, there may be other genes which contribute to breast cancer predisposition. Genetic linkage analysis was performed on families with early-onset breast cancer, who had no evidence of *BRCA1* mutation. A second gene, *BRCA2*, was identified on chromosome 13q (Wooster et al., 1995). Like *BRCA1*, *BRCA2* is a highly penetrant mutation with 80% of the females who carry the mutation, afflicted with the disease by age 70. Males carrying the *BRCA2* mutation are estimated to have a 6% risk over the general population for developing breast cancer by age 70 years (Easton et al., 1995). More recent findings have supported the contribution of *BRCA2* mutation to male breast cancer (Csokay et al., 1999).

BRCA1 and *BRCA2* mutation show a pattern similar to that observed in tumor suppressor genes such as retinoblastoma (*Rb*) and *p53*. Individuals inheriting a germ-line mutation in *BRCA1* or 2 are heterozygous at these particular loci, with one normal and one mutant allele. A predisposition

to breast cancer is dependent on a loss of heterozygosity at the *BRCA1/2* loci, which occurs by acquisition of a somatic mutation in the remaining normal allele of *BRCA1/2*. An important distinction between classic tumor suppressor genes and *BRCA1/2* is that the latter is thought not to directly promote tumor initiation but genomic instability (Kinzler and Vogelstein, 1997). Genetic instability facilitates the accumulation of somatic mutations, including mutations in genes, which may directly regulate cell division and death, thus resulting in tumor initiation.

More recently an additional gene that confers breast cancer susceptibility was identified. The cell cycle checkpoint kinase, Chk2, is involved in response to DNA damage and is thought to participate in the same pathway as BRCA1 and p53. A deletion within the *CHK2* gene resulting in a truncated, non-functional protein was observed in 5% of individuals with breast cancer (Meijers-Heifboer et al., 2002). Estimates indicated that female carriers of this mutation have a twofold greater risk over the general population of developing breast cancer, while male carriers have a tenfold increased risk. Unlike *BRCA1* and *BRCA2*, the *CHK2* mutation has relatively low penetrance. However, these findings are significant because the prevalence of the *CHK2* mutation in the general population is much higher (1.1%) than the estimated frequency of *BRCA1/2* mutations (0.12-0.29%) in the general population (Iau et al., 2001).

Homozygous mutation of the ataxia-telangiectasia (*AT*) gene produces the AT syndrome. Individuals with AT have a 100-fold increased risk for the development of cancer. Carriers of the *AT* gene mutation (heterozygotes) are not affected by the AT autosomal recessive syndrome but still have a higher risk for the development of cancer. Breast cancer is the most frequently associated form of cancer in heterozygous *AT* mutant individuals. Estimates predict that 30% of all female *AT* carriers will develop breast cancer by age 70. Furthermore, an elevated risk of male breast cancer is correlated with *AT* mutation carriers (Couch and Weber, 2001).

Multiple other genetic disorders have been related to an increased probability for the development of numerous forms of cancer, including breast. The predominant forms of cancer experienced by individuals with Li-Fraumeni syndrome and their first degree relatives are soft tissue sarcomas, bone and breast cancers. The underlying genetic defect in Li-Fraumeni syndrome is mutation of the *p53* tumor suppressor gene. Furthermore, mutation of the *PTEN* tumor suppressor gene, the Cowden's disease susceptibility gene, is associated with an increased risk to breast cancer. Cowden's disease is characterized by autosomal dominant inheritance of the mutant *PTEN*. Breast cancer has been observed in 30% of women with Cowden's disease (Iau et al., 2001). Cancer risk, including breast, is elevated in individuals with Muir-Torre and Peutz-Jeghers syndromes. These syndromes display autosomal dominant modes of inheritance and are linked to mutations in DNA mismatch repair genes (*MLH1/MSH2*) and a serine-threonine kinase (*STK11*), respectively (Couch and Weber, 2001). Lastly, the rare autosomal recessive disorder, Bloom syndrome, is accompanied by an increased incidence of breast cancer as well as leukemia, lymphoma and colon carcinomas. The underlying pathology of this disorder is not well characterized, but thought to be due to homozygosity at a particular locus on chromosome 15 that results in genomic instability.

Table 1. Inherited breast cancer susceptibility

Gene	Function	Penetrance	Associated Syndrome
<i>BRCA1</i>	Tumor Suppressor	85% *	None
<i>BRCA2</i>	Tumor Suppressor	80% *	None
<i>AT</i>	Tumor Suppressor	30% *	Ataxia Telangiectasia carriers
<i>P53</i>	Tumor Suppressor	15%	Li-Fraumeni
<i>PTEN</i>	Tumor Suppressor	30%	Cowden's
<i>MLH1/MSH2</i>	DNA Repair	N/A	Muir-Torre
<i>STK 11</i>	Signal Transduction	18%	Peutz-Jehgers
Chromosome 15		4%	Bloom

*Indicates penetrance for women by age 70 years. The remaining penetrance estimates were derived from observations of breast cancer incidence within groups of women (Cowden's) or men and women (Peutz-Jehgers and Bloom) of varied ages affected with the syndromes.

3. Somatic Mutations in Breast Cancer

A large portion (90-95%) of breast cancer incidences are not inherited but sporadic (American Medical Association, 2001). The somatic alterations present in breast cancer cells include amplifications, deletions and mutations. The carcinogenesis of breast cancer is a multi-step process, however the sequence of the genetic events leading to tumor progression is not clear. Somatic mutations in the *BRCA1* and *BRCA2* genes are rare, while inheritance of either is associated with an increased number of somatic mutations in other genes. This section will discuss the modifications of receptors, cell cycle regulators, transcription factors, signaling and cell adhesion molecules caused by known genetic alterations in breast cancer. Table 2 may be referred to for a summary of frequent somatic alterations observed in breast cancers.

Among the numerous genes with aberrant expression in breast cancer, the most frequent is the E-cadherin, *CDH1* gene. An estimated 60-70% of breast tumors display reduced or absent E-cadherin protein expression (Rimm et al., 1995; Palacios et al., 1995). E-cadherin is a calcium-dependent protein that functions in epithelial cell-cell interactions within normal adult mammary tissue. Reduced expression or loss of function of this cell adhesion molecule may facilitate invasive tumor cell growth. Nonsense and frameshift mutations in the *CDH1* gene produce truncated, non-immunohistochemically detectable E-cadherin protein molecules (Berx et al., 1995).

The *p53* tumor suppressor gene is mutated in half of all human cancers and in 15-34% of breast cancers (Ingvarsson, 1999). *P53* provides an example of a gene in which mutation may be inherited or sporadic. The majority of the somatic *p53* mutations are missense mutations created by a transversion of guanine:cytosine to thymine:adenine nucleotides. This transversion results in an amino acid substitution that for which produces a dominant negative or dysfunctional p53 protein (Saitoh et al., 2001). P53 is a transcription factor that regulates the G1 checkpoint of the

cell cycle. A function of p53 in response to DNA damage is transcriptional activation of the cyclin-dependent kinase inhibitor, p21, which halts cell cycle progression. At this pause in the cell cycle, DNA damage may be repaired or if damage is beyond repair, p53 may induce apoptosis via transcriptional activation of the bax apoptotic gene (Dahm-Daphi, 2000). In the absence of normal p53 function, cells may be allowed to progress to S-phase in spite of DNA damage, resulting in propagation of cells containing damaged DNA. In addition, the positive cell cycle regulator, cyclin D1, is reported to be amplified in 17% of breast tumors (Champeme et al., 1995).

Epidermal growth factor receptors (EGFR) are a family of receptor tyrosine kinases with proto-oncogenic properties. The EGFR and erbB-2 or HER-2/neu family members are reportedly overexpressed in 20-40% of breast cancers (Couch and Weber, 2001; Clark and McGuire, 1991). Increased expression results from amplification of the *EGFR* and *HER-2/neu* genes. Detection of HER-2/neu protein overexpression by immunohistochemistry correlated with gene amplification by fluorescence *in situ* hybridization (FISH) analysis (Lehr, 2001). Overexpression of either receptor leads to misregulation of signal transduction pathways and cell proliferation. Adapter proteins (Grb2 and Sos) couple HER-2/neu with the *ras* oncogene. Increased expression of HER-2/neu manifests in aberrant activation of *ras* and the downstream mitogen-activated protein kinase (MAPK) signal transduction pathway (Janes, 1994). While dysregulation of *ras* signal transduction pathways is common in breast cancer, *ras* mutation occurs in only approximately 5% of breast cancers (Clark and Deer, 1995).

Estrogen and progesterone receptors play an important role in the function of the mammary gland as well as in the development and progression of breast cancer. The absence of estrogen and progesterone hormone receptors (ER and PR, respectively), a feature in 1/3 of breast cancer cases, is associated with hormone independent growth, a less differentiated tumor phenotype and

poor prognosis (Lapidus et al., 1998). Alternatively, breast tumors that express the estrogen and progesterone receptors and retain hormone responsiveness have a better clinical outcome. Numerous genetic aberrations in both the estrogen and progesterone receptors are reported including point mutations and deletions that may alter receptor function, but generally do not result in a loss of expression. Evidence suggests that loss of expression is due to gene silencing through epigenetic modification of chromatin structure. Methylation of CpG islands within the *ER* promoter and first exon was observed in 25% of ER-negative breast tumor cells (Ottaviano et al., 1994). Subsequently, ER expression was rescued in ER-negative cells by treatment with demethylating agents (Ferguson et al., 1995). As in the case of the ER, CpG island methylation is a possible explanation for the loss of the PR receptor expression as well. Studies revealed a correlation between CpG island methylation within the *PR* promoter and first exon in approximately 40% of PR-negative breast tumor cells examined (Lapidus et al., 1996). Although, deletions, insertions, polymorphisms and rearrangements have been observed in the *ER* and *PR* genes, they are rare and not associated with ER and PR loss in breast cancer. Methylation of CpG islands is a feature shared by ER- and PR-negative breast cancers and is at least one mechanism for loss of receptor expression.

The *c-myc* proto-oncogene is a transcription factor that promotes cell proliferation as well as apoptosis. The principal role of *c-Myc* in cell proliferation is thought to involve transcriptional activation of genes which positively regulate the cell cycle and/or transcriptional repression of negative regulatory cell cycle genes (Liao and Dickson, 2000). Amplification of the *c-myc* gene is observed in approximately 18% of breast carcinoma biopsy specimens (Garcia et al., 1989; Berns et al., 1992). Inconsistent with amplification being the only means of increased *c-Myc* protein levels, *c-Myc* protein is reported to be overexpressed in 50-100% of breast cancer cases (Liao and Dickson, 2000). Rearrangements of the *c-myc* locus have been reported in a small

percentage of breast tumors (Bonilla et al., 1988). Despite the lack of a clearly defined underlying genetic mechanism, *c-myc* aberration appears to be important in the initiation/progression of breast cancer.

Table 2. Frequent somatic mutations in breast cancer

Gene	Product	Gene Alteration ➔	Consequence (protein)	Prevalance
<i>CDH1</i>	E-cadherin	Non-sense/Frameshift	Reduced/Absent	60-70%
<i>P53</i>	P53	Missense (G ➔T)	Non-functional	15-34%
<i>EGFR&HER-2/neu</i>	EGFR&HER-2/neu	Amplification	Overexpression	20-40%
<i>MYC</i>	Myc	Amplification	Overexpression	18%
<i>PRAD1</i>	CyclinD1	Amplification	Overexpression	17%

4. Breast Cancer Treatment

Current breast cancer treatment modalities include surgery, radiation and adjuvant therapies. The two major surgical options for local tumor control are lumpectomy and mastectomy. Surgical removal of only the tumor with preservation of healthy breast tissue or lumpectomy, is followed by daily doses of 1.8-2 Gy radiation for 5 to 6 weeks to reduce the risk of local recurrence (Perez and Brady, 1992). Radiation therapy may be advised following mastectomy in patients with advanced primary or node positive breast cancer to prevent local recurrence. Surgical oophorectomy (removal of ovaries) following local tumor control may be used to reduce the risk of systemic recurrence in pre-menopausal women.

Adjuvant therapies are used to reduce systemic recurrence risk depending on the stage of the disease and post-menopausal status of the patient. In patients with large tumors and/or nodal involvement, hormonal or chemo adjuvant therapies are usually prescribed. Tamoxifen, an anti-estrogen, is an adjuvant therapy option for tumors that are estrogen receptor (ER) positive, while ER negative tumors are treated with chemo adjuvant therapy (NIH Consensus Statement, 2000; Couch and Weber, 2001).

5. Radiation Therapy

5.1 Radiation Biology

The goal of radiation therapy is to precisely deliver a measured dose of radiation to a defined tumor resulting in tumor eradication with minimal damage to healthy tissue surrounding the tumor. For breast cancer therapy, radiation is used as a companion to surgical tumor excision. The effectiveness of radiation may be expressed in terms of the fraction of cells which survive the dose of radiation. Mammalian cell survival curves form a characteristic shape (Figure 1). In contrast to bacteria that for which have a linear slope, mammalian cell radiation survival curves

posses a "shoulder" followed by a logarithmic decline in survival. The shoulder is indicative of sub-lethal radiation injury. If the surviving fraction of cells is plotted on a logarithmic scale versus the dose of radiation on a linear scale the slope of the line has two separable components. The α component of the cell kill, which predominates in the 0-3 Gy range, forms a linear slope and represents non-repairable radiation damage. The β component has an initial slope that becomes progressively steeper as the radiation dose increases. The steeper slope of the β curve at high radiation doses represents the loss of cellular DNA repair mechanisms. Mammalian radiation survival curves are represented by the following linear quadratic equation:

$$\text{Log}_e S = \alpha D + \beta D^2$$

where S is the fraction of cells surviving the radiation dose, D, and α and β are the constants (Perez and Brady, 1992).

Figure 1. Radiation survival curve.

Mammalian cell radiation survival curves have two separable components: α and β . The α cell kill is caused by non-repairable DNA damage. The β component at less than 10Gy represents sub-lethal, repairable DNA damage, while at greater than 10Gy represents cell kill due to the loss of DNA repair mechanisms (Modified from Perez and Brady, 1992).

5.2 Radiation Induced Cellular Damage.

Radiation induced cell death is a result of the disruption of two distinct cellular targets: DNA and the plasma membrane. Through the generation of oxygen free radicals, ionizing radiation causes physical damage to membrane phospholipids. Damage to the cell membrane may activate signal transduction cascades important in the apoptosis pathway. One such example is the generation of ceramide in response to ionizing radiation (Haimovitz-Friedman et al., 1994). The sphingomyelinase signal transduction pathway was shown to be activated by ionizing radiation (Figure 2). In response to radiation, sphingomyelinase hydrolyzed sphingomyelin, resulting in the generation of ceramide. This lipid second messenger has been shown to induce apoptosis in response to ionizing radiation, tumor necrosis factor α (TNF- α) and Fas ligand (Haimovitz-Friedman et al., 1994; Obeid et al., 1993).

Another consequence of damage to the plasma membrane by ionizing radiation is the induction of anti-apoptotic signaling pathways. Membrane lipid peroxidation by ionizing radiation results in activation of protein kinase C (PKC) and PKC translocation from the cytosol to the membrane. Consistent with these findings, is the observation of a biphasic elevation in diacylglycerol (DAG), a PKC activator, in response to radiation (Nakajima and Yukawa, 1999). Additionally, PKC activation was shown to block radiation induced sphingomyelin hydrolysis and apoptosis (Haimovitz-Friedman et al., 1994). Thus, cell fate following exposure to ionizing radiation, is thought to teeter between apoptosis and survival, as influenced by conflicting pro- and anti-apoptotic signals from the sphingomyelin and PKC pathways, respectively (Haimovitz-Friedman, 1998).

The best documented target of ionizing radiation is DNA. Ionizing radiation is estimated to produce 1000 single- and 25-40 double-strand DNA breaks per cell per gray (Olive, 1998). Ionizing radiation may damage DNA through a direct interaction however, the predominant

mechanism for damage to DNA is by hydroxyl free radicals (Josephy, 1997). These reactive oxygen intermediates are generated by the interaction of high-energy radiation with intracellular water molecules. The reactive oxygen intermediates cause oxidative damage to nucleotides, single- and double-strand DNA breaks. Strand breaks in the DNA are caused by the free radical sequestration of a hydrogen atom at the deoxyribose C4 position, resulting in a loss of the phosphate linkage and a strand break (Ward, 1988). It has also been reported that in response to DNA damage by ionizing radiation, ceramide is produced by the alternative *de novo* ceramide synthase pathway (Liao et al., 1999).

Figure 2. Ceramide generation in response to ionizing radiation. Radiation activates membrane associated sphingomyelinase (SMase) which catalyzes the conversion of sphingomyelin (SM) to the second messenger, ceramide. Radiation induced damage of membrane lipids activates PKC. PKC activation blocks sphingomyelin hydrolysis. DNA damage by radiation activates the *de novo* ceramide synthase pathway. Ceramide acts as a signal for apoptosis, while PKC activation counteracts apoptotic signals.

5.3 Radiation Induced Gene Expression.

In addition to cellular membrane and DNA damage, ionizing radiation induces the expression of a number of genes and their products. Signal transduction pathways activated by radiation such as the stress activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) pathways converge in the nucleus, where they activate gene expression. The immediate-early response genes *c-jun*, *c-fos*, *c-myc* and *c-Ha-ras* were induced within one hour following exposure of transformed lymphoblastoid cells to low doses of radiation (Prasad et al., 1995). NF κ B DNA binding and NF κ B mRNA expression were increased in response to radiation (Brach et al., 1991). Low dose radiation induced transcription of genes encoding PKC and the EGF receptor while interleukin-1 beta and TNF α were induced by higher radiation doses (20 Gy) (Woloschack et al., 1990; Peter et al., 1993; Ishihara et al., 1993; Hallahan et al., 1989). While the particular signal transduction pathways that elicit radiation-induced gene expression are incompletely understood, it is clear that radiation induces both nuclear gene expression and cytoplasmic signal transduction.

5.4 Ionizing Radiation Damage and Repair.

Ionizing radiation induces transient arrests of the cell cycle at G1 and G2 checkpoints. G1 arrest in response to DNA damage is mediated by signals relayed from ATM (ataxia telangiectasia mutated) to p53 (Figure 3). Phosphorylation of p53 by ATM inhibits ubiquitin-dependent p53 degradation resulting in increased cellular p53 levels. P53 induction of p21 results in inhibition of cyclins regulating the G1/S transition. G2 arrest is dependent on inhibition of Cdc2 (cell division cycle protein 2) kinase activity. ATM activated Chk1 and Chk2 (checkpoint kinase 1 and 2) mediate Cdc2 activity via phosphorylation and subsequent nuclear export of the Cdc2 phosphatase (Cdc25C). Cdc2 kinase activity is dependent on Cdc25C phosphatase for the

removal of an inhibitory tyrosine-15 phosphate. In the absence of nuclear Cdc25, Cdc2 remains phosphorylated, hence inactive, and the G2/M transition is delayed (Dasika et al., 1999). In addition, a p53-dependent transcriptional repression of Cdc2 and cyclin B1 may also contribute to G2 arrest (Bernhard et al., 1995; Passalaris et al., 1999). Recent studies have identified a function for BRCA1 in the G2 arrest in response to radiation induced DNA damage. BRCA1 was shown to be an activator of Chk1 and thus essential for G2 arrest in response to DNA damage (Yarden et al., 2002).

Pauses in the cell cycle at G1 or G2 checkpoints permit time for repair of damaged DNA (Cohen-Jonathan et al., 1999). The majority of oxidized lesions in bases and sugars are effectively repaired by the base excision repair mechanism. This process recognizes and cleaves the damaged base from the sugar backbone, producing an intermediate single-strand break. The nucleotide gap in the DNA is filled and then ligated by DNA polymerase and DNA ligase, respectively (Norbury and Hickson, 2001). It is plausible that if two oxidized base lesions exist on opposing DNA stands and base excision repair is activated, the formation of two opposing single-strand break intermediates may result in a double-strand break (Wallace, 1998). In fact, the number of double-strand breaks induced by irradiation are not sufficient to account for the lethality of the radiation. It is proposed that additional double-strand breaks are generated during the repair processing of closely opposed lesions (Ward, 1988; Wallace, 1998).

Double-strand DNA breaks are the most genotoxic lesions induced by ionizing radiation. These breaks, which occur as a result of two opposing single-strand breaks in close proximity, may be repaired by one of three separate pathways. The mechanisms for double-strand break repair are homologous recombination, single strand annealing and nonhomologous end-joining (Norbury and Hickson, 2001). Among these, nonhomologous end-joining is the primary repair mechanism for ionizing radiation induced DNA damage in mammalian cells. The process

involves recruitment of Ku 70, Ku 80, DNA-dependent kinase, XRCC4 and ligase IV proteins to the site of the double-strand break (Olive, 1998). However, nonhomologous end-joining is not a perfect repair mechanism; deletions and misrejoined DNA fragments are associated with the process.

Damaged DNA which escapes repair challenges the fidelity of DNA replication. Unrepaired DNA damage may block DNA replication, thereby inhibiting further cell division. Alternatively, replication may proceed in the presence of damaged DNA but will amplify errors in the DNA and eventually cause cell death (Josephy, 1997). In either instance the lethality of the radiation induced DNA damage is dependent on the cells subsequent attempts at division. Thus faster proliferating cells are more sensitive to radiation than cells growing more slowly.

Figure 3. Cell cycle arrest in response to DNA damage.

ATM phosphorylates p53 in response to ionizing radiation (IR) induced DNA damage. P53 induces expression of p21. P21 acts on cyclin-dependent kinase 2 (Cdk2) to inhibit progression from G1 to S phase. ATM also activates checkpoint proteins 1 and 2 (Chk1 and Chk2). Chk1 and Chk2 phosphorylate the phosphatase, Cdc25C. Cdc25C is exported out of the nucleus. In the absence of Cdc25C, cell division cycle protein 2 (Cdc2) is inactive and the transition in cell cycle from G2 to M phase is arrested (Modified from Dasika et al., 1999).

6. Radiation Sensitivity

Different cell and tissue types vary in their sensitivities to radiation. Factors influencing cellular radiation sensitivity include cell cycle phase, proliferation rate, DNA repair mechanisms, apoptotic thresholds and tumor oxygenation. Based on the above factors, predictions regarding tumor cell sensitivity may be made. The late G2 and M phases of the cell cycle, where the DNA content is 4N, are associated with more radiosensitive cell populations, while the S and G1 phases are relatively radioresistant (Cox, 1994). Proliferating cells are more sensitive to radiation than non-proliferating cells (Perez and Brady, 1992). Cells with intact DNA repair systems are less sensitive to radiation than cells lacking components of their DNA repair mechanisms. An increased threshold for apoptosis reduces radiation induced apoptotic cell death and yields more radioresistant cells. Hypoxic tumor cells are radioresistant compared to more vascularized tumor cells with adequate oxygenation.

In 1952, Paterson recognized the differential radiation sensitivities of various tumor types. He divided all tumors into three groups based on their radiation sensitivities. The most radiosensitive were embryonic tumors followed secondly by the intermediately sensitive squamous cell and adenocarcinomas. The least sensitive group of tumor types included soft tissue and bone sarcomas and melanomas (Perez and Brady, 1992).

In fact, adenocarcinomas of the breast are highly refractive to radiation induced apoptosis. The response of breast tumor cells to radiation is growth arrest and reduced clonogenic survival (Gewirtz, 2000). Previous studies have shown that in response to 8 Gy radiation, MCF-7 breast tumor cells respond by mitotic arrest and giant cell formation (Strobl et al., 1998).

7. Radiation Sensitizing Agents

Radiosensitizers are chemical agents that increase the lethal effects of radiation through modulation of the biological factors influencing radiation sensitivity. In principal, selective radiosensitization of tumor cells allows for the effective treatment of tumors with lower doses of radiation which produce fewer side effects *in vivo*. In the following section agents designed to increase the sensitivity of cancer cells to radiation will be discussed.

7.1 DNA Analogues.

Under development since the 1960s, DNA-base analogs containing halogen atoms have been used as radiosensitizers. These analogs mimic cellular thymidine and are incorporated into DNA during the S-phase of the cell cycle and hence offer specificity toward proliferating cells. Incorporation of halogenated pyrimidines renders the cellular DNA more prone to radiation induced damage and impairs DNA double-strand break repair (Robertson JM et al., 1996). The halogenated pyrimidine, 5-bromodeoxyuridine has an increased susceptibility to free radical formation at the 5-position of uridine. Combining 5-bromodeoxyuridine with radiation results in permanent DNA damage (Shenoy and Singh, 1992).

More recently, nucleotide analogs depending on cellular metabolism for activity have been developed. Gemcitabine is an analog of cytosine arabinoside that upon phosphorylation forms the metabolite, difluorodeoxycytidine diphosphate (dFdCDP). dFdCDP inhibits ribonucleotide reductase, resulting in depletion of deoxyribonucleoside 5'-triphosphate (dNTP) pools. Exposure of cells to gemcitabine also results in concomitant cell cycle redistribution to S-phase. Radiosensitization is thought to occur by the concurrent dNTP pool depletion and S-phase redistribution. The combination of radiation and gemcitabine has shown clinical activity in head and neck and pancreatic cancers superior to either modality alone (Lawrence et al., 1999).

Five-fluorouracil (5-FU) is a uracil analog that is metabolized to fluorodeoxyuridine monophosphate (FdUMP). This metabolite inhibits the thymidylate synthase enzyme responsible for the conversion of uridylate to thymidylate. Inhibition of thymidylate synthase results in depletion of thymidine triphosphate (dTTP) pools, cell cycle arrest and DNA fragmentation. The mechanism that results in cellular radiosensitization is not known but is thought to involve the inhibition of thymidylate synthase (Robertson et al., 1996). Fluorodeoxyuridine (FdUrd) and 5-fluorocytosine (5-FC) are potent radiosensitizers that share the same FdUMP metabolite as 5-FU and similar mechanisms of action. Preclinical findings have shown radiosensitization of colon cancer cells by fluoropyrimidines (Khil et al., 1996). Clinical evaluation of fluoropyrimidines in combination with radiation have led to increased survival in patients with pancreatic and advanced breast cancers (Kaiser and Ellenberg, 1985; Kosma et al., 1997).

7.2 Hypoxic Cell Targeting Compounds.

Most solid tumors contain as much as 30% hypoxic cells (Denekamp et al., 1980). The oxygen deficit in tumor cells offers a major exploitable difference between normal and cancer cells. Hypoxic tumor cells are relatively radioresistant, because in the absence of oxygen, free radical induced DNA damage is not effective. Another important group of radiosensitizers is nitro compounds that replace oxygen in the hypoxic tumor cells. The first of these compounds, metronidazole and misonidazole, were entered into clinical trials in the 1970s. These drugs did enhance radiation sensitivity *in vivo* but with serious central neuropathy as a consequence (Perez and Brady, 1992). The second generation of these compounds including etanidazole and pimonidazole were effective but dose limiting toxicity due to peripheral neuropathy and acute central nervous system toxicity, respectively, were encountered. However, these two compounds have different-dose limiting toxicities. Lower doses of etanidazole and pimonidazole used in combination result in maximal radiosensitization with minimal toxicity (Taghian et al., 1991).

Perfluorocarbons also exploit tumor cell hypoxia, but through a mechanism different than that of the nitroimidazoles. The perfluorocarbons are able to absorb and release oxygen in high and low oxygen level environments, respectively. In hypoxic tumor cells, perfluorocarbons enhance circulation and oxygen distribution (Perez and Brady, 1992). Clinical use of a mixture of perfluorocarbons, fluosol, in combination with irradiation improved the overall survival of patients with advanced head and neck tumors (Lustig et al, 1989).

7.3 Alkylating Agents.

A relatively new class of radiosensitizers is the bioreductive alkylating agents. These compounds are preferentially metabolized by mitochondrial reductase enzymes in hypoxic tumor cells. Enzymatic reduction yields an alkylating agent, which targets and forms adducts with DNA. Mitomycin-C is an example of this type of compound and has been used clinically in combination with radiotherapy in patients with cancers of the head and neck (Sartorelli et al., 1994; Boyer, 1997).

7.4 Miscellaneous Compounds.

Finally, there are numerous other compounds and approaches used in tumor cell radiosensitization worthy of mention. Repair mechanisms for radiation-induced DNA damage are valid targets for radiosensitization. Three-aminobenzamide inhibits the repair enzyme, poly(ADP ribose) synthetase also known as PARP and increases cellular radiation sensitivity (Winer et al., 1995).

Camptothecin and its derivatives are topoisomerase I inhibitors. These compounds act as radiosensitizers but the mechanism for this action is not clear. It is proposed that camptothecin derivatives induce radiation sensitivity by inhibition of DNA repair (Falk and Smith, 1992). Alternatively, models predict that radiosensitization involves stabilization of the single-strand

cleavage complex produced by topoisomerase I which when combined with radiation becomes lethal (Chen et al., 1999).

Anthracyclines are potent inhibitors of topoisomerase II with radiosensitizing activity. Radiosensitization of human tumor cell lines has been achieved using aclacinomycin A and adriamycin (Bill et al., 1992; Lee et al., 1975).

Reduction of apoptotic threshold is another mechanism for radiosensitization. The farnesyl transferase inhibitor (FTI-277) blocks *ras* signal transduction and induces apoptosis in response to ionizing radiation (Bernhard et al., 1996).

Redistribution of the cell cycle from resistant to sensitive phases is another promising approach to increase radiation sensitivity. Paclitaxel, a microtubule stabilizer, arrests cells in the M phase of the cell cycle and has been shown to act as a radiosensitizer in a variety of human tumor cells (Kurdoglu et al., 1999).

Cellular membranes represent a target for radiation as well as for radiation sensitizers. In early studies, local anesthetics and phenothiazines were shown to sensitize bacterial cells to radiation. The observation that local anesthetics such as procaine caused radiosensitization was attributed to damage of bacterial membranes and subsequent leakage of small molecules and metal ions involved in DNA repair (Shenoy and Singh, 1992). Phenothiazines selectively sensitize hypoxic tumor cells. It is postulated that this selectivity is related to the differential Fe^{2+} and Fe^{3+} ion concentrations in normal versus tumor cells (Kale, 1996).

Compounds designed to inhibit intracellular glutathione pools are potential radiosensitizers. Glutathione counteracts the effects of ionizing radiation by reducing radiation-induced free radicals. Compounds such as diethylmaleate and buthionine sulfoximine bind or inhibit synthesis of glutathione, respectively (Perez and Brady, 1992). Radiosensitization in human breast cancer cells by buthionine sulfoximine is reported (Lehnert et al., 1990).

Breast cancer cells expressing the HER-2/neu receptor present a unique molecular target for radiation sensitizers. Herceptin is a monoclonal antibody designed to recognize and block the HER-2/neu receptor. Preclinical studies have shown that treatment of HER-2/neu expressing breast cancer cells with the combination of herceptin and radiation results in enhanced radiation sensitivity and reduced DNA repair (Pietras et al., 1999). A similar story has emerged for the ErbB-1 receptor which is expressed in a spectrum of cancers of epithelial cell origin. A monoclonal antibody recognizing ErbB-1, C225, was shown to increase apoptosis in response to radiation treatment of squamous carcinoma cells (Huang et al., 1999).

8. Protein Kinase C

8.1 Historical Perspective.

Protein kinase C (PKC) was initially discovered as a calcium activated enzyme (Inoue et al., 1977). Subsequently, the PKC enzyme was found to be activated by diacylglycerol (DAG) in a calcium- and phospholipid-dependent manner (Takai et al., 1979; Kishimoto et al., 1980). The pathway of agonist-induced turnover of phosphatidylinositol had been elucidated some years prior to the identification of PKC (Hokin and Hokin, 1958). Upon receptor stimulation by an agonist, phospholipase C is activated and catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂). The discovery of PKC proved to be fundamental in assigning a downstream cellular consequence to the agonist-induced inositol phospholipid pathway. The pathway was completed with the finding that the products of phosphatidylinositol hydrolysis, inositol 1,4,5-trisphosphate (IP₃), DAG and IP₃ stimulating intracellular calcium release, are responsible for PKC activation.

8.2 PKC Protein Structure.

PKC is an evolutionarily conserved group of serine/threonine kinases. The mammalian PKC family is composed of at least twelve PKC isoforms, which are distinguished on the basis of protein homology and cofactor utilization. PKC isoforms are divided into three subfamilies: classical (α , β I, β II, and λ), novel (δ , ϵ , η , θ , and μ) and atypical (ζ and ι/γ). Each of these isoforms is encoded by a unique gene with the exception of β I and β II, which share a common gene. Classical PKC isoforms possess a calcium binding domain and two cysteine-rich zinc fingers that are involved in DAG binding. While the novel PKC isoforms contain the DAG binding sites and two cysteine-rich zinc fingers, they lack the calcium binding domain and differ from the atypical isoforms, which require neither calcium nor DAG for activation and contain a single cysteine-rich zinc finger motif (Toker, 1998; Kuo, 1994). Atypical PKC isoforms are dependent on a unique set of activators including PIP_2 , phosphatidylinositol 3,4,5-trisphosphate (PIP_3), 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and ceramide (Toker, 1998). The amino termini of all PKC's contain a pseudosubstrate sequence, which keeps the enzyme in an inactive form in the absence of activators. The co-factor, phosphatidyl-L-serine promotes removal of the pseudosubstrate from the catalytic site on the carboxy terminus of the enzyme and an active PKC conformation (Toker, 1998). Evidence also suggests that displacement of the pseudosubstrate and calcium cause a translocation of inactive cytosolic PKC to the plasma membrane where it assumes an active conformation (Mosior and McLaughlin, 1991).

The majority of the PKC isoform protein homology differences reside in their regulatory domains. Classical PKC isoforms contain three variable regions within their regulatory domain, while the novel and atypical regulatory domains have two variable regions. The catalytic domain of all PKC isoforms has only one variable region. PKC isoforms are comprised of between 586

and 737 amino acid residues and exhibit molecular weights ranging from 67,200 to 83,492 (Hardie and Hanks, 1995).

8.3 PKC Activation.

Extracellular signals activate transmembrane receptors that activate PKC via the inositol phosphate pathway (Figure 4). The spectrum of agonists and receptors that stimulate this pathway includes hormones, growth factors, neurotransmitters and antigens (Kuo, 1994). G-protein coupled receptors, tyrosine kinase coupled receptors and receptor tyrosine kinases stimulate the inositol phosphate pathway.

The first evidence that phospholipase C activation was coupled to G-proteins came from experiments showing that G-proteins activators, such as GTP γ S or aluminum fluoride, stimulated phosphoinositide hydrolysis (Uhing et al., 1985; Rock and Jackowski, 1987). Additional evidence to support these findings was provided by experiments using pertussis toxin, a G_i- and G_o-protein inhibitor. Toxin treatment of a variety of cell types was shown to inhibit receptor-activated phosphoinositide hydrolysis (Kikuchi et al., 1986; Nakamura and Ui, 1985). G-proteins are divided into families based on their substrates where G_q and G_o family members mediate phospholipase C activation (Alberts et al., 1994). Agonist activation of G-protein coupled receptors results in dissociation of the G-protein heterotrimeric α , β and γ subunit complex into G α and G $\beta\gamma$ and the exchange of G α bound GDP for GTP. The GTP bound G α subunit then may activate phospholipase C. A number of receptors coupled with G_q-proteins, which stimulate phospholipase C have been identified and include the α 1-adrenergic, acetylcholine, angiotensin, metabotropic glutamate, muscarinic, thrombin and vasopressin receptors (Varma and Deng, 2000; Alberts et al., 1994; Sandberg and Ji, 2001; Bordin and Ugnoli, 1999; Brown et al., 1997; Clauser, 1995).

Tyrosine kinase receptors are also capable of stimulating phosphoinositide hydrolysis. Two independent studies showed that point mutations in either the epidermal growth factor (EGF) or platelet derived growth factor (PDGF) receptors, producing catalytically inactive receptors, attenuated phosphoinositide signaling in response to stimulation by the respective receptor ligands (Moolenaar et al., 1988; Escobedo et al., 1988). Immunoprecipitation studies revealed a physical association between phospholipase C and the EGF and PDGF receptors in response to receptor ligand stimulation (Margolis et al., 1989; Kumjian et al., 1989). The receptor tyrosine kinases, which are known to stimulate PKC include the EGF, PDGF and fibroblast growth factor (FGF) receptors (Noh et al., 1995).

There are several cell surface receptors, which do not contain tyrosine kinase activity but associate with non-receptor tyrosine kinases. These non-receptor tyrosine kinases, such as members of the src family, are known to stimulate DAG production. For example, the T-cell receptor, CD3, reportedly interacts with non-receptor tyrosine kinases, such as fyn or lck. Upon ligand stimulation of the CD3 receptor, phospholipase C and phosphoinositide hydrolysis are activated (June et al., 1990). In addition, $\alpha_2\beta_1$ integrin receptor activation by collagen (Keely and Parise, 1996).

PLC is not the sole enzyme responsible for DAG generation. Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to yield DAG, but through the generation of a phosphatidic acid intermediate (Kuo, 1994). Like PLC, PLD activity is mediated by G-protein coupled receptors, tyrosine kinase receptors and non-receptor tyrosine kinases (Newton, 1995). Phospholipase A2 catalyzes the generation of cis unsaturated fatty acids which enhance the activity of PKC in the presence of DAG. (Nishizuka, 1995).

In addition, there are other lipid second messengers and exogenous molecules that activate PKC independently of the previously mentioned DAG generating pathways. The tumor

promoting phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), acts as a substitute for DAG through competition for PKC activator and co-factor binding sites. However, fundamental differences between DAG and TPA in the activation of PKC exist. DAG but not TPA stimulates phosphatidylcholine and sphingomyelin degradation providing a possible explanation for differences observed between these two agents. Sphingosine generated by sphingomyelin degradation in response to DAG serves as a negative regulator of PKC (Kolesnick and Clegg, 1988). In addition, prolonged treatment with TPA first activates, then depletes cellular PKC. Short-term exposure, in the range of minutes, to TPA stimulates PKC activity and induces a translocation of cytosolic PKC to the plasma membrane. Extended exposure to 200 nM TPA is shown to abolish PKC enzyme activity by 24 hours as measured by ^{32}P i incorporation into PKC substrate (Rodriguez-Pena and Rozengurt, 1984). Of course, TPA mediates its effects only on the classical and novel PKC isoforms, which retain DAG sensitivity.

Figure 4. PKC activation by inositol phospholipid hydrolysis.

Following agonist induced receptor activation, phospholipase C (PLC) catalyzes the hydrolysis of phosphatidyl 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ stimulates Ca²⁺ release from the endoplasmic reticulum (ER). PKC is activated by DAG and Ca²⁺. Phospholipase D (PLD) and phospholipase A2 (PLA2) catalyze phosphatidylcholine hydrolysis (Modified from Kuo, 1994).

8.4 PKC Functions and Substrates.

PKC functions in a variety of normal cellular activities including proliferation, differentiation, intercellular interactions, cytoskeletal functions and apoptosis. PKC phosphorylates serine or threonine amino acid residues within its substrates. There are a plethora of physiological PKC substrates. In the following section a few of the well documented PKC substrates, their signaling pathways and cellular consequences will be discussed.

One mechanism whereby PKC mediates cellular growth and differentiation is through the mitogen-activated protein kinase (MAPK) pathway. This signaling pathway has been elucidated and consists of signals transduced from receptors through a series of cytoplasmic proteins: PKC/Ras/Raf/MAPK kinase/MAPK. The pathway results in translocation of MAPK to the nucleus where it phosphorylates target nuclear proteins such as Elk-1, SAP-1, c-Jun and c-Fos.

Phosphorylation of the nuclear membrane protein, lamin B occurs in response to the PKC activator bryostatin 1 (Fields et al., 1988). Lamin B phosphorylation preceeds nuclear lamina disassembly during mitosis. Further studies have shown that PKC activation by phorbol esters results in lamin B phosphorylation (Hornbeck et al., 1988).

Finally, the myristoylated alanine-rich C-kinase substrate MARCKS, is a specific substrate of PKC. MARCKS phosphorylation is commonly used to assess PKC activity. The physiological roles of activated MARCKS include growth factor-dependent mitogenesis and cell motility (Hartwig et al., 1992). There are numerous other potential PKC substrates including receptors (EGF receptor, acetylcholine receptor, transferrin receptor and insulin receptor), nerve cell proteins (neurogranin and neuromodulin), metabolic enzymes (glycogen synthase) and immune system proteins (lipocortin I and lipocortin II) (Hardie and Hanks, 1995).

8.5 PKC Tissue Distribution.

PKC is widely distributed in mammalian tissues. Some isoforms are expressed ubiquitously while the expression of other isoforms is restricted to specific tissues. For example, PKC γ expression is restricted to the central nervous system (CNS). The CNS, especially the brain is the most abundant source of PKC both in the quantity of the protein produced and in the number of isoforms present (Kuo, 1994). However, several of the 12 documented PKC isoforms are expressed ubiquitously, including PKC α , β I, β II, δ , ϵ and ζ (Liu and Heckman, 1998).

The PKC isoform profile determined in the human mammary epithelial cell line, MCF-7, includes PKC α , δ , ϵ , η , γ , ι , μ and ζ isoforms. MDA-MB-231 human mammary epithelial cell lines express a PKC isoform profile very similar to MCF-7 cells with the exception that PKC α is highly expressed in MDA-MB-231 cells and weakly expressed in MCF-7 cells (Morse-Gaudio et al., 1998; Ways et al., 1995). The PKC isoform expression in tumors may represent a deviation from the particular PKC isoforms and their levels of expression found in normal tissues.

8.6 PKC in Cancer.

PKC participates in abnormal growth processes such as carcinogenesis, tumor progression, and inflammation (Goekjian and Jirousek, 1999; Weinstein, 1990). As the primary intracellular receptor for the tumor promoting phorbol esters, PKC plays a role in stimulating early events in tumor formation. Proliferative signals converging on MAPK from G-protein coupled receptors and the receptor tyrosine kinases for epidermal- and platelet-derived growth factors are transduced through PKC (van Biesen et al., 1995) and could participate in tumor promotion. In addition, a role for PKC in the later stages of tumor development is suggested by the observation that the metastatic capacity of tumor cells and PKC activity are positively correlated (Carey et

al., 1999). Overexpression of the PKC α isoform in MCF-7 cells resulted in increased extracellular matrix attachments to lymph node and bone marrow receptor ligands and facilitated metastatic growth (Carey et al., 1999). Activation of PKC by 12-O-tetradecanoylphorbol-13-acetate (TPA) has been shown to increase extracellular matrix attachments to capillary endothelial cells and enhance metastatic growth, while reduction of these attachments and decreased metastatic activity have been observed in response to PKC inhibition (Herbert, 1993).

In breast cancer, overexpression and increased activity of PKC compared with normal mammary tissue have been observed (O'Brian et al., 1989; Gordge et al., 1996). Furthermore, there is a correlation between elevated PKC protein levels and aggressive breast cancer phenotypes, such as those that lack estrogen receptors (ER) and exhibit multidrug resistance (MDR) (Borner et al., 1987; Lee et al., 1992).

8.7 PKC in Response to Radiation.

PKC gene and protein expression are induced in response to γ -radiation (Kim et al., 1992). A dose dependent elevation in PKC mRNA levels was observed within one hour after ionizing radiation treatment (Woloschak et al., 1990). PKC activation opposes the pro-apoptotic effects of ceramide generation by sphingomyelin hydrolysis in response to radiation (Haimovitz-Friedman et al., 1994). Inhibition of PKC has been shown to increase the sensitivity of human squamous cancer (Hallahan et al., 1992), mouse embryo fibroblast tumor (Rocha et al., 2000), and human glioblastoma cell lines (Begemann et al., 1998) to radiation treatment. The specific role of PKC in radiation response and the individual isoforms involved are not well defined however, the use of PKC inhibitors, staurosporine, sangivamycin and H7 provided evidence that PKC activation was radioprotective (Begemann et al., 1998; Hallahan et al., 1992; Zhang et al., 1993). The role of individual PKC isoforms in the radiation response is not known.

8.8 Isoform Specific PKC Functions.

Relatively little is known about cell type specific functions of the individual isoforms in mammary epithelium. The consequences of PKC overexpression or inhibition in one cell type may be very different from that in other cell types. For example, overexpression of PKC δ in rat mammary adenocarcinoma cells stimulated anchorage independent growth (Kiley et al., 1999a), but inhibited growth of cells of ovarian origin (CHO) as well as of mouse NIH3T3 cell fibroblasts (Watanabe et al., 1992; Mischak et al., 1993). Furthermore, overexpression of PKC α stimulated MCF-7 breast tumor and C6 glioma cell growth (Ways et al., 1995; Baltuch et al., 1995), but overexpression of this same PKC isoform inhibited growth of bovine aortic endothelial cells and rat embryonic smooth muscle cells (Rosales et al., 1998; Wang et al., 1997). Thus, generalizations about the role of particular PKC isoforms without regard to cell context may not be valid. The cellular origins and the PKC isoform profiles are both important considerations in assigning cell type specific functional roles of PKC. In the following sections the cell-type specific functions of PKC α , δ and ζ with an emphasis on mammary cell specific functions will be addressed.

8.9 PKC Alpha (α).

The Ca^{2+} -dependent PKC α isoform has been shown to be involved with the acquisition of multi-drug resistant and estrogen receptor (ER) negative breast cancer cell phenotypes. MCF-7 breast cancer cells transfected with PKC α displayed cross-resistance to the anti-cancer drugs doxorubicin and vinblastine (Yu et al., 1991). The latter finding of a correlation between PKC α and ER status is evidenced by stable expression of PKC α in MCF-7 cells. MCF-7 cells overexpressing PKC α elicited increased proliferation rates and a reduction of ER expression and estrogen mediated gene expression. MCF-7-PKC- α cells also proliferated in suspension while

parental MCF-7 cells retained anchorage-dependence for growth (Ways et al., 1995). In response to PKC α overexpression PKC β protein levels were increased while PKC η levels were reduced relative to parental MCF-7 cells. These findings suggest that PKC α overexpression correlates with a more aggressive breast cancer phenotype. However, it is not clear if this is a direct effect of PKC α overexpression or a consequence of alterations in other PKC isoforms.

A possible explanation for the acquisition of anchorage-independent growth by MCF-7-PKC- α cells is provided by subsequent studies examining integrin receptor expression. Integrin receptors mediate extracellular matrix attachments and are composed of heterodimerized α and β subunits. The MCF-7-PKC- α cells displayed increased cell surface expression of $\alpha v \beta 3$ and reduced $\alpha v \beta 5$ integrins in contrast to parental MCF-7 cells. The altered expression of these αv -integrins in MCF-7-PKC- α cells correlated with an enhanced ability to adhere to vitronectin and osteopontin containing substrates. Vitronectin and osteopontin are integrin receptor ligands present in the lymph nodes and bone marrow, respectively. Enhanced attachment of MCF-7-PKC- α cells to vitronectin and osteopontin indicates an *in vivo* potential for metastasis to lymph nodes and bone marrow, the two most common sites of breast cancer metastases (Carey et al., 1999). These findings suggest that PKC α may be involved in breast cancer metastasis.

8.10 PKC Delta (δ).

PKC δ is a novel PKC isoform. This Ca^{2+} -independent isoform has been proposed to both regulate and serve as a substrate for caspases (Basu and Akkaraju, 1999). A possible mechanism for inhibition of growth by PKC δ is through its reported function as a caspase-3 substrate during apoptosis. Specifically, PKC δ is activated by caspase-3 during apoptosis in leukemia cells resulting in phosphorylation of lamin B (Cross et al., 2000), an event known to precede nuclear lamina disassembly during apoptosis. In response to TPA treatment, relocalization of

cytoplasmic PKC δ to the mitochondria of MCF-7 cells, preceding cytochrome c release has been observed (Majumder et al., 2000). These findings support a pro-apoptotic role for PKC δ .

In contrast, growth-promoting activities of PKC δ have also been observed in mammary tumor cells. PKC δ involvement in cytoskeleton-dependent processes is evident in MTLn3 mammary tumor cells. Expression of the inhibitory PKC δ regulatory domain (RD δ) inhibited growth in soft agar, cell motility and attachment (Kiley et al., 1999a). The invasive potential of MTLn3 cells was examined by migration of cells through an extracellular matrix coated filter toward FBS-supplemented growth medium. RD δ expression inhibited invasion measured by this assay. In animal studies, rats were injected with the MTLn3 tumor cells expressing the inducible RD δ construct and 25 days later metastases to the lung were assessed. The number of metastases to the lung from primary MTLn3 tumors with induced RD δ expression was significantly reduced (Kiley et al., 1999b). Furthermore, in highly metastatic mammary tumor cells derived from lung metastases, PKC δ and ζ protein and mRNA are significantly increased relative to the less metastatic primary tumor cells (Kiley et al., 1999a; Kiley et al., 1996). PKC δ may positively influence the progression of mammary tumor cells toward metastasis.

Ornithine decarboxylase (ODC) is an enzyme essential to cell proliferation. ODC is capable of inducing cell transformation when overexpressed and is frequently highly expressed in tumor cells. Dominant negative PKC δ expression in murine papilloma cells attenuates induction of ODC in response to oxidative damage (Otieno and Kensler, 2000). Thus, PKC δ might act to promote cell growth by facilitating the expression of ODC.

8.11 PKC Zeta (ζ).

The Ras/Raf signal transduction pathway has provided a link between PKC and the activation of MAPK. The atypical PKC isoform, ζ , has been coupled with Raf-1 by the 14-3-3 adapter protein

family, establishing a Ras independent mechanism for MAPK activation by PKC ζ in COS cells (van Der Hoeven et al., 2000). Consistent with PKC ζ functioning upstream of MAPK, PKC ζ dominant negatives impaired MAPK signaling (Berra et al., 1995). Taken together, these findings support the idea that PKC ζ , in response to growth factors, leads to MAPK activation and this may induce cell proliferation.

Nuclear factor κ B (NF κ B) is a pro-survival factor and counteracts apoptosis induction by ionizing radiation (Wang et al., 1996). NF κ B activity is dependent on phosphorylation of I κ B kinase (IKK) and the subsequent release of NF κ B from the NF κ B-I κ B complex. PKC ζ has been reported to play a critical role in NF κ B activation, through phosphorylation of IKK (Lallena et al., 1999).

Ceramide participates in a number of intracellular growth inhibitory processes such as apoptosis, cell cycle arrest and differentiation. A recent study provided evidence that in response to low concentrations (0.6 μ M) of ceramide, PKC ζ activity, measured by PKC ζ autophosphorylation, is increased. Conversely, high concentrations (>2.5 μ M) of ceramide inhibited PKC ζ activity and induced death in PC12 rat adrenal gland tumor cells. Overexpression of PKC ζ rescued cells from ceramide-induced mortality. Treatment of cells with low concentrations of ceramide correlated with increases in NF κ B and Jun N-terminal kinase (JNK) activities. These studies suggest that cell death in response to low concentrations of ceramide is counterbalanced by the activation of PKC ζ , NF κ B and JNK (Wang et al., 1999). Taken together all of the above evidence supports a pro-survival function of PKC ζ .

As previously mentioned, PKC ζ protein and mRNA are elevated in cells derived from lung metastases in contrast to cells derived from a local primary mammary tumor (Kiley et al.,

1999a). Apart from this finding, there are no further reports available concerning a role for PKC ζ in cells of mammary origin.

8.12 PKC Inhibitors.

Since the discovery of PKC, efforts have been made to identify inhibitors. To date there are a large number of identified inhibitors. The targets of PKC enzyme activity inhibitors include the DAG, lipid cofactor, ATP and substrate binding sites within the enzyme.

Isoquinolinesulfonamides, like H7, inhibit PKC through competition with the ATP binding. Because the mechanism of action for isoquinolinesulfonamides is through the ATP binding site, which is a highly conserved region in all PKC isoforms, they do not offer isoform specificity. These compounds effectively inhibit PKC activity in the range of 1-10 μM concentrations. However, within this concentration range cyclic-GMP- and cyclic-AMP-dependent kinases are inhibited as well (Kuo, 1994).

Another group of PKC antagonists are those that compete with endogenous substrates for binding to the PKC substrate binding domain. Chelerythine chloride, a benzophenanthridine alkaloid, is a potent and selective inhibitor of PKC. Fifty-percent maximal PKC inhibition is accomplished by a concentration of approximately 1 μM (Herbert et al., 1990).

The vast majority of PKC enzyme inhibitors are not selective for individual PKC isoforms. However, there are a few new classes of inhibitors, which offer some degree of PKC isoform specificity. Rottlerin selectively inhibits the PKC δ isoform (IC_{50} 3-6 μM) by interacting with the ATP binding site but also inhibits calmodulin kinase III (IC_{50} =5.3 μM) (Way et al., 2000).

Other groups of recently developed potent inhibitors, such as the staurosporine-derived indolocarbazoles and bisindolylmaleimides, offer specificity toward a subset of PKC isoforms. For example the indocarbazoles allow selective inhibition of the classical PKC isoforms (IC_{50}

0.02-0.03 μM), while members of the bisindolylmaleimide group inhibit classical PKC isoforms and PKC δ and ζ at nanomolar concentrations (Way et al., 2000).

Increasingly apparent is the lack of enzyme activity inhibitors, which offer selective inhibition of single PKC isoforms. DNA technology however, has provided a means to circumvent this problem through the use of PKC mRNA inhibitors. Oligonucleotides that target RNA are highly specific PKC isoform antagonists, and may be easier and less expensive to synthesize than traditional small molecule inhibitors targeted to the enzyme. In the following section antisense oligonucleotide inhibitors will be discussed.

9. Antisense Oligonucleotides

Antisense oligonucleotides are synthetic single-strand DNA (or RNA) molecules composed of 18 to 21 nucleotides, designed to selectively bind, through Watson-Crick base pairing, and inhibit a specific gene product. Oligonucleotides can inhibit mRNA translation by a number of different mechanisms including physical blocking of translation or activation of enzymes that cleave the mRNA species. In their native form, oligonucleotides consist of a phosphodiester backbone and are rapidly degraded by cellular nucleases. Chemical modification of the phosphodiester backbone has provided resistance to nuclease degradation. The fundamental chemical modifications as well as a few of the putative mechanisms of antisense oligonucleotides will be addressed.

9.1. Chemistry and Mechanism of Action.

In the first generation of antisense oligonucleotides, non-bridging oxygen atoms in the phosphate backbone were replaced with sulfur to improve nuclease stability. The phosphorothioate oligonucleotides are by far the most extensively studied although further modified second generation oligonucleotides are available. Second generation oligonucleotides

contain modifications at the 2'-position of the carbohydrate moiety and mixed phosphorothioate/phosphodiester backbones.

Theoretically, there are numerous mechanisms whereby the interactions of oligonucleotides with mRNA result in target mRNA depletion. The first and most simple mechanism involves physical blocking of translation or nascent mRNA processing. The majority of oligonucleotides have been designed to bind the translation start codon and arrest translation. However, the binding efficiency of these oligonucleotides is affected by 5'-capping of mRNA especially in mRNA species with lengthy 5'-untranslated sequences. As an alternative to this approach, oligonucleotides that bind to splicing junctions have been developed. These oligonucleotides block the spliceosome protein complexes necessary for splicing and mRNA maturation and cause the accumulation of splicing intermediates and a reduction of mature mRNA species.

The stability of mRNA is influenced by a number of structural features such as the 5'-methyl cap and the 3'-polyadenylation sequence. Oligonucleotides that interfere with these stabilizing features may promote mRNA degradation or inhibit translocation out of the nucleus. For example, oligonucleotides that bind near the 5'-cap site have been shown to be active. The exact mechanism for their activity has not been determined though.

A final strategy in the design of oligonucleotides has taken advantage of the endogenous RNase H enzyme. RNase H is a ubiquitous enzyme that recognizes RNA-DNA duplexes and catalyzes degradation of the RNA from the duplex. Hybridization of oligonucleotide DNA with target mRNA produces a sufficient substrate for RNase H. The efficiency of this mechanism is greatly affected by oligonucleotide backbone modifications. For instance, methylphosphonate modified oligonucleotides do not serve as RNase H substrates, while phosphorothioate oligonucleotides form excellent substrates for RNase H. Oligonucleotides designed to activate

RNase H have been the most valuable clinically (For review on antisense oligonucleotides: Nielson, 1999; Akhtar and Agrawal, 1997; Lebedeva and Stein, 2001).

9.2 Non-sequence Specific Effects.

Chemical modification of the native phosphodiester oligonucleotide backbone is somewhat of a tradeoff: it improves stability but produces non-sequence specific effects. This is especially true for phosphorothioate oligonucleotides. Reports cite that phosphorothioate oligonucleotides non-specifically bind several cellular proteins including growth factors and growth factor receptors producing growth inhibitory effects (Lebedeva and Stein, 2001). For example, phosphorothioate oligonucleotides have been demonstrated to interact with the EGF receptor and perturb receptor-ligand activation (Rockwell et al, 1997). Additional studies found that phosphorothioate oligonucleotides bind and displace fibroblast growth factor from the FGF receptor (Fennewald and Rando, 1995). It should be noted that in both of these independent studies, binding of the oligonucleotides to cellular proteins occurred at fairly high oligonucleotide concentrations (2.0 and 0.6 μM , respectively). These findings illustrate the importance of using appropriate controls in all experimental designs employing antisense oligonucleotides.

9.3 Delivery.

There are a wide variety of techniques available for the delivery of antisense oligonucleotides *in vitro*. Oligonucleotide delivery systems include liposomes, cationic lipids, peptides, starburst polyamidoamine (PAMAM) dendrimers, electroporation, conjugation with cholesterol, polycations and conjugation to cell surface ligands. Among these different approaches, cationic lipids are the most commonly used delivery system for adherent cell types. Typically in *in vivo* experiments, oligonucleotides are administered systemically by intravenous injection. Their

distribution is highly influenced by circulation with highest concentrations of oligonucleotides present in organs such as the kidney, liver, bone marrow and spleen.

9.4 Clinical Applications.

Antisense oligonucleotides have been extensively developed for use as therapeutic agents. Clinical trials employing antisense have been completed or are underway for the treatments of cancer, Crohn's disease and the HIV associated CMV retinitis. In terms of anti-cancer therapeutics, the targeted gene products in clinical trials include bcl-2, PKC α , *ras* and raf kinase. Phase I/II clinical trials utilizing the combination of bcl-2 antisense and chemotherapy are underway for the treatment of patients with lymphoma and malignant melanoma. These trials are expected to be expanded to patients with lung, prostate, renal and breast cancers (Gautshci et al., 2001).

PKC α has also shown promise clinically as an anti-cancer therapeutic target. Inhibition of PKC α by ISIS 3521 antisense oligonucleotide has shown anti-tumor activity in phase I and II clinical trials in solid tumors (Nemunaitis et al., 1999). ISIS 3521 is now undergoing phase III clinical trials and is pending approval by the U.S. Food and Drug Administration for the treatment of non-small cell lung cancer.

Aside from cancer therapy, the only thus far F.D.A. approved antisense oligonucleotide is fomivirsen also known as Vitravene (NovartisTM). Fomivirsen is a phosphorothioate oligonucleotide designed to inhibit replication of the human cytomegalovirus responsible for CMV retinitis in AIDS patients. Administration of this oligonucleotide is through direct injection into the affected eye(s). Fomivirsen has been successful in the treatment of previously resistant CMV retinitis.

10. Research Objectives

1. The first goal of this project was to test the following hypothesis:

Inhibition of individual PKC isoforms attenuates the survival of human breast tumor cells exposed to ionizing radiation treatment. Antisense oligonucleotide PKC inhibitors delivered to MCF-7 and MDA-MB-231 human mammary tumor cell lines were used to test this hypothesis.

The effect of the PKC antisense oligonucleotides in the absence of radiation treatment was also tested.

2. A second goal in this project was to examine the effects of the PKC oligonucleotides on normal mammary epithelial cells.

3. The comet assay was used to test the hypothesis that PKC δ and PKC ζ oligonucleotides produce DNA damage and enhance radiation induced DNA damage.

4. The final goal of this project was to confirm the PKC oligonucleotide results (goal #1) by using PKC δ and PKC ζ dominant negative plasmid constructs. The effect of PKC dominant negative plasmid expression on cell survival was tested.

II. MATERIALS AND METHODS

1. Materials

1.1 Cells.

MCF-7 cells were provided by Dr. Marc Lippman (Department of Medicine, University of Michigan). MDA-MB-231 cells were provided by Dr. Mike Miller (Department of Biochemistry and Molecular Pharmacology, West Virginia University). MCF10A cells were purchased from American Type Culture Collection (Manassas, VA). T47-D and MCF-7 cells transformed with *H-ras* oncogene were provided by Dr. Michael Moore (Department of Biochemistry, Marshall University) and Dr. Robert Dickson (Lombardi Cancer Center, Georgetown University), respectively.

1.2 Antisense Oligonucleotides.

PKC α , δ , ϵ , η or ζ methoxy-ethoxy modified antisense oligonucleotides (ISIS #9606 or #119406, #13513, #13518, #17000, #13516) and the respective nucleotide scrambled versions of the oligonucleotides (ISIS #13009, #13514, #13517, #13520, #128585) were obtained through a collaboration with Dr. Rob McKay (Isis Pharmaceuticals, Carlsbad, CA). Concentrated oligonucleotide solutions (1 mM) were diluted in PBS to yield 100 μ M working solutions. Oligonucleotide solutions were stored at -20°C. PKC oligonucleotides are presented in Table 3.

1.3 Drugs.

Rottlerin was purchased from Sigma Chemical Company (St. Louis, MO). Concentrated stock solutions (6 mM) were made by dissolving rottlerin in dimethyl sulfoxide (DMSO). Working solutions were prepared by diluting the stocks to 0.2 mM in water. Stock solution was stored at 4°C for 1 month; working solutions were freshly prepared for each experiment.

1.4 Plasmids.

PKC α , δ and ζ dominant negative plasmid constructs were generously provided by Dr. Bernard Weinstein (Herbert Irving Comprehensive Cancer Center, Columbia University).

1.5 Antibodies and Purified Proteins.

PKC α , δ or ϵ mouse monoclonal antibodies recognizing amino acids 270-427, 114-289 or 1-175, respectively were purchased from BD Transduction Laboratories (San Diego, CA) (#P16520, #P36520, #P14820). PKC ζ goat polyclonal Ab, PKC η rabbit polyclonal Ab, each recognizing carboxy terminal peptides and bcl-2 mouse monoclonal Ab (SC #216-G, #215, #509) were purchased from Santa Cruz (Santa Cruz, CA). The horse radish peroxidase-conjugated anti-mouse IgG (SC #2005), anti-rabbit (SC #2004) and anti-goat (SC #2020) secondary antibodies were also purchased from Santa Cruz. Purified PKC α , δ , ϵ , η and ζ proteins (#539674, #539673) were obtained from Calbiochem (San Diego, CA) and used as standards for western blotting. PKC antibodies are presented in Table 4.

Table 3. PKC oligonucleotides

PKC Isoform	Antisense ISIS#	Scrambled ISIS#
Alpha	9606, 119406	13009
Delta	13513	13514
Epsilon	13518	13517
Eta	17000	13520
Zeta	13516	128585

Table 4. PKC antibodies

PKC Isoform	Type	Recognition Site
Alpha	Mouse monoclonal	Amino acids 270-427 (hinge and ATP binding domain)
Delta	Mouse monoclonal	Amino acids 114-289 (cysteine rich repeat domain)
Epsilon	Mouse monoclonal	Amino acids 1-175
Eta	Rabbit polyclonal	Carboxy terminus
Zeta	Goat polyclonal	Carboxy terminus

2. Methods

2.1 Cell Culture.

MCF-7 (passage #39-50), MDA-MB-231, T47-D and MCF-7*ras* human mammary tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (Summit Biotechnology, Fort Collins, CO) and 0.04mg/ml gentamicin in a 7.5% CO₂, 37°C, humidified incubator. Cells were passaged weekly at 1:5 (MCF-7 and T47-D) or 1:10 ratios (MDA-MB-231 and MCF-7*ras*). MCF10A immortalized human mammary epithelial cells (passage #1-20) were maintained in Mammary Epithelial Cell Growth Medium (MEGM) (Bio Whittaker) and passaged weekly at a 1:4 ratio.

2.2 Clonogenic Survival.

MCF-7 cells were plated at 1×10^4 cells/100 mm² dish in 10 ml DMEM/10% FBS and maintained for seven days in a 7.5% CO₂, 37°C, humidified incubator. To visualize colonies, dishes were stained with 3 ml of 0.5% crystal violet, 5% formalin, 50% ethanol, 0.85% NaCl for 3 minutes, then rinsed with tap water. Colonies were scored using a Nikon Eclipse TS100 microscope (Nikon, Japan) at 100X magnification with ≥ 10 cells = 1 colony. For certain experiments, cells were treated with 1.5-9 μ M rottlerin (Sigma, St. Louis, MO) dissolved in DMSO with a final concentration of 0.1%-0.15% DMSO in the cell culture medium.

2.3 Antisense Oligonucleotides.

MCF-7 (2.2×10^5), MDA-MB-231 (1.1×10^5) or MCF10A (1.5×10^5) cells/35 mm² dish were treated for 4-5 hours with 100-200 nM PKC α , δ , ϵ , η or ζ methoxy-ethoxy modified antisense oligonucleotide, the respective nucleotide scrambled version of the oligonucleotide or the sense version of the PKC α oligonucleotide in 3 μ l Lipofectin (Invitrogen, Carlsbad, CA) transfection reagent/1 mL Opti-Mem I reduced serum medium (Invitrogen). Transfection mixture was

prepared by incubating Lipofectin and Opti-Mem at room temperature for 45 minutes. Oligonucleotides were added to 100 μ l of Opti-Mem followed by addition of 100 μ l of Lipofectin:Opti-Mem mixture and 15 minutes incubation. Eight hundred μ l of Opti-Mem were added to the Lipofectin:Opti-Mem:oligonucleotide mixture and the mixture was overlaid on cells in 35 mm² dishes washed one time with 1 ml of Opti-Mem. Transfection was stopped after 4-5 hours by medium exchange with DMEM/2% FBS or MEGM (MCF10A).

2.4 Radiation.

Cells were exposed to 0.75-9.5 Gy doses of γ -ionizing radiation delivered with a Cesium¹³⁷ source in a Gammacell 40 (Atomic Energy of Canada Ltd., Ottawa) at 108.7 rads/min or a Gammacell 1000 (Atomic Energy of Canada Ltd.) at 730.0 rads/min under ambient temperature and atmospheric conditions. The clonogenic survival curves generated at these two radiation rates were similar. After radiation, the culture medium was replaced with DMEM/10%FBS.

2.5 MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Assay.

Metabolism of MTS was used as index of cell viability. MCF-7, MDA-MB-231 or MCF10A cells were plated at 1000-2500 (MCF-7) or 250 (MDA-MB-231 and MCF-10A) cells/well in a 96-well plate in 225 μ l DMEM/10% FBS or MEGM (MCF-10A). After plating (7 or 5 days, respectively), fresh growth medium (100 μ l DMEM/5% FBS or MEGM + 20 μ l Cell Titer 96) (Promega, Madison, WI) was added. Conversion of MTS reagent to a colored formazan product was measured after a 2 hour incubation at 37°C by an increase in absorbance at 490 nm using a Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA). The MTS assay was linear under our assay conditions for 3 hours.

2.6 Western Blotting.

MCF-7 (7.0×10^5 cells/100 mm² dish), MDA-MB-231 (4.5×10^5 cells/100 mm² dish) or MCF10A (1.0×10^6 cells/100 mm² dish) were rinsed one time with 37°C phosphate buffered saline (PBS: 0.14 M NaCl, 2.7 mM KCl, 8.1 mM NaHPO₄7H₂O, 1.5 mM KH₂PO₄). Total cellular proteins were collected by syringe in 100-150 µl boiling lysis buffer (1% SDS, 10 mM Tris, pH 7.4) and chilled on ice. Proteins were boiled for 5 minutes then centrifuged at 4°C, 14,000rpm in an Eppendorf centrifuge 5415 C (Brinkmann Instruments Inc., Westbury, NY). Protease inhibitors, aprotinin, leupeptin and phenylmethyl sulfonyl fluoride at final concentrations of 1 mM, 1 mM and 100 µM, respectively were added to the supernatant. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Following the BCA assay, a final concentration of 1mM dithiothreitol was added and protein samples were diluted in 1X sample buffer (0.32 M Tris-HCl, 10% glycerol, 2% SDS, 0.2% bromphenol blue, 2.5% 2-mercaptoethanol, pH 6.8) and then resolved on 7.5% acrylamide gels at 100 V. Proteins were transferred to polyvinylidene difluoride membranes (Invitrogen) at 25 V for 2 hours. Purified PKC α , δ , ϵ , η and ζ proteins were used as standards. Membranes were blocked at 4°C, overnight in 3% non-fat dry milk/Tris buffered saline (TBS: 0.02 M Tris, pH 7.5, 0.5 M NaCl) + 0.05% Tween20. Membranes were washed three times, 5 minutes each wash with room temperature western wash solution (0.1% non-fat dry milk, 0.1% chick ovalbumin, 10% 10X PBS, 1% FBS and 0.2% Tween20). Membranes were incubated with PKC α , δ or ϵ mouse monoclonal antibody, PKC ζ goat polyclonal Ab, PKC η rabbit polyclonal Ab or bcl-2 mouse monoclonal Ab for 3 hours 15 minutes. All primary Ab were used at a 1:250 dilution of antibody:western wash solution. Following primary antibody hybridizations, membranes were washed three times, 5 minutes each

wash with western wash solution followed by one 5 minute wash in TBS. Membranes were hybridized with a 1:1000 dilution of anti-mouse, a 1:8000 dilution of anti-rabbit or a 1:10,000 dilution of anti-goat horse radish peroxidase-conjugated secondary antibody in TBS for 30 minutes. Primary and secondary antibody incubations were performed by placing membranes in Kapak heat sealable pouches (Kapak Corporation, Minneapolis, MN), adding antibody, heat sealing the pouch and placing the pouch on a rocker at room temperature for 3 hours and 15 minutes or 30 minutes, respectively. Signals were visualized by incubating the membrane with a chemiluminescent peroxidase substrate for 7 minutes (Super Signal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL) and exposing the membrane to film. Signals were quantitated by FluorChem (Alpha Innotech, San Leandro, CA) spot densitometry using automatic background subtraction and normalized to a 50kDa or 200kDa protein band on the Coomassie blue (Pierce) stained acrylamide gel.

2.7 Comet Analysis.

MCF-7 cells (1.0×10^5 /35 mm² dish) were harvested two days after plating in 5% FBS and then counted. Alternatively, MDA-MB-231 cells (1.1×10^5 cells/35 mm² dish) were treated with PKC δ or ζ oligonucleotides and harvested 24-48 hours after treatment. Cells were pelleted in a 15 ml conical tubes by centrifuging for 5 minutes at 1200 pm, washed with 1 ml ice cold PBS, centrifuged again and resuspended in ice cold PBS at a concentration of 1×10^5 cells/ml. Cells were maintained on ice and treated with 0-5.2 Gy radiation. Alternatively for 1 hour DNA repair time, cells were irradiated in 35 mm² dishes and incubated for 30 minutes at 37°C and 7.5% CO₂. Cells were then harvested (requires 30 minutes) as described above and resuspended in ice cold PBS for a final concentration of 1×10^5 cells/ml. Following irradiation, 50 μ l of PBS cell suspension was mixed with 500 μ l of low melting point agarose warmed to 42°C. Seventy-five

µl of cells in low melting point agarose were spread evenly onto a Comet Slide (#4250-050-03 Trevigen, Gathersburg, MD) and allowed to dry by laying the slide flat in the refrigerator (4°C and protected from light) for 30 minutes. Slides were then immersed in lysis solution (#4250-050-01, Trevigen) prechilled for 20 minutes at 4°C and incubated on ice for 45 minutes. Excess lysis solution was tapped off the slides and slides were transferred to freshly prepared alkali solution (300 mM NaOH, 1 mM EDTA-pH 8.0) for 45 minutes at room temperature, protected from light. Slides were transferred to the electrophoresis apparatus and aligned equidistant from the electrodes. Slides were electrophoresed for 30 minutes at 1 Volt/cm (33 V) and 300 mA. Slides were immersed in 70% ethanol for 5 minutes and then allowed to air dry overnight at room temperature. Fifty µl of SYBR Green stain [1000X] were added to slides and incubated for 7 minutes, protected from light immediately before data acquisition. Three to five drops of anti-fade solution were added to SYBR Green stain on slides. The antifade solution was made by dissolving one 20 mg p-phenylenediamine dihydrochloride tablet (Sigma #P7288) in 2 ml of PBS followed by addition of and 18 ml of glycerol. The anti-fade solution was stored in a 3 cc syringe with a 0.22 µm filter attached at -20°C and protected from light. The comets (nuclei) were visualized using a Nikon Eclipse TS100 microscope with 63X objective and FITC-filter cube. Comet images were captured and analyzed using the LAI Automated Comet Assay Analysis System (Loats Associates, Inc. Westminster, MD). The tail moment [(%DNA)(distance traveled)] was used for quantitative analysis of DNA damage for 80 comets per treatment per experiment.

2.8 Charcoal/Dextran Treated Serum.

A 10X dextran coated charcoal solution was prepared (2.5% charcoal, 0.25% dextran, 0.1 M Tris-pH 7.9) (Sigma D5251 and Fisher #AC404035000). Fetal bovine serum (FBS) was treated

with 2 units of sulfatase (S8504, Sigma) per ml of serum for 2 hours in 37°C water bath. Seven ml of the 10X dextran coated charcoal were centrifuged at 3000 rpm in the Sorvall swinging bucket rotor for the Beckman refrigerated centrifuge for 10 minutes in 50 ml conical tubes. The supernatant was removed and 35 ml of sulfatase treated FBS was added to the dextran charcoal pellet. This mixture was rocked for 5 hours at 4°C and then centrifuged at 3000 rpm for 10 minutes. Serum was transferred to freshly prepared charcoal pellets, rocked for 5 hours at 4°C and then centrifuged at 3000 rpm for 10 minutes. Serum was transferred to glass corex tubes and centrifuged at 9500 rpm in the Sorvall SS-34 rotor for 30 minutes. Serum was filtered through 0.45 µm then 0.2 µm bottle top filters (#290-3345, 290-3320, Nalg Nunc International, Rochester, NY). Serum was stored at -20°C. Before use serum was heat inactivated by incubation in 54°C water bath for 30 minutes.

For certain experiments, 3,3',5-triiodo-L-thyronine (T₃) (T-2877, Sigma) was dissolved in 0.1 M NaOH (70µl 0.01M NaOH/1.0mg T₃) and diluted to a 1 mM stock solution with saline solution (0.154 M NaCl). The T₃ stock solution was stored at -20°C. Stock solutions were diluted in DMEM/10% charcoal/dextran treated FBS to concentrations between 10 nM and 100 µM for addition to cell growth medium. Cells treated with 5.2 Gy radiation were grown in DMEM/10% charcoal/dextran treated FBS supplemented with 0.04-4000 nM T₃ for 7 days. Cell survival was estimated by the MTS assay.

2.9 Plasmids.

The HB101 strain of *E. coli* was transformed with PKC dominant negative mutant pcDNA3 plasmid constructs (Soh et al., 1999) using the Tris-Calcium-Thymidine method as follows. HB101 was inoculated into 1.5 ml of LB broth and grown overnight with shaking at 120 rpm at 37°C. Forty ml of LB broth in a 250ml flask were inoculated with 0.4 ml of the overnight

culture and incubated for 2 hours at 37°C with shaking at 120 rpm. Forty ml aliquots of bacterial culture were placed in 50 ml conical tubes and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed and bacterial pellets suspended in 10 ml of 4°C Tris-Calcium-Thymidine buffer (0.01 M Tris-pH 7.5, 0.74% CaCl₂, 0.05 mg/ml thymidine). Bacteria were collected and transferred to a 30 ml corex tube and incubated on ice for 15 minutes. Bacteria were centrifuged at 5000 rpm in Sorvall SS-34 rotor at 4°C for 5 minutes. The supernatant was removed and the bacteria were gently resuspended in 4 ml of Tris-Calcium-Thymidine buffer and maintained on ice. Two-hundred µl of competent bacteria were transferred to a 1.5 ml Eppendorf tube to which 2 ng of plasmid DNA were added. Bacteria and plasmid DNA were inverted to mix and incubated for 15 minutes on ice. Bacteria were heat shocked for 2 minutes in a 37°C water bath and then incubated for 10 minutes at room temperature. LB broth (0.5 ml) was added to the bacteria and bacteria were incubated for 50 minutes in a 37°C incubator. Top agar was liquified by warming in a microwave and then kept in a 48°C water bath. Three ml of top agar were aliquoted into glass tubes and placed in the 48°C water bath. Bacteria (0.7 ml) were added to the center of the top agar using a pasteur pipette and then immediately poured onto LB agar plates containing ampicillin (0.1 mg/ml). The agar solidified in 10 minutes at room temperature. Plates were placed upside down in a 37°C incubator and left overnight. On the next morning, single colonies were picked and streaked onto LB agar plates containing ampicillin. Plates were incubated at 37°C overnight. Rapid isolation of plasmid DNA was performed by inoculating 1.5 ml of LB broth containing ampicillin (0.04 mg/ml) with individual colonies picked from plates and incubating overnight at 37°C with shaking (120 rpm). The bacteria were pelleted by centrifuging the overnight culture and were resuspended in freshly prepared TENS buffer (0.01 M Tris, 1 mM EDTA-pH 8.0, 0.1 M NaOH, 0.5% SDS). The bacteria were placed

on ice and 150 μ l of 3 M sodium acetate, pH 5.2 were added. Bacteria cell membranes and denatured chromosomal DNA were pelleted and all of the supernatant was added to tubes containing -20°C 100% ethanol (900 μ l). Tubes were incubated for 45 minutes in a dry ice/ethanol slush bath. Plasmid DNA was pelleted by centrifugation for 5 minutes at 14,000 rpm, 4°C. The pellet was washed 2 times with -20°C 70% ethanol and the tubes were inverted to dry. The pellet was resuspended in 1X TE buffer (0.01 M Tris, 1 mM EDTA, pH 8.0). PKC inserts were excised from the plasmid by reacting 5 μ l of plasmid DNA with 10 units of *Xho I* restriction enzyme and 1.5 μ l of React 2 Buffer (1X final concentration) (#15231-012, Y92500, Life Technologies, Inc., Rockville, MD) for 1 hour 45 minutes in a 37°C water bath. Linearized DNA was electrophoresed in a 0.7% agarose gel at 50 V for 2 hours. Ethidium bromide staining and visualization by UV light were used to check for appropriate size fragments of linear plasmid and inserted DNA. Based on these results, bacterial clones containing plasmid constructs with expected inserts were selected for amplification and purification using the EndoFree Plasmid Maxi kit (#12362, Qiagen, Valencia, CA) in accordance with the manufacturers protocol.

2.10 Transformation.

MCF-7 cells (1×10^6 /100 mm² dish) were transformed with 10 μ g of the empty vector, 13.7 μ g of PKC δ dominant negative or 13.3 μ g of PKC ζ dominant negative (normalized for the neomycin resistance gene copy number) by the DOTAP transfection reagent (#1811177, Boehringer Mannheim, Germany). In accordance with the manufacturers protocol, 530 μ l of a 1:4 dilution of DOTAP reagent: 20 mM HEPES (#391333, Calbiochem) buffer (sterile, pH 7.4) were combined with HEPES buffer containing approximately 0.1 μ g/ml of plasmid DNA. The DNA/DOTAP mixture was incubated at room temperature for 15 minutes and then added directly to culture medium (9.5 ml DMEM/2% FBS). After 48 hours, medium was exchanged with 10 ml of

DMEM/5% FBS. On the following day, medium was exchanged with 10 ml DMEM/10% FBS + 400 µg/ml of G418 (#20039, Stratagene, La Jolla, CA). Following 18 days of growth in selection medium crystal violet staining was performed.

2.11 Statistical Analysis.

Statistically significant differences ($P < 0.05$) were determined using Student's t-test. For multiple comparisons one-way ANOVA with Tukey's multiple comparison test was used. For interactions between treatments two-way ANOVA with Bonferroni's post-test was used.

III. RESULTS

Chapter 1. Preliminary Results

1.1 Radiation Survival. Clonogenic survival performed in 100 mm² dishes is a highly sensitive assay for radiation dose response. Clonogenic survival assays measure the ability of cells to survive and form colonies at a low plating density. In this case cells were challenged with 0-8.8 Gy doses of γ -radiation prior to cloning. Radiation treatments were performed on proliferating cells released from confluent cultures by subculturing for approximately 24 hours prior to radiation treatment. MCF-7 cell clonogenic survival was measured using crystal violet staining and visual colony inspection (Figure 5). The radiation survival curve shows that radiation doses in the 0-2 Gy range have a minimal effect on cell survival indicative of radiation-induced DNA damage repair. Doses greater than 2 Gy produced a dose dependent decline in cell survival.

Mitochondrial metabolism of the substrate, MTS, was performed with cells growing in 96-well plates and is another measure of cell survival. Mitochondrial metabolism declined by 65% in MCF-7 cells seven days after a 5.2 Gy dose of γ -radiation as compared to un-irradiated cells. This radiation response was constant at varied plating densities of 1000 or 2500 cells/well with control cells reaching approximately 60% and 80% confluency, respectively, after seven days of growth. It should be noted that MCF-7 cells plated at a higher density (5000 cells/well) showed a reduced radiation response (32% decrease). This could be explained by reduced growth of control cells, which exceeded available nutrients during the seven day growth period. While the MTS assay is not as sensitive as clonogenic survival it offers broad applicability to many cell types and rapid data acquisition.

The MTS assay was also utilized as an index of MDA-MB-231 and MCF-10A cell survival in subsequent studies. Clonogenic survival is not a suitable endpoint for either of these cell lines since they do not form discrete colonies. Because MDA-MB-231 and MCF-10A cells have rapid doubling times in contrast to MCF-7 cells, MDA-MB-231 and MCF-10A cells were permitted only five days growth prior to end point analysis. Under these conditions all cell types were allotted appropriate time to complete at least three cell divisions.

Figure 5. MCF-7 cell clonogenic survival. MCF-7 cells (1×10^5 /35 mm² dish) 24 h post-subculture from confluency were treated with 0-8.8 Gy doses of γ -radiation. Following radiation exposure (20 h) cells were harvested and replated at a cloning cell density of 1×10^4 cells/100 mm² dishes. After 7 days of incubation cell survival was determined by the clonogenic survival assay. Survival of control cells was set =1. The efficiency of clonogenic survival in control cells was 9.0%. Data represent the mean $\pm$ SD of n=2 independent experiments.

1.2 PKC Expression in Human Breast Tumor Cells. PKC isoform content varies between different types of cells. Furthermore, even cells sharing the same origin, such as those derived from mammary tumors have striking differences in their PKC isoform profiles. To demonstrate this, western blot analysis of PKC α and PKC ζ protein in extracts prepared from a panel of human breast tumor cell lines was performed (Figure 6). Four cell lines commonly used as *in vitro* breast tumor cell models were chosen for this experiment: T47-D, MDA-MB-231, MCF-7*ras* and MCF-7. A prominent difference in PKC α expression was observed in MDA-MB-231 cells, which express high levels of PKC α relative to the other three cell types. PKC ζ expression was high in T47-D, MCF-7 and MCF-7*ras*, while PKC ζ expression was minimal in MDA-MB-231 cells. These experiments do not explain why these differences in PKC protein content occur. Experiments on extracts prepared from MCF-7 cells at 4 (early G1), 35 (S) and 60 (G2/M) hours after release from confluency showed no change in PKC δ or PKC ζ protein levels. The doubling times of these cell types are not the same. Ranked in order of shortest to longest doubling times are MCF-7*ras* (24 hours), MDA-MB-231 (27 hours), MCF-10A (38 hours), T47-D (44 hours), and MCF-7 (60 hours) (Melkounian, 2001).

Figure 6. PKC protein levels in human breast tumor cell lines. Whole cell extracts were prepared from T47-D, MDA-MB-231, MCF-7_{ras} and MCF-7 cells ($3.0 \times 10^6/100$ mm² dish) 24 h post-subculture from confluency. Proteins (40 µg/lane) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC α and PKC ζ. The PKC standards (STD) include purified PKC α (15 ng/lane) and PKC ζ (40 ng/lane). Data shown are typical of n=2 experiments.

1.3 MDA-MB-231 Cell Survival in Response to γ -radiation and PKC Oligonucleotides.

To identify individual PKC isoforms that positively regulate growth following γ -radiation treatment, antisense oligonucleotides were used as isoform specific PKC inhibitors. This initial screen utilized liposome delivered oligonucleotides targeted to five PKC isoforms representative of the classical (α), novel (δ , ϵ , η) and atypical (ζ) PKC subfamilies. The mitochondrial metabolism of MTS was used as an index of cell survival. Treatment of MDA-MB-231 cells with PKC δ or PKC ζ oligonucleotide significantly reduced cell survival following radiation insult compared to treatment with the respective nucleotide scrambled versions of PKC oligonucleotides (Figure 7). Liposome treatment alone had no effect on the survival of irradiated MDA-MB-231 cells however, some non-sequence specific effects of the oligonucleotides were observed. The nucleotide scrambled version of PKC ζ reduced survival relative to liposome treatment alone in irradiated cells. Both the isoform specific and nucleotide scrambled versions of PKC α and PKC ϵ oligonucleotides reduced cell survival following radiation. Illustrating that oligonucleotide treatment alone is insufficient to reduce cell survival, neither PKC η nor its nucleotide scrambled version had an effect on irradiated MDA-MB-231 cell survival.

Figure 7. MDA-MB-231 cell survival in response to γ -radiation +/- PKC antisense oligonucleotide treatment. MDA-MB-231 cells (1.1×10^5 /35 mm² dish) 24 h post-subculture from confluency were treated with 100 nM antisense oligonucleotides plus liposome that target PKC α , δ , ϵ , η and ζ (gray bars). Controls were treated with radiation alone (IR), radiation plus liposome (L), or radiation plus liposome plus scrambled nucleotide versions of these oligonucleotides (black bars). Post-transfection (24 h), cells were irradiated with 5.6 Gy of γ -radiation, harvested and replated in 96-well plates at a cloning density of 250 cells/well. After 5 days of incubation, cell survival was estimated using the MTS assay. Data are the mean $\pm$ SE of n=4 independent experiments performed with 5 replicates/treatment. Survival of cells treated with radiation alone was set =100%. Statistically significant differences between treatment groups receiving PKC antisense oligonucleotides versus nucleotide scrambled sequence PKC oligonucleotides are indicated (*P<0.05).

1.4 MCF-7 Cell Survival in Response to γ -radiation and PKC Oligonucleotides. In MCF-7 cells, liposome treatment alone elicited the most significant effect, reducing the survival of irradiated cells by approximately 50% (Figure 8). The magnitude of the liposome effect exceeded that of oligonucleotide treatment in affecting survival after γ -radiation treatment. Nevertheless, PKC δ and PKC ζ oligonucleotides significantly reduced the survival of MCF-7 cells after radiation treatment, thus confirming evidence from MDA-MB-231 cells of the importance of these two PKC isoforms in the radiation survival of human breast tumor cell lines. PKC η oligonucleotide treatment increased the survival of irradiated MCF-7 cells relative to its nucleotide scrambled version, implying a radioprotective effect by the PKC η oligonucleotide. Consistent with the results in MDA-MB-231 cells (Figure 7), both the isoform specific and nucleotide scrambled versions of PKC α and PKC ϵ reduced the survival of γ -irradiated MCF-7 cells. The results for PKC α and PKC ϵ in both cell types were inconclusive due to non-sequence specific effects of the nucleotide scrambled oligonucleotides. Isoform specific actions can only be distinguished when a nucleotide scrambled version of the oligonucleotide has no effect upon cell survival.

Figure 8. MCF-7 cell survival response to γ -radiation +/- PKC antisense oligonucleotide treatment. MCF-7 cells ($2.2 \times 10^5/35 \text{ mm}^2$ dish) 24 h post-subculture from confluency were treated with 200 nM antisense oligonucleotides plus liposome that target PKC α , δ , ϵ , η and ζ (gray bars). Controls were treated with radiation alone (IR), radiation plus liposome (L), or radiation plus liposome plus scrambled nucleotide versions of these oligonucleotides (black bars). Post-transfection (48 h), cells were irradiated with 5.6 Gy of γ -radiation, harvested and replated in 96-well plates at a cloning density of 2500 cells/well. After 7 days of incubation, cell survival was estimated using the MTS assay. Data are the mean $\pm$ SE of n=3 independent experiments performed with 5 replicates/treatment. Survival of cells treated with radiation alone was set =100%. Statistically significant differences between treatment groups receiving PKC antisense oligonucleotides versus nucleotide scrambled sequence PKC oligonucleotides are indicated (*P<0.05).

1.5 Treatment with PKC Oligonucleotides in the Absence of Liposome. MCF-7 cell survival is significantly impaired by treatment with liposome alone (Figure 8). In an effort to separate the effects of PKC oligonucleotides from those of liposomes, oligonucleotide treatments were performed in the absence of a lipid carrier. For these experiments, oligonucleotide concentrations were increased to 9 μ M, a concentration previously shown to produce a phenotype in MCF-7 cells consistent with *c-myc* depletion by *c-myc* oligonucleotides in the absence of liposome (Melkounian and Strobl, 2002). Treatment of MCF-7 cells with 9 μ M PKC δ , PKC ζ or the respective scrambled oligonucleotides had no effect on cell survival (Figure 9A). In addition, if oligonucleotide treatments were followed by exposure to 5.2 Gy radiation, no differences between PKC and scrambled oligonucleotides were observed, although a reduction in cell survival was observed in all cells that received any oligonucleotide compared to those treated with radiation alone. Furthermore, this same experiment was performed in MDA-MB-231 cells with no effect by the PKC δ or PKC ζ oligonucleotides (Figure 9B). In previous experiments, the combination of liposome and 100 nM PKC δ oligonucleotide produced a significant decrease in MDA-MB-231 cell survival. We conclude that 9 μ M PKC oligonucleotide without a lipid carrier was ineffective at reproducing the effects on cell survival of liposome delivered PKC δ or PKC ζ oligonucleotides. These findings suggest that these PKC oligonucleotides require complexation with liposomes for effective delivery.

Figure 9. PKC oligonucleotide treatment without liposome. (A) MCF-7 cells ($2.2 \times 10^5/35 \text{ mm}^2$ dish) 24 h post-subculture from confluency were treated with 9 μM PKC δ or PKC ζ antisense oligonucleotides and 48 h later, treated with 5.2 Gy IR, harvested and replated (2500 cells/well) in 96 well-plates. (B) MDA-MB-231 cells ($1.1 \times 10^5/35 \text{ mm}^2$ dish) were treated as in (A), except replated at a density of 250 cells/well 17 h post-oligonucleotide treatment. MTS activity was measured after 7 and 5 days (A and B, respectively) as an index of survival. Data are the mean $\pm$ SD of 5 replicates/treatment in a single experiment. Survival of control cells was set =100%.

Figure 9A. MCF-7

Figure 9B. MDA-MB-231

1.6 Immunoblot Analysis of PKC Oligonucleotide Treated Cells. To gain insight into the kinetics of oligonucleotide action on PKC protein levels, western blot analyses of extracts from MDA-MB-231 cells prepared at various times following PKC α oligonucleotide treatment were performed. Extracts from MDA-MB-231 cells prepared 5 or 24 hours after treatment with PKC α oligonucleotide did not show reductions in PKC α protein levels (Figure 10A). Cell extracts prepared at later time points (48-96 hours) following PKC α oligonucleotide treatment, yielded marked reductions in PKC α protein levels (Figure 10B). These results indicate that PKC α is a stable protein. PKC oligonucleotides likely produce rapid reductions in target mRNA levels and therefore prevent *de novo* PKC protein synthesis. However, pre-existing PKC protein is not affected by oligonucleotide action and its reduction is dependent on proteolytic degradation. The lag between inhibition of *de novo* PKC synthesis and degradation of pre-existing PKC appears to be in the range of 48 hours.

For additional evidence that oligonucleotides inhibit PKC protein levels, PKC ϵ protein levels were assessed. Whole cell lysates were prepared from MDA-MB-231 cells 52-72 h after PKC ϵ oligonucleotide treatment. Immunoblot analysis demonstrated reductions in PKC ϵ protein levels by the isoform specific oligonucleotide compared to controls (Figure 11). Extracts prepared from PKC ϵ oligonucleotide treated cells yielded dilute protein concentrations and this may account for the difference in mobility observed within the PKC ϵ signals. The conclusion from these studies is that PKC oligonucleotides are effective inhibitors of synthesis of PKC isoforms and require at least 48 hours for optimal PKC isoform protein reduction. These conclusions are consistent with PKC oligonucleotide studies performed by collaborators at Isis Pharmaceuticals.

Figure 10. Immunoblot analysis of PKC α antisense oligonucleotide treated cells.

MDA-MB-231 cells (6.0×10^5 /100 mm² dish) 24 h post-subculture from confluency were treated with lipofectin alone (L), 100 nM PKC α antisense oligonucleotide (PKC α) or the nucleotide scrambled version (SCR). Whole cell extracts were prepared from cells 5 h and 24 h (A) or 48-96 h (B) after treatments. Proteins (20 μ g/lane) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with PKC α antibody. The PKC standard (STD) is 4 ng of purified PKC α protein. The signal density for cells treated with the nucleotide scrambled version (A) or lipofectin alone (B) was set =100%. Signal densities were normalized to a 200 kDa protein band on the Coomassie blue-stained acrylamide gel. Results are from a single experiment.

Figure 10A.

Figure 10B.

Figure 11. Immunoblot analysis of PKC ε antisense oligonucleotide treated cells.

MDA-MB-231 cells (5.0×10^5 /100 mm² dish) 24 h post-subculture from confluency were treated with lipofectin alone (L), 100 nM PKC ε antisense oligonucleotide (PKCε), the nucleotide scrambled version (SCR) or no treatment (C). Whole cell extracts were prepared from cells 52-72 h after treatments. Proteins (50 μg/lane) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with PKC ε antibody. The PKC standard (STD) is 4 ng of purified PKC ε protein. Control signal densities were set =100%. Signal densities were normalized to a 200 kDa protein band on the Coomassie blue-stained acrylamide gel. Results are from a single experiment.

1.7 Radiation Induced DNA Damage. Radiation produces rapid DNA damage in the form of single- and double-strand DNA breaks. Alkaline electrophoresis of single cells following radiation treatment and cell lysis permits the separation of intact nuclear DNA from fragmented DNA, forming a discernible comet head and tail, respectively (Fairbiarn, 1995). Using the comet assay, experiments to quantify DNA damage and subsequent damage repair in MCF-7 cells were performed. For unirradiated control cells, the tail moment which measures the amount of damaged DNA in the tail and the electrophoretic distance traveled, was near zero (Figure 12). Following low dose radiation treatment (1.5 Gy), migration of DNA from the head of the comet was observed and is evidenced by the shift in the distribution frequencies toward higher tail moment values. In response to 5.2 Gy of radiation, the distribution of comets observed shifted toward higher values for the tail moment parameter. Interestingly, if MCF-7 cells were incubated for one hour at 37°C before lysis, DNA damage induced by 1.5 or 5.2 Gy irradiation was repaired. In conclusion, these experiments provide evidence that DNA damage and repair are rapid responses to γ -radiation treatment. The comet assay is a quick and sensitive method for detection and quantification of DNA damage.

Figure 12. Radiation induced DNA damage in MCF-7 cells. MCF-7 cells ($1 \times 10^5/35$ mm⁵ dish) 48 h post subculture from confluency were treated with 1.5 or 5.2 Gy γ -radiation or cells were incubated 1 h for repair of DNA damage post-irradiation (1.5Gy/5.2Gy + 1h) were analyzed by the comet assay as described in methods. The number of comets with tail moments in ranges between 0-2, 5-10, 10-20, 20-50, 50-100 and >100 are plotted for each treatment group. Data represent n=3 independent experiments with 80 comets scored per treatment/experiment. Statistically significant differences are indicated below (*P<0.05). The images shown are typical of the majority of comets for the respective treatment groups.

Statistical Analyses (ANOVA)

Comparison	P Value
*Control vs. 1.5Gy	<0.05
*Control vs. 5.2Gy	<0.05
*1.5Gy vs. 5.2Gy	<0.05
*1.5Gy vs. 1.5Gy +1h	<0.05
*5.2Gy vs. 5.2Gy +1h	<0.05

Control

1.5Gy

1.5Gy + 1h

5.2Gy

5.2Gy + 1h

1.8 Thyroid Hormone Manipulation *In Vitro*. Clinical studies have revealed that induction of hypothyroidism in patients with recurrent gliomas produces prolonged survival (Hercbergs, 2000; 1999). Additional evidence suggests that triiodothyronine (T_3) stimulates growth by mimicking estrogen in estrogen receptor positive breast cancer cell lines (Nogueira and Brentani, 1996). For these reasons, experiments were conducted to test if T_3 deprivation would impair breast tumor cell survival in response to γ -radiation treatment. Because selective depletion of thyroid hormone from serum is not a practical experimental design, fetal bovine serum (FBS) was charcoal/dextran and sulfatase treated to remove growth factors and hormones. MCF-7 cell growth in treated serum versus untreated FBS was inhibited in the presence or absence of radiation treatment (Figure 13). Addition of T_3 to charcoal/dextran treated growth medium did not stimulate MCF-7 cell proliferation in irradiated or unirradiated cells (Figure 14). In summary, the MCF-7 cells used in these experiments did not display T_3 responsiveness.

Figure 13. MCF-7 cell survival in charcoal/dextran treated FBS. MCF-7 cells ($1.1 \times 10^5/35 \text{ mm}^2$ dish) 24 h post-subculture from confluency were treated with 5.2 Gy γ -radiation. Post-irradiation (20 h) cells were harvested and replated in 96-well plates at a cloning density of 1500 cells/well. Cells were grown in DMEM/10% charcoal/dextran treated FBS or untreated FBS for 7 days. Cell survival was estimated using the MTS assay. Data are the mean $\pm$ SD of n=2 independent experiments performed with 5 replicates/treatment. Survival of cells grown in untreated FBS was set =100%.

Figure 14. MCF-7 cell response to T₃. MCF-7 cells (1.1×10^5 cells/35 mm² dish) 24 h post-subculture from confluency were treated with 5.2 Gy γ -radiation. Post-irradiation (20 h) cells were harvested and replated in 96-well plates at a cloning density of 1500 cells/well. Cells were grown in DMEM/10% charcoal/dextran treated FBS supplemented with increasing concentrations of 3,3',5-triiodo-L-thyronine (T₃) for 7 days. Cell survival was estimated using the MTS assay. Data are the mean of 5 replicates/treatment $\pm$ SD in a single experiment. Survival of cells grown in DMEM/10% charcoal/dextran treated FBS, without T₃ or radiation was set =100%.

Chapter 2. PKC δ is a Pro-survival Factor in Human Breast Tumor Cell Lines.

2.1 Radiation Survival Curves. Cell survival following exposure to increasing doses of γ -radiation was measured using mitochondrial metabolism of MTS as an end-point. This assay is rapid, reproducible and suitable for screening purposes. Using the MTS assay, the human mammary tumor cell lines, MDA-MB-231 and MCF-7, used for these studies showed similar radiation survival curves (Figure 15). However, at the radiation doses of 3.7 and 6.6 Gy the survival of MDA-MB-231 cells was significantly greater than that of the MCF-7 cells.

Figure 15. Radiation survival curves of MDA-MB-231 and MCF-7 cells. MDA-MB-231 and MCF-7 cells were treated with 0-9.5 Gy doses of γ -radiation. Following radiation exposure (20 h) cells were harvested and replated at cloning cell densities of 250 (MDA-MB-231) and 1500 (MCF-7) cells/well in 96-well plates. After 5 (MDA-MB-231) or 7 (MCF-7) days of incubation cell survival was determined by MTS assay. Control cell survival was set =100%. Data represent the mean $\pm$ SE of n=3 independent experiments performed with 5 replicates/treatment. Statistically significant differences between the two cell lines are indicated (*P <0.05).

2.2 Differential Expression of PKC Isoforms in MDA-MB-231 and MCF-7 Cells. Individual PKC isoforms are differentially expressed within human mammary tumor cells. Western blot analysis of MDA-MB-231 and MCF-7 cell extracts was performed to examine the relative protein levels of PKC α , PKC δ , PKC ϵ , PKC η and PKC ζ (Figure 16). The major difference in the PKC expression profile between these two cell lines is the PKC α isoform expression. PKC α is present at higher levels in the estrogen receptor-negative MDA-MB-231 cells than in estrogen receptor-positive MCF-7 cells. This finding is supported by previous investigations concluding a negative correlation between PKC α expression and estrogen receptor status in mammary tumor cells (Morse-Gaudio et al., 1998; Ways et al., 1995). MDA-MB-231 and MCF-7 cells also differed in their expression of the PKC η isoform. While MCF-7 cells contain two species of PKC η , a faster and slower migrating form, PKC η was undetectable in MDA-MB-231 cells. The correlation between PKC α overexpression and an absence of PKC η protein in MDA-MB-231 cells is consistent with previous reports where transformation of MCF-7 cells with PKC α results in reduction of PKC η protein (Ways et al., 1995). These results and previous findings support the correlation between PKC α overexpression and a less differentiated breast tumor cell phenotype. The expression of PKC δ , PKC ϵ and PKC ζ were similar between the two cell lines.

Figure 16. PKC protein levels in MDA-MB-231 and MCF-7 cells. Whole cell extracts were prepared from MDA-MB-231 ($3.5 \times 10^6/100 \text{ mm}^2$ dish) or MCF-7 ($2.5 \times 10^6/100 \text{ mm}^2$ dish) cells 24 h post-subculture from confluency. Proteins (40 $\mu\text{g/lane}$ - α , ϵ , η , ζ and 62 $\mu\text{g/lane}$ - δ) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC α , PKC δ , PKC ϵ , PKC η and PKC ζ . The PKC standards (STD) included purified PKC α (15 ng/lane), PKC δ (15 ng/lane), PKC ϵ (5 ng/lane), PKC η (3 ng/lane) and PKC ζ (40 ng/lane). Data shown are typical of $n=2$ experiments.

2.3 PKC δ Oligonucleotides Decreased Survival in Human Breast Tumor Cells. PKC gene and protein expression are induced following ionizing radiation (Woloschak et al., 1990; Kim et al., 1992). PKC inhibition radiosensitizes human squamous carcinoma, human colon adenocarcinoma, transformed mouse embryo fibroblasts and human glioblastoma cell lines (Hallahan et al., 1992; Zaugg et al., 2001; Rocha et al., 2000; Begemann et al., 1998). The goal of this project was to examine the roles of specific PKC isoforms on cell survival in response to radiation insult by 5.6 Gy. Oligonucleotides that targeted the novel PKC isoform, PKC δ were introduced into the two human breast tumor cell lines using liposomes. Treatment of MDA-MB-231 cells with PKC δ oligonucleotide decreased cell survival ($P < 0.05$) in the irradiated cells by 44% compared with cells that were treated with the nucleotide scrambled version of this oligonucleotide (Figure 17A). Liposome treatment alone or liposome treatment plus the nucleotide scrambled version of the PKC δ oligonucleotide had no effect on MDA-MB-231 survival indicating that the oligonucleotide sequence was critical to the decreased survival.

In MCF-7 cells (Figure 17B), liposome treatment alone reduced survival of irradiated cells by approximately 50%, and the magnitude of the liposome effect exceeded that of oligonucleotide treatment in affecting cell survival after γ -radiation. Nevertheless, PKC δ oligonucleotides significantly decreased the survival of MCF-7 cells after radiation treatment, thus providing confirmatory evidence for the importance of this PKC isoform in the radiation survival of human breast tumor cell lines. It was concluded that PKC influences breast tumor cell survival following ionizing radiation in an isoform specific manner and that PKC δ oligonucleotide effects on the survival of irradiated MDA-MB-231 and MCF-7 cells are quite specific.

Figure 17. Cell survival in response to γ -radiation +/- PKC δ oligonucleotide treatment. (A) MDA-MB-231 cells ($1.1 \times 10^5/35 \text{ mm}^2$ dish) were treated with 100 nM PKC δ (PKC δ) antisense oligonucleotide plus liposome. Controls were treated with radiation alone (IR), radiation plus liposome (L), or radiation plus liposome plus a scrambled nucleotide version of PKC δ oligonucleotide (SCR). Post-transfection (24 h), cells were irradiated with 5.6 Gy of γ -radiation, harvested and replated in 96-well plates at a cloning density of 250 cells/well. After 5 days of incubation, cell survival was estimated using the MTS assay. (B) MCF-7 cells ($2.2 \times 10^5/35 \text{ mm}^2$ dish) were treated with 200 nM PKC δ antisense oligonucleotide or controls as described in (A). Cells were irradiated with 5.6 Gy γ -radiation 48 h post-transfection, harvested and replated in 96-well plates at a cloning density of 2500 cells/well. After 7 days of incubation, cell survival was estimated using the MTS assay. Data are the mean $\pm$ SE of n=4 (A) or n=3 (B) independent experiments performed with 5 replicates/treatment. Survival of cells treated with radiation alone was set =100%. Statistically significant differences between groups treated with PKC δ antisense oligonucleotide versus scrambled version of PKC δ are indicated (*P<0.05).

Figure 17A. MDA-MB-231

Figure 17B. MCF-7

2.4 PKC δ Oligonucleotides Specifically Reduced PKC δ Protein Levels. To further analyze the role of PKC δ oligonucleotide in breast tumor cell survival post-irradiation, PKC δ protein levels in cells treated with oligonucleotide that targeted PKC δ were assessed. Whole cell extracts were prepared from MCF-7 and MDA-MB-231 cells either 52 h or 72 h after transfection with PKC δ oligonucleotide. These time points were chosen based preliminary studies that assessed the kinetics of PKC α antisense oligonucleotide and showed that optimal protein depletion required at least 48 hours. No PKC δ protein was detected in either cell line after 72 h (Figure 18 and 19). Untreated cells, cells treated with lipofectin alone or the inactive nucleotide scrambled version of the PKC δ oligonucleotide showed constant levels of PKC δ at 52 and 72 h. The PKC δ oligonucleotide transfection protocol, but not the control conditions used in the radiation survival experiments, depleted human breast tumor cell lines of PKC δ protein. To test whether the effect of PKC δ oligonucleotide was selective for the δ isoform of protein kinase C, protein levels of PKC ϵ and PKC ζ were assayed in the MCF-7 cell extracts (Figure 18). There was no effect of the PKC δ oligonucleotide on levels of either PKC ϵ or PKC ζ . Bcl-2 is an anti-apoptotic protein that is highly expressed in human breast tumor cells (Eissa et al., 1999). To test whether the effect of PKC δ oligonucleotide could be manifested as a decrease in bcl-2 protein levels immunoblot analysis for bcl-2 was performed. Figure 18 and 19 show that the levels of bcl-2 protein are constant in MCF-7 and MDA-MB-231 cells treated with the PKC δ oligonucleotide. Furthermore, the quantitated and normalized bcl-2 signal densities showed no significant differences ($n=3$, $P<0.05$) in response to control or PKC δ oligonucleotide treatments in either cell line. The decreased survival of irradiated cells following depletion of PKC δ protein with PKC δ oligonucleotide can not be attributed to a decrease in bcl-2 protein. We conclude that

reduced cell survival by PKC δ oligonucleotide is due to specific depletion of PKC δ protein from human breast tumor cells.

Figure 18. Immunoblot analysis of PKC δ oligonucleotide treated MCF-7 cells.

Whole cell extracts were prepared from MCF-7 cells (7.0×10^5 /100 mm² dish) 52-72 h after treatment with lipofectin alone (L), 100 nM PKC δ antisense oligonucleotide (PKC δ), the nucleotide scrambled version (SCR) or no treatment (C). Proteins (20 μ g/lane) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC δ , PKC ϵ , PKC ζ and bcl-2. The PKC standards (STD) included purified PKC δ (15 ng/lane), PKC ϵ (5 ng/lane) and PKC ζ (25 ng/lane). Data in graph are the mean $\pm$ SE of n=3 independent experiments. Control signal density was set =100%. PKC δ signal densities were normalized to a 200 kDa protein band on the Coomassie blue-stained acrylamide gel. Statistically significant differences between treatment groups receiving PKC δ antisense oligonucleotide versus nucleotide scrambled sequence of PKC δ oligonucleotide are indicated (*P<0.05). Blots are typical of n=3 independent experiments.

Figure 19. Immunoblot analysis of PKC δ oligonucleotide treated MDA-MB-231

cells. Whole cell extracts were prepared from MDA-MB-231 cells (5.0×10^5 /100 mm² dish) 52-72 h after treatment with lipofectin alone (L), 100nM PKC δ antisense oligonucleotide (PKC δ), a nucleotide scrambled version (SCR) or no treatment (C). Proteins (20 μ g/lane) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC δ and bcl-2. The PKC δ standard (STD) is 15 ng of purified PKC δ protein. Data in graph are the mean $\pm$ SE of n=3 independent experiments. Control signal density was set =100%. PKC δ signal densities were normalized to a 200 kDa protein band on the Coomassie blue-stained acrylamide gel. Statistically significant differences between treatment groups receiving PKC δ antisense oligonucleotide versus nucleotide scrambled sequence of PKC δ oligonucleotide are indicated (*P<0.05). Blots are typical of n=3 independent experiments.

2.5 PKC δ Protein Levels Are Elevated in Response to Ionizing Radiation Treatment.

Previous experiments supported the hypothesis that PKC δ is a survival factor in breast tumor cells. To evaluate whether PKC δ might augment survival following radiation treatment, PKC δ protein levels post-irradiation were examined. At early (3 h) and late time points (21 h) after treatment with 5.2 Gy γ -radiation, PKC δ protein levels were significantly (n=3, P<0.05) elevated in extracts prepared from irradiated cells compared to non-irradiated controls (Figure 20). In addition, experiments performed in parallel showed no change in PKC ζ protein levels following radiation treatment (Figure 31). These findings support the hypothesis that PKC δ also acts as a survival factor in response to ionizing radiation-induced DNA damage.

Figure 20. PKC δ protein levels in MCF-7 cells following exposure to ionizing radiation. Whole cell extracts were prepared from MCF-7 cells 3 h and 21 h after exposure to 5.2 Gy radiation. Proteins (25 μ g/lane) were resolved on 7.5% acrylamide gels, transferred to filters and probed with an antibody to PKC δ . A Coomassie blue-stained protein is shown as the loading control. Data shown are representative of n=3 independent experiments.

2.6 Rottlerin Reduced Cell and Clonogenic Survival. To test the hypothesis that PKC δ is a survival factor in breast tumor cell lines, clonogenic survival assays with MCF-7 cells following exposure to rottlerin, a PKC δ selective PKC inhibitor were performed. Clonogenic survival assays are a sensitive measure of drug activity, and the MCF-7 cells were highly responsive to rottlerin under these conditions. Clonogenic survival of MCF-7 cells exposed to 3 μ M rottlerin for seven days, was reduced by 78% compared to control cells exposed to the solvent alone (Figure 21). The published IC_{50} value for rottlerin inhibition of purified PKC δ is 3-6 μ M (Gschwendt et al., 1994). These results support the hypothesis that PKC δ is a survival factor in breast tumor cells. The effect of rottlerin on the survival of MCF-7 and MDA-MB-231 cells following γ -radiation was also tested (Figure 22A and B). MCF-7 cells were exposed to either 1.5 or 5.2 Gy of γ -radiation. MDA-MB-231 cells were tested only at the 5.2 Gy radiation dose. After radiation, the cells were harvested and plated into 96-well dishes containing medium with varying concentrations of rottlerin. MCF-7 cell radiation survival measured by the MTS assay was reduced by treatment with 3 μ M rottlerin. MDA-MB-231 cells were even more sensitive to 3 μ M rottlerin in the presence or absence of radiation (Figure 22B) than MCF-7 cells. In either cell type 3 μ M was the minimum concentration of rottlerin required to produce a statistically significant decrease in cell survival at each radiation dose with no significant interaction detected between the radiation and rottlerin treatments.

Figure 21. Effect of rottlerin on clonogenic survival. MCF-7 cells (1.0×10^4 /100 mm² dish) were treated with indicated concentrations of rottlerin. Cells were left undisturbed for 7 days then plates were stained with crystal violet to visualize colonies. The cloning efficiency for control cells was 11.0% and this was set =100%. The mean ($\pm$ SE) clonogenic survival was determined for n=3 independent experiments. Statistically significant differences between control and treatment groups are indicated (*P<0.05).

Figure 22. Effect of rottlerin on cell survival. (A) MCF-7 cells (3.0×10^5 / 35 mm² dish) were treated 1.5 or 5.2 Gy γ -radiation (IR), harvested, replated at a cloning density of 2500 cells/well and treated with indicated concentrations of rottlerin. Cells were grown for 7 days and cell survival was determined by MTS assay. (B) MDA-MB-231 cells (2.0×10^5 /35 mm² dish) were treated as in (A) but with 5.2 Gy IR, replated at a cloning density of 250 cells/well and grown for 5 days. Data are the mean $\pm$ SE of n=3 independent experiments performed with 5 replicates/treatment. Survival of control cells was set =100%. Statistically significant differences between control and rottlerin treatments are indicated (*P<0.05).

Figure 22A. MCF-7

Figure 22B. MDA-MB-231

2.7 The Effects of PKC δ Oligonucleotide and Low Dose γ -Radiation on Breast Tumor Cell

Survival. Breast cancer radiation therapy is delivered in the range of 1-2 Gy/treatment. To determine whether PKC δ oligonucleotide would decrease breast tumor cell survival *in vitro* in this radiation dose range, cells were exposed to 1.5 Gy radiation 17 h after transfection with oligonucleotides. Control, non-irradiated cells were treated in parallel with PKC δ oligonucleotide or its nucleotide scrambled version. PKC δ oligonucleotide treatment reduced the survival of MDA-MB-231 treated with a 1.5 Gy dose of radiation (Figure 23A). Consistent with earlier results (Figure 17A), a 40% decrease in cell survival over five days was observed in non-irradiated MDA-MB-231 cells treated with the PKC δ compared with the nucleotide scrambled oligonucleotide (Figure 23A). Survival of the MDA-MB-231 cells exposed to the low dose of γ -radiation (1.5 Gy) alone was decreased approximately 20% compared to non-irradiated controls. Pre-treatment of the cells with the PKC δ oligonucleotide caused cell survival to decrease by approximately 70%. Cell survival in irradiated cells pre-treated with the PKC δ antisense oligonucleotide was statistically significantly reduced compared with the cells that were pre-treated with the nucleotide scrambled version of the oligonucleotide. In conclusion, PKC δ depletion by antisense oligonucleotides impairs the survival of MDA-MB-231 human breast tumor cells.

To evaluate the effects of PKC δ antisense oligonucleotide on the response of MCF-7 cells to a therapeutic dose of γ -radiation, MCF-7 cells pre-treated with PKC δ antisense oligonucleotide were exposed to 1.5 Gy radiation. Non-irradiated control cells were treated with PKC δ antisense oligonucleotide in parallel. The survival of MCF-7 cells treated with PKC δ antisense oligonucleotide was not reduced compared with liposome treatment alone (Figure 23B). PKC δ antisense oligonucleotide did not reduce MCF-7 cell survival post-1.5 Gy radiation. The

discrepancy in the response of MCF-7 and MDA-MB-231 cells to PKC δ antisense oligonucleotide might be attributed to differences between the two cell types. In contrast to MCF-7 cells, MDA-MB-231 cells are caspase-3 positive, p53 mutant, ER negative and have a rapid proliferation rate. It is conceivable that inhibition of a survival factor such as PKC δ would have a greater effect on the survival of rapidly proliferating cells like MDA-MB-231.

Figure 23. The effect of PKC δ oligonucleotides and low dose γ -radiation on the survival of human breast tumor cells. (A) MDA-MB-231 cells were transfected with PKC δ oligonucleotides and 17 h later irradiated with 1.5 Gy γ -radiation (IR), harvested and replated (250 cells/well) in 96 well-plates. (B) MCF-7 cells were transfected with 200 nM PKC δ oligonucleotides and 48 h later irradiated with 1.5 Gy γ -radiation, harvested and replated (2500 cells/well) in 96 well-plates. MTS activity was measured after 5 (A) or 7 (B) days as an index of survival. The survival of unirradiated control cells was set =100%. Data are the mean $\pm$ SE of n=4 (A) or n=3 (B) independent experiments performed with 5 replicates/treatment. Statistically significant differences between the oligonucleotide treatments are indicated (*P <0.05).

Figure 23A. MDA-MB-231

Figure 23B. MCF-7

2.8 Expression of PKC δ Dominant Negative Impaired MCF-7 Cell Survival. To determine if inhibition of PKC δ through an alternative approach could reduce MCF-7 survival, PKC δ dominant negatives were utilized. A point mutation was introduced into the full length coding sequence of PKC δ that produced a single amino acid substitution within the ATP binding domain. Mutant PKC δ was incorporated into a plasmid vector containing a neomycin resistance gene and a cytomegalovirus promoter that yields high plasmid expression in human cells. This mutation was sufficient to create a PKC δ protein with dominant negative activity (Soh et al., 1999).

Transformed MCF-7 cells expressing the neomycin resistance gene were selected for by growth in the presence of G418. The neomycin gene conferred resistance to G418 as evident by visualization of MCF-7 cell colony formation (pcDNA3-neo) (Figure 24). In the absence of the neomycin resistance gene, G418 acted as an 80S ribosome subunit inhibitor, interfered with protein synthesis and was toxic to MCF-7 cells (“mock”). Transformation of MCF-7 cells with PKC δ dominant negative (pcDNA3-neo-PKC δ dn) reduced visual colony numbers compared to transformation with the plasmid vector alone. These results indicate that expression of a dominant negative form of PKC δ attenuates MCF-7 cell survival. Furthermore, these finding support the hypothesis that PKC δ functions as a survival factor in breast tumor cells.

Figure 24. MCF-7 cell transformation with PKC δ dominant negatives. MCF-7 cells ($1 \times 10^6/35 \text{ mm}^2$ dish) were transfected with pcDNA3-neo, pcDNA3-neo-PKC δ dn or “mock” transfected. Post-transfection (72 h) culture medium was exchanged with DMEM/10% FBS containing 400 $\mu\text{g/ml}$ G418. After 18 days of growth in selection medium, cell colonies were stained with crystal violet. Results are from a single experiment.

2.9 PKC δ Oligonucleotide Treatment Resulted in Loss of DNA Integrity. Single cell gel electrophoresis or “comet analysis” performed under alkali conditions permits sensitive and quantitative analysis of single- and double-strand DNA damage (Fairbairn et al., 1995). The electrophoretic mobility and relative intensity of fragmented nuclear DNA are incorporated into the tail moment parameter. This assay was used to test the effects of PKC δ oligonucleotide treatment on cellular DNA. MDA-MB-231 cells were treated with PKC δ oligonucleotides for 24-48 hours. Following this treatment, cells were exposed to γ -radiation and immediately prepared for comet analysis. Results are presented as frequency distribution of comet tail moments in Figure 25A. In control cells the frequency of comets with tail moments less than 2 was 60%. These are representative of cells with undamaged nuclear DNA. Treatment of MDA-MB-231 cells with PKC δ oligonucleotide caused the comet frequency distribution to shift toward larger tail moments and this result is indicative of damaged DNA. The scrambled nucleotide treatment did not result in increased DNA damage. Treatment with 1.5 Gy γ -radiation caused an increase in the frequency of tail moments greater than 2, similar to the effect of PKC δ oligonucleotide treatment. The combination of PKC δ oligonucleotide and radiation produced a further increase in the number of comets displaying DNA damage. Similar effects on the comet tail moments were observed when cells were analyzed 48 hours after treatment with PKC δ oligonucleotide (Figure 25B).

Table 5 summarizes the comet analysis results for 24 and 48 hours after treatment of MDA-MB-231 cells with PKC δ oligonucleotide. The percentage of comets with tail moments greater than 2 and greater than 20 are shown. Tail moments of greater than 2 are an index of all DNA damage, while tail moments greater than 20 are an index of severe DNA damage. These experiments provide evidence that reduced cell survival by depletion of PKC δ is accompanied

by a loss of DNA integrity. Furthermore, PKC δ oligonucleotide treatment alone is sufficient to produce DNA damage in the absence of radiation treatment. It is possible that PKC δ oligonucleotide treatment directly damages DNA, but this is difficult to reconcile with the observation that the scrambled oligonucleotide is inactive at causing DNA damage. Rather, it is hypothesized that PKC δ is involved in pathways regulating genome integrity or during DNA synthesis. Furthermore, we hypothesize that PKC δ also contributes to the repair of DNA following DNA damage.

Figure 25A. Comet analysis of MDA-MB-231 cells treated with PKC δ oligonucleotide +/- radiation. MDA-MB-231 cells (1.1×10^5 /35 mm² dish) were treated with nothing (control), lipofectin (L), scrambled oligonucleotide (SCR) or PKC δ oligonucleotide (PKC δ). Following transfection (24 h), cells were irradiated (1.5 Gy IR) and prepared for comet analysis. The number of comets with tail moments in ranges between 0-2, 5-10, 10-20, 20-50, 50-100 and >100 are plotted for each treatment group. Data represent n=3 independent experiments with 80 comets scored per treatment/experiment. Statistically significant differences are indicated below (*P<0.05).

Statistical Analyses (ANOVA)

Comparison	P Value
*Control, L and SCR vs. PKC δ	<0.05
*Control, L, SCR and PKC δ vs. IR, L+IR, SCR+IR and PKC δ +IR	<0.05
*IR and SCR+IR vs. PKC δ +IR	<0.05

Figure 25B. Comet analysis of MDA-MB-231 cells treated with PKC δ oligonucleotide +/- radiation. MDA-MB-231 cells (1.1×10^5 /35 mm² dish) were treated with nothing (control), lipofectin (L), scrambled oligonucleotide (SCR) or PKC δ oligonucleotide (PKC δ). Following transfection (48 h), cells were irradiated (1.5 Gy IR) and prepared for comet analysis. The number of comets with tail moments in ranges between 0-2, 5-10, 10-20, 20-50, 50-100 and >100 are plotted for each treatment group. Data represent n=3 independent experiments with 80 comets scored per treatment/experiment. Statistically significant differences are indicated below (*P<0.05).

Statistical Analyses (ANOVA)

Comparison	P Value
*Control, L and SCR vs. PKC δ	<0.05
*Control vs. PKC δ +IR	<0.05
*L vs. IR, L+IR and PKC δ +IR	<0.05
*SCR vs. PKC δ +IR	<0.05
*IR, L+IR and SCR+IR vs. PKC δ +IR	<0.05

Tail Moment:	24h		48h	
	>2,	>20	>2,	>20
Control	39.6%, 2.5%		38.3%, 10.0%	
scr	32.5%, 5.8%		32.5%, 7.9%	
PKCδ	60.4%, 16.3%		54.6%, 23.3%	
IR	60.4%, 17.9%		58.3%, 21.7%	
scr+IR	59.2%, 17.1%		39.2%, 10.0%	
PKCδ+IR	70.4%, 27.1%		70.0%, 28.3%	

Table 5. Comet analysis summary. Data summarized are comet assay results obtained in MDA-MB-231 cells treated with PKC δ oligonucleotide for 24 and 48 hours after treatment (Figure 25). Data are presented as fraction comets with tail moments greater than 2 and greater than 20.

2.10 PKC Isoform Expression MCF-7 and MCF-10A Cell Lines. MCF-10A cells are an immortalized cell line derived from normal mammary epithelium. The expression of individual PKC isoforms in normal versus tumor mammary epithelial cells was of interest. Western blot analyses of MCF-7 and MCF-10A whole cell extracts were performed and the relative levels of PKC α , PKC δ , PKC ϵ , PKC ζ and bcl-2 were compared. Neither MCF-7 nor MCF-10A expressed immunodetectable PKC α (Figure 26). In contrast, MDA-MB-231 mammary tumor epithelial cells overexpress PKC α (Figure 6). PKC δ , PKC ϵ and PKC ζ were detected in MCF-7 and MCF-10A cells. Bcl-2 protein expression levels presented the most striking difference between the two cell lines. The anti-apoptotic bcl-2 protein was detected at a greater intensity in MCF-7 cell extracts than in extracts from MCF-10A cells. These findings demonstrate that in contrast to normal mammary cells, breast cancer cells contain increased PKC isoform and bcl-2 protein expression.

Figure 26. PKC protein levels in MCF-7 and MCF-10A cells. Whole cell extracts were prepared from MCF-7 (7.5×10^5) or MCF-10A (1.0×10^6) cells/100 mm² dish 24 h post-subculture from confluency. Proteins (40 μ g/lane- α , δ , ϵ , bcl-2 and 20 μ g/lane- ζ) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC α , PKC δ , PKC ϵ , PKC ζ and bcl-2. The PKC standards (STD) included purified PKC α (15 ng/lane), PKC δ (20 ng/lane), PKC ϵ (4 ng/lane) and PKC ζ (25 ng/lane). Data shown are typical of n=2 experiments.

2.11 Treatment of Normal Mammary Cells with PKC δ Oligonucleotides. The finding that PKC δ antisense oligonucleotide treatment reduced the survival of breast tumor cells provided a rationale to test the effects of this oligonucleotide in normal mammary cells. Neither the nucleotide scrambled sequence of PKC δ oligonucleotide nor lipofectin alone produced a reduction in MCF-10A cell survival (Figure 27). While the difference was not statistically significant, MCF-10A cells treated with PKC δ antisense oligonucleotide displayed reduced survival compared to treatment with the nucleotide scrambled sequence. The magnitude of the reduction was less than that observed in MDA-MB-231 cells treated with PKC δ antisense oligonucleotide (Figure 23A). These results indicate that the growth inhibitory effects of PKC δ depletion by oligonucleotides are not limited to breast tumor cells. However, breast tumor cells exhibited greater sensitivity to PKC δ antisense oligonucleotide treatment than normal mammary cells.

In addition, the effects of γ -radiation on MCF-10A cell survival were evaluated. In response to 1.5 Gy irradiation survival was reduced by approximately 5%, while 5.2 Gy irradiation reduced survival by 50%. The reduction in cell survival by radiation in MCF-10A cells was similar to that seen in MCF-7 and MDA-MB-231 cell lines, indicating that these normal cells do not have an increased sensitivity to radiation in comparison to the two tumor cell lines tested.

Figure 27. MCF-10A cell survival in response to PKC δ oligonucleotide or γ -radiation treatment. MCF-10A cells were treated with 100 nM PKC δ oligonucleotide plus liposome (PKC δ). Controls were treated with no treatment (C), liposome alone (L), liposome plus scrambled nucleotide sequence of the PKC δ oligonucleotide (SCR) or radiation alone (1.5 Gy or 5.2 Gy). Post-transfection (24 h), cells were harvested and replated in 96-well plates at a cloning density of 250 cells/well. After 5 days of incubation, cell survival was estimated using the MTS assay. Data are the mean $\pm$ SE of n=3 independent experiments performed with 5 replicates/treatment. Survival of control cells was set =100%. There were no statistically significant differences between groups treated with PKC δ antisense oligonucleotide versus nucleotide scrambled PKC δ oligonucleotide (SCR) (P<0.05).

Chapter 3. PKC ζ is a Pro-survival Factor.

3.1 Survival of Breast Tumor Cells Treated with PKC ζ Antisense Oligonucleotides and

Radiation. PKC ζ has been cited by numerous independent studies to promote cell proliferation and survival (Introduction 8.11). Consistent with these reports, MDA-MB-231 cell survival in response to radiation was significantly decreased by pre-treatment with PKC ζ antisense oligonucleotides compared to pre-treatment with the nucleotide scrambled sequence oligonucleotide ($P < 0.05$) (Figure 28A). In this pair of oligonucleotides, the nucleotide scrambled version of PKC ζ (SCR) reduced cell survival compared to liposome treatment alone, indicating these sequences exert some non-specific effects on cell survival. PKC ζ antisense oligonucleotide resulted in a marked overall decrease in cell survival of irradiated cells from 91% (liposome treatment alone) to 32% (liposome plus PKC ζ antisense oligonucleotide). In conclusion, both PKC ζ antisense and nucleotide scrambled sequence oligonucleotides induce tumor cell death in response to radiation. The mechanism of this response is explored in more detail in subsequent experiments (Figures 31 and 33).

In MCF-7 cells, as noted previously, liposome treatment alone significantly impaired cell survival (Figure 28B). PKC ζ antisense oligonucleotide treatment did however, significantly reduce the survival of irradiated MCF-7 cells in comparison to treatment with the nucleotide scramble sequence oligonucleotide (SCR). Because the scrambled sequence oligonucleotide did not affect MCF-7 cell survival, the specific effects of the PKC ζ antisense oligonucleotide may be distinguished from non-sequence specific effects. These results confirm the participation of PKC ζ in the survival of radiation treated breast tumor cells.

Figure 28. Cell survival in response to γ -radiation +/- PKC ζ oligonucleotide treatment. (A) MDA-MB-231 cells ($1.1 \times 10^5/35 \text{ mm}^2$ dish) 24 h post-subculture from confluency were transfected with liposome plus 100 nM PKC ζ antisense (PKC ζ) or scrambled sequence oligonucleotide (SCR) plus liposome. Controls received either no treatment or liposome (L) alone. Post-transfection (24 h), cells were irradiated (IR) with 5.6 Gy of γ - radiation, harvested and replated in 96-well plates at a cloning density of 250 cells/well. After 5 days of incubation, cell survival was estimated using the MTS assay. (B) MCF-7 cells ($2.2 \times 10^5/35 \text{ mm}^2$ dish) 24 h post-subculture from confluency were transfected with liposome plus 200 nM PKC ζ antisense (PKC ζ) or scrambled sequence oligonucleotide (SCR) plus liposome. Controls were as described above. Post-transfection (48 h), cells were irradiated with 5.6 Gy γ -radiation 48 h post-transfection, harvested and replated in 96-well plates at a cloning density of 2500 cells/well. After 7 days of incubation, cell survival was estimated using the MTS assay. Data are the mean $\pm$ SE of n=4 (A) or n=3 (B) independent experiments performed with 5 replicates/treatment. Survival of cells treated with radiation alone was set =100%. Statistically significant differences between PKC antisense oligonucleotide treatment versus nucleotide scrambled sequence PKC oligonucleotide (SCR) treatment are indicated (*P<0.05).

Figure 28A. MDA-MB-231

Figure 28B. MCF-7

3.2 Treatment of Breast Tumor Cells with the Combination of Low Dose γ -radiation and PKC ζ Oligonucleotide. To evaluate the ability of PKC ζ antisense oligonucleotide to reduce cell viability in response to a therapeutically relevant dose of radiation, experiments were performed using cells treated with 1.5 Gy radiation. MDA-MB-231 cell survival was impaired by treatment with either the isoform specific (PKC ζ) or nucleotide scrambled version (SCR) of the PKC ζ antisense oligonucleotide (Figure 29A). There was no statistically significant difference in survival of cells treated with the PKC ζ antisense oligonucleotide versus the scrambled nucleotide version. In MCF-7 cells, survival was attenuated by treatment with liposome or PKC ζ oligonucleotide (Figure 29B), but there was no statistically significant reduction in survival in MCF-7 cells treated with the PKC ζ antisense oligonucleotide versus the scrambled sequence oligonucleotide.

Figure 29. The effect of PKC ζ oligonucleotides on cells treated with low dose γ -radiation. (A) MDA-MB-231 cells ($1.1 \times 10^5/35 \text{ mm}^2$ dish) 24 h post-subculture from confluency were transfected with 100 nM PKC ζ oligonucleotides and 17 h later irradiated with 1.5 Gy γ -radiation (IR), harvested and replated (250 cells/well) in 96 well-plates. MTS activity was measured after 5 days as an index of survival. (B) MCF-7 cells ($2.2 \times 10^5/35 \text{ mm}^2$ dish) 24 h post-subculture from confluency were treated with 200 nM PKC ζ oligonucleotides and 48 h later irradiated with 1.5 Gy γ -radiation, harvested and replated (2500 cells/well) in 96 well-plates. MTS activity was measured after 7 days as an index of survival. Data are the mean $\pm$ SE of n=4 (A) or n=3 (B) independent experiments performed with 5 replicates/treatment. Survival of cells treated with radiation alone was set =100%. There were no statistically significant differences between the PKC ζ antisense and scrambled sequence oligonucleotide treatments ($P < 0.05$).

Figure 29A. MDA-MB-231

Figure 29B. MCF-7

3.3 PKC ζ Protein Levels in PKC ζ Oligonucleotide Treated Cells. To test whether PKC ζ antisense oligonucleotide treatment resulted in reductions in PKC ζ protein levels, western blot analyses were conducted using extracts prepared from MCF-7 cells 48 hours after PKC ζ antisense oligonucleotide treatment. PKC ζ antisense oligonucleotide treatment caused a 50% reduction in PKC ζ protein level and neither liposome alone nor the nucleotide scrambled version (SCR) of the oligonucleotide had an effect on the PKC ζ protein levels (Figure 30A).

PKC ζ protein levels were examined in extracts prepared from MDA-MB-231 cells 24 hours after treatment with PKC ζ oligonucleotide. PKC ζ protein levels were reduced by PKC ζ antisense oligonucleotide treatment (Figure 30B). Liposome alone or control oligonucleotide did not affect PKC ζ protein levels in MDA-MB-231 cells. These results indicate that the reduction in cell survival by the nucleotide scrambled version of this oligonucleotide (Figures 28A and 29A) was not due to a depletion of PKC ζ protein. It is possible that this oligonucleotide manifests non-sequence specific activity on cellular factors, which influence survival such as those previously described (Introduction 9.2). Alternatively, the scrambled nucleotide sequence might act as an antisense oligonucleotide on another target mRNA species within the MDA-MB-231 cells and absent from MCF-7 cells.

The magnitude of the reduction by the PKC ζ oligonucleotide was less in MDA-MB-231 cells than in MCF-7 cells and is likely due to the lower antisense oligonucleotide concentration (50 nM) and shorter incubation time of 24 hours between oligonucleotide treatment and extract preparation. Perhaps, a greater reduction would have been observed in extracts prepared 48 hours after 100 nM PKC ζ oligonucleotide treatment. Immunodetection of PKC ζ in MCF-7 and MDA-MB-231 cells confirm that PKC ζ oligonucleotide is an inhibitor of PKC ζ protein and

support that reduced MCF-7 cell survival by PKC ζ antisense oligonucleotide involves PKC ζ protein reduction.

Figure 30. PKC ζ protein levels in PKC ζ oligonucleotide treated cells. (A) Extracts were prepared from MCF-7 cells 48 h after treatment with lipofectin alone (L), 100 nM PKC ζ antisense oligonucleotide (PKC ζ), the nucleotide scrambled version (SCR) or no treatment (C). Proteins (45 μ g/lane) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with an antibody to PKC ζ . Control signal density was set =100%. Signal densities were normalized to a 200 kDa protein band on the Coomassie blue-stained acrylamide gel. Data in graph are the mean $\pm$ SE of n=3 independent experiments. Statistically significant differences between treatment groups receiving PKC ζ oligonucleotides versus nucleotide scrambled sequence of PKC ζ oligonucleotides are indicated (*P<0.05). Blot is typical of n=3 independent experiments. (B) Protein extracts (55 μ g/lane) were prepared from MDA-MB-231 cells 24 h after treatment with lipofectin alone (L), 50 nM PKC ζ oligonucleotide (PKC ζ), the nucleotide scrambled version (SCR) or no treatment (C). Results are from a single experiment.

Figure 30A. MCF-7

Figure 30B. MDA-MB-231

3.4 PKC ζ Protein Levels in MCF-7 Cells Following Exposure to Ionizing Radiation. To determine if PKC ζ protein level elevation is a response to γ -radiation treatment, PKC ζ protein levels were examined following exposure to 5.2 Gy radiation. Extracts prepared from MCF-7 cells at either 3 or 21 hours after radiation exposure showed constant levels of PKC ζ protein (Figure 31). Previous experiments evidenced that PKC ζ is a survival factor in breast tumor cells. The observation that PKC ζ protein levels are unaltered in response to radiation suggests that changes in PKC ζ enzyme activity may be occurring as a response to radiation exposure.

Figure 31. PKC ζ protein levels in MCF-7 cells following exposure to ionizing radiation. Whole cell extracts were prepared from MCF-7 cells 3 h and 21 h after exposure to 5.2 Gy radiation. Proteins (30 μ g/lane) were resolved on 7.5% acrylamide gels, transferred to filters and probed with an antibody to PKC δ . A Coomassie blue-stained protein is shown as the loading control. Data shown are representative of n=2 independent experiments.

3.5 Expression of PKC ζ Dominant Negative Inhibited MCF-7 Cell Survival. For further confirmation that inhibition of PKC ζ abrogates cell survival, MCF-7 cells were transformed with a dominant negative form of PKC ζ . As described previously (Results 2.8), dominant negative PKC species were generated by introducing a point mutation into the ATP binding domain. MCF-7 cells expressing the neomycin resistance gene were selected for by the addition of G418 to the culture medium. The product of the neomycin resistance gene is an aminoglycoside phosphotransferase that inactivates G418 and confers cellular resistance. Following 18 days of growth in the presence of G418, the survival of MCF-7 cells transformed with the neomycin resistance gene is evidenced by colony formation (Figure 32). Colony formation was inhibited if MCF-7 cells were transformed with the PKC ζ dominant negative (pcDNA3-neo-PKC ζ dn) in comparison to MCF-7 cells transformed with vector alone (pcDNA3-neo). “Mock” transfected MCF-7 cells were not able to survive in the presence of G418. We conclude that PKC ζ is a survival factor in breast tumor cells and that inhibition of PKC ζ reduces MCF-7 cell survival. These results are consistent with previous findings that PKC ζ antisense oligonucleotide reduced MCF-7 cell survival (Figures 28B and 29B).

Figure 32. MCF-7 cell transformation with PKC ζ dominant negatives. MCF-7 cells ($1 \times 10^6/35 \text{ mm}^2$ dish) were transfected with pcDNA3-neo, pcDNA3-neo-PKC ζ dn or “mock” transfected. Post-transfection (72 h) culture medium was exchanged with DMEM/10% FBS containing 400 $\mu\text{g/ml}$ G418. After 18 days of growth in selection medium, cell colonies were stained with crystal violet. Results are from a single experiment.

3.6 DNA Damage Analysis Following PKC ζ Oligonucleotide Treatment. To analyze the effects of PKC ζ depletion on DNA integrity in MDA-MB-231 cells, PKC ζ antisense oligonucleotide was added. Following treatment (24 hours), cells were exposed to 1.5 Gy radiation and prepared for comet analysis. Neither PKC ζ antisense oligonucleotide nor the scrambled nucleotide control (SCR) produced significant DNA damage in MDA-MB-231 cells as measured by the tail moment parameter (Figure 33). Exposure to 1.5Gy γ -radiation did produce significant increases in the frequency of comets containing damaged DNA. The combination of PKC ζ antisense oligonucleotide and 1.5 Gy radiation did not increase DNA damage greater than radiation alone. In conclusion, PKC ζ antisense oligonucleotide treatment did not affect DNA integrity in MDA-MB-231 cells.

Figure 33. Comet analysis of MDA-MB-231 cells treated with PKC ζ oligonucleotide +/- radiation. MDA-MB-231 cells ($1.1 \times 10^5/35 \text{ mm}^2$ dish) were treated with lipofectin alone (Control), scrambled oligonucleotide (SCR) or PKC ζ antisense oligonucleotide (PKC ζ). Following treatment (24 h), cells were treated with 1.5 Gy radiation (IR) and prepared for comet analysis. The number of comets with tail moments in ranges between 0-2, 2-5, 5-10, 10-20, 20-50, 50-100 and >100 were plotted for each treatment group. Data represent n=3 independent experiments with 80 comets scored per treatment/experiment. Statistically significant differences are indicated below (*P<0.05).

Statistical Analyses (ANOVA)

Comparison	P Value
*Control, SCR and PKC ζ vs. IR, SCR+IR and PKC ζ +IR	<0.05

3.7 Treatment of Normal Mammary Cells with PKC ζ Oligonucleotides. To test if the growth inhibitory effects of PKC ζ antisense oligonucleotide are restricted to breast tumor cells, normal mammary tumor cells were treated with PKC ζ oligonucleotides. Treatment of MCF-10A cells with PKC ζ antisense oligonucleotide produced no specific reduction in survival compared to treatment with the scrambled sequence oligonucleotide (Figure 34). Non-specific effects were evident by the impairment of survival in MCF-10A cells treated with the scrambled nucleotide version of the PKC ζ oligonucleotide. The PKC ζ oligonucleotide did however, reduce survival relative to treatment with liposome alone. While these results do not separate sequence specific from non-sequence specific effects of the PKC ζ oligonucleotides, they do show that the survival of normal mammary cells is affected by PKC ζ oligonucleotide treatment.

Figure 34. MCF-10A cell survival in response to PKC ζ oligonucleotide treatment.

MCF-10A cells were treated with 100nM PKC ζ oligonucleotide plus liposome (PKC ζ). Controls were treated with no treatment (C), liposome alone (L) or liposome plus a scrambled nucleotide sequence of the PKC ζ oligonucleotide (SCR). Post-transfection (24 h), cells were harvested and replated in 96-well plates at a cloning density of 250 cells/well. After 5 days of incubation, cell survival was estimated using the MTS assay. Data are the mean $\pm$ SE of n=3 independent experiments performed with 5 replicates/treatment. Survival of control cells was set =100%. There were no statistically significant differences between groups treated with PKC ζ antisense oligonucleotide versus nucleotide scrambled PKC ζ oligonucleotide (SCR) ($P < 0.05$).

IV. DISCUSSION

Protein kinase C (PKC) participates in a variety of cellular processes. Of importance to this discussion is the role that PKC plays in cancer. As a regulator of signal transduction pathways which control cell proliferation and death and as the primary intracellular receptor for tumor promoting phorbol esters, PKC represents a potentially important molecular target for anti-cancer therapeutics. PKC is a family of serine/threonine kinases composed of twelve unique isoforms. Since the discovery of PKC more than 20 years ago, studies are only recently revealing the functions of individual PKC isoforms. Thus far, the roles of PKC isoforms in cancer have proven to be complex and often contradictory. Conclusions on the functions of individual PKC isoforms must be assigned within their appropriate cell contexts as many of their functions are cell-type specific.

Relatively little information exists pertaining to the functions of individual PKC isoforms in breast cancer. However, PKC has previously been shown to have increased activity and expression in breast cancer versus normal mammary tissue (O'Brian et al., 1989; Gordge et al., 1996). The present study was aimed at identifying specific PKC isoforms that facilitate breast tumor cell survival. Gamma radiation was used to challenge survival of the breast tumor cells in these studies because the results of earlier experiments with the PKC inhibitors, staurosporine, sangivamycin and H7 provided evidence that PKC activation was radioprotective (Begemann et al., 1998; Hallahan et al., 1992; Zhang et al., 1993). Our preliminary results showed that specific inhibition of PKC δ and PKC ζ suppressed the survival of two radiation treated breast tumor cell lines. Subsequent studies provided evidence that in the absence of radiation treatment, PKC δ

and PKC ζ antisense oligonucleotides reduced cell survival. Hence, PKC δ and PKC ζ isoform antagonists are not radiosensitizers, but are inhibitors of survival.

The main approaches to isoform selective PKC inhibition are site selective enzyme inhibition or suppression of the mRNA levels. Oligonucleotides that target RNA are highly specific PKC isoform antagonists, and may be easier and less expensive to synthesize than traditional small molecule inhibitors targeted to the enzyme. A few small molecule inhibitors offer PKC isoform specific inhibition. The small molecule inhibitor, rottlerin, is selective for the PKC δ isoform (IC_{50} 3-6 μ M) but also inhibits calmodulin kinase III (IC_{50} =5.3 μ M) (Way et al., 2000). Alternatively, one other approach to isoform selective PKC inhibition is through the use of dominant negatives. PKC δ and PKC ζ coding sequences containing mutations within the ATP binding domains encode PKC proteins with dominant negative inhibitory activity on wild-type PKC δ and PKC ζ , respectively (Soh et al., 1999). Our approach to elucidate the role of particular PKC isoforms involved in mammary tumor cell survival following radiation has been to use antisense oligonucleotides, dominant negatives and where possible, small molecule inhibitors.

The experiments performed in this project using PKC antisense oligonucleotides were complicated by some non-specific effects. Delivery of the PKC oligonucleotides was mediated by the cationic liposome, Lipofectin. MCF-7 cells in particular were highly sensitive to treatment with the lipid carriers and exhibited reduced survival as a consequence (Figure 8). The nature of liposome mediated toxicity has been investigated previously and was reported to involve the generation of reactive oxygen intermediates. Examination of cellular toxicity produced by liposomes showed a positive correlation between the net positive charge of the liposome and toxicity. Cationic liposomes triggered the generation of reactive oxygen intermediates in a

charge and dose-dependent manner (Dokka et al., 2000). These findings provide a possible explanation for the reduction in MCF-7 cell survival observed in response to liposome treatment, but do not account for the differences in sensitivity to liposomes observed between MCF-7 and MDA-MB-231 cells. Perhaps differences in anti-oxidant capabilities of these two cell lines exist to explain this difference. PKC oligonucleotide delivery in the absence of liposome was attempted but was not an effective method in either cell type (Figure 9).

PKC oligonucleotides also displayed some non-sequence specific activity on cell survival. Sequence specific effects of antisense oligonucleotides are those effects due solely to the depletion of the targeted mRNA. Conversely, non-sequence specific effects are those due to oligonucleotide interference with cellular components (mRNA or protein) other than their specific targets. Non-sequence specific activities are inherent problems with oligonucleotides and are thought to be a consequence of the chemical modifications required for nuclease resistance (Nielson, 1999). In the case of PKC ζ oligonucleotides, the non-sequence specific effects observed on cell survival (Figure 7, 28A, 29A) were not manifested as alterations of PKC ζ protein levels (Figure 30) indicating that the scrambled sequence oligonucleotide was affecting a cellular factor other than PKC ζ . PKC oligonucleotides were effective and specific inhibitors of target protein expression, while control scrambled sequence oligonucleotides had no effect on PKC protein expression levels (Figures 18 and 19). Certain antisense oligonucleotides, such as PKC δ , exhibited only sequence specific effects on cell survival while the scrambled nucleotide sequence had no influence on survival (Figures 7, 17 and 23).

Several studies have correlated overexpression of the α PKC isoform with more aggressive breast tumor cell phenotypes, such as estrogen receptor-negative and multi-drug resistant phenotypes (Ways et al., 1995; Yu et al., 1991). Comparisons of PKC isoform content between

different breast tumor cell types supported these findings. MDA-MB-231 cells, which are estrogen receptor-negative and tumorigenic, displayed markedly higher levels of PKC α than the estrogen receptor-positive, non-tumorigenic MCF-7 cells (Figures 6 and 16). In fact, previous studies have shown that transformation of MCF-7 cells with PKC α results in loss of estrogen receptors, enhanced proliferation, anchorage-independent growth and increased tumorigenicity (Ways et al., 1995). These findings provided a rationale to test whether PKC α inhibition would reduce cell survival. As expected, PKC α antisense oligonucleotide impaired survival in radiation treated MDA-MB-231 cells, but not in MCF-7 cells relative to liposome treatment (Figures 7 and 8). However, the scrambled nucleotide control sequence reduced survival to the same extent as the PKC α antisense oligonucleotide. In subsequent experiments utilizing a different sequence, the effect of nucleotide scrambled control was persistent. The non-specific activity of the control sequence on cell survival prevented conclusions regarding sequence specific activity of PKC α antisense oligonucleotide on cell survival.

MCF-10A are an immortalized, non-transformed and non-tumorigenic cell line. Other studies have shown that stable transformation of MCF-10A with PKC α resulted in increased motility and adhesion, characteristics consistent with a metastatic phenotype (Sun and Rotenberg, 1999). Our findings confirm the absence of PKC α expression in MCF-10A cells (Figure 26). In addition, MCF10A cells express reduced levels of all PKC isoforms tested when compared to MCF-7 cells, indicating that increased PKC protein levels in breast tumor cells may be a deviation from the PKC protein content of normal mammary cells.

MCF-10A cells were further utilized to examine the effects PKC δ and PKC ζ oligonucleotides on non-tumorigenic mammary cells. Ideally, anti-cancer therapeutics should be tumor selective. Treatment of MCF-10A normal mammary cells with PKC δ antisense oligonucleotide (Figure

27) indicated that these cells were less sensitive to PKC δ inhibition than the tumorigenic MDA-MB-231 cells (Figure 23). Treatment of MCF-10A cells with PKC ζ antisense oligonucleotide did not produce any distinguishable sequence specific effects on cell survival (Figure 34). To reduce the effects of PKC oligonucleotides on normal mammary cells a tumor targeted delivery system for PKC oligonucleotides might be advantageous. The receptor tyrosine kinase, Her-2/neu, is overexpressed in a significant number of breast cancers and associated with poor prognosis. Her-2/neu presents a unique target that may be exploited for tumor selective therapy. Liposomes conjugated to Her-2/neu monoclonal antibodies that target Her-2/neu positive breast tumor cells offer an example of a targeted delivery system (Hong et al., 1999). This type of approach may be useful in future studies with PKC antisense oligonucleotides.

In conclusion, the present study showed that selective inhibition of the PKC δ and PKC ζ isoforms through the use of antisense oligonucleotides, small molecule inhibitors and dominant negatives decreased human mammary tumor cell survival. Coinciding with reduced cell survival, PKC δ depletion by antisense oligonucleotide caused an increase in cells containing damaged DNA (Figure 25). Cell death (necrosis) was evidenced by propidium iodide uptake following treatment (24-48 hours) with the PKC δ inhibitor, rottlerin. These findings suggest that PKC δ and PKC ζ positively regulate survival in breast cancer cells. This work begins to clarify the role of individual PKC isoforms that mediate breast tumor cell survival. Additional work is needed to elucidate the cell death pathways that are regulated by PKC δ and PKC ζ .

The clinical success of ISIS 3521, a PKC α antisense oligonucleotide, in solid tumor treatment illustrates the importance of PKC isoforms as anti-cancer therapeutic targets (Nemunaitis et al., 1999). Overall, our findings support the use of tumor targeted PKC antisense oligonucleotides to

breast tumors. Furthermore, the expansion of isoform selective PKC inhibitors to other tumor types will be a valuable application in the future development of novel anti-cancer therapeutics.

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VI. CURRICULUM VITAE

Meredith A. McCracken

ADDRESS: PO Box 9142
Biochemistry and Molecular Pharmacology
R.C. Byrd Health Sciences Center
West Virginia University
Morgantown, WV 26506
Phone: (304) 293-7102, Fax: (304) 293-6846
e-mail: mmccrack@wvu.edu

BIRTH DATE: 12.22.1972
Huntington, WV

EDUCATION:

1990		Fairmont Senior High School
1995	B.S.	Biology, Fairmont State College
May, 2002	Ph.D.	Genetics and Developmental Biology, West Virginia University

PROFESSIONAL HISTORY:

1991-1997	Wait Staff	China Garden Restaurant, Fairmont, WV
1994-1995	Laboratory Assistant	Biology Department, Fairmont State College, Fairmont, WV
1997-1998	Graduate Teaching Assistant	Biology Department, West Virginia University, Morgantown, WV
1998-present	Graduate Research Assistant	Biochemistry and Molecular Pharmacology Department, West Virginia University, Morgantown, WV

SUPPORT/AWARDS:

1998-1999	Association for Women in Science- West Virginia Chapter "Induction of radiosensitization by antisense oligonucleotide gene therapy" \$1,900 total award. M McCracken, P.I. and J Strobl, Co-P.I.
1999-2002	Pre-doctoral Fellowship, U.S. Army Department of Defense, DAMD17-99-1-9449 "Induction of radiosensitization by antisense oligonucleotide gene therapy" \$65,116 total award. M McCracken, P.I. and J Strobl, Co-P.I.

July, 2001

Granted attendance to the American Association for Cancer Research,
Pathobiology of Cancer Workshop.

RESEARCH EXPERIENCE:

Cell Culture/Experiments: Culture of human tumor and normal mammary epithelial cell lines. Fluorescence activated cell sorting, cell proliferation and clonogenic survival assays, transient transfections, immunohistochemistry and radiation survival.

DNA: Single cell gel electrophoresis, mini-preps, maxi-preps and restriction digests.

Protein: Protein isolation, BCA assay and Western blots.

Software: Sigma Plot, GraphPad, Adobe Photoshop, Loats Associates High Capacity
Slide
Analysis System, ImageQuant, MS Word, Excel, Power Point

Equipment: Loats Associates comet analysis system, Zeiss LSM 510 Scanning Laser
Microscope, Densitometer, Alpha Innotech Fluorochem, Spectra Max 340PC
Microtiter plate reader.

RESEARCH INTERESTS:

Cancer biology, cancer genetics, translational cancer research, radiosensitization, pharmacogenomics, gene therapy.

RESEARCH PROJECT:

The goal of my research project was 1) to identify individual protein kinase C (PKC) isoforms that function in breast tumor cell survival and 2) to enhance radiation induced cell death in breast tumor cells. I hypothesized that isoform specific inhibition of PKC with antisense oligonucleotides would enhance radiation induced cell death and reduce human breast tumor cell survival. Five PKC isoform specific oligonucleotides were introduced into breast tumor cells lines by lipofectin. Treatment with oligonucleotides targeted to the δ and ζ PKC isoforms resulted in reduced survival of breast cancer cell lines. Furthermore, an additive reduction in cell survival was observed when the individual oligonucleotides were combined with radiation. Western blotting was used to confirm that PKC antisense oligonucleotides are both effective and specific inhibitors of PKC isoform expression. Subsequent experiments using single cell gel electrophoresis showed that PKC δ antisense specific oligonucleotide treatment is associated with a loss of DNA integrity. My data provide evidence that PKC δ and PKC ζ are pro-survival factors in human mammary tumor cell lines.

ABSTRACTS/PUBLICATIONS:

1. **McCracken MA**, Miraglia LJ, McKay RA and Strobl JS: PKC delta is a pro-survival factor in human breast tumor cell lines. Manuscript in preparation.
2. Zhou Q, **McCracken MA** and Strobl JS: Control of mammary tumor cell growth *in vitro* by novel cell differentiation and apoptosis agents. *Breast Cancer Research and Treatment*, accepted March, 2002.

3. Liu C, **McCracken MA**, Strobl JS, Schilling JK and Kingston DGI: Design and synthesis of steroid linked taxol analogs: A new route toward selective delivery of cytotoxic taxanes. *43rd Annual Meeting of the American Society of Pharmacognosy and 3rd Monroe Wall Symposium*, 2002.
4. Martirosyan A, Zhou Q, Bata R, **McCracken M**, Freeman A, Morato-Lara C and Strobl JS: New pharmacologic agents promote cell differentiation in human breast tumor cells. *Proc. of the 41st Annual American Society for Cell Biology Meeting*, Abstract#762, 2001.
5. Melkounian Z, **McCracken MA** and Strobl JS: Suppression of *c-myc* protein and induction of cellular differentiation in human breast cancer cells but not in normal human breast epithelial cells by quinidine. *Proc. of the 41st Annual American Society for Cell Biology Meeting*, Abstract#70, 2001.
6. Gahr S, Kocamis H, **McCracken MA** and Killefer J: The expression of TGF-beta family members in MCF-7 and MDA-MB-231 breast tumor cell lines. *Proc. Experimental Biology*, 2001.
7. **McCracken MA**, McKay RA, and Strobl JS: Protein kinase C delta involvement in radiation-induced mammary tumor cell death. *Proc. of the 92nd American Assoc. Cancer Res.*, Vol. 42:Abstract# 3583, 2001.
8. **McCracken MA**, Miraglia L and Strobl JS: The effects of protein kinase C antisense oligonucleotides on mammary tumor cell lines. *Proc. of the 91st American Assoc. Cancer Res.*, Vol. 41:Abstract# 4491, 2000.
9. **McCracken MA**: Combination radiation and antisense oligonucleotide gene therapy for breast cancer. *Proc. Susan G. Komen Foundation Meeting*, 2000.
10. **McCracken MA**, Miraglia L and Strobl JS: Sensitization of breast cancer cells to ionizing radiation by protein kinase C inhibition. *Proc. of the 90th American Assoc. Cancer Res.*, Vol. 40:Abstract# 4216, 1999.

PROFESSIONAL MEETINGS ATTENDED:

July, 2001	American Association for Cancer Research, Pathobiology of Cancer Workshop, Keystone, CO
March, 2001	American Association for Cancer Research, New Orleans, LA
September, 2000	Susan G. Komen Breast Cancer Foundation, Washington, DC
April, 2000	American Association for Cancer Research, San Francisco, CA
December, 1999	American Society for Cell Biology, Washington, DC
April, 1999	American Association for Cancer Research, Philadelphia, PA

REFERENCES:

- Jeannine Strobl, Ph.D. Professor of Biochemistry and Molecular Pharmacology
West Virginia University
Phone: (304) 293-7151
Fax: (304) 293-6846
e-mail: jstrobl@hsc.wvu.edu
- Robert Stitzel, Ph.D. Associate Chair of Biochemistry and Molecular Pharmacology
West Virginia University
Phone: (304) 293-5761
Fax: (304) 293-6846
e-mail: rstitzel@wvu.edu
- Sharon Wenger, Ph.D. Director of Cytogenetics
West Virginia University
Phone: (304) 293-3212
Fax: (304) 293-397
e-mail: swenger@hsc.wvu.edu