THE EXPRESSION AND REGULATION OF PROLIFERATION-LINKED GENES IN CANCER

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ABSTRACT

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Cancer is a disease characterized by altered regulation of cell growth and proliferation. Studies were conducted which examined the expression and regulation of different proliferation-linked genes in non-transformed and transformed cells. These genes included the matrix metalloproteinases (MMPs), which mediate metastasis, survivin, which is an inhibitor of apoptosis protein (IAP), and ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyltransferase (SSAT), both of which are integral in polyamine metabolism. The effects of flavonoid-enriched fractions from lowbush blueberry (*Vaccinium angustifolium*) on MMP regulation was examined in DU145 human prostate cancer cells. It was shown that MMP-2 and MMP-9 were down-regulated by lowbush blueberry fractions, and that this regulation was protein kinase C (PKC) and/or mitogen activated protein (MAP) kinase dependent. Survivin expression in 10T½ and NR3 cells was examined in response to the mitogenic growth factors, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF). Survivin expression was induced in NR3 cells by all growth factors, and results show that survivin induction by each growth factor was phoshatidylinositol-3 (PI3) kinase dependent, while PDGF and EGF also used competent PKC and MAP kinase pathways to induce survivin. The expression of ODC and SSAT in 10T½ and NR3 cells was examined in response

to insulin-like growth factor (IGF)-1 and -2. Results show that IGF-1 and IGF-2 induce both ODC and SSAT, and that both IGF-1- and IGF-2-mediated induction of these enzymes was PKC and MAP kinase dependent. These results illustrate the complex regulation of important proliferation-linked genes in cancer cells, and may provide further insight into the mechanisms involved in the selective growth advantage observed in cancer cells.

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LIST OF ABBREVIATIONS

AIF	apoptosis inducing factor
α-MEM	alpha minimal essential medium
AN	anthocyanin-enriched fraction
AP	alkaline phosphatase
Apaf-1	apoptosis protease-activating factor
AZ	antizyme
AZI	antizyme inhibitor
bFGF	basic fibroblast growth factor
BCIP	5-bromo-4-chloro-3-indolylphosphate
BE-3-3-3	N^1, N^{11} –bis(ethyl)norspermine
BESpm	N^1, N^{12} –bis(ethyl)spermine
BIR	Baculovirus IAP repeat
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CB	crude blueberry fraction
c-FLIP	cellular FLICE inhibitory protein
CHAPS	3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate
dcSAM	decarboxylated S-adenosylmethionine
DIABLO	direct inhibitor of apoptosis binding protein with low pI
DM	defined medium
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallocatechin-3-gallate
EGF	epidermal growth factor
ERK	extracellular receptor kinase
FADD	Fas-associated death domain protein
FBS	fetal bovine serum
FLICE	FADD-like interleukin-1 converting enzyme
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HGF	hepatocyte growth factor
IAP	inhibitor of apoptosis protein
IFN	interferon
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGF-R	insulin-like growth factor receptor
IL	interleukin
IR	insulin receptor
LDH	lactate dehydrogenase
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
NADH	nicotinamide adenine dinucleotide
NBT	nitro blue tetrazolium
NF-KB	nuclear factor kappa B

NGF	nerve growth factor
NO	nitric oxide
ODC	ornithine decarboxylase
PAC	proanthocyanidin-enriched fraction
PAO	polyamine oxidase
PDGF	platelet derived growth factor
PI3K	phosphatidylinositol-3 kinase
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMF	polyamine modulated factor
PMSF	phenylmethylsulfonylfluoride
PRE	polyamine response element
RASSF1C	Ras-associated domain family-1 protein
SAMDC	S-adenosylmethionine decarboxylase
Smac	second mitochondria-derived activator of caspases
SSAT	spermidine/spermine N^1-acetyltransferase
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween-20
TGF-ß	transforming growth factor-beta
TIMP	tissue inhibitor of matrix metalloproteinases
TNF	tumor necrosis factor
Tris	1-[bis(2,3-dibromopropoxy)phosphinoyloxy]-2,3-dibromo-propane
VEGF	vascular endothelial growth factor

1. CHAPTER ONE

Literature Review

1.1 Cancer

Cancer is a term used to encompass any neoplastic (tumorigenic) disease characterized by the formation and development of tumors. Tumorigenesis is a complex process which can be attributed to any number of factors. The onset and rate of tumor progression cannot be attributed to a single factor, making the study of tumor development a challenging task. One characteristic factor attributed to tumorigenesis is genetic mutations, which has led to the identification and characterization of tumor suppressor genes and oncogenes involved in cancer susceptibility (Cahill et al. 1999, Pupa et al. 2002). This view focuses on the development of neoplastic cells at the cellular level, and how DNA damage leads to improper regulation of such cellular processes as proliferation, differentiation, and apoptosis, or programmed cell death (Pupa et al. 2002).

Various recent studies have viewed tumorigenesis in a completely different manner. In particular, one theory proposes tumors as functional tissues intimately interconnected with the micro-environment (Radisky et al. 2001). The relationship between the tumor and the micro-environment, which includes an insoluble extracellular matrix (ECM), a stroma composed of (1) cells (such as fibroblasts), (2) adipose tissue, (3) vasculature and (4) resident immune cells, and a host of cytokines and growth factors, is a dynamic interaction with many consequences (Pupa et al. 2002). By acknowledging that tumors exhibit tissue-like interactions with the micro-

environment of the host organism, recent research investigations have studied the interactions of various cancer cells and their corresponding micro-environment (Maeda-Yamamoto et al. 1999, Manes et al. 1999, Bhatia and Agarwal 2001, Taraboletti et al. 2002).

1.2. Cancer: a selective growth advantage

1.2.1. Proteolysis of the extracellular matrix (ECM) and metastasis

Many aspects of cancer enhance cell proliferation and survival. These characteristics separate and define cancer as a unique and particularly devastating disease. The extracellular matrix (ECM) is a fluid-mosaic mixture of complex proteins such as collagen, elastin, laminin, and fibronectin that provides structural and mechanical support to cells and tissues, while exerting a profound effect on many biological activities (Matrisian 1990). The ECM influences such basic cellular processes as proliferation, differentiation, adhesion, migration, as well as tissue morphogenesis (Ingber and Folkman 1989, Juliano and Haskill 1993, Assoian and Marcantonio 1996). ECM components can, through interactions with various cellular surface receptors, influence cell shape, behaviour, and the response of cells to soluble signalling molecules such as cytokines and growth factors (Lin and Bissell 1993). Because of the importance of the ECM in cellular behaviour, morphology, and general biology, recent approaches point to the contribution of stromal components of the ECM to oncogenic signals that mediate deleterious genomic and phenotypic changes in epithelial cells (Tlsty and Hein 2001). The importance of the integrity of

the ECM cannot be understated, since proteolysis of the ECM, which facilitates tumor cell invasion, is a crucial early step in the mechanism of metastasis (Stetler-Stevenson and Yu 2001).

Dynamic interactions between tumor cells and the ECM are a requirement for tumor progression (Pupa et al. 2002). A fundamental event of tumor progression is the remodeling of the micro-environment, which requires the active proteolysis of the ECM by various proteases (Pupa et al. 2002). This allows for tumor growth and development, while maintaining a viable micro-environment to facilitate communication. ECM remodeling is not restricted to tumors, since normal physiological processes such as wound healing and embryonic development exhibit this phenomenon (Pupa et al. 2002). Repeated ECM modification, however, can have a profound negative effect on cell survival, proliferation, and migration (Pupa et al. 2002).

Neoplasms, in order to become metastatic, must first penetrate various connective tissue barriers, such as the basement membrane (BM) or interstitial stroma, which separate tumors and existing blood vessels (Stetler-Stevenson and Yu 2001). Although the invasive behaviour of tumor cells has contributions from many sources, certain families of proteases, namely the serine proteases (plasmin, urokinase-type plasminogen activator (μPA), elastase), cysteine proteases (cathepsin B), aspartic proteinases (cathepsin D), and the matrix metalloproteinases (MMPs), are thought to be primary players in invasion (Heck et al. 1990, Andreasen et al. 1997).

Consequently, understanding the complex relationship between the ECM and the ECM proteolytic enzymes is fundamental in understanding metastasis.

1.2.2. Flavonoids and cancer

The flavonoids are a diverse group of natural phenolic compounds which are present in nearly all vascular plants (Kandaswami and Middleton 1997). They are loosely divided into ten different classes, depending on the arrangement of certain functional groups (Kandaswami and Middleton 1997, Kahkonen and Heinonen 2003). All flavonoid compounds are aromatic compounds which are naturally produced by plants from two of the aromatic amino acids, phenylalanine and tyrosine, and acetate units (Kahkonen and Heinonen 2003). All flavonoids are structurally related to the parent compound, flavone (2-phenylbenzopyrone), and most only differ in the arrangement of their functional groups (Yang et al. 2001). Recent studies have expanded on the various antioxidant properties of flavonoids to determine their effects in cancer models. Green and black tea polyphenols, especially epigallocatechin-3-gallate (EGCG), have been reported in numerous studies as possessing anti-carcinogenic properties. Such effects include modulating gene expression of known cancer-related genes, inhibiting invasive properties of various cancer cell types, and inducing endogenous cell processes (such as apoptosis) which control the proliferation and differentiation of cancer cells (Bomser et al. 1996, Gupta et al. 1999, Luceri et al. 2002).

Lowbush blueberry (*Vaccinium angustifolium*) has been shown to have high levels of flavonoids as compared to other fruits and vegetables (Kalt et al. 2000). Many recent studies have evaluated the effects of flavonoid-rich extracts from these fruits in various cancer models. Flavonoid-rich fractions from *Vaccinium* species inhibit the tumor promoter phorbol 12-myristate 13-acetate (PMA) induced increase of ODC, while inducing the xenobiotic detoxification enzyme quinone reductase (QR) *in vitro*, which is one of several enzymes that inactivate electrophilic forms of carcinogens providing a mechanism for the inhibition of carcinogenesis (Bomser et al. 1996). Nobiletin, a flavonoid prevalent in various *Vaccinium* species as well as citrus fruits, has been reported to inhibit the invasive behaviour of human fibrosarcoma HT1080 cells *in vitro* (Sato et al. 2002). Furthermore, it was shown that nobiletin inhibits the expression of two MMPs (MMP-9 and pro-MMP-1) while inducing expression of TIMP-1 in these cells *in vitro*. Moreover, flavonoids (anthocyanins) from bilberry (*Vaccinium myrtillus*) were shown to induce apoptotic cell bodies and nucleosomal DNA fragmentation in HL60 human leukemia cells, thereby inhibiting cell proliferation and development (Katsube et al. 2003). These studies suggest that flavonoids may possess inherent anti-carcinogenic properties.

1.2.3. Evasion of apoptosis

Cellular proliferation and differentiation are highly regulated processes (Guo and Hay 1999, Hengartner 2000). At the level of the organism, cellular division must be tightly controlled to maintain homeostatic conditions and prevent abnormal

development (Hickman et al. 2002). The critical balance between cell growth and death is essential for proper growth and development (Guo and Hay 1999, Hengartner 2000). A functional loss of either process is severe and can result in uncontrolled cell proliferation and a neoplastic phenotype (Guo and Hay 1999). In many cases, neoplastic cells have an increased resistance to regulatory cell death, or apoptosis (Guo and Hay 1999). The ability of neoplastic cells to evade the cell death machinery responsible for initiating apoptosis has been a focus of research for many years (Hickman et al. 2002, Gaur and Aggarwal 2003). Extracellular and intracellular signals mediate the initiation or repression of each process and each of these mechanisms are well documented as playing important roles in maintaining homeostatic cell proliferation (Hengartner 2000). However, signalling mechanisms do not control those transformed cells whose signal transduction pathways and proliferation- and/or survival-promotion pathways are mutated (Guo and Hay 1999, Hengartner 2000, Hickman et al. 2003). Compensatory communication between the cell proliferation and cell death machinery *via* signals is an alternative in these cells, and evidence that each process influences the other has been previously reported (Gross et al. 1999).

The family of inhibitor of apoptosis proteins (IAPs) are important cell death regulators (Salvesen and Duckett 2002). The list of IAPs continues to grow, and to date the following members have been identified: NIAP (neuronal IAP), HIAP (human IAP), cIAP1 and cIAP2 (cellular IAP 1 and 2), XIAP (X chromosome-linked IAP), BRUCE (BIR repeat containing ubiquitin-conjugating enzyme), livin, and

survivin (Jaattela 1995). Survivin has been studied extensively with respect to its role in cancer, and its expression is tightly controlled. Survivin expression is differential in cancer *versus* non-cancer tissues, and over-expression of survivin has been demonstrated in many human tumors. Given this evidence, understanding the regulation of expression of genes important in cell proliferation and death, such as survivin, is crucial to the development of new therapeutic opportunities in cancer chemotherapy.

1.2.4. Enhanced cell proliferation and polyamines

The polyamines (putrescine, spermidine, and spermine) are ubiquitous low-molecular weight aliphatic amines that play roles in both cell proliferation and differentiation (Heby and Persson 1990, Urdiales et al. 2001). These molecules are highly charged cations at physiological pH, allowing them to stabilize nucleic acids, proteins, and membranes, and therefore influence macromolecular biosynthesis and maintain cell growth (Urdiales et al. 2001). Being aliphatic amines, the polyamines have been suggested to contribute to the neutralization of the negative charge on the DNA backbone (Heby and Persson 1990). Because of their ability to intimately interact with DNA, the polyamines have been shown to distort the secondary structure of the DNA backbone, and may even cause a conformational change in the secondary structure from the common B-form DNA to the more unusual A- or Z-forms, underlining the influence that the polyamines can have on DNA-protein interaction in the cell (Heby and Persson 1990). Polyamines can also interact with

other negatively charged double-helical structures, such as tRNA, rRNA and mRNA, through stabilization of either their stem/loop secondary structure (rRNA and tRNA), or through binding to specific sites to stabilize different conformations (mRNA) (Heby and Persson 1990). These interactions may be the basis for the stimulatory effects by polyamines on DNA replication, RNA synthesis (transcription), and protein synthesis (translation) (Heby and Persson 1990).

Putrescine, spermidine, and spermine are the natural polyamines found in cells, and cellular polyamine levels are maintained within a narrow range, emphasizing their importance in overall cellular biology (Shappell et al. 1993). High levels of polyamines have been shown to be cytotoxic, whereas low polyamine levels retard normal cell growth and proliferation (Shappell et al. 1993, Urdiales et al. 2001). Consequently, rapid and specific control of intracellular polyamine pools is necessary (Carper et al. 1991, Shappell, et al. 1993, Urdiales et al. 2001).

The polyamines have been implicated in growth and differentiation processes (Bachrach 2004). Polyamine levels fluctuate during the cell cycle, with increases in polyamine content at the G1 and G2 stages and prior to mitosis (Wallace et al. 2003). The relationship between polyamine levels and the development and progression of cancer is well documented (Wallace and Caslake 2001). Intratumor polyamine concentrations are increased in a large number of solid tumors, and levels of circulating polyamines in both urine and blood samples from cancer patients are increased (Kingsnorth et al. 1984a, Kingsnorth et al. 1984b, Wallace and Caslake 2001).

Increased levels of polyamines are the direct result of altered polyamine metabolism (Wallace et al. 2003). The major enzymes involved in polyamine metabolism are ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyltransferase (SSAT). ODC is the rate-limiting enzyme in the biosynthesis of polyamines, while SSAT is the major activity in polyamine catabolism. Furthermore, SSAT can interconvert polyamines, thereby increasing polyamine levels when needed. Consequently, the regulation of ODC and SSAT is tightly controlled (Wallace et al. 2003). Many tumors exhibit altered expression of ODC and SSAT, leading to a marked increase in polyamines in these tumors (Wallace et al. 2003). Understanding the regulatory mechanisms of polyamine metabolism may provide further insight into the selective growth mechanisms displayed in tumor cells.

1.2.5. The insulin-like growth factor (IGF) signalling system and cancer

The insulin-like growth factor (IGF) family of ligands, binding proteins and receptors is an important growth factor system involved in both overall development and maintenance of normal cellular functions (LeRoith and Roberts 2003). The IGF system is implicated in many pathophysiological conditions, and its role in carcinogenesis is particularly prominent (LeRoith and Roberts 2003). Many studies have shown that high levels of circulating IGF-1 constitutes a risk factor in the development of many different cancers, such as breast, prostate, and colon cancer (Cohen 1998, Hankinson et al. 1998, Ma et al. 1999). IGF-1 and IGF-2 are commonly expressed by tumor cells and may act as autocrine growth factors (LeRoith and

Roberts 2003). Circulating IGF-1 and IGF-2 may also induce hypoglycemia in target tissues (Cohen 1998). Furthermore, the IGF-1 receptor has been shown to be over-expressed in many cancers, ultimately providing a selective growth advantage for tumors through altered IGF signalling (Ma et al. 1998). Further understanding of the IGF system may prove beneficial in understanding the altered signalling mechanisms observed in many tumors.

1.2.6. H-ras and metastatic potential

Metastasis is a well known process that results in tumor growth in foreign tissue sites (Mulcahy et al. 1985). Oncogenes are genes which are normally responsible for controlling such regular cellular processes as proliferation, differentiation, and communication (Mulcahy et al. 1985). The mutation or disregulation of oncogenes is a critical event in cellular transformation and the metastatic process (Mulcahy et al. 1985). H-ras is a well-known oncogene which contributes to the metastatic or malignant potential of neoplastic cells (Egan et al. 1988). It has been reported that H-ras expression is directly correlated with the formation of metastases, and that H-ras transformed cells have an increased malignant potential (Egan et al. 1988). Using non-transformed and H-ras transformed cells, comparative studies into the involvement of H-ras in the altered regulation of many proliferation-linked genes may provide a further understanding of the complex regulation of these genes in cancer.

1.3. Research objectives and general hypothesis

The objectives of the research conducted in this study include:

- Examination of the effects of fractions isolated from lowbush blueberry (*Vaccinium angustifolium*) on matrix metalloproteinase (MMP) activity in DU145 human prostate cancer cells.

- Elucidation of the mechanism(s) by which fractions from lowbush blueberry alter MMP activity in DU145 human prostate cancer cells.

- Examination of the effects of mitogenic growth factors on the expression of survivin in H-ras transformed mouse fibroblasts, and elucidation of possible mechanism(s) involved in these effects.

- Examination of the effects and elucidation of mechanisms involved in the alteration of expression of ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyltransferase (SSAT) in H-ras transformed cells by insulin-like growth factor (IGF)-1 and -2.

The following is the general hypothesis:

Through modulation of signal transduction mechanisms involved in both cellular proliferation and cellular apoptosis, cancer cells acquire a selective growth advantage.

2. CHAPTER TWO

Blueberry flavonoids inhibit matrix metalloproteinase activity in DU145 human prostate cancer cells

2.1. Abstract

Regulation of the matrix metalloproteinases (MMPs), which are the major mediators of extracellular matrix (ECM) degradation, is crucial to regulate ECM proteolysis which is important in metastasis. This study examined the effects of three flavonoid-enriched fractions on MMP activity in DU145 human prostate cancer cells *in vitro*. The fractions were a "crude" fraction, an anthocyanin-enriched fraction and a proanthocyanidin-enriched fraction, which were prepared from lowbush blueberries (*Vaccinium angustifolium*). Using gelatin gel electrophoresis, MMP activity was evaluated from cells following a 24 hour exposure to blueberry fractions. All fractions exhibited an ability to decrease the activity of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9). Of the fractions tested, the proanthocyanidin-enriched fraction was found to be the most effective regarding inhibition of MMP activity in these cells. No induction of either necrotic or apoptotic cell death was noted in these cells in response to treatment with these blueberry fractions. These findings indicate that flavonoids from blueberry possess the ability to effectively decrease MMP activity which may decrease overall ECM degradation which may be important in controlling tumor metastasis formation.

2.2. Introduction

The role of extracellular matrix (ECM) integrity and ECM degrading enzymes such as the matrix metalloproteinases (MMPs) (also designated matrixins) in the process of cancer metastasis has been shown to be substantive since ECM degradation is essential for tumor metastasis to occur (Stetler-Stevenson et al. 2001, Pupa et al. 2002, Lynch and Matrisian 2002, Freije et al. 2003). MMP expression and activity are tightly regulated processes indicating their importance in regular cellular dynamics and interactions (Pupa et al. 2002, Visse and Nagase 2003). Although not the only contributing factor to tumor metastasis, it has been clearly shown that many cancer cells demonstrate a pronounced increase in MMP activity and that over-expression of MMPs may be an important factor in both tumor invasion and tumor angiogenesis into foreign tissues (Stetler-Stevenson 2001, Pupa et al. 2002). Due to their important and pivotal role in tumorigenesis and metastasis formation, MMP expression and regulation may prove to be a strategic target for the development of methods to combat and to treat cancer (Pupa et al. 2002). Recent studies have shown that lowbush blueberries (*Vaccinium angustifolium*) have beneficial effects against certain chronic diseases (Bomser et al. 1996, Knekt et al. 2002). Blueberries contain relatively high concentrations of polyphenolic compounds such as flavonoids and phenolic acids (Smith et al. 2000, Kalt et al. 2002). Flavonoids have been shown to have antioxidant effects in many different models ranging from free-radical quenching to protection during hypoxia-ischemia insults (Kandaswami et al. 1995, Sweeney et al. 2002, Kahkonen and Heinonen 2003). Furthermore, flavonoids from

Vaccinium species (lingonberry, bilberry, cranberry, and lowbush blueberry) possess anti-carcinogenic properties through the regulation of ornithine decarboxylase whose activity or expression is altered in many tumor types (Bomser et al. 1996). Green tea (*Camellia sinensis*) flavonoids such as epigallocatechin have also been shown to modify MMP activity and to modulate ornithine decarboxylase activity in *in vitro* studies (Gupta et al. 1999, Garbisa et al. 2001).

In this study, three fractions from lowbush blueberry were tested to examine their effects on MMP activity in DU145 human prostate cancer cells. These fractions represented the major groups of flavonoids in these fruits, namely a "crude" fraction (containing all flavonoids), an anthocyanin-enriched fraction, and a proanthocyanidin-enriched fraction. The anthocyanins and the proanthocyanidins are groups of flavonoids that are believed to be the major active agents responsible for the anti-carcinogenic properties possessed by many flavonoid-containing fruits and vegetables (Bomser et al. 1996, Sartor et al. 2002, Sato et al. 2002). Therefore, in this study, we propose that the gelatinolytic activity of MMPs in DU145 cells will be inhibited following exposure to the various flavonoid containing fractions indicating that these flavonoid-enriched fractions isolated from lowbush blueberry can affect the activity of MMPs and in so doing regulate their activity.

2.3. Materials and methods

2.3.1. Cell culture

Human DU145 prostate adenocarcinoma cells (ATCC, Rockville, MD) were cultured on 100 mm Falcon plastic tissue culture dishes (Becton Dickinson Labware, Franidin Lakes, NJ) in alpha minimal essential medium (MEM) (Invitrogen, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone/VWR Canlab, Mississauga, ON) at 37°C in 5% CO_2. Defined medium (DM), which is a serum-free medium, was also used. DM consisted of alpha MEM supplemented with transferrin (Sigma, Oakville, ON) and insulin (Sigma, Oakville, ON). Blueberry-enriched DM was prepared by dissolving each of the fractions from lowbush blueberry, namely "crude" (CB), "anthocyanin-enriched" (AN), and "proanthocyanidin-enriched" (PAC) in DM to a final concentration of 0.1, 0.5 and 1.0 mg/ml, respectively. The blueberry fractions used were prepared by Dr. W. Kalt (Agriculture and Agri-Food Canada, Kentville, NS) and Dr. S. MacKinnon (NRC Halifax, NS). All analyses of fraction contents were performed by Dr. S. MacKinnon (NRC Halifax, NS). Subconfluent cells were exposed to blueberry enriched DM for 24 hours and the resulting conditioned medium was analyzed for MMP activity.

2.3.2. Gelatin gel electrophoresis

Gelatinolytic activity was analyzed using gelatin gel electrophoresis, or zymography (Heussen and Dowdle 1980, Samuel et al. 1992). An aliquot of conditioned medium was mixed (4:1) with sample buffer which consisted of 10%

SDS, 0.1% bromophenol blue in 0.3 M Tris-HCl, pH 6.8 and then incubated at 37°C for 5 minutes. Aliquots of each sample were loaded into wells of a 5% poly-acrylamide stacking gel and resolved at a constant current at ambient temperature. The 10% poly-acrylamide resolving gel contained Type A gelatin (Sigma, Oakville, ON) to a final concentration of 1 mg/ml. Following electrophoresis, gels were washed in 0.05 M Tris-HCl, pH 7.4, 2% Triton X-100 for one hour at room temperature followed by a 30 minute wash in 0.05 M Tris-HCl, pH 7.4 at room temperature. Gels were then incubated at 37°C for 24 hours in a substrate buffer containing 0.05 M Tris-HCl, pH 7.4, 1% Triton X-100 and 0.005 M $CaCl_2$. Following this incubation period, gels were stained with 0.1% Coomassie Blue R-250 in a solution of acetic acid, methanol, and water (5:10:85 v/v/v). Pre-stained molecular weight markers (Bio-Rad, Mississauga, ON) were also resolved on the same gel. Gelatinase activity appeared as zones of clearing (due to gelatin degradation) against a blue background. As a loading control, identically loaded complementary Coomassie Brilliant Blue stained polyacrylamide gels without gelatin are used (Oetken et al. 1992). Staining of these gels produces a number of protein bands for each lane. The intensity of these stained bands is used to ensure an equal amount of protein was added per lane for each individual zymogram (data not shown).

2.3.3. Necrotic cell death

Conditioned medium from cells exposed to various concentrations of blueberry fractions was assayed for the presence of lactate dehydrogenase (LDH),

which was used as an indicator of necrotic cell death. Most cells have LDH present in the cytosol to catalyse the fermentative reaction that reduces pyruvate to lactate in anaerobic metabolism. The presence of LDH in the conditioned medium indicates lysis of the cellular membrane due to necrotic cell death (Gay et al., 1968). A lactate dehydrogenase (LDH-L) assay (Diagnostic Chemicals Ltd. [DCL], Charlottetown, PE) was used to assay for the presence of LDH in conditioned medium. The LDH-L assay detects LDH through NADH formation, which has a moderate molar absorptivity at 340 nm ($\varepsilon = 6.22$). LDH reduces NAD^+ to NADH while oxidizing lithium lactate (L-lactate) to pyruvate, and NADH formation is proportional to LDH activity. Conditioned medium from DU145 cells was mixed 1:100 with warm (37°C) LDH-L reagent (7.5 mM NAD^+, 55.5 mM L-lactate, buffer [pH 9.05 at 25 ^{0}C], stabilizers and preservatives; DCL) and the mixture was incubated at 37 ^{0}C for 90 seconds. The mixture was then transferred to a 12.5 mm plastic cuvette and the absorbance was measured at one minute intervals over a span of three minutes. LDH activity was expressed as units per litre (U/L) and calculated from the equation:

$$\text{LDH} = (\Delta \text{ Absorbance / minute}) \times 16238$$

(16238 is a constant derived from all constant factors in the assay, such as assay volume, sample volume, absorbance coefficient, and light path). Group means were compared using a one-way analysis of variance (ANOVA), and sample sizes were n = 5.

2.3.4. Apoptotic cell death

Treated cells were assayed for the presence of caspase-3 in the cytosol, which was used as an indicator of apoptotic cellular death. Caspase-3 is a member of the CED-3 subfamily of caspases (cysteine-requiring aspartate proteases) and is one of the critical enzymes of apoptosis. Caspase-3 specifically cleaves most of the key proteins involved in apoptosis, and is integral in mediating nuclear apoptosis including chromatin condensation and DNA fragmentation (Cohen 1997). The caspase-3 colorimetric assay (Sigma, Oakville, ON) is based on the hydrolysis of the polypeptide acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline moiety, which has a high absorptivity at 405 nm ($\varepsilon = 10.5$). Immediately following treatment with blueberry fractions, DU145 cells were removed from tissue plates using trypsin (BD Biosciences, Mississauga, ON) and centrifuged to remove the supernatant. The pellet was reconstituted in 1X phosphate buffered saline (PBS), and the mixture was centrifuged. The pellet was reconstituted in 100 µl of cell lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT; Sigma) and incubated on ice for 15-20 minutes. After cell lysis, the mixture was centrifuged at 14,000 *g* for 10-15 minutes. Cell lysate was removed and mixed 1:100 with a mixture of 98% assay buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT; Sigma) and 1% Ac-DEVD-pNA (20 mM in DMSO; Sigma), which served as a substrate for the reaction. Caspase-3 (100 µg/ml caspase-3 in 50 mM HEPES, pH 7.4, 0.1% CHAPS, 10 mM DTT, 100 mM NaCl, 1 mM EDTA, 10% sucrose; Sigma) was substituted for substrate and served as a

positive control, while a caspase-3 inhibitor (2 mM Ac-DEVD-CHO in dimethylsulfoxide [DMSO]; Sigma) was added to the above mixture to serve as a negative control. Immediately after substrate (or substitute) addition, samples were incubated at 37 ^{0}C for 1.5-2 hours. Samples were then transferred to a quartz cuvette, and absorbance was measured at 405 nm. Caspase-3 activity was calculated as μmol pNA released per minute per ml of sample, using the equation:

$$\mu\text{mol pNA/min/ml} = \Delta\text{Absorbance} / 10.5$$

(10.5 is the absorbance coefficient of pNA at 405 nm). Group means were compared using a one-way analysis of variance (ANOVA), and sample sizes were n = 5.

2.4. Results

2.4.1. Effects of lowbush blueberry fractions on MMP activity

Gelatinolytic activity of conditioned medium from DU145 cells following 24 hour treatment with crude fraction was determined. DU145 cells were exposed to 0.1, 0.5 and 1.0 mg/ml crude blueberry fraction (CB). As shown in Figure 2.1, in control cells not exposed to CB fraction, discrete bands of gelatinolytic activity were noted at about 92 kDa and ~62-72 kDa. These gelatinolytic activities correspond to MMP-9, activated forms of MMP-2 and pro-MMP-2. Activity of MMP-9 was unaffected by 0.1 mg/ml CB fraction but was progressively inhibited by 0.5 and 1.0 mg/ml CB fraction (Figure 2.1). The proteolytic activity of both the pro-enzyme form of MMP-2 and activated isoforms of MMP-2 decreased in a dose-dependent manner in response to exposure to CB fraction. In this regard, 1.0 mg/ml crude fraction results in a

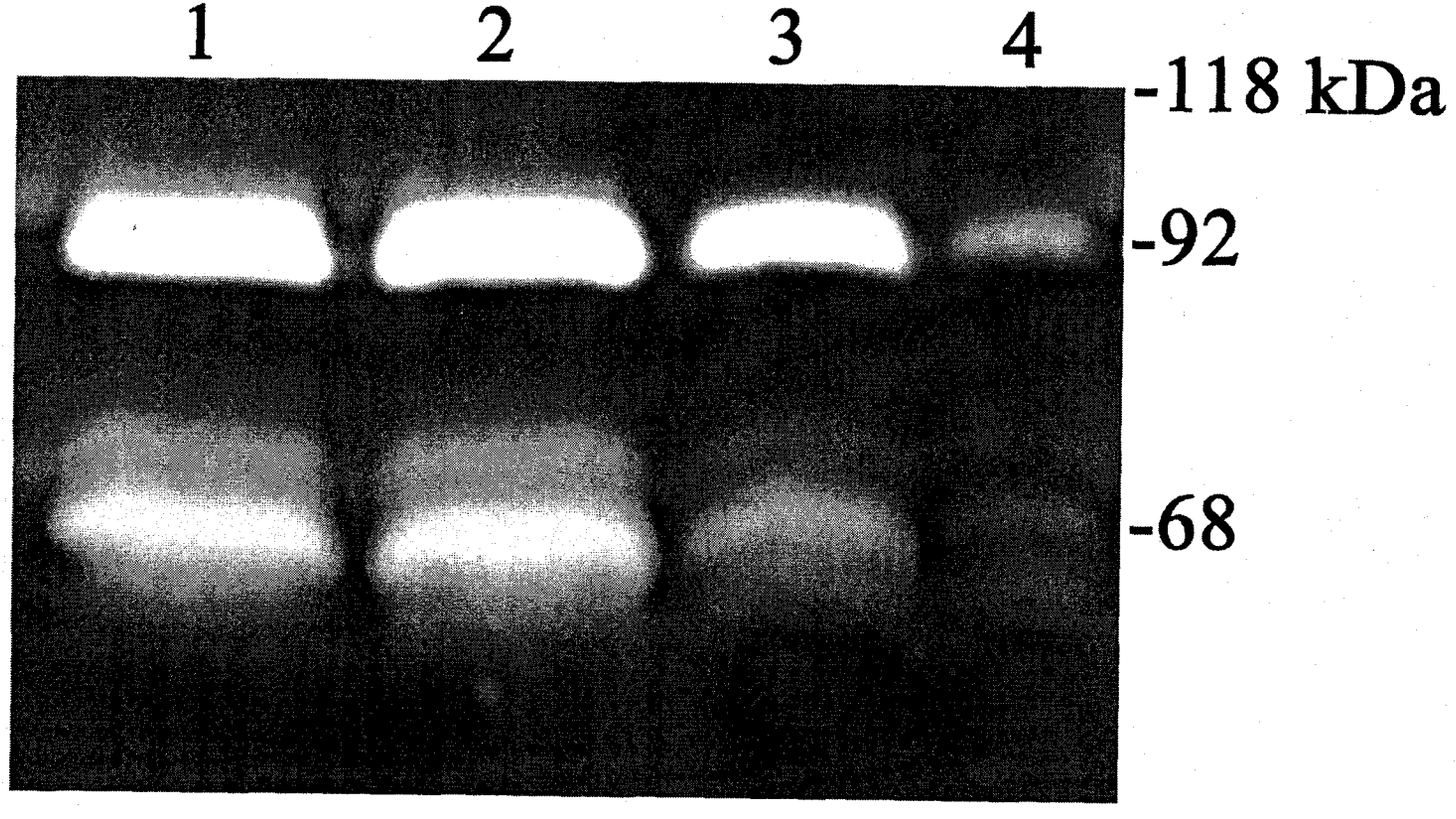

Figure 2.1. Effects of crude fraction from lowbush blueberry (CB) on MMP activity in DU145 cells. Gelatin gel electrophoresis was performed on aliquots of conditioned medium from DU145 cells following 24 hours exposure to CB fraction. Gelatinolytic activity in cells cultured in the absence of CB fraction is shown in (1) and gelatinolytic activity in cells following 24 hours exposure to 0.1 mg/ml CB fraction is shown in (2), to 0.5 mg/ml CB fraction is shown in (3) and to 1.0 mg/ml CB fraction is shown in (4).

pronounced decrease in gelatinolytic activity of the activated isoforms of MMP-2 and a complete inhibition of the activity of pro-MMP-2 (Figure 2.1).

Gelatinolytic activity of conditioned medium from DU145 cells following 24 hour treatment with 0.1, 0.5 and 1.0 mg/ml anthocyanin-enriched fraction (AN) is shown in Figure 2.2. Figure 2.2 shows that MMP activity is differentially modulated following 24 hour exposure to AN fraction from lowbush blueberry. MMP-9 activity was unaffected following exposure to either 0.1 or 0.5 mg/ml AN fraction but was inhibited in the presence of 1.0 mg/ml AN fraction. MMP-2 activity is unaffected by 0.1 mg/ml AN fraction. MMP-2 activity is affected by 0.5 and 1.0 mg/ml AN fraction. In this regard, pro-MMP-2 activity is virtually abolished in the presence of 1.0 mg/ml AN fraction.

Gelatinolytic activity of conditioned medium from DU145 cells following 24 hour exposure to 0.1, 0.5 and 1.0 mg/ml proanthocyanidin-enriched fraction (PAC) from lowbush blueberry is shown in Figure 2.3. Figure 2.3 shows that all MMP activity is dramatically reduced in the presence of the PAC fraction. Activity of MMP-9 is markedly reduced in the presence of 0.1 mg/ml of PAC and this activity is completely abolished in response to 0.5 and 1.0 mg/ml PAC. MMP-2 activity is also virtually eliminated following exposure to 0.1 mg/ml PAC and is completely abolished in response to 0.5 and 1.0 mg/ml PAC treatments.

To further characterize the effect of the PAC fraction on MMP activity the effects of the PAC fraction at lower concentrations was evaluated. Figure 2.4 shows the effect of a 24 hour exposure to 0.01 mg/ml and to 0.05 mg/ml PAC fraction on

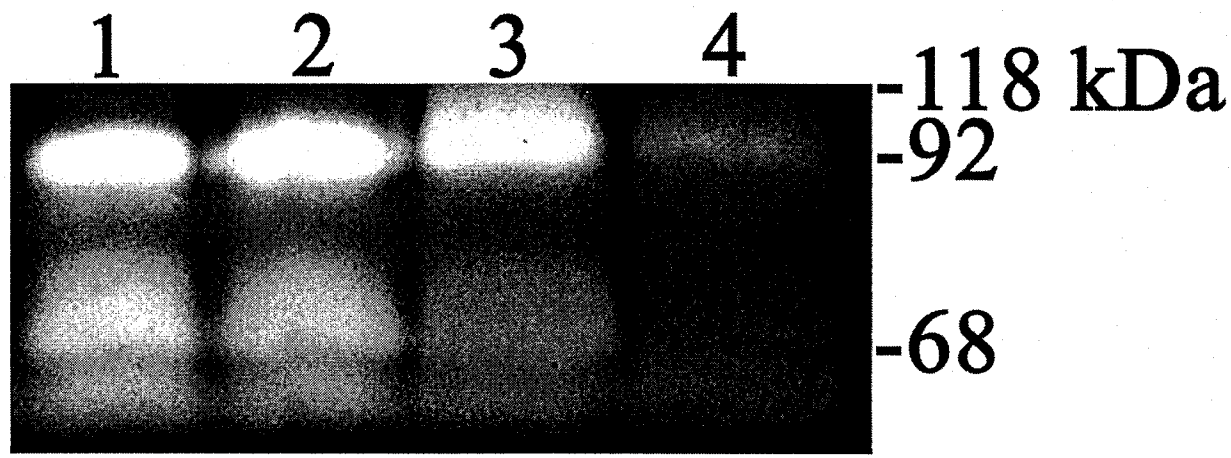

Figure 2.2. Effects of anthocyanin-enriched fraction from lowbush blueberry (AN) on MMP activity in DU145 cells. Gelatin gel electrophoresis was performed on aliquots of conditioned medium from DU145 cells following 24 hours exposure to AN fraction. (1) control cells and cells exposed to 0.1 mg/ml AN (2), to 0.5 mg/ml AN (3) and to 1.0 mg/ml AN fraction (4).

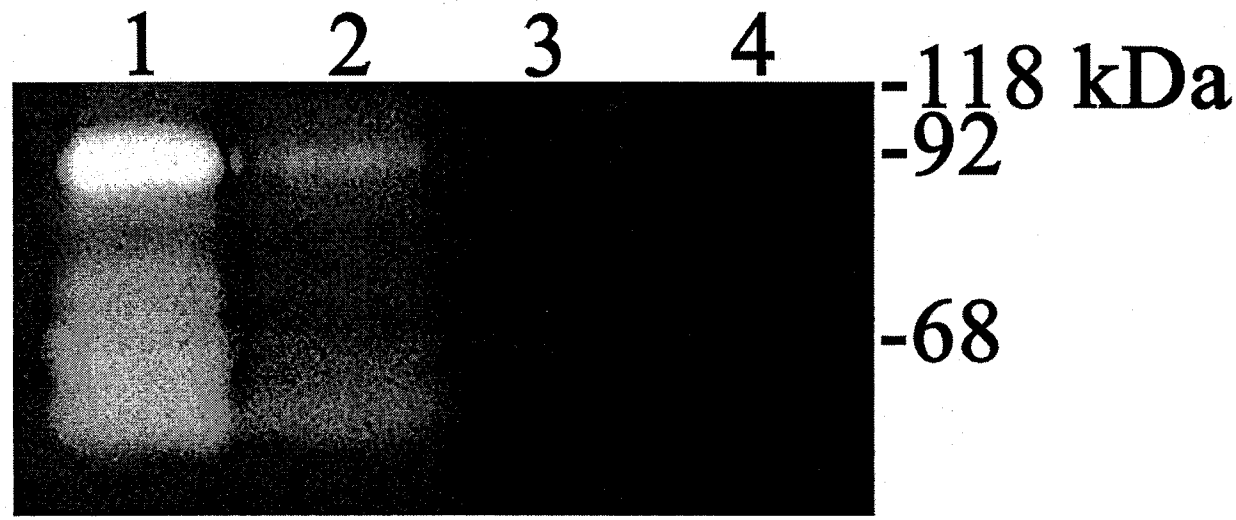

Figure 2.3. Effects of proanthocyanidin-enriched fraction from lowbush blueberry (PAC) on MMP activity in DU145 cells. Gelatin gel electrophoresis was performed on aliquots of conditioned medium from DU145 cells following 24 hours exposure to PAC fraction. (1) control cells and cells exposed to 0.1 mg/ml PAC (2), to 0.5 mg/ml PAC (3) and to 1.0 mg/ml PAC fraction (4).

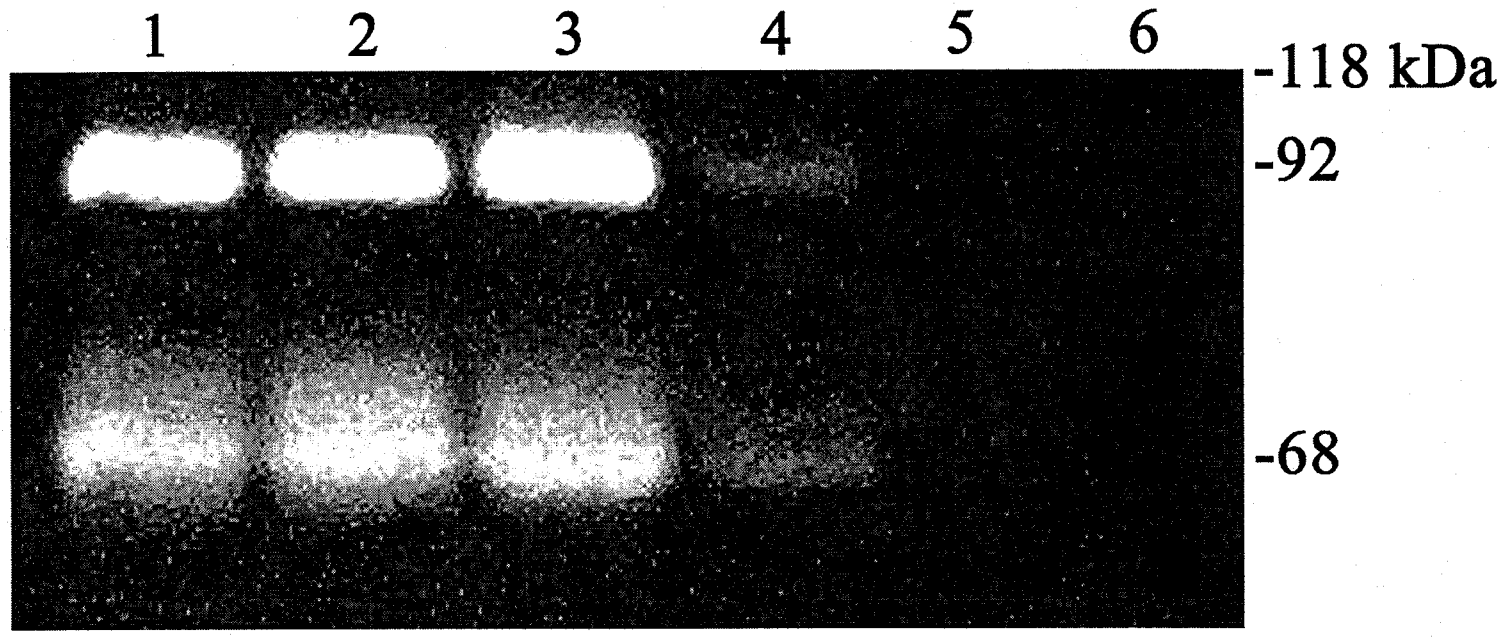

Figure 2.4. Effects of lower concentrations of PAC fraction from lowbush blueberry on MMP activity in DU145 cells. Gelatin gel electrophoresis was performed on aliquots of conditioned medium from DU145 cells following 24 hours exposure to various concentrations of PAC fraction. Gelatinolytic activity in control cells (1) and gelatinolytic activity in cells exposed to 0.01 mg/ml (2), 0.05 mg/ml (3), to 0.1 mg/ml (4), to 0.5 mg/ml (5) and to 1.0 mg/ml(6) PAC fraction, respectively.

MMP activity in DU145 cells. At these concentrations, PAC fraction was unable to inhibit MMP activity in these cells suggesting a threshold value of PAC fraction is required for the inhibitory effect to be expressed. For comparison, the remaining lanes illustrated in Figure 2.4 are replicate lanes of 0.1, 0.5 and 1.0 mg/ml PAC fraction, respectively, as shown also in Figure 2.3. Results presented in Figures 2.1-2.4 respectively are representative of observations noted from, at a minimum, 3 separate experiments.

2.4.2. Necrotic and apoptotic cell death in DU145 cells in response to blueberry fractions

To determine whether the decrease in MMP activity in response to treatment with the blueberry fractions was due to a toxic effect on the cells, the contribution of generalized cell death due to cellular necrosis or programmed cell death due to cellular apoptosis was evaluated. LDH and caspase-3 activities were used as indicators of necrotic cell death and apoptotic cell death, respectively. Biochemical assays were performed to quantify the presence and the activity of LDH in DU145 cells exposed to the highest concentration of CB, AN and PAC fractions from lowbush blueberry, namely 1.0 mg/ml. Figure 2.5 shows LDH activity in DU145 cells in the absence and in the presence of these fractions. Treatment of these cells with CB, AN and PAC fractions (1.0 mg/ml) for 24 hours did not result in increased LDH activity compared to control cells indicating that increased cell death due to necrosis was apparently not a contributing factor to the reduction in MMP activity noted in

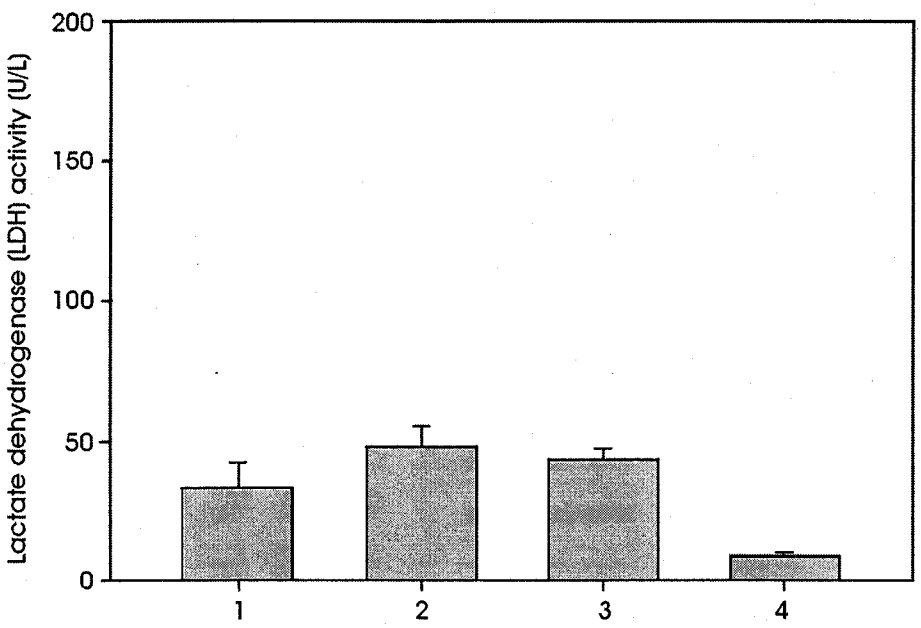

Figure 2.5. Lactate dehydrogenase (LDH) activity as a measure of necrotic cell death. LDH activity was measured in DU145 cells following a 24 hour exposure to defined medium (control cells) (1), defined medium supplemented with CB fraction (1.0 mg/ml) (2), defined medium supplemented with AN fraction (1.0 mg/ml) (3), and defined medium supplemented with PAC fraction (1.0 mg/ml) (4). Results indicated are from duplicate experiments with 5 culture plates per condition tested, $p = 0.067$, ANOVA.

these cells in response to treatment with these blueberry fractions ($p = 0.067$). Figure 2.6 shows caspase-3 activity in DU145 cells in the absence and in the presence of blueberry fractions. Treatment of DU145 cells with CB, AN and PAC fractions (1.0 mg/ml) did not result in increased caspase-3 activity compared to control cells indicating that cell death due to apoptosis was also apparently not a contributing factor to the reduction in MMP activity noted in these cells in response to treatment with these blueberry fractions ($p = 0.827$). These observations suggested that the decreased expression of MMPs observed in DU145 cells in response to treatment with these blueberry fractions were target-directed and not due to cellular death.

2.5. Discussion

This study has demonstrated that MMP activity from human prostate cancer cells, specifically DU145 cells, decreases following exposure to flavonoid-enriched fractions from lowbush blueberry. These results are in keeping with other studies investigating the effects of flavonoids in cancer models (Garbisa et al. 2001, Sartor et al. 2002, Sato et al. 2002). Although the gelatinolytic activity of the MMPs in DU145 cells decreases in response to treatment with blueberry fractions, the effects of each flavonoid-enriched fraction on MMP activity is varied. This is not unexpected since although the anthocyanins and proanthocyanidins are both flavonoids, they comprise distinct flavonoid subclasses, and as such, have different chemical structures. The crude fraction contains approximately 75% anthocyanins and 8.5% proanthocyanidins which may have had synergistic or additive effects. To the best of my knowledge, this

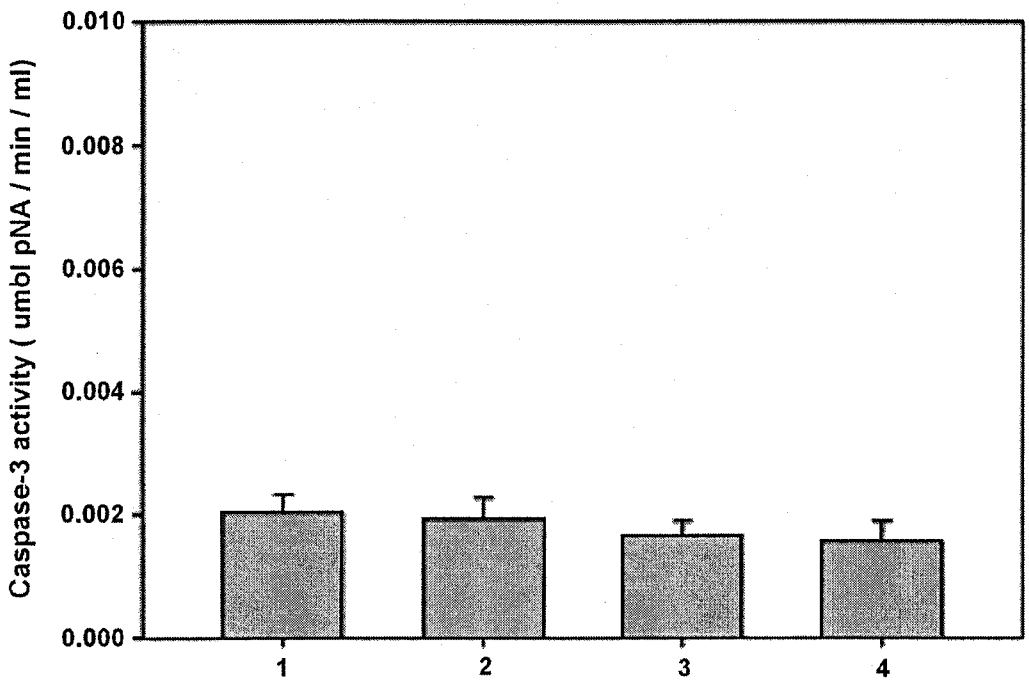

Figure 2.6. Caspase-3 activity as a measure of apoptotic cell death. Caspase-3 activity was measured in DU145 cells following a 24 hour exposure to defined medium (control cells) (1), defined medium supplemented with CB fraction (1.0 mg/ml) (2), defined medium supplemented with AN fraction (1.0 mg/ml) (3), and defined medium supplemented with PAC fraction (1.0 mg/ml) (4). Results indicated are from duplicate experiments with 5 culture plates per condition tested, $p = 0.827$, ANOVA.

study is the first to demonstrate a link between "bioactive" containing fractions isolated from lowbush blueberry and the inhibition of MMP expression. The PAC fraction has been shown to contain a range of proanthocyanidins from monomers to decamers (Appendix Figure A.1). The AN fraction has been found to contain a number of potentially "bioactive" compounds including 4 main glucosides (malvidin, petunidin, delphinidin and quercetin), quercetin-3-galactoside and quercetin-3-rhamnoside, amongst others (Appendix Figure A.1). Anthocyanins and proanthocyanidins from other sources have been shown to affect MMP activity. Delphinidin has been shown to decrease the activity of MMP-2 (in human neuroblastoma cells) and MMP-9 (in HT-1080 human fibrosarcoma cells) (Sartor et al. 2002). The green tea catechins, which are very similar to proanthocyanidins, have been shown to down-regulate MMP activity in cancer cells (Gupta et al. 1999, Garbisa et al. 2001). Epigallocatechin-3-gallate (EGCG) has also been reported to inhibit MMP-2 and MMP-9 activity (Sartor et al. 2002). Myricetin, which is a proanthocyanidin, also effectively inhibits the activity of MMPs (Sartor et al. 2002). Additionally, the proanthocyanidin nobiletin has been shown to decrease the activity of MMP-1 and MMP-9 *in vitro* (Sato et al. 2002). The results presented in this study are consistent with these observations. Studies are currently ongoing to define the exact nature of the "bioactive" compounds found in these fractions isolated from lowbush blueberries.

It is important to note that these blueberry fractions did not induce necrotic cell death in these cells. These findings imply specificity between the action of the

"bioactive" components contained in these blueberry fractions and the inhibition of MMP (MMP-2 and MMP-9) activity in DU145 cells. In this regard, studies are presently ongoing to understand the mechanism(s) involved in this inhibitory process. MMPs are the major mediators of basement membrane degradation and as such are possible relevant targets for the development of novel anti-cancer treatments. Our findings demonstrate that flavonoid-enriched fractions from lowbush blueberries can down-regulate the activities of specific MMPs in a target-directed manner suggesting that further understanding of the complex properties of flavonoids, in particular from lowbush blueberry, may allow for the further development and refinement of the potential role of flavonoids in the prevention of carcinogenesis and metastasis.

In conclusion, our results are the first to describe a potential regulatory effect of flavonoids isolated from lowbush blueberry on human prostate cancer cells. This regulatory effect was directed to the activity of matrix metalloproteinase-2 and -9. These activities may be one of the several targets of the potential anti-proliferative effects associated with flavonoid containing fractions isolated from lowbush blueberries.

3. CHAPTER THREE

Inhibition of matrix metalloproteinase activity in DU145 human prostate cancer cells by flavonoids from lowbush blueberry (*Vaccinium angustifolium*) involves protein kinase C and mitogen activated protein kinase mediated events

3.1. Abstract

Regulation of the matrix metalloproteinases (MMPs) is crucial to regulate extracellular matrix (ECM) proteolysis which is important in metastasis. This study investigated the mechanism(s) by which three flavonoid-enriched fractions from lowbush blueberry (*Vaccinium angustifolium*) down-regulate MMP activity in DU145 human prostate cancer cells. MMP activity was evaluated from cells exposed to "crude", anthocyanin-enriched (AN) and proanthocyanidin-enriched (PAC) fractions. Differential down-regulation of MMPs was observed. The activity of the endogenous tissue inhibitors of metalloproteinases (TIMPs) from these cells was also evaluated. Increases in TIMP-1 and TIMP-2 activity were observed in response to these fractions. The involvement of protein kinase C (PKC) and mitogen activated protein (MAP) kinase pathways in the flavonoid-mediated decreases in MMP activity was observed. These findings indicate that blueberry flavonoids may use multiple mechanisms in down-regulating MMP activity in these cells.

3.2. Introduction

Prostate cancer (CaP) is a malignant disease which is one of the leading causes of death due to cancer among males in the United States and Canada. Recent cancer statistics state that projected new cases of CaP for 2004 in the USA (230,110) and Canada (20,100) will total more than a quarter-million, making CaP the most prevalent cancer among males in these countries (Jemal et al. 2004, National Cancer Institute of Canada, 2004). The development of malignant CaP continues to be the primary cause of death due to CaP.

The ability of prostate cancer cells to metastasize and invade foreign tissues starts with the proteolytic degradation of the basement membrane (Pupa et al. 2002, Sauer et al. 2004). The major mediators of basement membrane degradation are the matrix metalloproteinases (MMPs) (Stetler-Stevenson and Yu 2001, Pupa et al. 2002). The MMPs are a family of zinc (Zn^{2+})-dependent endopeptidases which can collectively cleave all major extracellular matrix (ECM) proteins such as collagens, proteoglycans, laminin, fibronectin, and elastin (Matrisian 1990). The MMPs are secreted as inactive zymogens into the extracellular milieu, and require both a Zn^{2+} ion in the active site and cleavage of the pro-enzyme domain through endogenous cascades, such as the plasminogen cascade, or by other MMPs to become activated (Stetler-Stevenson and Yu 2001, Klein et al. 2004). MMP expression and activity are highly regulated processes indicating their importance in regular cellular dynamics and interactions (Pupa et al. 2002). MMP expression is regulated post-translationally by, among other things, the endogenous tissue inhibitors of metalloproteinases

(TIMPs) (Stetler-Stevenson and Yu 2001). The TIMPs act to specifically inhibit MMP activity in the ECM, which emphasizes their importance in the overall regulation of the MMPs (Stetler-Stevenson and Yu 2001, Pupa et al. 2002). Although not the sole factor in tumor metastasis, it has been shown that many cancer cells demonstrate a pronounced increase in MMP activity and that over-expression of MMPs may be an important factor in both tumor invasion and tumor angiogenesis in foreign tissues (Klein et al. 2004). Due to their important role in tumorigenesis and metastasis, MMP expression and regulation may prove to be a strategic target for the development of novel methods to combat and treat cancer (Canning et al. 2001, Pupa et al. 2002).

Recent studies have shown that lowbush blueberries (*Vaccinium angustifolium*) have beneficial effects against certain chronic diseases (Bomser et al. 1996, Knekt et al. 2002). These berries contain relatively high concentrations of polyphenolic compounds such as flavonoids and phenolic acids (Kalt et al. 2000, Smith et al. 2000, Schmidt et al. 2004). Flavonoids have been shown to have antioxidant effects in many different models, ranging from free radical quenching to protection during hypoxia-ischemia insults (Sweeney et al. 2002, Kahkonen and Heinonen 2003). Furthermore, Bomser *et al.* (1996) have shown that flavonoids from *Vaccinium* species (lingonberry, bilberry, cranberry, and lowbush blueberry) possess anti-carcinogenic properties through the regulation of ornithine decarboxylase (ODC) which is an important enzyme in carcinogenesis (Bomser et al. 1996). Green tea (*Camellia sinensis*) flavonoids such as epigallocatechin-3-gallate (EGCG) have been

shown to modify MMP activity, along with modulating ODC activity, in various *in vitro* studies (Gupta et al. 1999, Garbisa et al. 2001, Sartor et al. 2002, Vayalil and Katiyar 2004). Amongst these studies, Vayalil and Katiyar (2004) have shown that EGCG inhibits the activity of MMP-2 and MMP-9 by inhibiting the activation of mitogen-activated protein (MAP) kinase, c-jun, and nuclear factor-κB (NF-κB) in DU145 human prostate cancer cells (Vayalil and Katiyar 2004). Previous work in our laboratory has shown that fractions from lowbush blueberry inhibit MMP activity in DU145 cells (Matchett et al. 2005).

Given their ability to down-regulate MMP activity, this study focussed primarily on the mechanism(s) by which lowbush blueberry fractions inhibit MMP activity in these cells. Based on previous data showing MMP inhibition in DU145 cells following 24 hours exposure to fractions from lowbush blueberry, we evaluated the effects of earlier exposure times (1, 3, and 6 hours) on MMP activity in these cells. Furthermore, the ability of these fractions to induce the activity of the TIMPs was determined. Moreover, using specific inhibitors of cellular signal transduction pathways, the signalling mechanism(s) by which the lowbush blueberry fractions mediate their effects were determined. These findings may provide novel insights as to the efficacy of nutritional 'bioactives' against known markers for the possible prevention of prostate cancer.

3.3. Materials and methods

3.3.1. Cell culture and treatment

Human DU145 prostate adenocarcinoma cells (ATCC, Rockville, MD) were cultured on 100 mm plastic tissue culture dishes (Becton Dickinson Labware, Franidin Lakes, NJ) in α-minimal essential medium (MEM) (Invitrogen, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone/VWR Canlab, Mississauga, ON) and were grown at 37°C in 5% CO_2. A defined medium (DM) which is a serum-free medium, was also used. DM consisted of α-MEM supplemented with transferrin (Sigma, Oakville, ON) and insulin (Sigma, Oakville, ON). Blueberry-enriched DM was prepared by dissolving "crude" (which represents the whole unfractionated extract), anthocyanin-enriched, and proanthocyanidin-enriched extracts from lowbush blueberry in DM to a final concentration of 0.1, 0.5, and 1.0 mg/ml, respectively. Subconfluent cells were exposed to blueberry-enriched DM for 1, 3, 6, and 24 hours and the resulting conditioned medium was analysed for MMP activity. To evaluate the possible signal transduction pathways involved, cells were pre-treated for 1 hour with either phosphatidylinositol-3 (PI-3) kinase inhibitors (wortmannin and LY294002) (Sigma), protein kinase C (PKC) inhibitor (calphostin C) (Sigma), and mitogen activated protein (MAP) kinase inhibitor (PD98059) (Sigma), respectively, prior to exposure to blueberry fractions.

3.3.2. Gelatin gel electrophoresis

Gelatinolytic activity was analyzed by gelatin gel electrophoresis (zymography) essentially as described in section 2.1.3.2. Briefly, an aliquot of conditioned medium was mixed (4:1) with sample buffer which consisted of 10% SDS, 0.1 % bromophenol blue in 0.3 M Tris-HCl, pH 6.8 and incubated at 37°C for 5 minutes. Aliquots of each sample were loaded into wells of a 5% stacking gel and resolved by electrophoresis at a constant current at ambient temperature. The 10% resolving gel contained Type A gelatin (Sigma, Oakville, ON) at a final concentration of 1 mg/ml. Following electrophoresis, gels were washed in 0.05 M Tris-HCl, pH 7.4, containing 2% Triton X-100 for one hour at room temperature followed by a 30 minute wash in 0.05 M Tris-HCl, pH 7.4, at room temperature. Gels were then incubated at 37°C for 24 hours in a substrate buffer containing 0.05 M Tris-HCl, pH 7.4, 1% Triton X-100 and 0.005 M $CaCl_2$. Following this incubation period, gels were stained with 0.1 % Coomassie Blue R-250 in a solution of acetic acid, methanol, and water (5:50:45 v/v/v) and de-stained in a solution of acetic acid, methanol, and water (5:10:85 v/v/v). Pre-stained molecular weight markers (Biorad) were also resolved on the same gel. Gelatinase activity appeared as zones of clearing (due to gelatin degradation) against a blue background. As a loading control, identically loaded complementary Coomassie Brilliant Blue stained polyacrylamide gels without gelatin are used (Oetken et al. 1992). Staining of these gels produces a number of protein bands for each lane. The intensity of these stained bands is used to ensure an equal amount of protein was added per lane for each individual zymogram (data not shown).

3.3.3. Reverse zymography

Reverse zymography was used to assay for the presence of the endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) (Edwards et al. 1996). This technique allows for detection of the TIMPs in conditioned medium through the presence of dark bands against a clear background (as TIMPs will inhibit MMP-induced gelatinolytic activity). Aliquots of each sample were mixed 4:1 with sample buffer (10% SDS, 0.1 % bromophenol blue in 0.3 M Tris-HCl, pH 6.8) and were loaded into wells of a 5% poly-acrylamide stacking gel and resolved at a constant current at ambient temperature. The 10% poly-acrylamide resolving gel contained Type A gelatin (Sigma, Oakville, ON) to a final concentration of 1 mg/ml and conditioned medium from NR3 ras-transfected mouse fibrosarcoma cells (Egan et al. 1987, Voskas et al. 2001a). The conditioned medium was used as a source of gelatinases, and was obtained from a culture of NR3 cells grown in the presence of DM for 24 hours. Following electrophoresis, gels were washed using the washing protocol described above. Gels were then incubated at 37°C in substrate buffer (0.05 M Tris-HCl, pH 7.4, 1% Triton X-100 and 0.005 M $CaCl_2$) for 30-36 hours. Following incubation, gels were stained using the staining procedure described above. Dark bands were indicative of TIMP activity.

3.4. Results

3.4.1. Effects of lowbush blueberry fractions on MMP activity

Gelatinolytic activity of conditioned medium from DU145 cells following 24 hour treatment with 0.1, 0.5 and 1.0 mg/ml "crude" blueberry fraction was determined. As shown in Figure 3.1, in control cells (no exposure to "crude" fraction), discrete bands of gelatinolytic activity were noted at about 92 kDa and 62-72 kDa. These gelatinolytic activities correspond to MMP-9, activated isoforms of MMP-2 and pro-MMP-2. Activity of MMP-9 was unaffected by 0.1 mg/ml "crude" fraction but was progressively inhibited by 0.5 and 1.0 mg/ml "crude" fraction (Figure 3.1). The proteolytic activity of both the pro-enzyme form of MMP-2 and activated isoforms of MMP-2 decreased in a dose dependent manner in response to exposure to "crude" blueberry fraction. In this regard, 1.0 mg/ml "crude" fraction results in a pronounced decrease in gelatinolytic activity of the activated isoforms of MMP-2 and a complete inhibition of the activity of pro-MMP-2 (Figure 3.1).

Gelatinolytic activity of conditioned medium from DU145 cells following 24 hour treatment with 0.1, 0.5 and 1.0 mg/ml anthocyanin-enriched blueberry fraction is shown in Figure 3.2A. MMP activity is differentially modulated following 24 hour exposure to anthocyanin-enriched (AN) fraction from lowbush blueberry. MMP-9 activity was unaffected following exposure to either 0.1 or 0.5 mg/ml AN fraction butwas inhibited in the presence of 1.0 mg/ml AN fraction. MMP-2 activity is unaffected by 0.1 mg/ml AN fraction. MMP-2 activity is affected by 0.5 and 1.0 mg/ml AN fraction. In this regard, the activities of pro-MMP-2 and activated

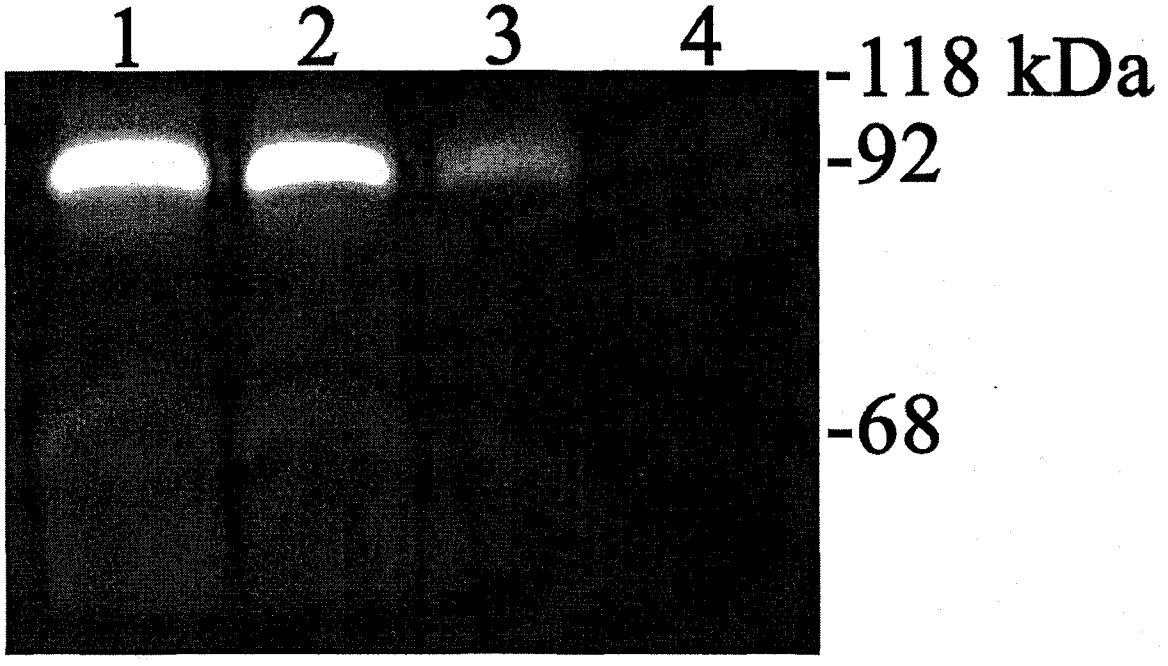

Figure 3.1. Effects of "crude" fraction from lowbush blueberry on MMP activity in DU145 cells. Gelatinolytic activity in cells cultured in the absence of "crude" blueberry fraction is shown in (1) and in cells following 24 hour exposure to 0.1 mg/ml (2), 0.5 mg/ml (3), and 1.0 mg/ml (4) "crude" fraction is shown.

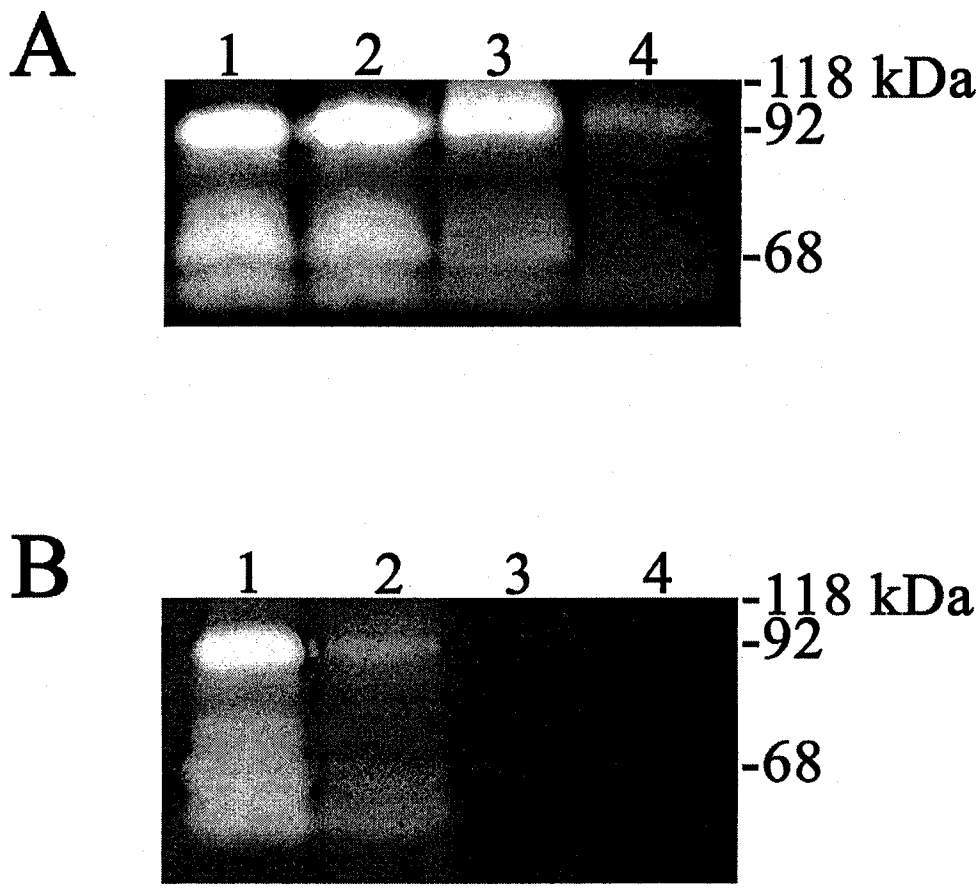

Figure 3.2. Effects of flavonoid-enriched fractions from lowbush blueberry on MMP activity. A: Effects of anthocyanin-enriched (AN) fraction. (1) Control cells and cells exposed for 24 hours to 0.1 mg/ml AN (2), 0.5 mg/ml AN (3), and 1.0 mg/ml AN (4). B: Effects proanthocyanidin-enriched (PAC) fraction. (1) Control cells and cells exposed for 24 hours to 0.1 mg/ml PAC (2), 0.5 mg/ml PAC (3), and 1.0 mg/ml PAC (4).

isoforms of MMP-2 were virtually abolished in the presence of 1.0 mg/ml AN fraction.

Gelatinolytic activity of conditioned medium from DU145 cells following 24 hour treatment with 0.1, 0.5 and 1.0 mg/ml proanthocyanidin-enriched blueberry (PAC) fraction is shown in Figure 3.2B. Figure 3.2B shows that all MMP activity is dramatically reduced in the presence of the PAC fraction. Activity of MMP-9 is markedly reduced in the presence of 0.1 mg/ml PAC fraction and this activity is completely abolished in response to 0.5 and 1.0 mg/ml PAC fraction. MMP-2 activity is also virtually eliminated following exposure to 0.1 mg/ml PAC fraction and is completely abolished in response to 0.5 and 1.0 mg/ml PAC fraction treatments.

Gelatinolytic activity of conditioned medium from DU145 cells following 1, 3, and 6 hour treatments with 0.1, 0.5 and 1.0 mg/ml "crude" blueberry fraction is shown in Figure 3.3A. Figure 3.3A shows that the activity of MMP-9 is reduced in response to 1 hour exposure to 0.5 and 1.0 mg/ml "crude" fraction, and that MMP-9 activity is progressively decreased following 3 and 6 hour exposure to 0.5 and 1.0 mg/ml "crude" fraction, respectively. In this regard, MMP-9 activity was virtually abolished following 6 hour treatment with 1.0 mg/ml "crude" fraction. The activities of pro-MMP-2 and activated isoforms of MMP-2 were unchanged following 1, 3 and 6 hour exposure to 0.1 mg/ml "crude" fraction but was decreased in response to 1, 3 and 6 hour exposure to 0.5 and 1.0 mg/ml "crude" fraction. MMP-2 activity was markedly decreased in response to 6 hour treatment with 1.0 mg/ml "crude" fraction.

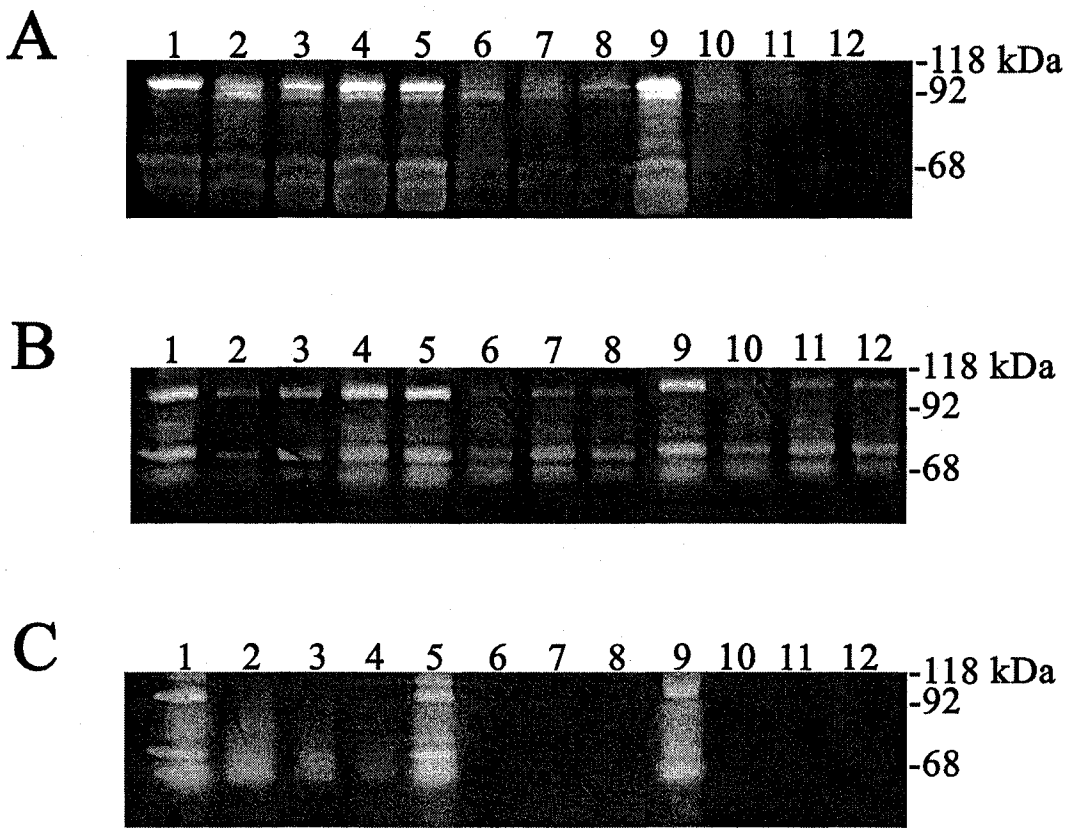

Figure 3.3. Effects of varying exposure times of fractions from lowbush blueberry on MMP activity in DU145 cells. **A:** Effects of "crude" fraction. Control cells cultured for 1 (1), 3 (5), and 6 hours (9) is shown. Cells exposed for 1 hour to 0.1 mg/ml (2), 0.5 mg/ml (6), and 1.0 mg/ml (10) "crude" fraction are shown. Cells exposed for 3 hours to 0.1 mg/ml (3), 0.5 mg/ml (7), and 1.0 mg/ml (11) "crude" fraction are shown. Cells exposed for 6 hours to 0.1 mg/ml (4), 0.5 mg/ml (8), and 1.0 mg/ml (12) "crude" fraction are shown. **B:** Effects of anthocyanin-enriched (AN) fraction. Cells exposed for 1 hour to 0.1 mg/ml (2), 0.5 mg/ml (6), and 1.0 mg/ml (10) AN fraction are shown. Cells exposed for 3 hours to 0.1 mg/ml (3), 0.5 mg/ml (7), and 1.0 mg/ml (11) AN fraction are shown. Cells exposed for 6 hours to 0.1 mg/ml (4), 0.5 mg/ml (8), and 1.0 mg/ml (12) AN fraction are shown. **C:** Effects of proanthocyanidin-enriched (PAC) fraction. Cells exposed for 1 hour to 0.1 mg/ml (2), 0.5 mg/ml (6), and 1.0 mg/ml (10) PAC fraction are shown. Cells exposed for 3 hours to 0.1 mg/ml (3), 0.5 mg/ml (7), and 1.0 mg/ml (11) PAC fraction are shown. Cells exposed for 6 hours to 0.1 mg/ml (4), 0.5 mg/ml (8), and 1.0 mg/ml (12) PAC fraction are shown.

Gelatinolytic activity of conditioned medium from DU145 cells following 1, 3, and 6 hour treatments with 0.1, 0.5 and 1.0 mg/ml anthocyanin-enriched (AN) blueberry fraction is shown in Figure 3.3B. Figure 3.3B shows that the activity of MMP-9 is initially reduced in response to 1 hour exposure to 0.1 mg/ml AN fraction, but that the activity returns to basal levels following 6 hour treatment with 0.1 mg/ml AN fraction. MMP-9 activity is markedly reduced following 1, 3 and 6 hour treatment with 0.5 and 1.0 mg/ml AN fraction. The activities of pro-MMP-2 and activated isoforms of MMP-2 are initially reduced in response to 1 hour exposure to 0.1 mg/ml AN fraction, but the activities return to basal levels following 6 hour treatment with 0.1 mg/ml AN fraction. The activity of pro-MMP-2 is decreased following 3 and 6 hour exposure to 0.5 and 1.0 mg/ml AN fraction, while the activated isoforms of MMP-2 are markedly decreased following similar treatments.

Gelatinolytic activity of conditioned medium from DU145 cells following 1, 3, and 6 hour treatments with 0.1, 0.5 and 1.0 mg/ml proanthocyanidin-enriched (PAC) blueberry fraction is shown in Figure 3.3C. Figure 3.3C shows that the activity of MMP-9 is virtually abolished following 1 hour exposure to 0.1 mg/ml PAC fraction, and that it remained completely abolished following all other treatments with PAC fraction. MMP-2 activity is drastically reduced following 1 hour exposure to 0.1 mg/ml PAC fraction, and this activity continually decreases in a time-dependent manner following 3 and 6 hour exposures to PAC fraction. All MMP-2 activity is completely abolished following 3 and 6 hour exposures to 0.1, 0.5 and 1.0 mg/ml

PAC fraction. Results presented in Figures 3.1-3.3 are representative of observations noted from, at a minimum, 3 separate experiments.

3.4.2. Effects of lowbush blueberry fractions on TIMP activity

The effects of 24 hour exposure to "crude" fraction from lowbush blueberry on TIMP activity in DU145 cells is shown in Figure 3.4A. Figure 3.4A shows that in control cells (no exposure to "crude" fraction), discrete bands were noted at about 28 kDa and 21 kDa. These bands correspond to TIMP-1 and TIMP-2. As shown in Figure 3.4A, TIMP-1 and TIMP-2 activity is modestly increased in a dose-dependent manner in response to increasing concentrations of "crude" fraction.

The effects of 24 hour exposure to anthocyanin-enriched (AN) fraction from lowbush blueberry on TIMP activity in DU145 cells is shown in Figure 3.4B. As shown in Figure 3.4B, the activity of TIMP-1 is markedly induced following treatment with 0.1 mg/ml AN fraction, and this activity remains elevated following treatments with 0.5 and 1.0 mg/ml AN fractions, respectively. TIMP-2 remains unaffected following treatment with 0.1 mg/ml AN fraction. However, TIMP-2 activity is substantially increased following treatment with 0.5 and 1.0 mg/ml AN fraction.

The effects of 24 hour exposure to proanthocyanidin-enriched (PAC) fraction from lowbush blueberry on TIMP activity in DU145 cells is shown in Figure 3.4C. Figure 3.4C shows that the TIMP-1 activity remains unaffected following treatment with 0.1 mg/ml PAC fraction. TIMP-1 activity is increased following treatments with

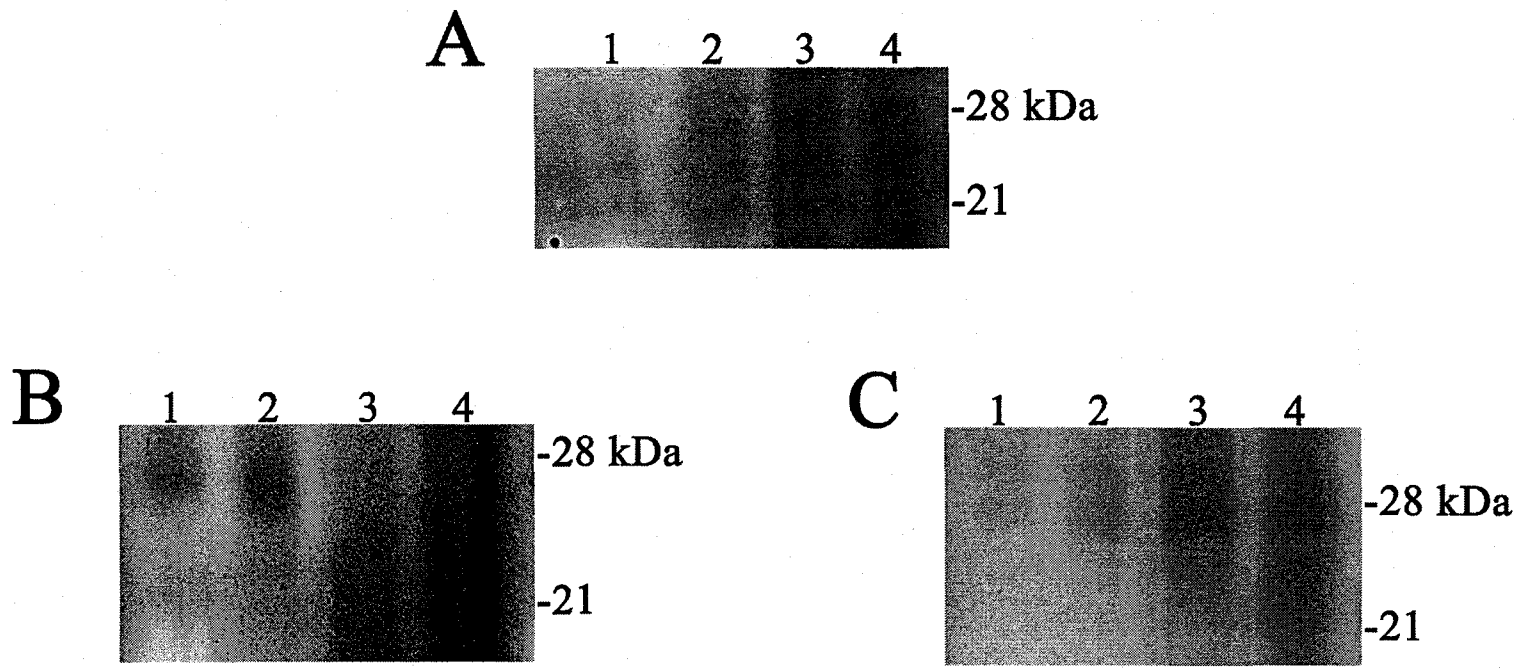

Figure 3.4. Effects of lowbush blueberry fractions on TIMP activity in DU145 cells. **A:** Effects of "crude" fraction. TIMP activity in control cells is shown in (1) and TIMP activity in cells following 24 hour exposure to 0.1 mg/ml (2), 0.5 mg/ml (3), and 1.0 mg/ml (4) "crude" fraction is shown. **B:** Effects of anthocyanin-enriched (AN) fraction. TIMP activity in control cells is shown in (1) and TIMP activity in cells following 24 hour exposure to 0.1 mg/ml (2), 0.5 mg/ml (3), and 1.0 mg/ml (4) AN fraction is shown. **C:** Effects of proanthocyanidin-enriched (PAC) fraction. TIMP activity in control cells is shown in (1) and TIMP activity in cells following 24 hour exposure to 0.1 mg/ml (2), 0.5 mg/ml (3), and 1.0 mg/ml (4) PAC fraction is shown.

0.5 and 1.0 mg/ml PAC fraction, respectively. TIMP-2 activity is unaffected following treatment with 0.1 and 0.5 mg/ml PAC fraction. TIMP-2 activity is slightly increased following treatment with 1.0 mg/ml PAC fraction. Results presented in Figure 3.4 are representative of observations noted from, at a minimum, 3 separate experiments.

3.4.3. Effects of cellular signalling inhibitors on blueberry flavonoid mediated decreases in MMP activity

Specific inhibitors of particular cellular signalling pathways were used to evaluate the possible mechanism(s) involved in blueberry flavonoid mediated decreases in MMP activity in these cells. For these mechanistic experiments, anthocyanin-enriched (AN) and proanthocyanidin-enriched (PAC) fractions were used as they are representative of two distinct subclasses of flavonoids found in lowbush blueberry. Gelatinolytic activity of conditioned medium from DU145 cells pre-treated for 1 hour with PKC inhibitor calphostin C (500 nM) (Chen et al. 1998) prior to 3 hour treatment with 1.0 mg/ml anthocyanin-enriched (AN) and proanthocyanidin-enriched (PAC) fraction from lowbush blueberry is shown in Figure 3.5A. As shown in Figure 3.5A, the inhibition of PKC by calphostin C alleviates the AN fraction-mediated decrease in MMP-2. Decreased MMP-9 activity by 1.0 mg/ml AN fraction from blueberry remains unchanged following PKC inhibition by calphostin C. PKC inhibition by calphostin C does not affect PAC-mediated decreases in either MMP-2 or MMP-9.

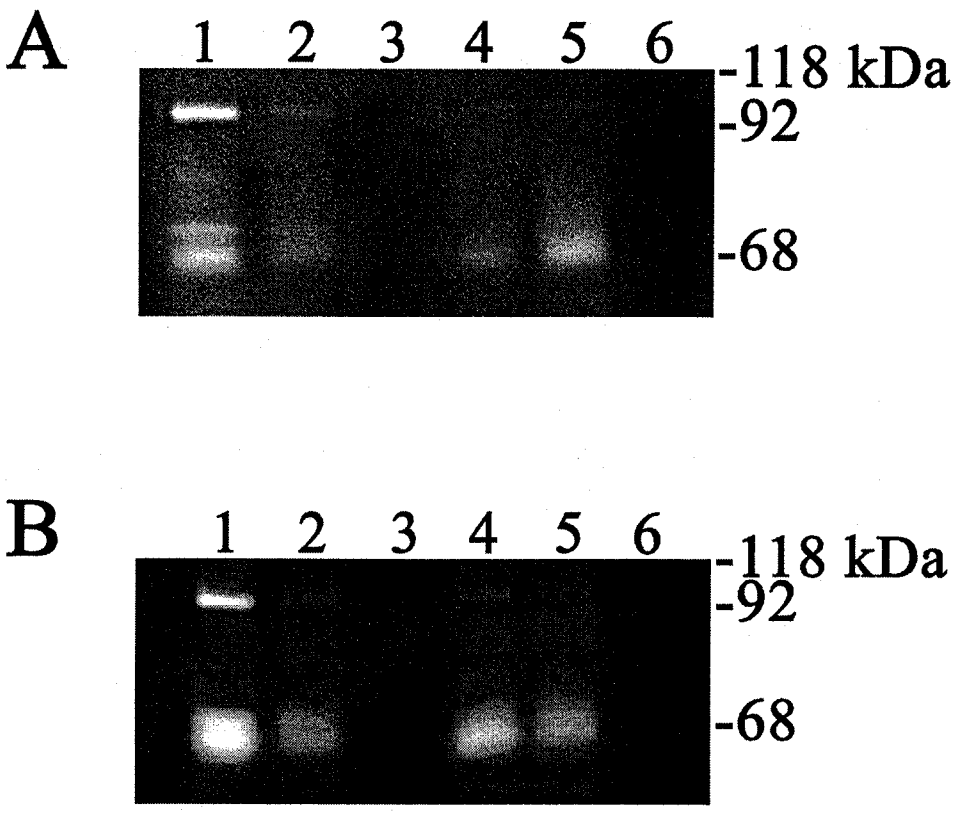

Figure 3.5. Effects of calphostin C and PD98059 treatment on anthocyanin-enriched (AN) fraction-mediated and proanthocyanidin-enriched (PAC) fraction-mediated decreases in MMP activity in DU145 cells. **A:** (1) control cells (vehicle alone), cells exposed for 3 hours to 1.0 mg/ml AN fraction (2) and 1.0 mg/ml PAC fraction (3), cells treated with 500 nM calphostin C (4), and cells treated with 500 nM calphostin C for 1 hour prior to 3 hour exposure to 1.0 mg/ml AN fraction (5) and 1.0 mg/ml PAC fraction (6). **B:** (1) control cells (vehicle alone), cells exposed for 3 hours to 1.0 mg/ml AN fraction (2) and 1.0 mg/ml PAC fraction (3), cells treated with 50 μM PD98059 (4), and cells treated with 50 μM PD98059 for 1 hour prior to 3 hour exposure to 1.0 mg/ml AN fraction (5) and 1.0 mg/ml PAC fraction (6).

Gelatinolytic activity of conditioned medium from DU145 cells pre-treated for 1 hour with MAP kinase inhibitor PD98059 (50 μM) (Segawa et al. 2001) prior to 3 hour treatment with 1.0 mg/ml anthocyanin-enriched (AN) and proanthocyanidin-enriched (PAC) fraction from lowbush blueberry is shown in Figure 3.5B. Figure 3.5B shows that MAP kinase inhibition by PD98059 alleviates the AN fraction-mediated decrease in MMP-2. Decreased MMP-9 activity by 1.0 mg/ml AN fraction remains unchanged following MAP kinase inhibition by PD98059. MAP kinase inhibition by PD98059 does not affect PAC-mediated decreases in either MMP-2 or MMP-9.

Gelatinolytic activity of conditioned medium from DU145 cells pre-treated for 1 hour with PI-3 kinase inhibitor wortmannin (300 nM) (Jiang et al. 2002) prior to 3 hour treatment with 1.0 mg/ml anthocyanin-enriched (AN) and proanthocyanidin-enriched (PAC) fraction from lowbush blueberry is shown in Figure 3.6. Figure 3.6 shows that decreases in MMP-2 and MMP-9 activity following 3 hour treatment with 1.0 mg/ml AN and PAC fraction from blueberry remain unchanged following inhibition of PI-3 kinase by wortmannin.

Since the PAC fraction from blueberry showed particular efficacy in down-regulating MMP activity in these cells, similar mechanistic experiments as outlined above were performed with lower concentrations of PAC fraction. Gelatinolytic activity of conditioned medium from DU145 cells pre-treated for 1 hour with

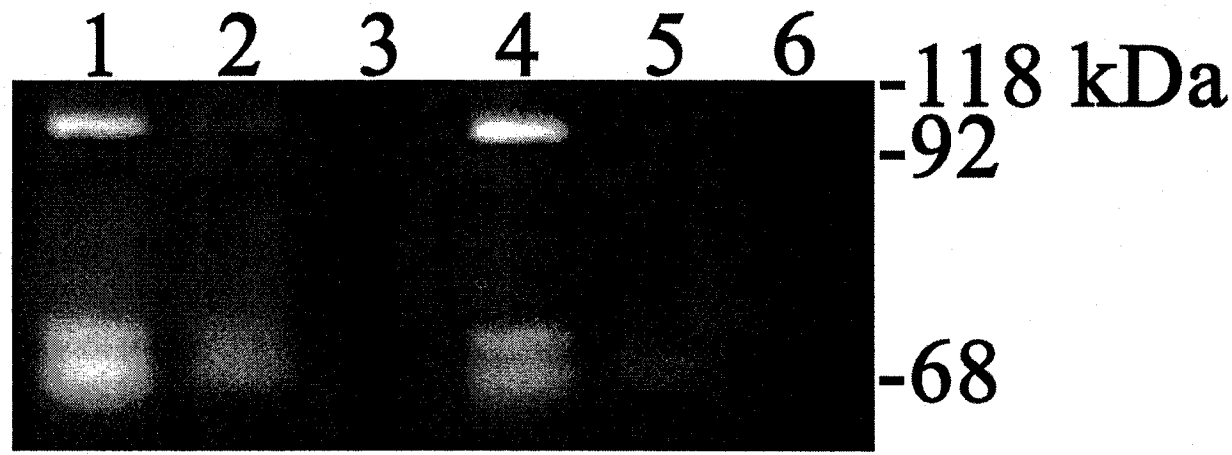

Figure 3.6. Effects of wortmannin treatment on anthocyanin-enriched (AN) fraction-mediated and proanthocyanidin-enriched (PAC) fraction-mediated decreases in MMP activity in DU145 cells. (1) control cells (vehicle alone), cells exposed for 3 hours to 1.0 mg/ml AN fraction (2) and 1.0 mg/ml PAC fraction (3), cells treated with 300 nM wortmannin (4), and cells treated with 300 nM wortmannin for 1 hour prior to 3 hour exposure to 1.0 mg/ml AN fraction (5) and 1.0 mg/ml PAC fraction (6).

PD98059 prior to 3 hour exposure to 0.1 mg/ml PAC fraction is shown in Figure 3.7A. As shown in Figure 3.7A, PAC-mediated decreases in both MMP-2 and MMP-9 were alleviated following inhibition of MAP kinase with PD98059. In particular, MMP-9 activity is drastically increased following MAP kinase inhibition.

Gelatinolytic activity of conditioned medium from DU145 cells pre-treated for 1 hour with PI-3 kinase inhibitor LY294002 (10 μM) (Sato et al. 2002) prior to 3 hour exposure to 0.1 mg/ml PAC fraction is shown in Figure 3.7B. Figure 3.7B shows that PAC-mediated decreases in both MMP-2 and MMP-9 remained unchanged following inhibition of PI-3 kinase with LY294002. Results presented in Figures 3.5-3.7 are representative of observations noted from, at a minimum, 3 separate experiments.

Additionally, caffeic acid phenyl ester (CAPE) (EMB Biosciences) (Natarajan et al. 1996) and 6-22 amide (EMB Biosciences) (Kemp et al. 1998) were used as specific inhibitors of the nuclear factor κB (NF-κB) and protein kinase A (PKA) signalling pathways, respectively in order to determine their possible involvement in these blueberry fraction mediated decreases in MMP activity. Following treatment of DU145 cells with either CAPE or 6-22 Amide the blueberry fraction mediated decreases in MMP activity remained unchanged suggesting that these blueberry fractions do not apparently use either the PKA- or NF-κB-mediated pathways in down-regulating MMP activity in these cells (data not shown).

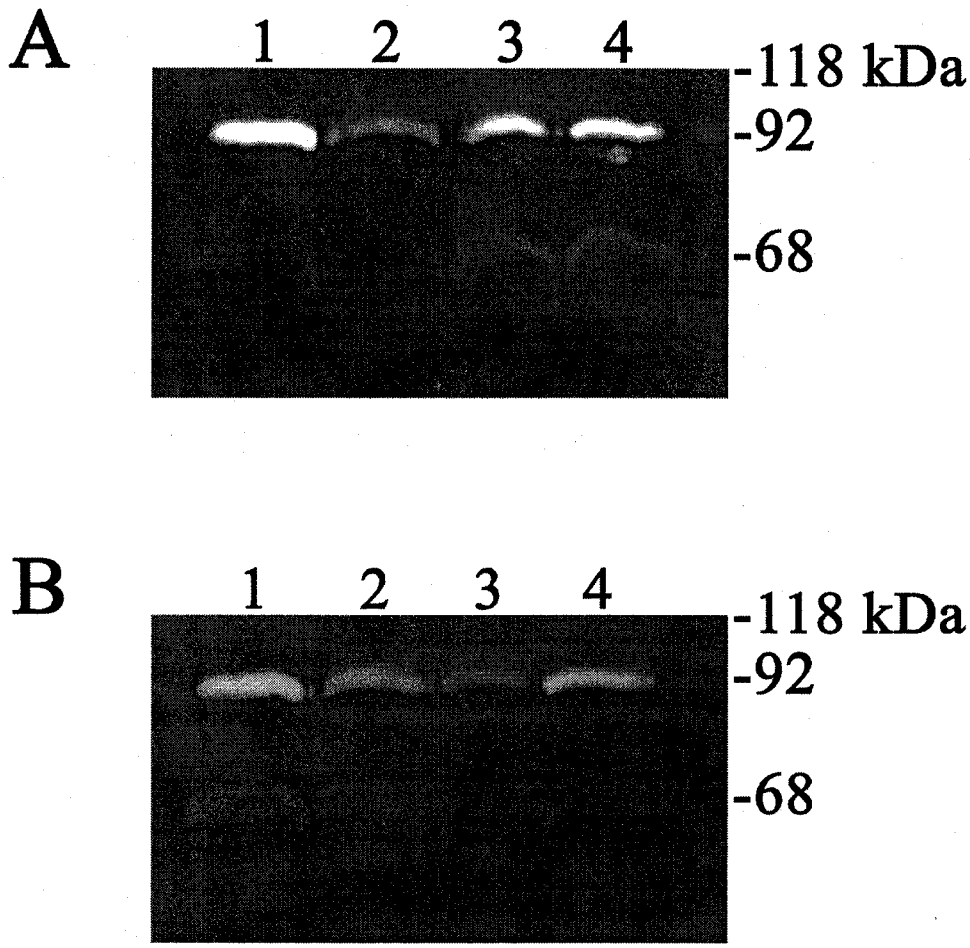

Figure 3.7. Effects of PD98059 and LY294002 treatment on proanthocyanidin-enriched (PAC) fraction-mediated decreases in MMP activity in DU145 cells. **A:** (1) control cells (vehicle alone), cells exposed for 3 hours to 0.1 mg/ml PAC fraction (2), cells treated with 50 μM PD98059 for 1 hour prior to 3 hour exposure to 0.1 mg/ml PAC fraction (3) and cells treated with 50 μM PD98059 (4). **B:** (1) control cells (vehicle alone), cells exposed for 3 hours to 0.1 mg/ml PAC fraction (2), cells treated with 10 μM LY294002 for 1 hour prior to 3 hour exposure to 0.1 mg/ml PAC fraction (3) and cells treated with 10 μM LY294002 (4).

3.5. Discussion

This study has demonstrated that MMP activity from DU145 human prostate cancer cells decreases following exposure to flavonoid-enriched fractions from lowbush blueberry, which is consistent with other studies investigating the effects of flavonoids in cancer models (Garbisa et al. 2001, Sartor et al. 2002, Sato et al. 2002). In particular, this study outlines a series of possible mechanisms involved in the blueberry flavonoid mediated decrease in MMP activity in these cells. Time course studies with "crude", AN and PAC show that there are differential responses in both MMP-2 and MMP-9 activity in response to 1, 3 and 6 hour treatments with these fractions. These results are not unexpected since the AN and PAC fraction each represent distinct subclasses of flavonoids, namely anthocyanins and proanthocyanidins, and the "crude" fraction contains all flavonoids from blueberry, which in itself may have synergistic or additive consequences. Furthermore, these results show that TIMP expression, and in particular TIMP-1, is increased following treatment with these fractions. These results are in keeping with previous results which demonstrate that EGCG and genistein, both flavonoids, also increased TIMP expression in HT1080 fibrosarcoma cells *in vitro* (Yan et al. 1999, Garbisa et al. 2001).

The use of specific inhibitors of known cellular signalling pathways demonstrates that the down-regulation of MMP activity by blueberry flavonoids observed in these cells involve specific pathways. In particular, AN fraction seems to use a competent mitogen activated protein (MAP) kinase pathway to down-regulate

MMP-2 activity in these cells. These results are similar to previous studies which report that EGCG inhibits MMP-2 *via* inhibition of MAP kinase in DU145 cells (Vayalil and Katiyar 2004). These results also demonstrate that AN fraction uses a competent protein kinase C (PKC) pathway to down-regulate MMP-2. Given that the PKC signalling cascade is upstream of MAP kinase, this demonstrates that AN fraction may mediate its effect using a complete PKC-MAP kinase signal cascade. Mechanistic studies with PAC fraction from blueberry were performed with a much lower effective concentration of PAC fraction, considering that PAC fraction is considerably more efficacious at down-regulating MMP activity in these cells. These experiments demonstrate that PAC fraction down-regulates both MMP-2 and MMP-9 activity using a competent MAP kinase pathway. This result is in keeping with previous results showing that grape seed proanthocyanidins (GSP) inhibit the expression of MMP-2 and MMP-9 in DU145 cells through inhibition of MAP kinase (Vayalil et al. 2004). Studies with inhibitors of the PI-3 kinase signal pathway were included to examine the possible use of this other common signalling cascade. These results demonstrate that neither AN nor PAC fraction from blueberry rely on a competent PI-3 kinase pathway to mediate their effects.

To the best of our knowledge, this study is the first to elucidate the possible mechanisms by which flavonoid-enriched fractions from lowbush blueberry down-regulate MMP activity in DU145 cells. Previously, it has been reported that the AN fraction used in this study contained particular anthocyanins which have been shown to be "bioactive" such as malvidin, delphidin and chlorogenic acid (Matchett et al.

2005). In this regard, Sartor et al. (2002) have demonstrated that delphinidin specifically inhibits both MMP-2 and MMP-9 activity *in vitro* (Sartor et al. 2002). EGCG, which is structurally similar to the proanthocyanidins, has been shown to effectively down-regulate MMP activity in cancer cells (Gupta et al. 1999, Garbisa et al. 2001). Furthermore, proanthocyanidins isolated from grape seed have been shown to specifically inhibit MMP activity in DU145 cells, indicating that there may be common proanthocyanidins between grape seeds and lowbush blueberries (Vayalil et al. 2004). Moreover, nobiletin, a polymethoxy flavone structurally similar to proanthocyanidins, has been shown to specifically inhibit MMP activity by inducing TIMP expression in human fibrosarcoma cells (Sato et al. 2002). Studies are ongoing to investigate the exact nature of the particular compounds in AN and PAC fractions from lowbush blueberry.

Previous results from our laboratory have shown that these blueberry fractions do not induce apoptotic or necrotic cell death in these cells, indicating that these fractions act in a target-directed manner which can not be attributed to cellular death (Matchett et al. 2005). This further supports the results presented here, namely that these fractions use specific signal transduction pathways to down-regulate MMP activity in these cells.

In conclusion, our results describe the mechanisms involved in the regulation of human prostate cancer cells by lowbush blueberry flavonoids. In particular, this regulation is the down-regulation of MMP activity and up-regulation of TIMP activity in these cells. By elucidating the mechanisms involved in this regulation,

increased understanding of the manner in which flavonoids modulate the complex regulatory mechanisms in these cells may lead to the development of potential novel anti-proliferative and anti-carcinogenic treatments for prostate cancer.

4. CHAPTER FOUR

Growth factor-mediated induction of survivin, an inhibitor of apoptosis protein (IAP), in H-ras transformed mouse fibroblasts involves phosphatidylinositol-3 kinase-, mitogen activated protein kinase-, and protein kinase C-mediated events

4.1. Abstract

The inhibitors of apoptosis (IAP) proteins have been identified as possible targets for chemotherapeutic treatment of certain cancers. A novel member of the IAP family, survivin, has been of recent interest to researchers and is thought to be one of the most important IAPs in cancer. Many previous studies have reported a correlation between survivin expression and the cancer phenotype. The current study aimed to investigate the correlation between survivin expression and the expression of a well known oncogene, H-ras, in growth factor-mediated regulation of survivin. Using H-ras transformed mouse fibroblasts, survivin protein levels were evaluated in response to basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF). Results show that basal levels of survivin expression correlated with H-ras expression, and that H-ras transformed cells showed a dose- and time-dependent induction of survivin following serum stimulation. Survivin expression was differentially induced by bFGF, PDGF, and EGF. The involvement of different signal transduction pathways was observed in growth factor-mediated survivin induction. In particular, bFGF induced survivin using a competent phosphatidylinositol-3 kinase (PI3 kinase) signaling pathway. PDGF and EGF both

used functional PI3 kinase, mitogen activated protein (MAP) kinase, and protein kinase C (PKC) signaling pathways to induce survivin in these cells. These results suggest that the survivin pathway may be a relevant target for cancer therapy.

4.2. Introduction

4.2.1. Apoptosis

Apoptosis can be defined as genetic cellular suicide characterized by such morphological characteristics as membrane blebbing, chromatin condensation, and formation of apoptotic bodies (Kerr et. al 1972). Apoptosis is crucial in the developing organism during both embryonic and fetal growth (Meier et al. 2000, Altieri 2004). The mediators of apoptosis are caspases, a family of cysteine proteases that are specifically activated in apoptotic cells (Hengartner 2000, Altieri 2004). All caspases possess a cysteine residue in the active site, and specifically cleave substrates after aspartic acid residues, hence C (cysteine) – ASP (aspartic acid) – ASE (protease) (Hengartner 2000). There have been many subfamilies of caspases identified, and each caspase's distinct substrate specificity is determined by the four amino acid residues amino-terminal to the active site (Hengartner 2000).

Two main apoptotic pathways have been identified (Hengartner 2000). The death receptor pathway, or "extrinsic pathway", is initiated by members of the death-receptor superfamily, including CD95 and tumor necrosis factor receptor-I (Hengartner 2000, Altieri 2004). Binding of the CD95 ligand (CD95L) to the CD95 death receptor (CD95) results in the aggregation of the adaptor molecule FADD (Fas-

associated death domain protein) near the cytoplasmic tail of CD95, thereby recruiting multiple procaspase-8 molecules (Hengartner 2000). These procaspase-8 molecules are then activated through induced proximity to form multiple active caspase-8 molecules, known also as FLICE (FADD-like interleukin-1 converting enzyme) molecules (Hengartner 2000). Caspase-8/FLICE activation can be blocked by the caspase homologue c-FLIP (cellular FLICE inhibitory protein) (Altieri 2004). Activated caspase-8/FLICE then activates caspase-3, an effector caspase, resulting in apoptosis (Hengartner 2000).

An "intrinsic pathway", or mitochondrial pathway, has also been identified, and is used more extensively in response to internal cues such as irreparable DNA damage (Altieri 2004). When such cues are detected by the cell, pro-apoptotic members of the Bcl-2 family of proteins localize near the surface of the mitochondria and trigger the release of key pro-apoptotic molecules such as cytochrome *c*, AIF (apoptosis inducing factor), and Smac/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI) (Hengartner 2000). Released cytochrome *c* interacts with Apaf-1 (apoptosis protease-activating factor-1) and procaspase-9 to form a holoenzyme complex known as the apoptosome (Hengartner 2000). The apoptosome then activates caspase-3, resulting in apoptotic body formation and apoptosis (Altieri 2004). Smac/DIABLO release allows apoptosis to proceed by inhibiting the activity of the inhibitor of apoptosis proteins (IAPs) (Hengartner 2000).

4.2.2. Survivin: an inhibitor of apoptosis protein (IAP)

The family of inhibitor of apoptosis proteins (IAPs) are important cell death regulators (Salvesen and Duckett 2002). The defining characteristic of the IAPs is the presence of a Baculovirus IAP repeat (BIR), which is a zinc finger motif of approximately 70 amino acids (Altieri 2004). IAPs function in both the protection of cells from apoptosis and in the regulation of cell division, and most IAPs perform the former by acting as endogenous caspase inhibitors (Salvesen and Duckett 2002). The list of IAPs continues to grow, and to date the following members have been identified: NIAP (neuronal IAP), HIAP (human IAP), cIAP1 and cIAP2 (cellular IAP 1 and 2), XIAP (X chromosome-linked IAP), BRUCE (BIR repeat containing ubiquitin-conjugating enzyme), livin, and survivin (Jaattela 1995).

Survivin is structurally unique compared to other IAPs, organized as a stable dimer containing a single BIR (Verdecia et al. 2000). Survivin expression is tightly controlled at the transcriptional level, and post-translational regulation is complex and involves proteasome- and ubiquitin-mediated degradation (Kobayashi et al. 1999, Li and Altieri, 1999, Zhao et al. 2000). Much evidence has accrued to support the role of survivin in suppressing cell death. Survivin over-expression inhibits both extrinsic and intrinsic pathway-mediated apoptosis (Kobayashi et al. 1999, Islam et al. 2000). Also, molecular antagonists of survivin such as antisense, siRNA oligonucleotides and dominant-negative mutants resulted in enhanced apoptotic stimuli and caspase-dependent cell death (Li et al. 1999, Olie et al. 2000, Kanwar et al. 2001).

The cytoprotective ability of survivin is unique, and evidence suggests that this cytoprotection is more selective than other members of the IAP family (Altieri 2004). Survivin has been shown to form a complex with caspase-9 and prevent its activation within a functional apoptosome (O'Connor et al. 2000a). Moreover, survivin antagonists induce apoptosis consistent with cytochrome *c* release, apoptosome-dependent caspase-9 activation, and increased permeability of the mitochondrial membrane (O'Connor et al. 2000a, Mesri et al. 2001, Beltrami et al. 2004, Liu et al. 2004).

4.2.3. Survivin and cancer

Among members of the IAP family, survivin has generated the most interest in apoptotic-resistant cancer cells (Jaattela 1995, Chiou et al. 2003). Survivin expression is differential in cancer *versus* non-cancer tissues (Ambrosini et al. 1997). Dramatic over-expression of survivin has been demonstrated in many human tumors using *in situ* hybridization, RT-PCR, immunohistochemistry, and Western blotting (Altieri 2003). Selective over-expression of survivin in cancer appears to involve cancer-specific transcription of the survivin promoter, along with deregulation of many oncogenic pathways, including growth factor receptor signalling, STAT activation, PI3 kinase/Akt signalling, ras expression, and loss of p53 tumor suppressor molecules (Tran et al. 1999, Hoffman et al. 2001, Bao et al. 2002, Mirza et al. 2002, Aoki et al. 2003, Sommer et al. 2003, Dan et al. 2004).

Many growth factors and cytokines have been shown to regulate survivin expression in both normal and cancerous cells and tissues (Jaattela 1995, Chiou et al. 2003). Survivin expression in human umbilical vein endothelial cells (HUVECs) was shown to be induced by the mitogenic growth factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (O'Connor et al. 2000b). In this same study, the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) elicited no change in survivin expression, while IL-11 increased survivin gene transcription (thereby increasing survivin mRNA) in HUVECs (O'Connor et al. 2000b, Mahboubi et al. 2001). This emphasizes the differential and cytokine/growth factor-specific regulation of survivin in these cells.

4.2.4. H-ras and metastatic potential

H-ras is a well-known oncogene that is believed to contribute to the metastatic or malignant potential of neoplastic cells (Egan et al. 1988). It has been reported that H-ras expression is directly correlated with the formation of metastases, and that H-ras transformed cells have an increased malignant potential (Egan et al. 1988). Given the above reports, the current study was aimed at investigating the effects of mitogenic growth factors on the expression of survivin in transformed mouse lung fibroblast cell lines. These cell lines differ in the expression of the H-ras oncogene, and therefore display different cellular phenotypes (Egan et al. 1988). The working hypothesis is that mitogenic growth factors will up-regulate survivin expression, and that this up-regulation will correlate with H-ras expression. Furthermore, using

specific inhibitors of common cellular signal transduction pathways, possible mechanism(s) involved in the growth factor-mediated induction of survivin in these cells will be investigated.

4.3. Materials and methods

4.3.1. Cell culture and treatment

Normal and H-ras transformed mouse fibroblasts (10T½ and NR3) were used in this study. The properties of these cells represent different stages of neoplastic development. 10T1/2, which has no H-ras expression, represents the "normal" cellular phenotype, and NR3, which has low levels of H-ras expression, represents the cellular phenotype consistent with benign tumour formation (with no metastatic potential) (Egan et al. 1988). Cells were routinely cultured on 100 mm plastic tissue culture dishes (Becton Dickinson Labware, Franidin Lakes, NJ) in α-minimal essential medium (MEM) (Invitrogen, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone/VWR Canlab, Mississauga, ON) and were grown at 37°C in 5% CO_2. Cells were cultured to subconfluence, and were then exposed to defined medium (DM), a serum-free medium, which contained 4.0 μg/ml transferrin (Sigma, Oakville, ON) and 2.0 μg/ml insulin (Sigma, Oakville, ON) in α-MEM, for 24 hours. Afterwards, cells were exposed to DM with or without FBS (0, 10, or 20% vol/vol) or the growth factors bFGF (10 ng/ml), PDGF (5 ng/ml), or EGF (20 ng/ml) (R&D Systems, Inc., Minneapolis, MN) for predetermined times. Cells were removed from the surface of tissue culture plates using trypsin (Sigma, Oakville, ON). To

evaluate the possible signal transduction pathways involved, cells were pre-treated for 1 hour with either the phosphatidylinositol-3 (PI-3) kinase inhibitor LY294002 (Sigma, Oakville, ON), the protein kinase C (PKC) inhibitor calphostin C (Sigma, Oakville, ON), or the mitogen activated protein (MAP) kinase inhibitor PD98059 (Sigma, Oakville, ON), respectively, prior to exposure to growth factors.

4.3.2. SDS-PAGE and Western analysis

Western analysis was performed to analyse the changes in survivin protein expression (Burnette 1981). Cells were harvested and lysed in cell lysate buffer (10 mM Tris-base, 1 mM PMSF). Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Mississauga, ON). Equal amounts of protein (between 50-100 μg) were electrophoresed on 10% SDS-polyacrylamide gels and transferred for 1.5-2 hours onto 0.2 μm nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON). A protein enhancer kit (BioLynx, Brockville, ON) was used to enhance protein binding. Western blots were blocked overnight (4°C) in Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl) containing 1% bovine serum albumin (BSA), followed by incubation in TBS supplemented with 0.05% Tween-20 (Sigma, Oakville, ON) (TBS-T) and survivin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:100 dilution. Blots were washed with TBS-T and incubated in 1:500 diluted alkaline phosphatase (AP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1-2 hours at room temperature. Following a second wash in TBS-T, blots were developed using

standard colorimetric protocol in bicarbonate buffer (0.213 mM $MgCl_2$, 10 mM $NaHCO_3$, pH 9.8) containing nitro-blue tetrazolium (NBT) (Sigma, Oakville, ON) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma, Oakville, ON). As a loading control, the housekeeping protein β-actin was used. Identically loaded complementary membranes were incubated with β-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:100 dilution followed by incubation in 1:500 β-actin secondary and processed as described above.

4.4. Results

4.4.1. Serum stimulation of survivin protein expression

Many cell types are growth-stimulated in the presence of serum. The presence of many different growth factors and cytokines in serum accounts for much of this stimulatory effect. Serum stimulation of survivin protein expression in 10T1/2 and NR3 cells was investigated to determine if the correlation between H-ras expression and survivin protein expression was evident following serum stimulation. Figure 4.1 shows the level of survivin protein in 10T1/2 cells following 48 hour stimulation with 0, 10, and 20% fetal bovine serum (FBS). As seen in Figure 4.1, survivin protein expression remains unchanged following exposure to 0, 10, or 20% FBS, respectively, for 48 hours. Additionally, survivin protein expression in 10T1/2 cells following 24 hour exposure to FBS was investigated, and was shown to be unresponsive following exposure to 10 and 20% FBS (data not shown). Conversely, Figure 4.2 shows the level of survivin protein expression in NR3 cells in response to 24 hour serum

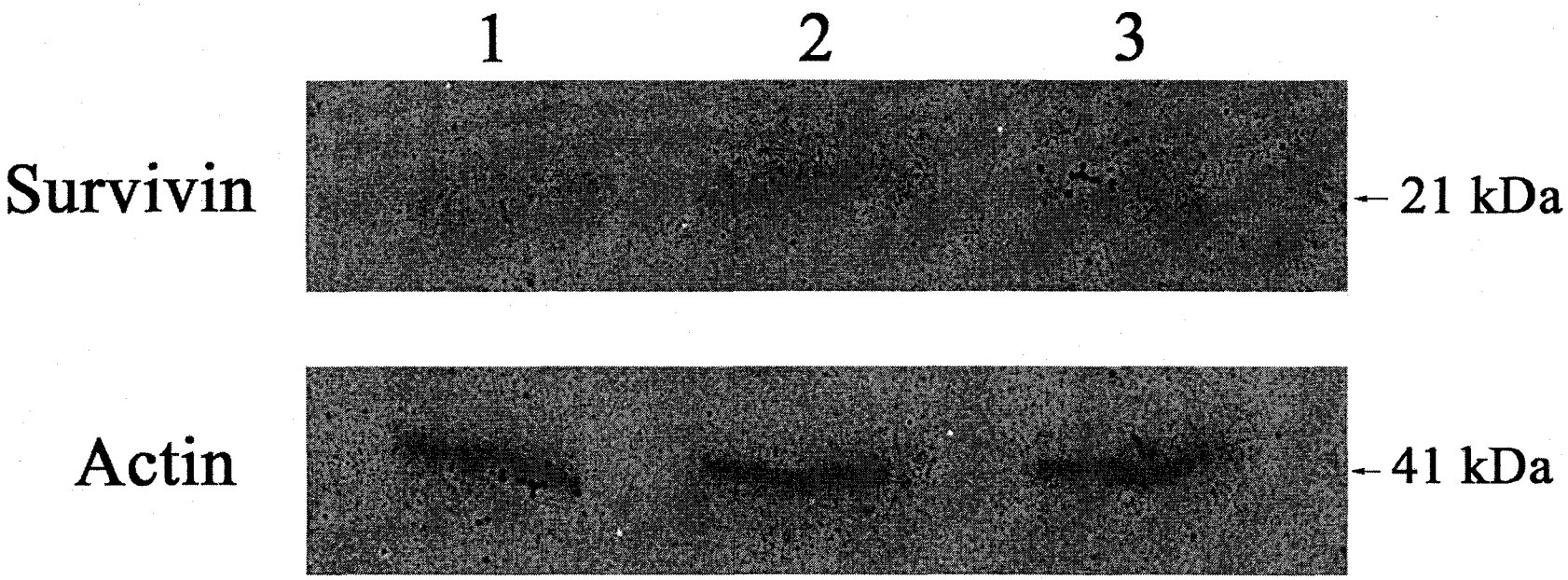

Figure 4.1. Western blot showing survivin expression in 10T1/2 cells following 48 hour treatment with serum (FBS). Survivin expression in cells cultured in the absence of serum is shown in (1) and survivin expression in cells following 24 hour exposure to 10% serum is shown in (2), and 20% serum is shown in (3). 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

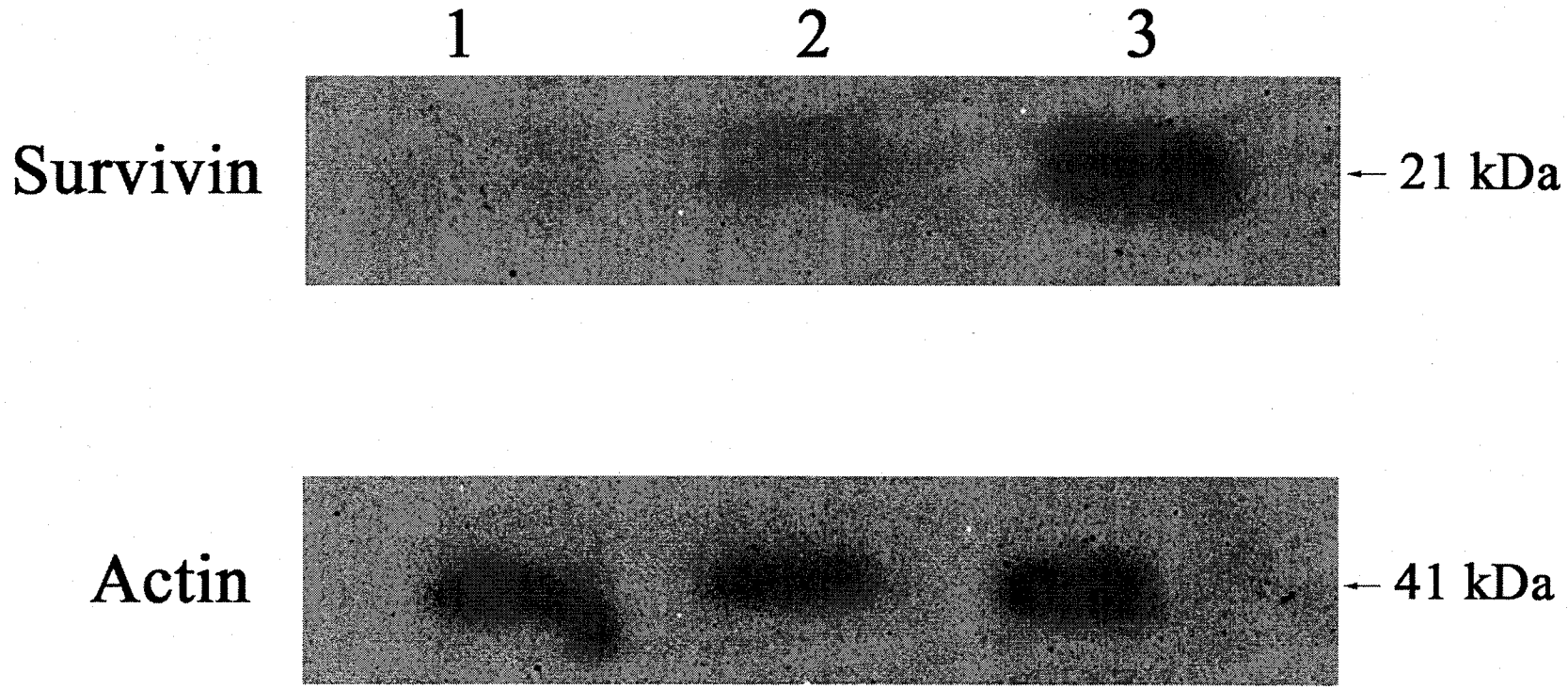

Figure 4.2. Western blot showing survivin expression in NR3 cells following 24 hour treatment with serum (FBS). Survivin expression in cells cultured in the absence of serum is shown in (1) and survivin expression in cells following 24 hour exposure to 10% serum is shown in (2), and 20% serum is shown in (3). 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

stimulation. As seen in Figure 4.2, survivin protein expression is markedly induced following 24 hour exposure to 10% FBS, while 24 hour exposure to 20% FBS resulted in a dramatic induction of survivin in these cells. It is also important to note that basal levels of survivin protein expression in 10T1/2 cells (Figure 4.1, Lane 1) and NR3 cells (Figure 4.2, Lane 1) show that the H-ras transformed NR3 cell line has a higher level of basal survivin protein expression, further supporting the correlation between ras expression and survivin protein expression. Survivin protein expression in NR3 cells following exposure to 20% serum for 1, 3, and 6 hours is shown in Figure 4.3. Figure 4.3 demonstrates that survivin protein levels are rapidly and markedly induced following 1 hour exposure to 20% FBS, and that this induction continues to occur following 3 and 6 hour exposure to FBS, in a time-dependent manner.

4.4.2. Effects of mitogenic growth factors on survivin protein expression

Many different growth factors and cytokines act to synergistically account for the stimulatory effect of serum on cells. This study investigated the effects of three particular mitogenic growth factors present in serum, bFGF, PDGF, and EGF, on survivin protein expression in NR3 cells. bFGF is an angiogenic growth factor with many cell type-specific functions (Teh et al. 2004). In cancer cells, bFGF serves as a vital modulator of cell survival and is secreted during tumor growth and angiogenesis (Teh et al. 2004). PDGF is an important angiogenic growth factor, with a plethora of biological functions (Levitzki 2004). PDGF and its receptor (PDGF-R) play an

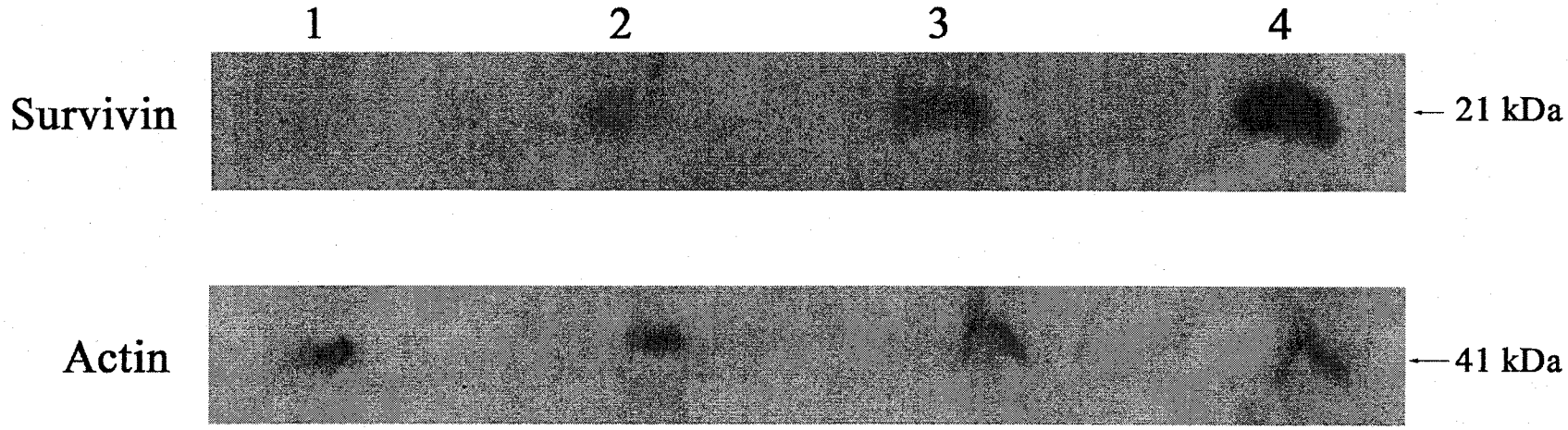

Figure 4.3. Western blot showing survivin expression in NR3 cells following 1, 3, and 6 hour treatment of serum (FBS). Survivin expression in cells cultured in the absence of serum is shown in (1) and survivin expression in cells exposed to 20% serum for 1 hour is shown in (2), 3 hours is shown in (3), and 6 hours is shown in (4). 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

important role in many cancers, such as lung and prostate cancer, through increased signalling activity in the PDGF/PDGF-R axis (Levitzki 2004). EGF is crucial in the formation and propagation of many tumors through its effects on cell signalling pathways, cellular proliferation, control of apoptosis, and angiogenesis (Lockhart and Berlin 2005). As such, EGF is an attractive target for the development of anti-cancer therapies (Lockhart and Berlin 2005).

The effects of 6 hour treatment of NR3 cells with bFGF (10 ng/ml), PDGF (5 ng/ml), and EGF (20 ng/ml) on survivin protein levels in these cells is shown in Figure 4.4. Survivin protein expression is markedly and differentially increased by bFGF, PDGF, and EGF. PDGF appears to be the most efficacious at inducing survivin protein expression, with a marked induction of survivin protein levels observed compared to control.

4.4.3. Effects of cellular signalling inhibitors on growth factor-mediated induction of survivin

Specific inhibitors of common cellular signalling pathways were used to evaluate the possible mechanism(s) involved in growth factor-mediated induction of survivin in these cells. Survivin protein expression in NR3 cells pre-treated for 1 hour with MAP kinase inhibitor PD98059 (50 μM) (Segawa et al. 2001), PKC inhibitor calphostin C (500 nM) (Chen et al. 1998), and PI3 kinase inhibitor LY294002 (10 μM) (Sato et al. 2002) prior to 6 hour treatment with 10 ng/ml bFGF is shown in Figure 4.5. As shown in Figure 4.5, the inhibition of PI3 kinase by LY294002

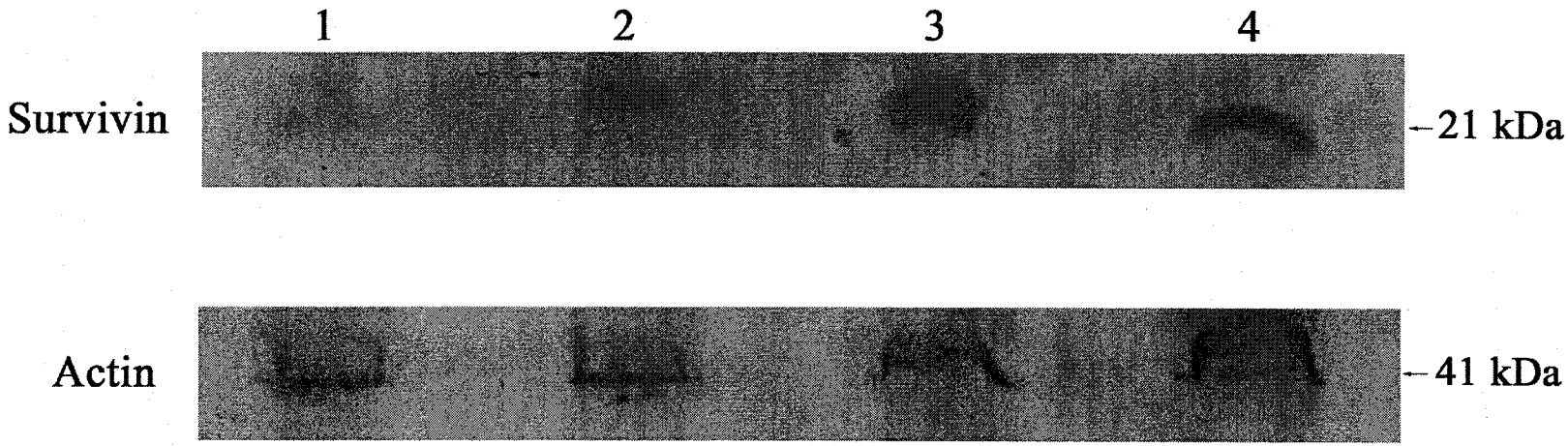

Figure 4.4. Western blot showing survivin expression in NR3 cells following 24 hour exposure to bFGF, PDGF, and EGF. Survivin expression in cells cultured in the absence of growth factor is shown in (1) and survivin expression in cells exposed to 10 ng/ml bFGF is shown in (2), 5 ng/ml PDGF is shown in (3), and 20 ng/ml EGF is shown in (4). 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

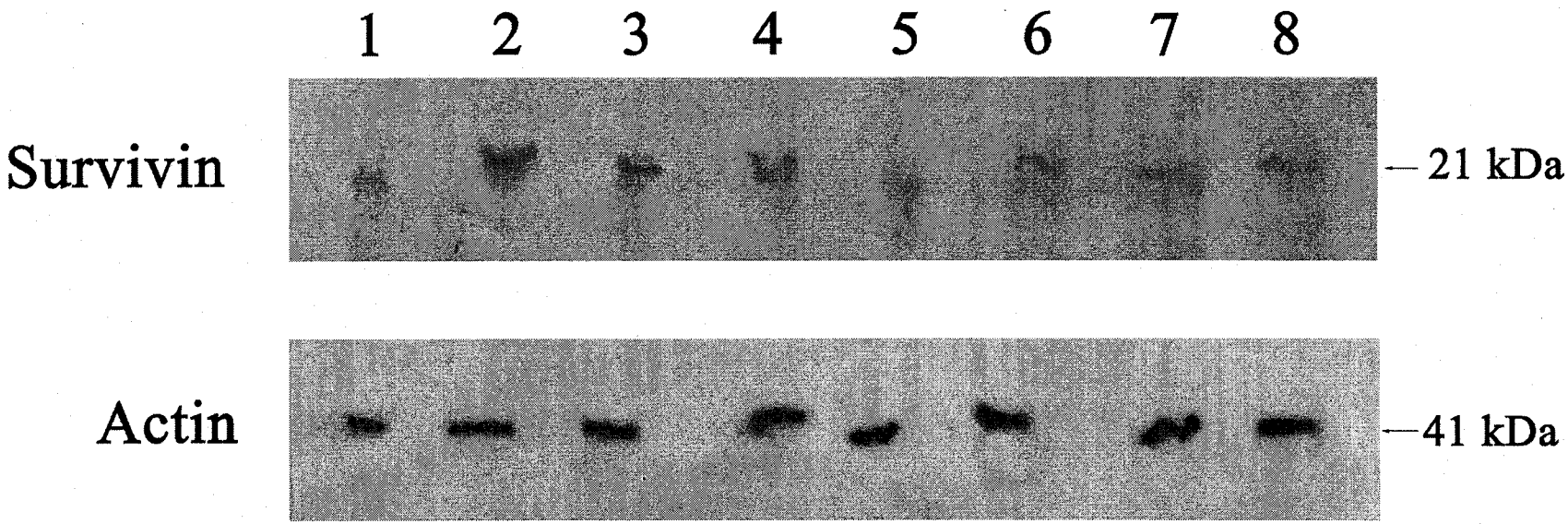

Figure 4.5. Western blot showing the effects of signal transduction inhibitors on bFGF-mediated induction of survivin in NR3 cells. Survivin expression in NR3 cells pre-treated with PD98059 (50 μM), calphostin C (500 nM), and LY294002 (10 μM) prior to exposure to bFGF (10 ng/ml) is shown. (1) control cells (vehicle alone), 6 hour exposure to bFGF (2), cells pre-treated for 1 hour with PD98059 prior to 6 hour treatment with bFGF (3), cells pre-treated for 1 hour with calphostin C prior to 6 hour treatment with bFGF (4), cells pre-treated for 1 hour with LY294002 prior to 6 hour treatment with bFGF (5), and cells treated with PD98059 alone (6), calphostin C alone (7), and LY294002 alone (8). 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

effectively abolishes the bFGF-mediated induction of survivin in these cells. Elevated survivin expression is still noted in these cells in response to bFGF following inhibition of PKC and MAP kinase. These results suggest that only a functional PI3 kinase pathway is involved in the bFGF-mediated induction of survivin in these cells, and that PKC and MAP kinase signalling pathways are apparently not involved in this induction.

Survivin protein expression in NR3 cells pre-treated for 1 hour with PD98059 (50 μM), calphostin C (500 nM), and LY294002 (10 μM) prior to 6 hour treatment with 5 ng/ml PDGF is shown in Figure 4.6. As shown in Figure 4.6, the involvement of a functional PI3 kinase signalling pathway is necessary for PDGF-mediated survivin induction in these cells. Furthermore, following inhibition of MAP kinase by PD98059, an alleviation of PDGF-mediated survivin induction is also noted, demonstrating the involvement of MAP kinase in PDGF-mediated regulation of survivin. Finally, the minor involvement of a functional PKC pathway is evident, with a slight alleviation of PDGF-mediated survivin induction noted following pre-treatment with calphostin C.

Survivin protein expression in NR3 cells pre-treated for 1 hour with PD98059 (50 μM), calphostin C (500 nM), and LY294002 (10 μM) prior to 6 hour treatment with 20 ng/ml EGF is shown in Figure 4.7. As shown in Figure 4.7, the involvement of a functional PI3 kinase signalling pathway is necessary for EGF-mediated survivin induction in these cells. Furthermore, following inhibition of MAP kinase by PD98059, an alleviation of EGF-mediated survivin induction is also noted,

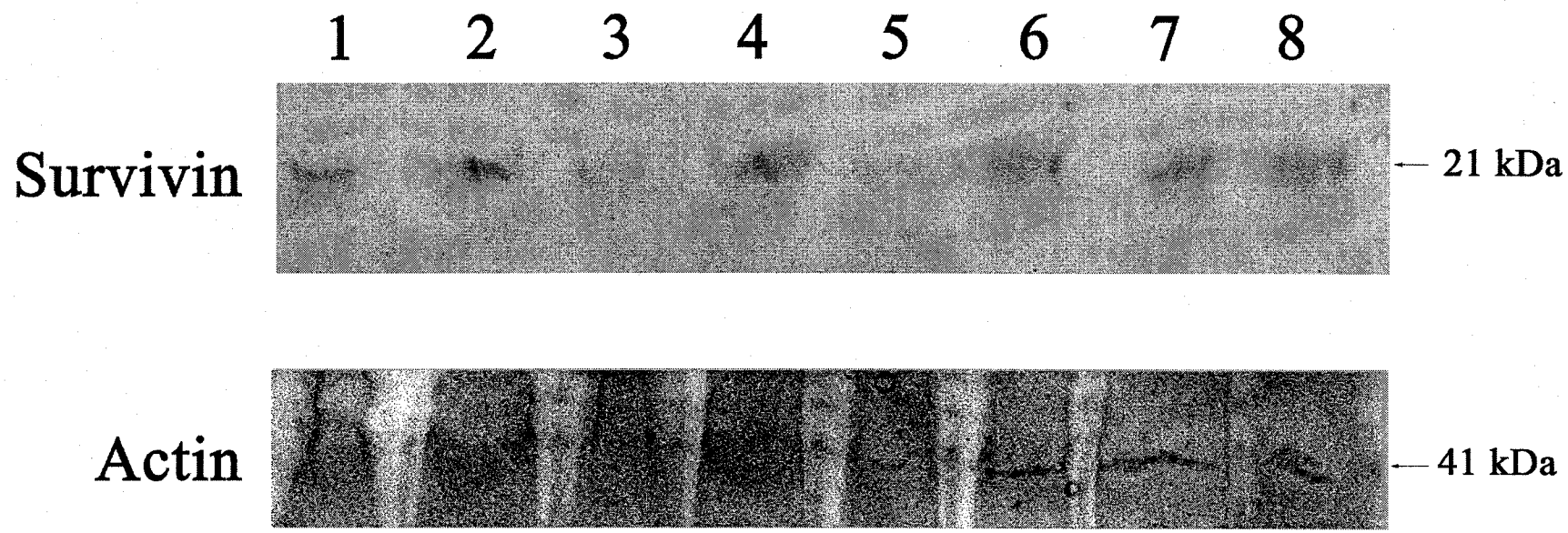

Figure 4.6. Western blot showing the effects of signal transduction inhibitors on PDGF-mediated induction of survivin in NR3 cells. Survivin expression in NR3 cells pre-treated with PD98059 (50 μM), calphostin C (500 nM), and LY294002 (10 μM) prior to exposure to PDGF (5 ng/ml) is shown. (1) control cells (vehicle alone), 6 hour exposure to PDGF (2), cells pre-treated for 1 hour with PD98059 prior to 6 hour treatment with PDGF (3), cells pre-treated for 1 hour with calphostin C prior to 6 hour treatment with PDGF (4), cells pre-treated for 1 hour with LY294002 prior to 6 hour treatment with PDGF (5), and cells treated with PD98059 alone (6), calphostin C alone (7), and LY294002 alone (8). 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

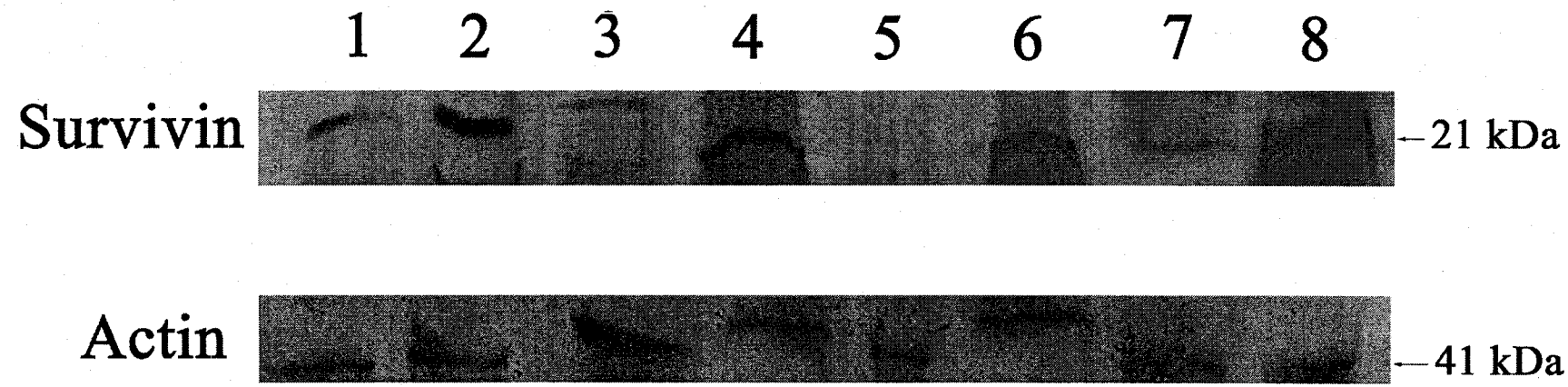

Figure 4.7. Western blot showing the effects of signal transduction inhibitors on EGF-mediated induction of survivin in NR3 cells. Survivin expression in NR3 cells pre-treated with PD98059 (50 μM), calphostin C (500 nM), and LY294002 (10 μM) prior to exposure to EGF (20 ng/ml) is shown. (1) control cells (vehicle alone), 6 hour exposure to EGF (2), cells pre-treated for 1 hour with PD98059 prior to 6 hour treatment with EGF (3), cells pre-treated for 1 hour with calphostin C prior to 6 hour treatment with EGF (4), cells pre-treated for 1 hour with LY294002 prior to 6 hour treatment with EGF (5), and cells treated with PD98059 alone (6), calphostin C alone (7), and LY294002 alone (8). 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

demonstrating the involvement of MAP kinase in EGF-mediated regulation of survivin. Finally, the minor involvement of a functional PKC pathway is evident, with a slight alleviation of EGF-mediated survivin induction noted following pre-treatment with calphostin C.

4.5. Discussion

The aims of this study were to determine the interaction between H-ras and different mitogenic growth factors in the regulation of survivin expression, and it is evident that a relationship is indeed present within these cells. In particular, when comparing the data presented in Figure 4.1 (serum stimulation of survivin in 10T1/2 cells) and Figure 4.2 (serum stimulation of survivin in NR3 cells), a correlation between H-ras and survivin expression is evident, as basal levels of survivin in10T1/2 cells (Figure 4.1, Lane 1) are much lower than those in NR3 cells (Figure 4.2, Lane 1). Furthermore, survivin expression is highly induced by serum in NR3 cells, while no response was noted in 10T1/2 cells. Given the unresponsiveness of survivin in the non-transformed 10T1/2 cell line after 24 and 48 hour stimulation with serum, no further experiments were conducted with these cells, and the focus shifted entirely to the transformed NR3 cells. Following shorter exposure times (1, 3, and 6 hours) to 20% serum, survivin expression in NR3 cells exhibited a time-dependent induction, with appreciable induction occurring after 1 hour exposure to serum.

The effect of bFGF, PDGF, and EGF on survivin expression in NR3 cells was investigated, since these growth factors are major mitogenic factors present in serum.

As reported in Figure 4.3, survivin expression in NR3 cells is highly induced by bFGF, PDGF, and EGF. These results are in keeping with previous work that has shown that bFGF, along with vascular endothelial growth factor (VEGF), can induce survivin expression up to 16-fold in HUVEC cells (O'Connor et al. 2000b). Furthermore, PDGF has been shown to up-regulate survivin expression in vascular smooth muscle cells (VSMCs), thereby promoting angiogenesis and vessel-wall remodelling (Blanc-Brude et al. 2002). Moreover, EGF has been shown on many occasions to stimulate cell growth and suppress apoptosis, through the over-expression of the EGF receptor (EGF-R) and increased EGF signalling (Boonstra et al. 1995, Murphy et al., 2001, Matsuda et al. 2002).

The use of specific inhibitors of known cellular signalling pathways demonstrates that the bFGF-, PDGF-, and EGF-mediated up-regulation of survivin in NR3 cells involve specific pathways. In particular, all growth factors appear to regulate survivin expression using a competent PI3 kinase pathway. These results are consistent with numerous studies which show the involvement of the PI3 kinase/Akt pathway in the regulation of survivin and apoptosis in other cell types (Papapetropoulos et al. 2000, Dan et al. 2004). Akt (also known as protein kinase B) is a serine-threonine protein kinase which is a critical mediator of cell survival and has been implicated in normal cell growth, cell cycle progression, and apoptosis (Datta et al. 1999). There are more than 30 Akt substrates identified to date, and these phosphorylation events mediate the biological effects observed in Akt-mediated signalling (Kim et al. 2005). Since Akt is a downstream effector protein of PI3

kinase, it is suggested that impaired PI3 kinase function would alter Akt-mediated cellular survival (Chaudhary and Hruska 2001). Furthermore, the link between expression of the ras oncogene and the PI3 kinase/Akt pathway has been well examined (Kauffman-Zeh et al. 1997, Jin et al. 2003). Suppression of apoptosis by another oncogene, c-myc, has been reported in mesenchymal cells by over-expression of ras *in vitro* (Kauffmann-Zeh et al. 1997).

The involvement of the MAP kinase signalling pathway is also evident in the bFGF-mediated induction of survivin expression in NR3 cells. In particular, bFGF was shown to use a competent MAP kinase pathway to induce survivin expression in these cells. This result reflects previous findings, where bFGF-mediated survivin induction in MCF-7 human breast cancer cells was shown to involve a MAP kinase-dependent signal transduction event (Teh et al. 2004). Also, bFGF was shown to modulate survivin expression in human osteoblastic cells by activating MAP kinase (Chaudhary and Hruska 2001).

PDGF-mediated survivin induction was alleviated by the MAP kinase inhibitor, PD98059, supporting the role of MAP kinase in PDGF-mediated survivin regulation in NR3 cells. Similar results have been previously reported, where PDGF induced survivin expression in human osteoblastic cells through PI3 kinase/Akt-dependent MAP kinase activation (Chaudhary and Hruska 2001). Furthermore, a minor involvement of PKC was noted in the PDGF-mediated induction of survivin in these cells, since survivin induction was slightly alleviated following treatement with calphostin C.

In summary, this study reports a link between survivin expression and the mitogenic growth factors bFGF, PDGF, and EGF in H-ras transformed mouse fibroblasts. Specifically, it is shown here that the PI3 kinase/Akt signal transduction pathway is integral in bFGF-, PDGF-, and EGF-mediated induction of survivin in H-ras transformed mouse fibroblasts, and that involvement of the MAP kinase and PKC pathways are also important in PDGF- and EGF-mediated induction of survivin in these cells. These results suggest that survivin, and more specifically the survivin-inducing PI3 kinase/Akt, MAP kinase, and PKC pathways, may be relevant targets for anti-cancer and pro-apoptotic interventions.

5. CHAPTER FIVE

Insulin-like growth factor (IGF)-1- and -2-mediated induction of ornithine decarboxylase (ODC) and spermidine/spermine N^1–acetyltransferase (SSAT) in H-ras transformed mouse fibroblasts involves protein kinase C- and mitogen activated protein kinase-mediated events

5.1. Abstract

The polyamines (putrescine, spermidine, and spermine) are ubiquitous low-molecular weight aliphatic amines that play roles in both cell proliferation and differentiation. Polyamine biosynthesis is accomplished through the activity of ornithine decarboxylase (ODC), a key rate-limiting enzyme in the polyamine biosynthetic pathway. Conversely, spermidine/spermine N^1-acetyltransferase (SSAT) is a rate-limiting enzyme in the catabolism of polyamines. The insulin-like growth factor (IGF) system plays an important role in the growth and development of many tissues and regulates overall growth and has also been implicated in tumorigenesis. The aim of this study was to investigate the possible link between IGF-mediated signalling during H-ras mediated cellular transformation and the expression of ODC and SSAT. Using untransformed and H-ras transformed mouse fibroblast cells (10T1/2 and NR3), ODC and SSAT protein expression was analyzed in response to exposure to IGF-1 and IGF-2 *in vitro*. Results show that ODC and SSAT protein levels are differentially induced in response to exposure to IGF-1 and IGF-2 for 1-24 hours in NR3 cells, while no response was observed in 10T1/2 cells. Furthermore,

studies showed that involvement of protein kinase C (PKC) and mitogen activated protein (MAP) kinase are necessary in IGF-mediated induction of ODC and SSAT in NR3 cells. These results indicate a part of the altered growth regulatory program which results following ras-mediated cellular transformation.

5.2. Introduction

5.2.1. Polyamine biosynthesis

In mammalian cells, *de novo* synthesis of polyamines is accomplished from the polyamine precursor amino acid, ornithine. Ornithine is an amino acid which is not commonly found in mammalian proteins, and is notable for its role in the metabolism of nitrogen and the formation of urea (urine) (Urdiales et al. 2001). Putrescine (the initial polyamine) is directly synthesized in mammalian cells from ornithine through the activity of ornithine decarboxylase (ODC; EC 4.1.1.17), a key rate-limiting enzyme in the polyamine biosynthetic pathway. ODC is a pyridoxal phosphate (PLP)–requiring enzyme with a monomeric structure consisting of an N-terminal β/α-barrel and a C-terminal β-sheet (Hillary and Pegg 2003). Mammalian ODC is an obligate homodimer with two identical active sites formed at the interface between each 51 kDa dimer (Auvinen 1997, Hillary and Pegg 2003). ODC has a very short half-life, and the major player in ODC turnover is the protein antizyme (AZ) (Coffino 2001, Hillary and Pegg 2003).

The conversion of ornithine to putrescine is accomplished by ODC through the decarboxylation of ornithine, which produces putrescine and releases CO_2 (Shantz

and Pegg 1999). Putrescine accumulation drives forward the reactions responsible for synthesis of the other polyamines, spermidine and spermine (Shantz and Pegg 1999). These reactions are simple transfers of aminopropyl groups to each precursor polyamine from an aminopropyl donor, decarboxylated S-adenosylmethionine (dcSAM) (Shantz and Pegg 1999). dcSAM is generated in cells from the amino acid methionine through the activity of S-adenosylmethionine decarboxylase (SAMDC). SAMDC is another important regulatory enzyme in the biosynthetic pathway of polyamines, and its regulation is a key determinant in the overall *de novo* synthesis of polyamines (Shantz and Pegg 1999, Urdiales et al. 2001, Hillary and Pegg 2003).

5.2.2. Polyamine interconversion and degradation

Another way to increase intracellular polyamine levels is through the polyamine interconversion pathways (Shappell et al. 1993, Shantz and Pegg 1999, Hillary and Pegg 2003). The interconversion of spermine to spermidine and spermidine to putrescine represents an alternative method for generating specific polyamines when required (Urdiales et al. 2001). The interconversion of polyamines is essentially a two-part cycle, the first is a part of the polyamine biosynthetic pathway outlined above (Shappell et al. 1993, Shantz and Pegg 1999, Hillary and Pegg 2003). This portion of the interconversion pathway revolves around the activity of spermidine and spermine synthase, which are both considered to be irreversible reactions, and ultimately results in the synthesis of spermidine from putrescine and spermine from spermidine, respectively (Shantz and Pegg 1999, Hillary and Pegg

2003). The second part of the cycle proceeds in the opposite direction, generating spermidine from spermine and putrescine from spermidine, respectively (Shappell et al. 1993, Shantz and Pegg 1999). These reactions occur through the activity of two enzymes: spermidine/spermine N^1–acetyltransferase (SSAT; EC 2.3.1.57) and polyamine oxidase (PAO).

The first enzyme in this pathway, SSAT, is a key regulatory enzyme in the regulation of intracellular polyamine pools (Shappell et al. 1993). Mammalian SSAT is a tetramer with four identical subunits of molecular weight 20000 (Parry et al. 1995). SSAT is a transferase which acetylates polyamines (spermine or spermidine) producing acetylated derivatives of these polyamines (Shappell et al. 1993). Basal levels of SSAT in many mammalian cells are extremely low (>100 times lower than those of ODC and SAMDC). This presents a challenge when trying to characterize the expression and regulation of SSAT (McCloskey et al. 1999). It also underscores the important role that SSAT plays in polyamine regulation (Shappell et al. 1993, McCloskey et al. 1999). By catalysing the acetylation of spermidine and spermine into *N*–acetylspermidine and *N*–acetylspermine, respectively, SSAT prepares these polyamines for further interconversion or export out of the cell (Urdiales et al. 2001). SSAT regulation is multi-levelled and complex, and further discussion in this review will demonstrate this point (Shappell et al. 1993, Vujcic et al. 2000, Wang et al. 2001). Upon formation of the acetylated polyamine derivatives by SSAT, the final part of the interconversion pathway relies on the activity of polyamine oxidase (PAO), to generate spermidine from spermine and putrescine from spermidine, respectively

(Urdiales et al. 2001). PAO activity is not as tightly controlled as that of SSAT, indicating that the rate-limiting step in polyamine interconversion is that catalysed by SSAT (Shappell et al. 1993).

5.2.3. Regulation of polyamine metabolism

5.2.3.1. Regulation of polyamine biosynthesis – ODC regulation

As previously stated, the ability of cells to maintain polyamine levels within a narrow range is integral to regulate cell growth and proliferation. Specific regulation of key processes in polyamine metabolism is necessary to achieve regulation of the system as a whole. One of the major regulatory processes in polyamine metabolism is the activity of ODC in the biosynthesis of polyamines (Heby and Persson 1990, Hillary and Pegg 2003).

5.2.3.2. Transcriptional and post-transcriptional regulation of ODC

At the transcriptional level, many cytokines have been shown to mediate ODC gene expression and ODC mRNA synthesis. The interleukin (IL) family of cytokines has a plethora of functions in mammalian cells, from inducing cellular growth and differentiation to mediating inflammatory responses (Goldsby 2000). IL-1β was shown to induce ODC activity in insulin-producing rat cells, through either a direct increase in ODC mRNA production or through IL-1β-mediated induction of the ODC protein (Smismans et al. 2000). Also, IL-3 was shown to induce ODC gene transcription in murine myeloid cells through the activity of c-Myc, which initiates

transcription (Packham and Cleveland 1997). Furthermore, IL-1β and tumor necrosis factor (TNF)-α have been shown to individually induce ODC gene transcription in mouse tissues, and together create a synergistic effect that further augments ODC mRNA levels (Endo et al. 1988, Endo 1989). In contrast, IL-1α has been shown to induce antizyme expression, by increasing antizyme mRNA, and thereby decreasing ODC protein synthesis and activity in human melanoma cells (Yang et al. 1997).

ODC regulation is also mediated at the transcriptional level by an increasing number of growth factors. Platelet-derived growth factor (PDGF) is a growth factor that has many functions in mammalian cells, such as affecting membrane flexibility in capillaries (Levitzki 2004). PDGF was shown to induce ODC gene transcription in mouse fibroblast cells by increasing ODC mRNA (Vicenzi et al. 1985). Also, PDGF was shown to induce ODC expression in H-ras transformed mouse fibroblasts by increasing ODC gene transcription and stabilizing ODC mRNA (Voskas et al. 2001a). Nerve growth factor (NGF), which is essential for the survival of sympathetic and sensory neurons, has been shown to increase ODC activity in primary neuronal cultures by increasing ODC mRNA synthesis (Marschall and Feinstein 1995, Gilbert 2003). Hepatocyte growth factor (HGF) is a growth factor that can, among other things, return hepatocytes to the cell cycle, which is important in liver regeneration (Gilbert 2003). HGF can induce ODC gene transcription in mouse melanoma cells, by triggering the AP-1 pathway (Bianchi et al. 2002). AP-1 is the protein product of two peptides, c-Fos and c-Jun, and once synthesized acts to initiate transcription of key genes (Gilbert 2003). Another growth factor, epidermal growth factor (EGF),

promotes the proliferation of a number of epidermal and epithelial cells in many organisms (Dealy et al. 1998). EGF induces ODC expression in human epidermoid carcinoma cells, through transcriptional and post-transcriptional events (Yazigi et al. 1989). Transforming growth factor (TGF-β) is an important growth factor which has a broad range of biological functions and affects cellular proliferation and differentiation in a cell-type specific manner, and TGF-β_1 was shown to induce ODC expression in H-ras transformed mouse fibroblasts by increasing ODC mRNA levels and increasing ODC mRNA stability (Hurta et al., 1993).

The effects of phorbol ester tumor promoters on ODC transcriptional regulation have also been studied. Phorbol ester tumor promoters are pharmacological probes that mimic the action of the lipid second messenger diacylglycerol (DAG), and that exert a variety of cellular effects including changes in proliferation, malignant transformation, cell death, and differentiation (Caloca et al. 1997). The phorbol ester tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA or PMA) is a potent tumor promoter, and has been shown to induce ODC activity by increasing ODC gene transcription while unaffecting ODC mRNA half-life in mouse skin tumors (Verma 1988). Furthermore, PMA has been shown to induce ODC mRNA synthesis in human keratinocyte cells (Xue et al. 1996). PMA also increases both ODC transcription and stability of ODC mRNA in H-*ras* transformed mouse fibroblasts (Voskas et al. 2001b).

Since ODC functions primarily to increase intracellular polyamine levels by synthesizing putrescine, this then suggests that the polyamines themselves may have

an effect on ODC activity in the cell. Polyamines are able to induce antizyme (AZ) synthesis in intestinal epithelial crypt (IEC-6) cells, thereby decreasing ODC activity by increasing AZ-mediated ODC degradation (Yuan et al. 2001). Furthermore, the efficacy of each of the polyamines in inducing AZ is differential. Spermine is greater than spermidine which is greater than putrescine at mediating AZ induction (Yuan et al. 2001). Moreover, C55.7 Chinese hamster ovary (CHO) cells, which produce a metabolically inactive ODC protein, exhibit a pronounced increase in ODC synthesis in the absence of polyamines (Svensson and Persson 1996). However, corresponding ODC mRNA levels in these cells are not increased, indicating that polyamines mediate ODC synthesis in a post-transcriptional manner in these cells (Svensson and Persson 1996).

5.2.3.3. Translational and post-translational regulation of ODC

ODC expression is also highly regulated at the translational and post-translational level by a number of factors. Studies have shown that translational / post-translational regulation of ODC by certain factors results in an increase in ODC expression. Asparagine, a common amino acid, has been shown to induce ODC expression in DF-40 cells (which have an amplified ODC gene) by increasing ODC mRNA translation and suppressing ODC protein degradation (Chen and Chen 1992). Moreover, asparagine was shown to interact with EGF in inducing ODC expression in IEC-6 cells by contributing a factor that facilitates ongoing ODC protein synthesis (Ray et al. 1999).

Alternatively, some reports indicate that translational / post-translational regulation of ODC can result in a decrease in ODC expression. Studies have shown that nitric oxide (NO), induced simultaneously by tumor necrosis factor (TNF)-α and interferon (IFN)-γ, effectively down-regulates ODC activity in transformed kidney proximal tubule cells through nitrosylation of a cysteine in the interface between ODC homodimers (Satriano et al. 1999, Bauer et al. 2001). Furthermore, in Swiss 3T3 mouse fibroblasts, antizyme inhibitor (AZI), which has no ODC activity can release enzymatically active ODC from AZ suppression *in vitro* by competing for the active site in AZ and replacing ODC (Nilsson et al. 2000). This may represent a means of rescuing ODC molecules that have been inactivated and tagged for degradation by AZ (Nilsson et al. 2000).

5.2.3.4. ODC regulation – implications in cancer

The importance of ODC expression in the development of cancer and metastatic potential has been studied extensively. H-*ras* transformed mouse fibroblasts exhibited cyclic AMP (cAMP)-induced ODC expression which correlated with malignant potential through transcriptional, post-transcriptional, and translational events (Hurta and Wright 1994). Furthermore, platelet-derived growth factor (PDGF) was shown to modulate ODC expression in H-ras transformed cells (Voskas et al. 2001a). In separate studies, H-ras transformed cells were shown to exhibit increased ODC expression in response to the tumor promoter PMA, basic fibroblast growth factor (bFGF), and transforming growth factor (TGF)-β_1 (Hurta et

al. 1996, Hurta et al. 2001, Voskas et al. 2001a, Voskas et al. 2001b). Moreover, ODC over-expressing MCF-7 human breast cancer cells exhibit an increased hormone independence and altered growth and cellular signalling (Manni et al. 1995). Over-expression of ODC in mouse 10T1/2 fibroblasts triggers mitogen-activated protein (MAP) kinase activity, which in turn implies a proportional increase in invasiveness (Kubota et al.1997). Furthermore, increased ODC expression results in increased invasive ability through a synthetic reconstituted basement membrane and increased secretion of matrix metalloproteinase (MMP)-2, a 72 kDa pro-gelatinase essential to extracellular matrix (ECM) degradation (Kubota et al.1997).

5.2.3.5. Regulation of polyamine catabolism – SSAT regulation

The catabolic pathways which interconvert and metabolize polyamines are highly regulated steps in the overall metabolism of polyamines (Seiler 1990). As previously mentioned, a key enzyme in the catabolism of polyamines is SSAT (Carper et al. 1991). SSAT activity is highly regulated at many levels, including at the levels of both transcription and translation (Casero and Pegg 1993).

5.2.3.6. Mechanisms of transcriptional control of SSAT expression

A mechanism for SSAT regulation at the level of transcription has been proposed which involves specific transcription factors and elements which directly affect SSAT gene transcription (Wang et al. 1998, Wang et al. 1999, Wang et al. 2001, Husbeck et al. 2003, Marverti et al. 2004). The polyamine-responsive element (PRE),

a 9-base pair sequence in the midst of a 31-base pair stretch within the SSAT transcriptional start site, is integral to the process (Wang et al 1998). The PRE was shown to bind constitutively to an NF-E2-related transcription factor (Nrf-2), which is a transcription factor related to the globin gene transcription factor NF-E2 (Wang et al. 1998, Wang et al. 2001). The binding of the PRE to Nrf-2 was shown to mediate increased SSAT gene transcription by the polyamine analogue N^1, N^{11}-Bis(ethyl)norspermine (BESpm) (Wang et al. 1998). Secondly, a new transcriptional cofactor, polyamine-modulated factor-1 (PMF-1), was characterized as a partner protein of Nrf-2 that combines with Nrf-2 to bind to the PRE and ultimately regulate SSAT gene transcription (Wang et al. 1999). The mechanism of interaction between PMF-1 and Nrf-2 involves the binding of the leucine-zipper region of Nrf-2 with a C-terminal coiled-coil region of PMF-1 that does not contain a leucine zipper (Wang et al. 2001). Transcriptional regulation is evident in MCF-7 human breast cancer cells transfected with human thioredoxin-1 (Trx-1) showed a marked decrease in SSAT expression resulting from decreased SSAT mRNA levels (Husbeck et al. 2003). This decrease was mediated by decreased Nrf-2/PMF-1 binding to PRE, without a change in protein expression of Nrf-2 or PMF-1 (Husbeck et al. 2003).

5.2.3.7. Regulation of SSAT expression by polyamines and polyamine analogues

An overwhelming proportion of studies concerning SSAT regulation employ the natural polyamines and synthetic polyamine analogues to induce SSAT gene expression. The efficacy of each of the natural polyamines to induce SSAT

expression is differential, with the general scheme being that spermine is greater than spermidine which is greater than putrescine at inducing SSAT gene expression, as shown by increased SSAT activity and half-life of SSAT protein (Erwin and Pegg 1986, Fogel-Petrovic et al. 1996b). In addition to the natural polyamines, synthetic polyamine analogues have been shown to induce an even further increase in SSAT gene expression (Fogel-Petrovic et al. 1993a, Fogel-Petrovic et al. 1993b). Treatment of human large cell lung carcinoma (NCI H157) cells with BESpm, a polyamine analogue, results in a 600-fold increase in SSAT activity (Casero et al. 1990). Induction of SSAT was shown to be a result of both increased gene transcription and protein synthesis (Casero et al. 1990). SSAT gene expression was increased in mouse LA-4 lung adenoma cells by 2.3 and 6.5-fold following treatment with spermine and BESpm, respectively (Fogel-Petrovic et al. 1993a). Furthermore, MALME-3M human melanoma cells exhibit an increased SSAT expression of 200-fold following treatment with BESpm, accompanied by a 45-fold increase in SSAT mRNA (Fogel-Petrovic et al. 1993b). SSAT mRNA was stabilized and the resulting half-life of SSAT mRNA increased from 17 to 64 hours following treatment with BESpm in these cells (Fogel-Petrovic et al. 1993b). Studies report that SSAT induction by polyamine analogues is the result of decreased or inhibited SSAT protein turnover (Parry et al. 1995, Coleman and Pegg 2001). BESpm increased SSAT activity by 380-fold in simian virus (SV)-40 transformed kidney fibroblast (COS-7) cells without any increase in SSAT mRNA, and SSAT protein turnover was increased from 20 minutes to 13 hours following BESpm treatment in these cells (Parry et al. 1995). Moreover,

two polyamine analogues, BESpm and N^1, N^{11}-bis(ethyl)norspermine (BE-3-3-3), prevented the efficient polyubiquitination of SSAT, leading to both increased SSAT protein and SSAT activity (Coleman and Pegg 2001).

The natural polyamines also play an integral role in SSAT regulation regardless of basal levels of SSAT expression. MALME-3M human melanoma cells treated with inhibitors of protein synthesis show a marked increase in SSAT mRNA without a corresponding increase in SSAT activity, while introduction of spermidine and spermine to these cells restored SSAT activity without increasing SSAT message (Fogel-Petrovic et al. 1996a). Furthermore, differential induction of SSAT occurs in polyamine analogue-responsive human non-small lung carcinoma (H157) cells compared to polyamine analogue-unresponsive human small-cell carcinoma (H82) cells (Xiao and Casero 1996). These findings indicate that cells must have the physiological requirements to respond to polyamine analogues, and that these analogues do not induce constitutive SSAT expression in all cells (Xiao and Casero 1996).

5.2.3.8. Regulation of SSAT expression by physiological factors

The differential regulation of SSAT by many physiological factors such as growth factors, cytokines, and amino acids has been reported. (Desiderio et al. 1998, Aubel et al. 2003). Agmatine, which as previously mentioned, serves as a source of polyamines in most bacteria and plants, has been shown to regulate SSAT expression in certain mammalian cells. Treatment of both primary cultured hepatocytes and rat

hepatoma HTC cells with agmatine results in a 12-fold increase in SSAT protein expression in each cell type (Vargiu et al. 1999).Furthermore, agmatine induced SSAT expression without increasing SSAT mRNA in mouse liver and kidney tissue *in vivo*, suggesting an involvement of post-transcriptional events in this specific regulation (Dudkowska et al. 2003). Moreover, deprivation of the amino acids arginine, methionine, and leucine resulted in an increased SSAT expression of between 2 and 5-fold in human cervical carcinoma (HeLa) cells (Aubel et al. 2003).

Evidence has been presented which indicates that growth factors and cytokines play a role in the regulation of SSAT in certain cell types. The specific paracrine growth factors hepatocyte growth factor (HGF) and interleukin (IL)-1β have been reported to induce SSAT expression at the transcriptional level in human hepatocarcinoma (HepG2) cells, by increasing SSAT gene transcription (Desiderio et al. 1998). Furthermore, TNF-α was less effective in inducing SSAT compared with HGF and IL-1β, although TNF-α did induce a slight increase in SSAT mRNA synthesis (Desiderio et al. 1998).

5.2.3.9. SSAT regulation – implications in cancer

In terms of cancer relevance, reports have shown that altered SSAT expression can influence cellular proliferation and may even mediate cellular transformation. SSAT expression in Yoshida AH-130 hepatoma cells increases as a function of tumor growth (Desiderio and Bardella 1994). SSAT mRNA and SSAT activity levels also correlate with increased levels of H-ras expression in mouse

fibrosarcoma cell lines of increasing metastatic potential (Hurta unpublished observations). Furthermore, SSAT expression was increased in correlation with malignant human prostate cancer prostatectomy specimens compared with normal tissue (Bettuzzi et al. 2000). Human breast cancer tissue biopsies exhibited an increase in SSAT expression compared to normal tissue, and this increase is accompanied by a decreased expression of PAO, indicating that acetylated polyamines may be involved in human breast cancer progression (Wallace et al. 2000). Moreover, transgenic mice over-expressing SSAT show a 10-fold increase in the number of epidermal tumors developed compared to controls (Coleman et al. 2002).

5.2.4. The insulin-like growth factor system

The insulin-like growth factor system plays a critical role in the development of many tissues and regulates overall growth (LeRoith and Roberts 2003). The IGF-1 system includes the IGF ligands (IGF-1 and IGF-2), the IGF-related receptors, including the IGF-1 receptor (IGF-1R), the IGF-2 receptor (IGF-2R), and the insulin receptor (IR) (LeRoith and Roberts 2003). Much evidence has accrued to suggest that high levels of circulating IGF-1 constitutes a risk factor in the development of many different cancers, such as breast, prostate, lung, and colon cancer (LeRoith and Roberts, Jr. 2003). Insulin, insulin-like growth factor (IGF)-1 and an estrogen (17β-estradiol) have been shown to cooperatively induce ODC expression in estrogen-responsive MCF-7 human breast cancer cells, by decreasing ODC enzyme turnover

(Huber and Poulin 1996). Furthermore, IGF-1 induced a marked increase in SSAT mRNA production in the human endometrial carcinoma cell line, Hec-1-A (Green et al. 1998).

5.2.5. H-ras and metastatic potential

H-ras is a well-known oncogene which contributes to the metastatic or malignant potential of neoplastic cells (Egan et al. 1988). It has been reported that H-ras expression is directly correlated with the formation of metastases, and that H-ras transformed cells have an increased malignant potential (Egan et al. 1988). The current study was aimed at investigating the effects of IGF-1 and IGF-2 on the expression of ODC and SSAT in transformed mouse lung fibroblast cell lines. These cell lines differ in the expression of the H-ras oncogene, and therefore display different cellular phenotypes (Egan et al. 1988). The working hypothesis is that IGF-1 and IGF-2 will modulate ODC and SSAT expression in correlation with H-ras expression. Furthermore, using specific inhibitors of common cellular signal transduction pathways, possible mechanism(s) involved in the IGF-mediated modulation of ODC and SSAT in these cells will be investigated.

5.3. Materials and methods

5.3.1. Cell culture and treatment

H-ras transformed mouse fibroblasts (NR3) and non-transformed mouse fibroblasts (10T½) were used in this study. The properties of these cells represent

different stages of neoplastic development, as explained in Chapter 4.1.3.1.(Egan et al. 1988). Cells were routinely cultured on 100 mm plastic tissue culture dishes (Becton Dickinson Labware, Franidin Lakes, NJ) in α-minimal essential medium (MEM) (Invitrogen, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone / VWR Canlab, Mississauga, ON) and were grown at 37°C in 5% CO_2. Cells were cultured to subconfluence, and were then exposed to defined medium (DM), a serum-free medium, which contained 4.0 μg/ml transferrin (Sigma, Oakville, ON) and 2.0 μg/ml insulin (Sigma, Oakville, ON) in α-MEM, for 24 hours. Cells were then exposed to DM with or without insulin-like growth factor-1 or -2 (IGF-1 or IGF-2) to a final concentration of 10 ng/ml (R&D Systems, Inc., Minneapolis, MN) for pre-determined times. Cells were removed from the surface of tissue culture plates using trypsin solution (Sigma, Oakville, ON). To evaluate the possible signal transduction pathways involved, cells were pre-treated for 1 hour with either the phosphatidylinositol-3 (PI-3) kinase inhibitor LY294002 (Sigma, Oakville, ON), the protein kinase C (PKC) inhibitor calphostin C (Sigma, Oakville, ON), or the mitogen activated protein (MAP) kinase inhibitor PD98059 (Sigma, Oakville, ON), respectively, prior to exposure to growth factors.

5.3.2. SDS-PAGE and Western analysis

Western analysis was performed essentially as described in Chapter 4.1.3.2. (Burnette 1981). Cells were harvested and lysed in cell lysate buffer (10 mM Tris-base, 1 mM PMSF). Protein concentrations were determined using the Bio-Rad

Protein Assay Kit (Bio-Rad Laboratories, Mississauga, ON). Equal amounts of protein (between 50-100 μg) were electrophoresed on 10% SDS-polyacrylamide gels and transferred for 1.5-2 hours onto 0.2 μm nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON). A protein enhancer kit (BioLynx, Brockville, ON) was used to enhance protein binding. Western blots were blocked overnight (4°C) in Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl) containing 1% bovine serum albumin (BSA), followed by incubation in TBS supplemented with 0.05% Tween-20 (Sigma, Oakville, ON) (TBS-T) and either ODC antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:100 dilution or SSAT antibody (a generous gift from Dr. A. E. Pegg, PSU, USA) at 1:200 dilution. Blots were washed with TBS-T and incubated in 1:5000 (for ODC) or 1:2000 (for SSAT) alkaline phosphatase (AP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1-2 hours at room temperature. Following a second wash in TBS-T, blots were developed using standard colorimetric protocol in bicarbonate buffer (0.213 mM $MgCl_2$, 10 mM $NaHCO_3$, pH 9.8) containing nitro-blue tetrazolium (NBT) (Sigma, Oakville, ON) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma, Oakville, ON). As a loading control, the housekeeping protein β-actin was used. Identically loaded complementary membranes were incubated with β-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:100 dilution followed by incubation in 1:500 β-actin secondary and processed as described above. Blots were quantified using the Bio-Rad GS-800 Calibrated Densitometer with Quantity One 1-D Analysis Software v. 4.5.1 (both Bio-Rad Laboratories, Mississauga, ON).

5.4. Results

5.4.1. Effects of IGF-1 and IGF-2 on ODC and SSAT protein expression in 10T1/2 cells

The effects of IGF-1 and IGF-2 on ODC and SSAT protein expression in non-transformed 10T1/2 cells were investigated. Figure 5.1 shows the protein expression of ODC and SSAT in 10T1/2 cells following 24 hour treatment with 10, 25, and 50 ng/ml IGF-1. As shown in Figure 5.1, IGF-1 does not affect the protein expression of either ODC or SSAT. The effects of IGF-2 on ODC and SSAT protein expression in 10T1/2 cells are shown in Figure 5.2. As shown in Figure 5.2, ODC protein expression remains unaffected following 24 hour exposure to 10, 25, and 50 ng/ml IGF-2. Similarly, SSAT protein expression is also unresponsive following treatment with these concentrations of IGF-2. These results indicate that IGF-1 and IGF-2 do not play a role in the regulation of ODC and SSAT protein expression in 10T1/2 cells under these experimental conditions. Additionally, ODC and SSAT protein expression in 10T1/2 cells following 48 hour exposure to IGF-1 and IGF-2 was investigated, and neither ODC nor SSAT was shown to be responsive following exposure to either 10 ng/ml IGF-1 or 10 ng/ml IGF-2 (data not shown).

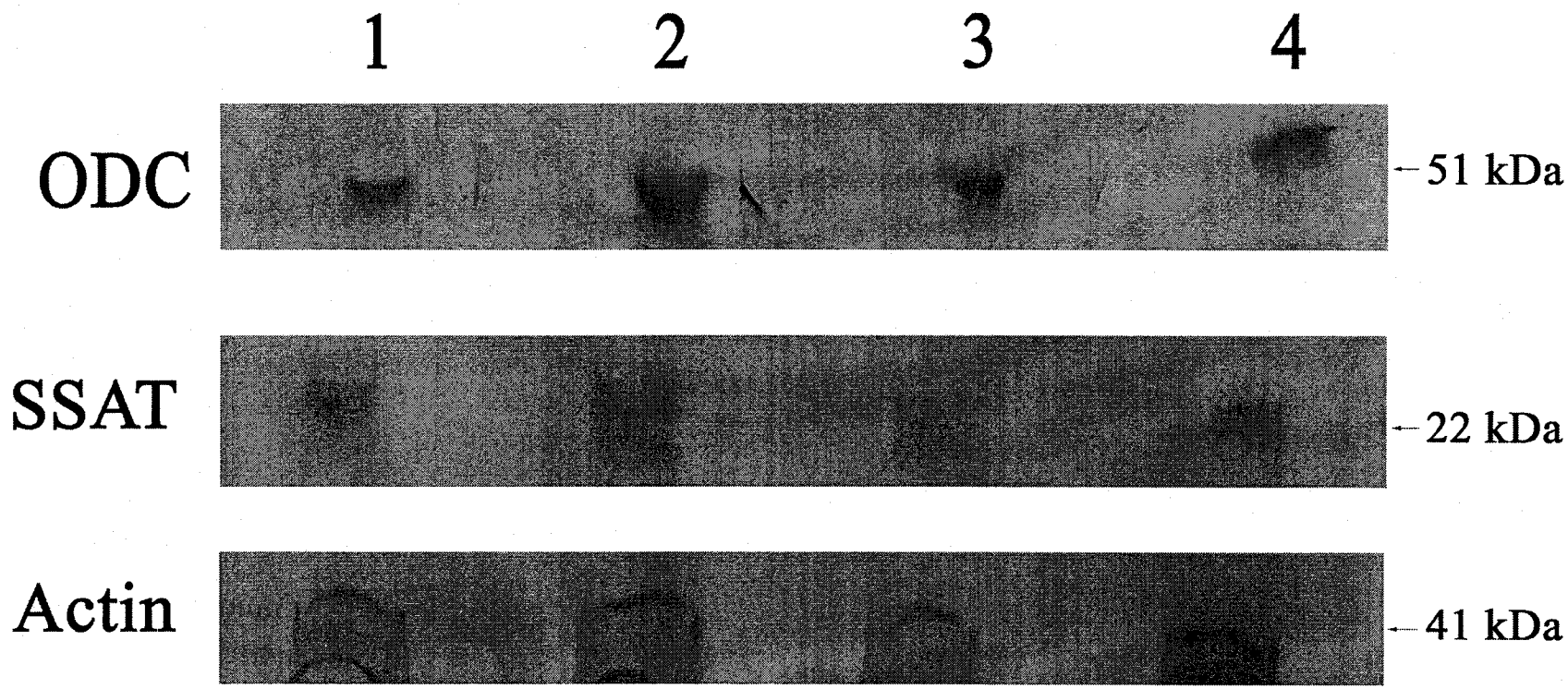

Figure 5.1. Western blot showing ODC and SSAT expression in 10T1/2 cells following 24 hour treatment with IGF-1. Control cells treated with vehicle alone are shown in (1) and ODC/SSAT expression in cells following 24 hour exposure to 10 ng/ml (2), 25 ng/ml (3), and 50 ng/ml IGF-1 are shown. 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

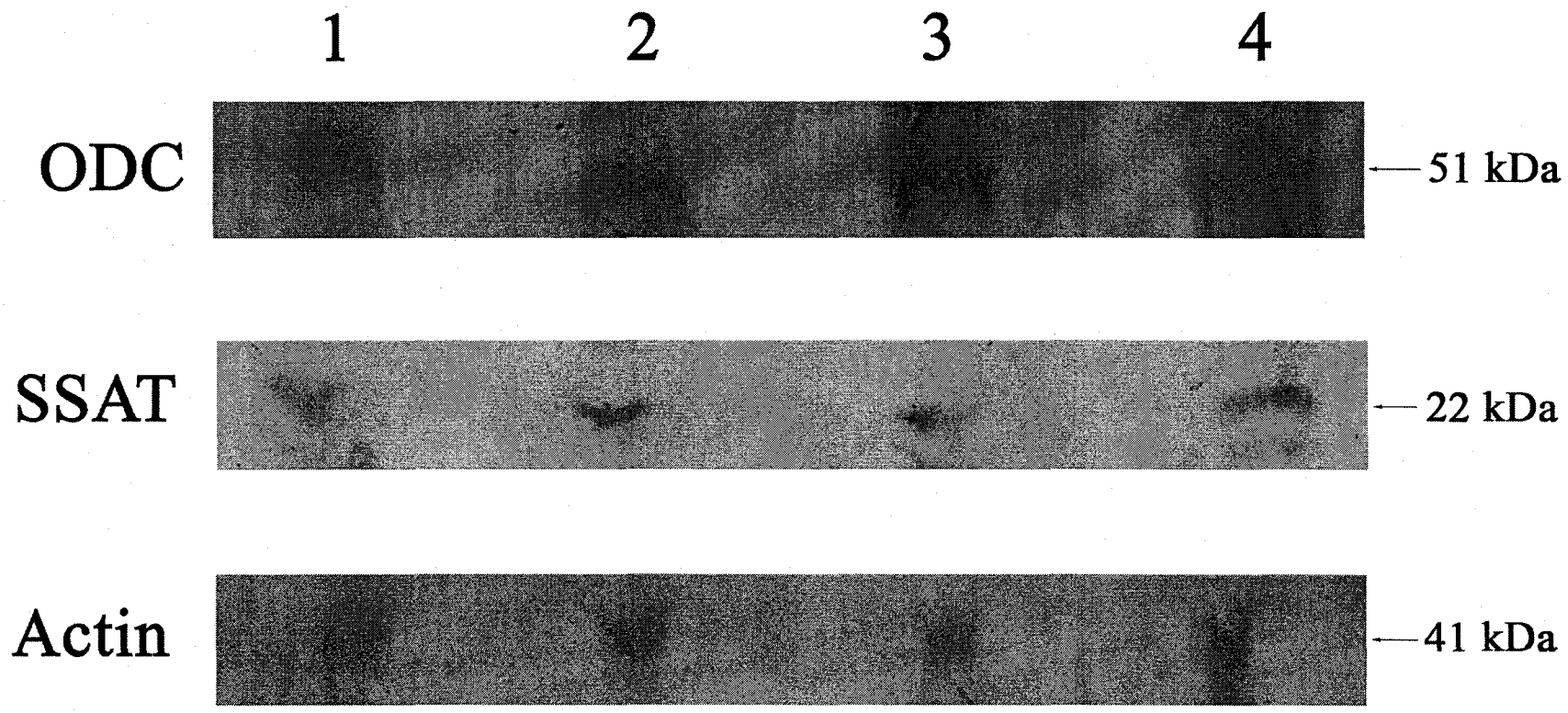

Figure 5.2. Western blot showing ODC and SSAT expression in 10T1/2 cells following 24 hour treatment with IGF-2. Control cells treated with vehicle alone are shown in (1) and ODC/SSAT expression in cells following 24 hour exposure to 10 ng/ml (2), 25 ng/ml (3), and 50 ng/ml IGF-2 are shown. 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

5.4.2. Effects of IGF-1 and IGF-2 on ODC and SSAT protein expression in NR3 cells

5.4.2.1 Effects of IGF-1 on ODC and SSAT protein expression in NR3 cells

The effects of IGF-1 on ODC and SSAT protein expression in ras-transformed NR3 cells were investigated. Figure 5.3 shows ODC and SSAT protein expression in NR3 cells following 24 hour treatment with 10, 25, and 50 ng/ml IGF-1. As shown in Figure 5.3, ODC protein expression remains relatively unchanged following exposure to 10 ng/ml IGF-1 for 24 hours. However, ODC protein expression is induced following exposure to 25 ng/ml IGF-1, and following exposure to 50 ng/ml IGF-1 ODC protein expression is markedly induced. Furthermore, Figure 5.3 shows that SSAT protein expression is elevated following exposure to 10 ng/ml IGF-1 for 24 hours and, following exposure to 25 and 50 ng/ml IGF-1, SSAT protein expression is markedly increased.

The ability of IGF-1 to modulate ODC and SSAT protein expression in NR3 cells following lower exposure times was also investigated. Figure 5.4 shows ODC and SSAT protein expression in NR3 cells following exposure to 25 ng/ml IGF-1 for 1, 3, and 6 hours respectively, since 25 ng/ml IGF-1 was shown to markedly increase ODC and SSAT protein expression in NR3 cells following 24 hours exposure. Figure 5.4 shows that IGF-1 induces both ODC and SSAT protein expression in a time-dependent manner, with induction of protein expression occurring as early as 1 hour post-IGF-1 exposure for ODC and SSAT, respectively.

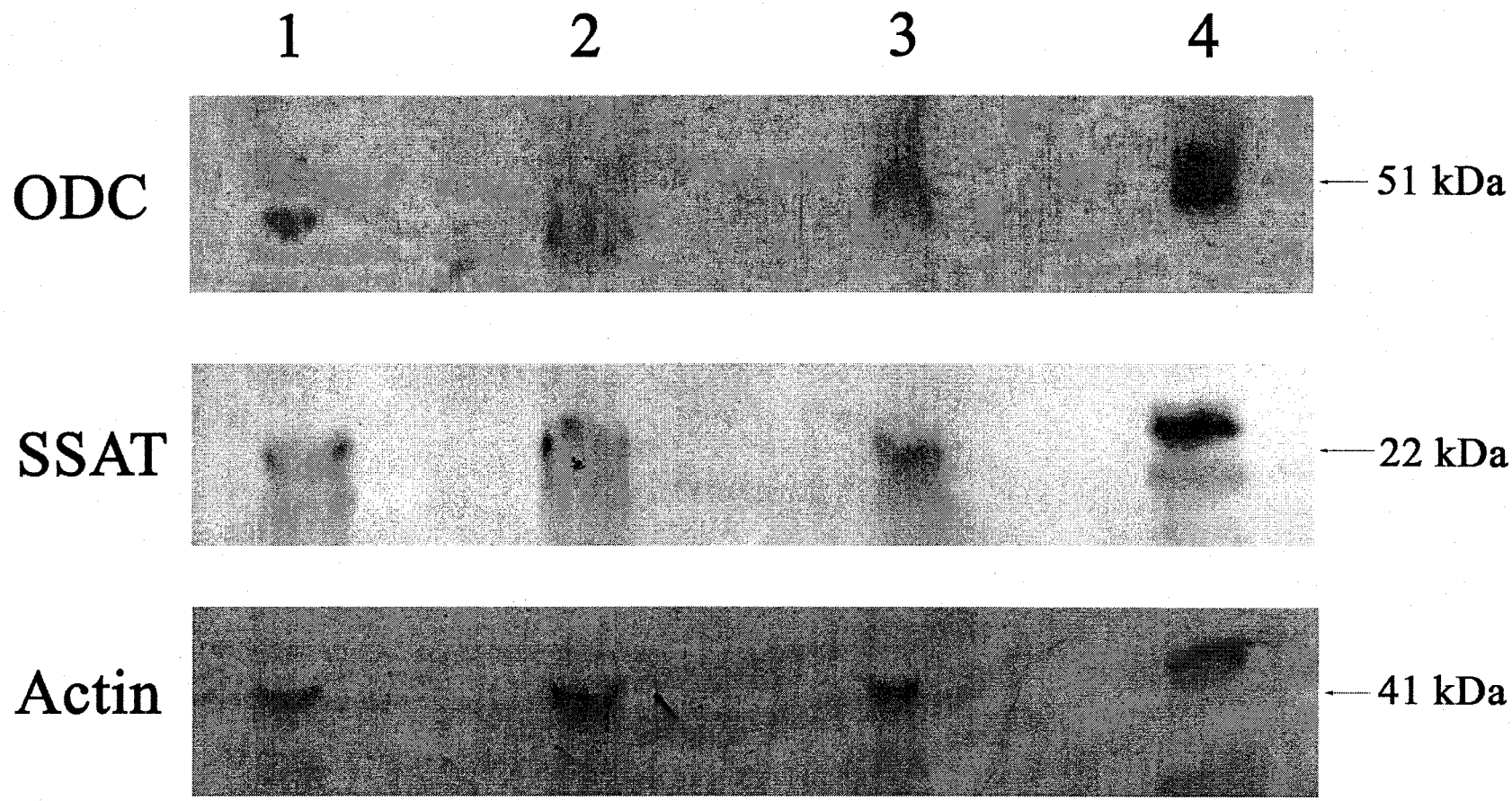

Figure 5.3. Western blot showing ODC and SSAT expression in NR3 cells following 24 hour treatment with IGF-1. Control cells treated with vehicle alone are shown in (1) and ODC/SSAT expression in cells following 24 hour exposure to 10 ng/ml (2), 25 ng/ml (3), and 50 ng/ml IGF-1 are shown. 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

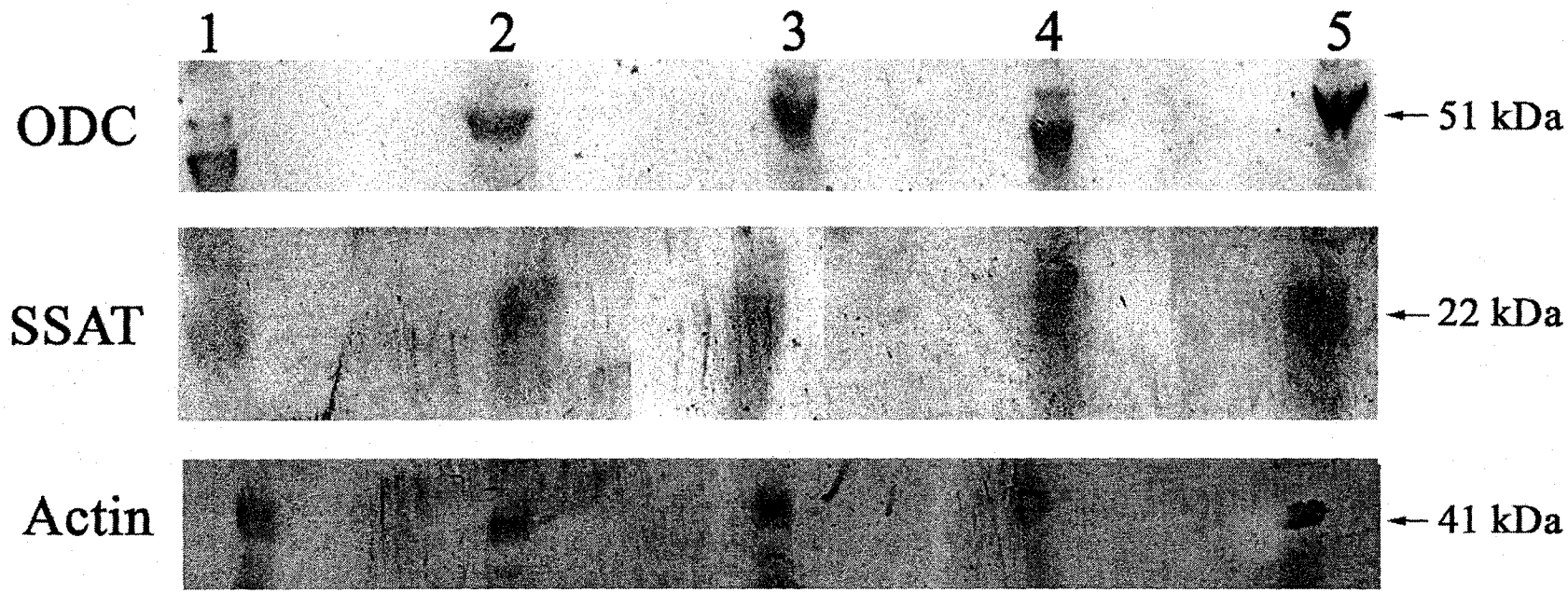

Figure 5.4. Western blot showing ODC and SSAT expression in NR3 cells following 1, 3, 6, and 24 hour exposure to IGF-1. ODC and SSAT expression in cells cultured in the absence of IGF-1 (vehicle alone) is shown in (1) and ODC and SSAT expression in cells exposed to 25 ng/ml IGF-1 for 1 hour (2), 3 hours (3), 6 hours (4), and 24 hours (5) are shown. 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

5.4.2.2. Effects of IGF-2 on ODC and SSAT protein expression in NR3 cells

The effects of IGF-2 on ODC and SSAT protein expression in NR3 cells were investigated. Figure 5.5 shows ODC and SSAT protein expression in NR3 cells following 24 hour treatment with 10, 25, and 50 ng/ml IGF-2, respectively. As shown in Figure 5.5, ODC and SSAT protein expression remains unchanged following 24 hour exposure to 10 ng/ml IGF-2, while 25 and 50 ng/ml IGF-2 induces ODC and SSAT protein expression, resulting in a substantial induction of both ODC and SSAT following 24 hour exposure to 50 ng/ml IGF-2.

To further investigate the effects of IGF-2 on ODC and SSAT protein expression in NR3 cells, ODC and SSAT protein expression was examined in these cells following early time exposure to IGF-2. ODC and SSAT protein expression in NR3 cells following exposure to 25 ng/ml IGF-2 for 1, 3, and 6 hours are shown in Figure 5.6, since 25 ng/ml IGF-2 was shown to markedly increase ODC and SSAT protein expression in NR3 cells following 24 hours exposure. Figure 5.6 shows that ODC protein expression remains unchanged following 1, 3, and 6 hour exposure to 25 ng/ml IGF-2. ODC protein expression is markedly induced, however, following 24 hour exposure to 25 ng/ml IGF-2. Induction of SSAT protein expression in response to 25 ng/ml IGF-2 occurs in a time-dependent manner, with induction of SSAT occurring as early as 1 hour post-IGF-2 exposure (Figure 5.6).

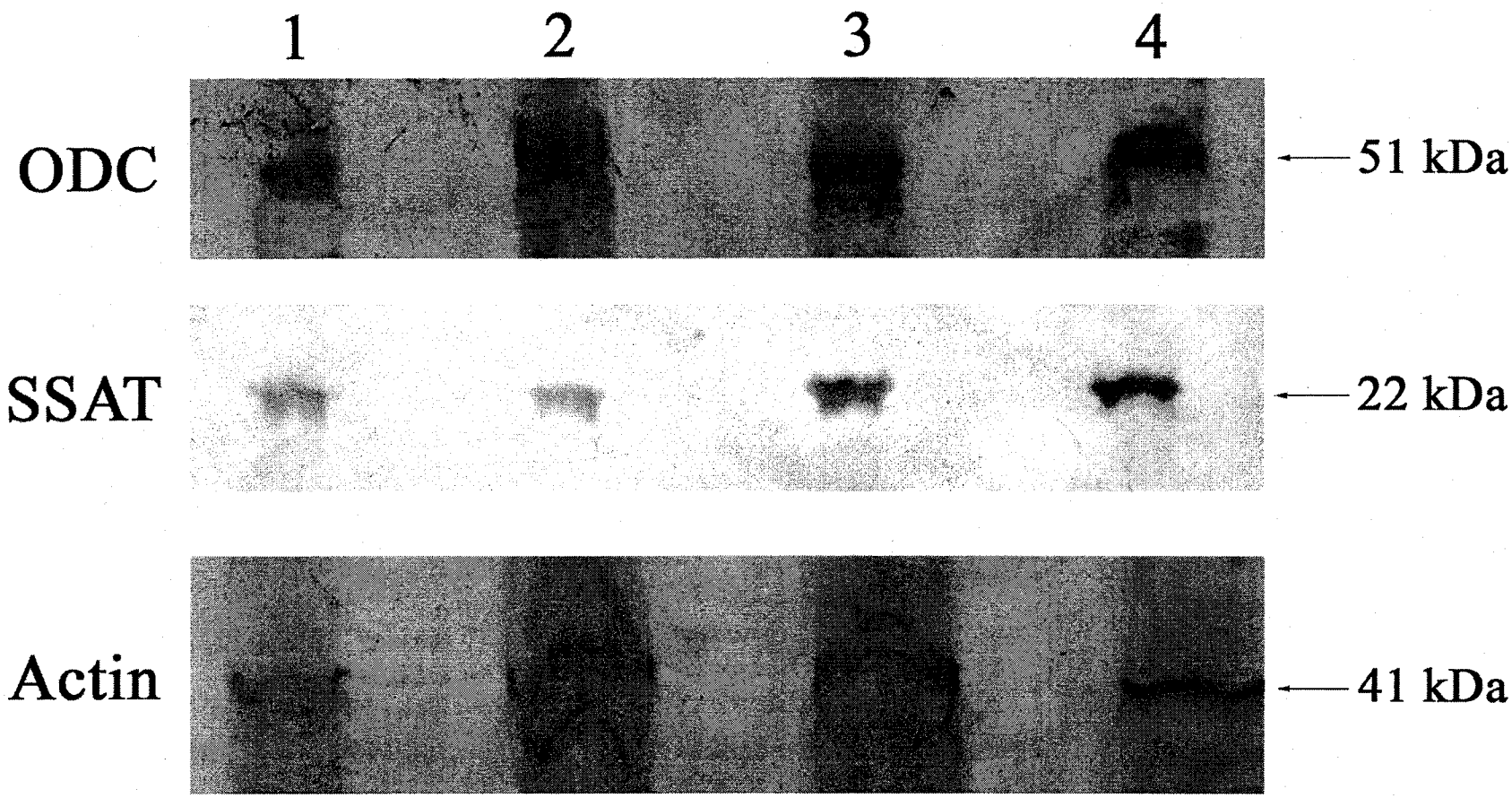

Figure 5.5. Western blot showing ODC and SSAT expression in NR3 cells following 24 hour treatment with IGF-2. Control cells treated with vehicle alone are shown in (1) and ODC/SSAT expression in cells following 24 hour exposure to 10 ng/ml (2), 25 ng/ml (3), and 50 ng/ml IGF-2 are shown. 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

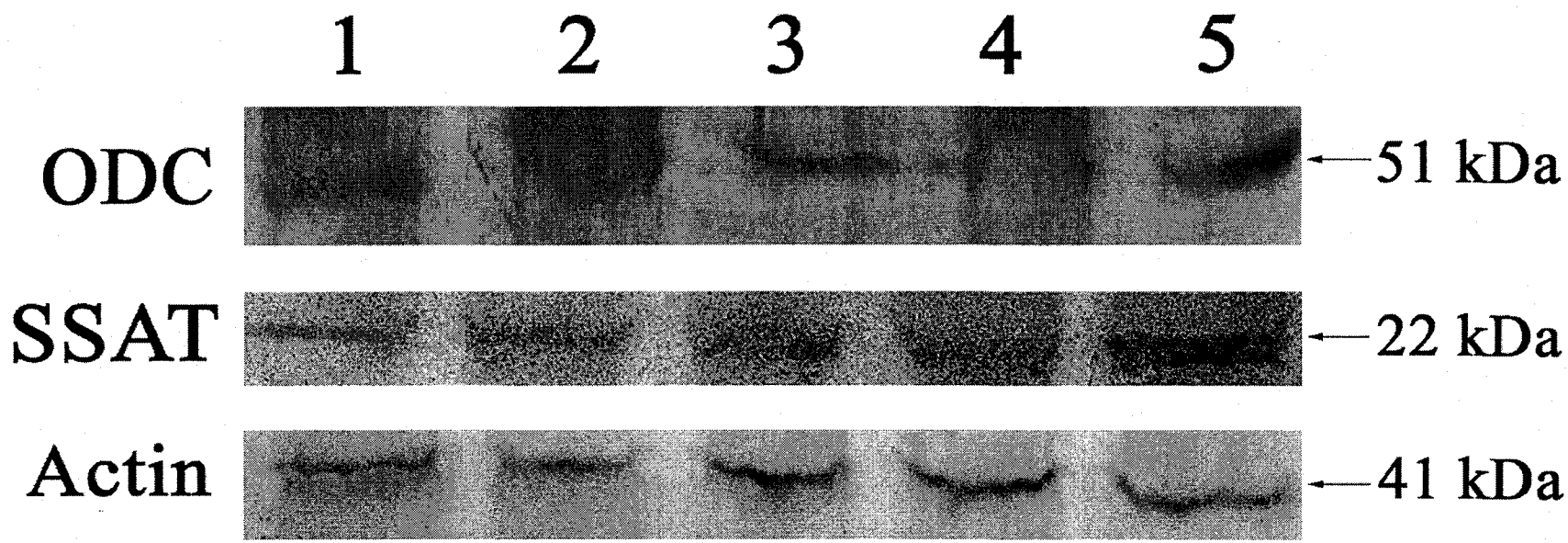

Figure 5.6. Western blot showing ODC and SSAT expression in NR3 cells following 1, 3, 6, and 24 hour exposure to IGF-2. ODC and SSAT expression in cells cultured in the absence of IGF-2 (vehicle alone) is shown in (1) and ODC and SSAT expression in cells exposed to 25 ng/ml IGF-2 for 1 hour (2), 3 hours (3), 6 hours (4), and 24 hours (5) are shown. 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.

5.4.3. Effects of cellular signalling inhibitors on IGF-mediated induction of ODC and SSAT protein expression in NR3 cells

5.4.3.1. Effects of cellular signalling inhibitors on IGF-1-mediated induction of ODC and SSAT protein expression in NR3 cells

Using specific inhibitors of common cellular signalling pathways, possible mechanism(s) involved in IGF-1-mediated induction of ODC and SSAT in NR3 cells was investigated. ODC and SSAT protein expression in NR3 cells pre-treated for 1 hour with PKC inhibitor calphostin C (500 nM), MAP kinase inhibitor PD98059 (50 μM), and PI3 kinase inhibitor LY294002 (10 μM) prior to 6 hour treatment with 25 ng/ml IGF-1 is shown in Figures 5.7 and 5.8, respectively. As shown in Figures 5.7 and 5.8, the inhibition of PKC by calphostin C abolishes the IGF-1-mediated induction of both ODC and SSAT protein levels in these cells. Furthermore, the IGF-1-mediated induction of ODC and SSAT protein is also abolished noted following treatment with PD98059, indicating the possible involvement of the MAP kinase pathway in this induction. No alleviation of induced ODC or SSAT protein expression is noted in these cells following inhibition of PI3 kinase, indicating that the PKC and MAP kinase pathways are involved in the IGF-1-mediated induction of ODC and SSAT protein in these cells, and that the PI3 kinase signalling pathway is not involved in this induction.

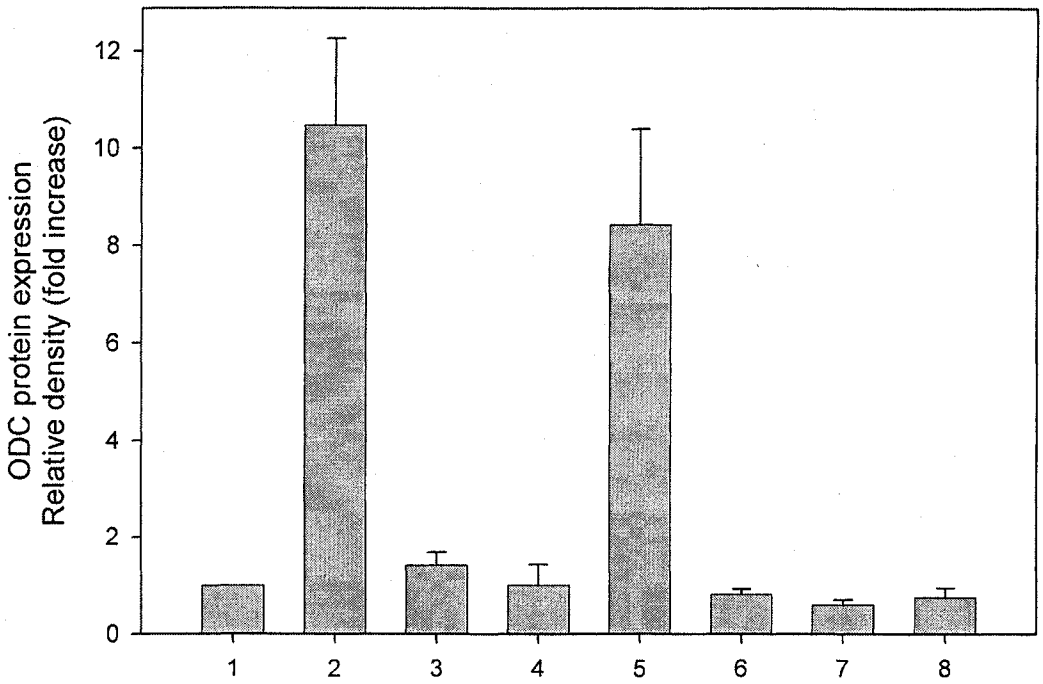

Figure 5.7. Histogram showing the effects of signal transduction inhibitors on IGF-1-mediated induction of ODC in NR3 cells. ODC expression in NR3 cells pre-treated with calphostin C (500 nM), PD98059 (50 μM), and LY294002 (10 μM) prior to exposure to IGF-1 (25 ng/ml) is shown as relative density values (pixels/mm^2). (1) control cells (vehicle alone), 6 hour exposure to IGF-1 (2), cells pre-treated for 1 hour with calphostin C prior to 6 hour treatment with IGF-1 (3), cells pre-treated for 1 hour with PD98059 prior to 6 hour treatment with IGF-1 (4), cells pre-treated for 1 hour with LY294002 prior to 6 hour treatment with IGF-1 (5), and cells treated with calphostin C alone (6), PD98059 alone (7), and LY294002 alone (8). 100 μg of total protein was loaded in each sample, and results indicated are representative of duplicate experiments with 3 culture plates per condition tested.

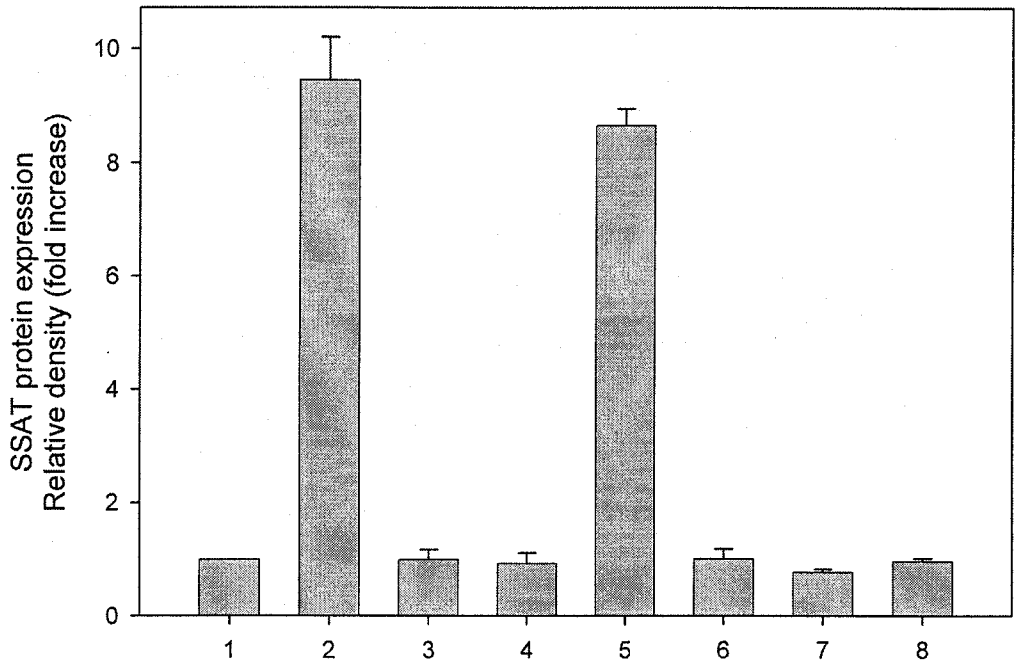

Figure 5.8. Histogram showing the effects of signal transduction inhibitors on IGF-1-mediated induction of SSAT in NR3 cells. SSAT expression in NR3 cells pre-treated with calphostin C (500 nM), PD98059 (50 μM), and LY294002 (10 μM) prior to exposure to IGF-1 (25 ng/ml) is shown as relative density values (pixels/mm^2). (1) control cells (vehicle alone), 6 hour exposure to IGF-1 (2), cells pre-treated for 1 hour with calphostin C prior to 6 hour treatment with IGF-1 (3), cells pre-treated for 1 hour with PD98059 prior to 6 hour treatment with IGF-1 (4), cells pre-treated for 1 hour with LY294002 prior to 6 hour treatment with IGF-1 (5), and cells treated with calphostin C alone (6), PD98059 alone (7), and LY294002 alone (8). 100 μg of total protein was loaded in each sample, and results indicated are representative of duplicate experiments with 3 culture plates per condition tested.

5.4.3.2. Effects of cellular signalling inhibitors on IGF-2-mediated induction of ODC and SSAT protein expression in NR3 cells

Using specific inhibitors of common cellular signalling pathways, possible mechanism(s) involved in IGF-2-mediated induction of ODC and SSAT in NR3 cells was investigated. ODC and SSAT protein expression in NR3 cells pre-treated for 1 hour with PKC inhibitor calphostin C (500 nM), MAP kinase inhibitor PD98059 (50 μM), and PI3 kinase inhibitor LY294002 (10 μM) prior to 6 hour treatment with 25 ng/ml IGF-2 is shown in Figures 5.9 and 5.10, respectively. As shown in Figures 5.9 and 5.10, the inhibition of PKC by calphostin C abolishes the IGF-2-mediated induction of both ODC and SSAT protein in these cells. Furthermore, an abolishment of IGF-2-mediated induction of ODC and SSAT protein is noted following treatment with PD98059, indicating the possible involvement of MAP kinase in this induction. No alleviation of induced ODC or SSAT protein expression is noted in these cells following inhibition of PI3 kinase, indicating that the PKC and MAP kinase pathways are involved in the IGF-2-mediated induction of ODC and SSAT protein in these cells, and that the PI3 kinase signalling pathway is not involved in this induction.

5.5. Discussion

The IGF signalling system plays an important role in tumorigenesis, and this study investigated the role of the IGF system in ras-mediated cellular transformation. By using both transformed and non-transformed cells, these results indicate an

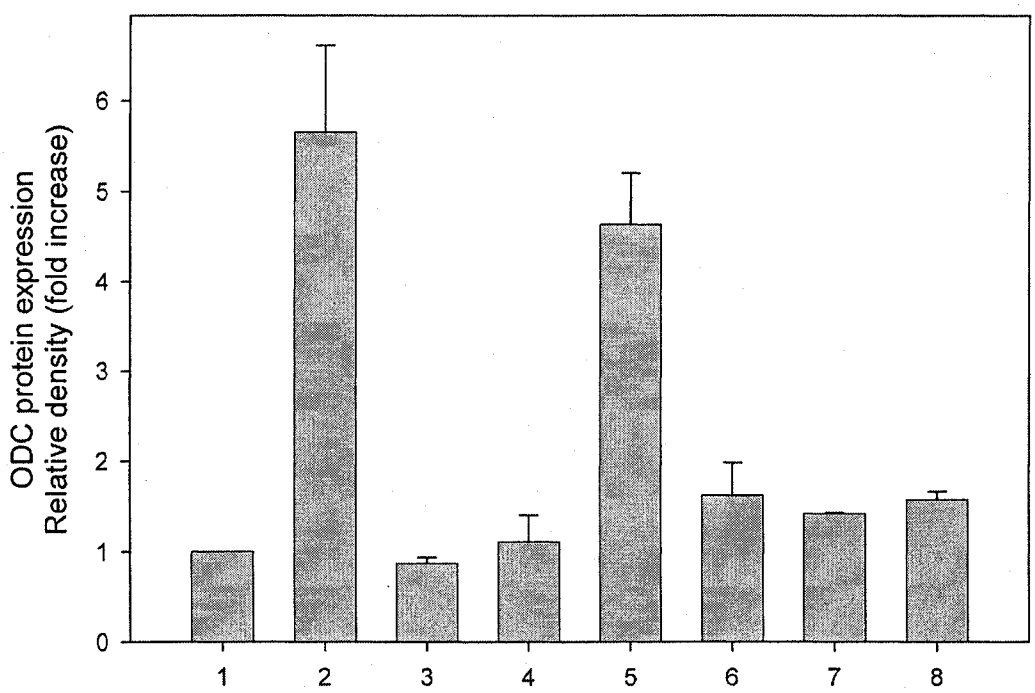

Figure 5.9. Histogram showing the effects of signal transduction inhibitors on IGF-2-mediated induction of ODC in NR3 cells. ODC expression in NR3 cells pre-treated with calphostin C (500 nM), PD98059 (50 μM), and LY294002 (10 μM) prior to exposure to IGF-2 (25 ng/ml) is shown as relative density values (pixels/mm^2). (1) control cells (vehicle alone), 6 hour exposure to IGF-2 (2), cells pre-treated for 1 hour with calphostin C prior to 6 hour treatment with IGF-2 (3), cells pre-treated for 1 hour with PD98059 prior to 6 hour treatment with IGF-2 (4), cells pre-treated for 1 hour with LY294002 prior to 6 hour treatment with IGF-2 (5), and cells treated with calphostin C alone (6), PD98059 alone (7), and LY294002 alone (8). 100 μg of total protein was loaded in each sample, and results indicated are representative of duplicate experiments with 3 culture plates per condition tested.

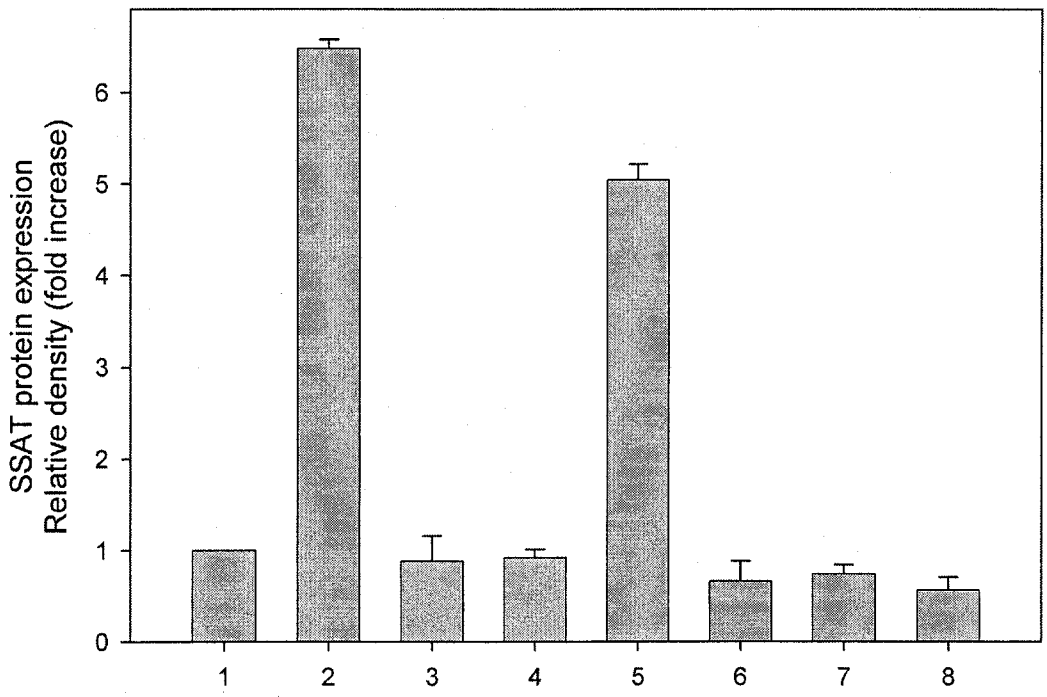

Figure 5.10. Histogram showing the effects of signal transduction inhibitors on IGF-2-mediated induction of SSAT in NR3 cells. SSAT expression in NR3 cells pre-treated with calphostin C (500 nM), PD98059 (50 μM), and LY294002 (10 μM) prior to exposure to IGF-2 (25 ng/ml) is shown as relative density values (pixels/mm^2). (1) control cells (vehicle alone), 6 hour exposure to IGF-2 (2), cells pre-treated for 1 hour with calphostin C prior to 6 hour treatment with IGF-2 (3), cells pre-treated for 1 hour with PD98059 prior to 6 hour treatment with IGF-2 (4), cells pre-treated for 1 hour with LY294002 prior to 6 hour treatment with IGF-2 (5), and cells treated with calphostin C alone (6), PD98059 alone (7), and LY294002 alone (8). 100 μg of total protein was loaded in each sample, and results indicated are representative of duplicate experiments with 3 culture plates per condition tested.

obvious relationship between IGF-mediated signalling and ras-mediated cellular transformation. In the 10T1/2 non-transformed cell line, IGF-mediated signalling was unable to elicit a change in the expression of the major polyamine-related enzymes, ODC and SSAT. As such, further studies with 10T1/2 cells and IGF-1/IGF-2 were not undertaken. Interestingly, both IGF-1 and IGF-2 induced ODC and SSAT protein expression in NR3 transformed cells. The ability of IGF-1 to induce ODC and SSAT was pronounced, and results show that the expression of both enzymes was induced in both a dose- and time-dependent manner by IGF-1. On the other hand, IGF-2 was more selective in inducing ODC and SSAT in these cells, as evidenced by the inability of IGF-2 to induce ODC expression following 1, 3, or 6 hour exposure times, respectively. This differential result indicates that IGF-1 may be more intimately involved than IGF-2 in the regulation of ODC in ras-transformed cells. Conversely, IGF-1 and IGF-2 regulated SSAT expression identically, indicating that ras-mediated SSAT regulation has similar responsiveness to both IGF-1 and IGF-2.

These results suggest that the IGF system is an important regulatory system in ras-mediated expression of ODC and SSAT in transformed NR3 cells. The IGF-I receptor, which is an essential effector of the IGF system, is over-expressed in many cancer cells, thereby conferring enhanced survival to malignant cells (Resnicoff et al. 1995). Enhanced IGF signalling is of critical importance to ras transformation, since cellular transformation using ras, among other oncogenes, was impossible in IGF-1 receptor-defective mouse fibroblasts (Sell et al. 1994). Recently, a ras effector protein, RASSF1C (Ras-associated domain family-1 protein), was shown to interact with IGF

binding protein-5 (IGFBP-5) (Amaar et al. 2005). The IGFBPs affect half-lives and bioavailability of IGFs, and are integral in IGF-mediated effects (LeRoith and Roberts, Jr. 2003). The study also showed that when RASSF1C was inhibited, IGFBP-5-mediated phosphorylation of MAP kinase/Erk kinase was abolished, indicating that ras (through RASSF1C) regulates IGF-mediated signalling using, at least in part, a MAP kinase/Erk kinase pathway (Amaar et al. 2005). These results underscore the complex relationship between IGF signalling and ras-mediated cellular transformation.

Results in the current study are in keeping with these results, where IGF-1 and IGF-2 were shown to induce ODC and SSAT expression in ras-transformed NR3 cells. In each case, by inhibiting MAP kinase with its specific inhibitor, PD98059, IGF-mediated induction of both ODC and SSAT was abolished. Furthermore, IGF-mediated induction of ODC and SSAT was abolished following treatment of cells with the PKC inhibitor calphostin C, indicating the role of PKC in this induction. Conversely, PI3 kinase inhibition in these cells did not affect IGF-mediated induction of ODC or SSAT in these cells, indicating that the involvement of the PI3 kinase signalling cascade is not necessary in this induction.

The PKC/MAP kinase axis is a known cell signalling pathway with an important role in the pathophysiology of many diseases (Vincent and Feldman 2002). The MAP kinases are a series of serine-threonine protein kinases that are activated by among others, the IGF-1 receptor (Vincent and Feldman 2002). Activated IGF-1 receptor initiates a signalling cascade which ultimately leads to activation of the ras-

ERK pathway (Kim et al. 1998). In the current study, IGF signalling lead to an increase in ODC and SSAT expression, indicating that these enzymes are regulated by activated ras-ERK signalling. This is consistent with other reports, since NR3 cells, which have increased expression of ras, showed a marked induction of ODC and SSAT expression compared to the non-transformed, non-ras expressing 10T1/2 cell line. More specifically, IGF-1 was shown in this study to be more efficient at inducing ODC expression than IGF-2, as shown in Figures 5.5 and 5.6. These data demonstrate the ability of IGF-1 to induce ODC as early as 1 hour post-treatment, whereas IGF-2 did not induce ODC until 24 hours post-treatment. These data are consistent with previous findings, when the structure and function of IGF-1 and IGF-2 is further examined (LeRoith and Roberts, Jr. 2003). Both IGF-1 and IGF-2 are peptides consisting of domains which have a high degree of homology to insulin (LeRoith and Roberts, Jr. 2003). Initially, it was hypothesized that both ligands mediate their effects through IGF-1 receptor signalling cascades, and each ligand's ability to stimulate proliferation was dependent on IGF-1 receptor expression. However, work with knockout mice revealed that IGF-2 could mediate its effect through the insulin receptor (Rother and Accili 2000). These results indicate that IGF-1 may have a more substantial role in binding to the IGF-1 receptor (Rother and Accili 2000). In the current study, IGF-1 was more efficient at inducing ODC and SSAT expression. These data suggest that increased ODC and SSAT protein expression observed in this study may be a function of increased signalling in the IGF

system, and that IGF-1, and to a lesser extent IGF-2, play an integral role in the induction of the proliferation-linked enzymes ODC and SSAT in these cells.

6. CHAPTER SIX

6.1 General discussion

The research documented in this thesis summarizes some of the different mechanisms involved in the selective growth advantage possessed by many neoplastic cells. High endogenous levels of MMPs, which are major mediators of metastasis, were shown in DU145 cells, and the effects of natural flavonoid-enriched fractions isolated from lowbush blueberry on MMP activity *in vitro* were investigated (Chapter 2 and 3). The findings reported here suggest that the flavonoid-enriched fractions down-regulated MMP activity in a target-directed manner in these cells (Chapter 2 and 3). In another study, the ability of cancer cells to evade apoptosis can be attributed, in part, to increased expression of the inhibitor of apoptosis proteins (IAPs). Research conducted in this thesis examined the expression of survivin, an IAP, in both non-transformed and ras-transformed cells in response to mitogenic growth factors (bFGF, PDGF, and EGF) (Chapter 4). Results indicated that survivin expression correlated with ras expression and that specific induction of survivin by each growth factor involved one or more specific signal transduction cascades (Chapter 4). Finally, cancer cells possess altered polyamine metabolism, which promotes growth and proliferation, and findings reported that up-regulation of the major enzymes in polyamine metabolism, ODC and SSAT, was present in ras-transformed cells but not in non-transformed cells (Chapter 5).

Taken together, these data outline the complex processes involved in the enhanced growth observed in cancer cells. By up-regulating MMP activity, cancer

cells promote not only metastasis, but angiogenesis, a complex process which recruits host blood vessels to the tumor site (Rak et al. 2000). The angiogenic process remodels tissue and basement membrane to facilitate and enhance tumor growth by providing oxygen and nutrients from the blood (Rak et al. 2000). This aspect of the selective growth advantage observed in cancer cells is an important one, since MMP up-regulation and angiogenesis are necessary pre-requisites to metastasis (Stetler-Stevenson and Yu 2001). In this study, evidence shows that lowbush blueberry flavonoids specifically inhibit MMP activity in DU145 cells in a MAP kinase pathway-dependent and PKC pathway-dependent manner, suggesting that these compounds may possess specific anti-cancer, and in particular anti-metastatic, properties.

Basal levels of survivin in non-transformed 10T1/2 cells reported here were minimal, while ras-transformed NR3 cells showed relatively high endogenous levels of the protein (Chapter 4). Survivin levels in normal tissue are very low (Altieri 2003). Conversely, survivin levels in cancer cells and tumors are elevated (Altieri 2003). Cancer cells have the ability to manipulate their growth responses to induce survivin, and thereby evade apoptosis (Sommer et al. 2003). In this thesis, NR3 cells displayed altered mitogenic signalling systems compared to 10T1/2 cells. In particular, serum stimulated NR3 cells showed a marked induction of survivin, while similar experiments with 10T1/2 cells showed no induction of survivin. Upon further examination, the mitogenic growth factors bFGF, PDGF, and EGF all induced survivin expression in NR3 cells, and the involvement of specific signal cascades is

outlined (Chapter 4). These results indicate that the ras-transformed NR3 cell line possesses an altered growth response, which promotes growth and suppresses apoptosis, and that survivin may prove to be a viable target in the development of anti-cancer therapies.

Altered polyamine metabolism is a hallmark characteristic of many cancers (Hillary and Pegg 2003). By increasing intracellular polyamine levels, cancer cells promote growth and proliferation, which drives tumor growth (Casero Jr. and Pegg 1993). Results in this thesis demonstrate that ras-transformed NR3 cells exhibit altered polyamine metabolism compared to non-transformed 10T1/2 cells (Chapter 5). In particular, the ability of IGF-1 and IGF-2 to induce the major enzymes in polyamine metabolism, ODC and SSAT, was restricted solely to NR3 cells, indicating that these cells may have altered IGF signalling systems. In particular, IGF-1- and IGF-2-mediated induction of ODC and SSAT was found to involve MAP kinase and PKC signal transduction events. These results suggest that the IGF signalling system as an important mediator of the altered growth response in ras-transformed cells.

6.2. Future research

Research reported in this thesis provides information on some of the mechanisms involved in the selective growth advantage observed in cancer cells. In all cases, cancer cells were shown to have altered cellular processes which promoted cell growth and survival, while suppressing cell death and apoptosis. Future

directions should focus on the particular mechanisms reported here, and further investigate the role of these mechanisms in other aspects of proliferation-linked gene expression.

Continuing studies on MMP activity in response to flavonoids from lowbush blueberry should involve the examination of MMP protein levels (possibly by Western blotting) and MMP mRNA levels (possibly by Northern blotting). The flavonoid-enriched fractions used in this study contained several different flavonoids and flavonoid-related compounds, and future work should investigate the possible involvement of flavonoids other than anthocyanins and proanthocyanidins in MMP regulation in DU145 cells. Also, further studies should examine the role of flavonoids from lowbush blueberry in MMP regulation in other cancer cell types, such as human breast cancer cells or human cervical carcinoma cells. Studies of this nature will provide a better understanding of the complex and diverse properties of flavonoids, along with their involvement in MMP regulation in many cancer cells.

Future research on survivin expression should examine the levels of survivin mRNA and the levels of survivin protein activity in both transformed and non-transformed cells in response to the mitogenic growth factors used in this study (bFGF, PDGF, EGF). Furthermore, the effects of other growth factors, such as transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF), on survivin expression should be conducted. Studies should be conducted to examine the ability of mitogenic growth factors to suppress apoptosis *in vitro*, since evidence shown in this study suggests that the expression of survivin, an inhibitor of apoptosis,

is up-regulated in transformed cells by bFGF, PDGF, and EGF. These proposed studies will provide a physiological link between survivin expression and apoptosis *in vitro*.

Future studies on ODC and SSAT will provide a better understanding of the complex regulation of polyamine metabolism. Studies to examine the effects of IGF-1 and IGF-2 on ODC and SSAT mRNA and activity levels will further characterize the regulation of these important enzymes. Moreover, the effects of insulin on ODC and SSAT expression at the mRNA, protein, and activity levels will compare and contrast the physiological roles of insulin and IGF-1 / IGF-2. Expression of the IGF-1 receptor in transformed and non-transformed cells will provide further data on the IGF signalling system and its role in transformed cells. Furthermore, studies to investigate the effects of IGF-1 and IGF-2 on other important enzymes in polyamine metabolism, such as S-adenosylmethionine decarboxylase (SAMDC), polyamine oxidase (PAO), and antizyme (AZ), will better characterize the holistic effects of these growth factors on polyamine metabolism in transformed and non-transformed cells.

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8. APPENDIX

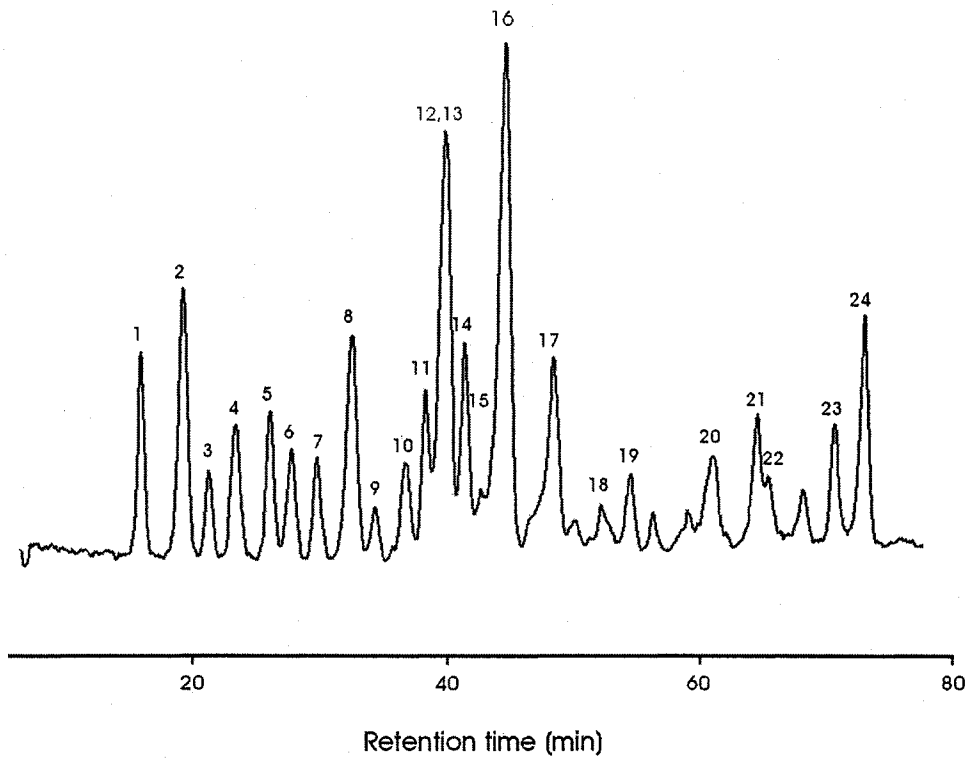

Figure A.1. LC/MS selective ion monitoring (SIM) analysis of "colored fraction #1" showed the presence of the following 21 anthocyanins and three flavonol glycosides: (1) delphinidin-3-galactoside, (2) delphinidin-3-glucoside, (3) cyanidin-3-galactoside, (4) delphinidin-3-arabinoside, (5) cyanidin-3-glucoside, (6) petunidin-3-galactoside, (7) cyanidin-3-arabinoside, (8) petunidin-3-glucoside, (9) peonidin-3-galactoside, (10) petunidin-3-arabinoside, (11) quercetin-3-glucoside, (12) malvidin-3-galactoside, (13) peonidin-3-glucoside, (14) quercetin-3-galactoside, (15) peonidin-3-arabinoside, (16) malvidin-3-glucoside, (17) malvidin-3-arabinoside, (18) quercetin-3-rhamnoside, (19) delphinidin-6-acetyl-3-galactoside, (20) cyanidin-6-acetyl-3-glucoside, (21) petunidin-6-acetyl-3-glucoside, (22) malvidin-6-acetyl-3-galactoside, (23) peonidin-6-acetyl-3-glucoside, (24) malvidin-6-acetyl-3-glucoside.

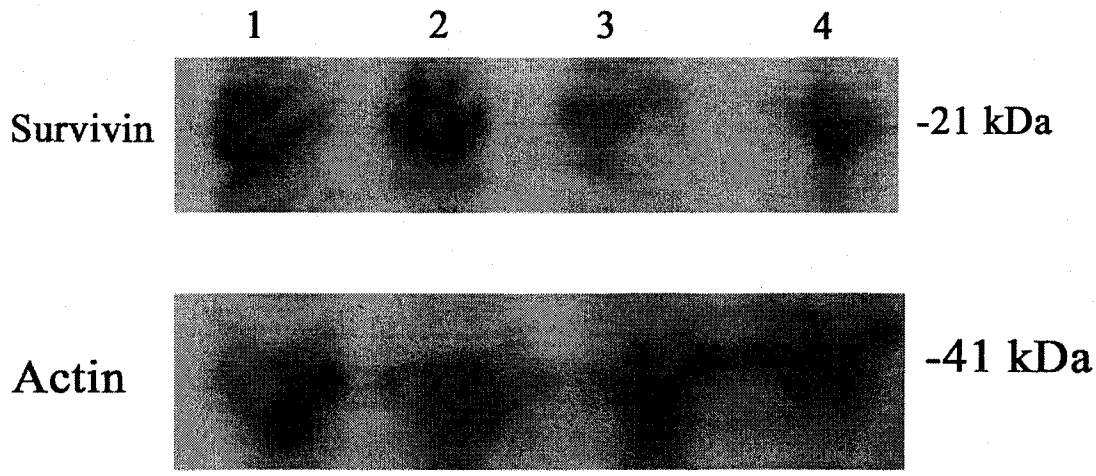

Figure A.2. Western blot showing survivin expression in 10T1/2 cells following 24 hour treatment with serum (FBS). Survivin expression in cells cultured in the absence of serum is shown in (1) and survivin expression in cells following 24 hour exposure to 10% serum is shown in (2), and 20% serum is shown in (3). 100 μg of total protein was loaded in each lane. β-Actin served as a loading control.