THE ROLE OF VITAMIN E SUCCINATE IN REGULATION OF GROWTH AND CYCLOOXYGENASE EXPRESSION IN B16 MURINE MELANOMA CELLS.

THESIS

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ABSTRACT

This study was undertaken to determine the effects and possible mechanism of action of vitamin E succinate supplementation on B16 murine melanoma cell growth *in vitro*. Studies revealed that supplementation of 5, 7 and 10μg/ml of this vitamin significantly inhibited growth of B16 cells. Non-malignant LLCMK cells supplemented with the same concentrations of vitamin E succinate resulted in similar inhibition of cell growth.

The actual mechanism by which vitamin E succinate inhibits B16 cell growth is unclear, though there has been much speculation about its possible role as an antioxidant. Vitamin E succinate is not a physiological antioxidant and for this ester to behave as an antioxidant, cleavage of the ester bond must occur, releasing the antioxidant vitamin E part of the molecule. To determine whether the observed inhibitory effects on B16 cell growth were due to the intact vitamin E succinate or the vitamin E cleavage product, cleavage studies were undertaken. Results from these studies revealed that in B16 cells vitamin E succinate cleavage did not occur suggesting that the observed inhibitory effects of vitamin E succinate on B16 cells were due to the intact compound. In contrast vitamin E succinate cleavage was shown to occur in LLCMK cells, suggesting that these cells may contain an esterase capable of liberating succinic acid and vitamin E.

Further studies focussed on the possible role of vitamin E succinate in regulation of cyclooxygenase activity in B16 cells as vitamin E succinate was found to effect the activity of various enzymes involved in the arachidonic acid cascade, notably cyclooxygenase, the rate-limiting enzyme in prostaglandin synthesis. Time course studies were used to determine when the cyclooxygenase protein was being produced, thus allowing an estimation of when the gene was being 'switched on'. These studies revealed that vitamin E succinate does not significantly effect cyclooxygenase activity in B16 cells over a period of 2 to 12 hours as compared to the OE control cultures. Further studies using RNA techniques investigated whether vitamin E succinate was having an effect on cyclooxygenase activity at a molecular level. These investigations

were unsuccessful for the 6 day supplementation for a number of possible reasons, the main reason being RNA stability. Subsequent studies revealed an increase in COX mRNA after 2 hours, suggesting that the gene was 'switched on' soon after supplementation with vitamin E succinate, and further increases in COX mRNA were observed after 8 to 12 hours. The molecular studies were, however, inconclusive.

Previous studies suggested that vitamin E succinate was indirectly causing growth inhibition of B16 cells via regulation of cyclooxygenase activity, however, this study does not support these findings and it would seem unlikely that regulation of cyclooxygenase expression in B16 cells by vitamin E succinate has a role to play in the mechanism by which vitamin E succinate inhibits growth in B16 cells.

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God for building character.

CHAPTER 1

LITERATURE REVIEW

1.1 CANCER

BACKGROUND

Cancer is a generic term used to refer to a number of malignant tumours rather than a single disease with a specific cause or cure. It can be broadly categorized into three major groups, namely, a) carcinomas - which arise in the epithelia, b) sarcomas - which arise in the fibrous tissue and blood vessels, and c) leukaemia and lymphomas - which arise in the blood forming cells of the bone marrow and lymph nodes.[1] Under the influence of chemicals in the environment, radiation and other factors, DNA in normal cells may be transformed in such a way that normal control mechanisms which restrict cell growth fall away. The body deals with this by using repair enzymes to restore DNA structure and the immune system to eliminate cancer cells. If these control mechanisms fail, cancer cells may reproduce uncontrollably, invade surrounding healthy tissue and eventually spread by the process of metastasis to form secondary growths. Cancer is thus particularly difficult to treat.[2-4]

The mechanism(s) by which cells undergo transformation is at present unclear, although for many cancers certain causative and predisposing factors have been defined. These factors include chemical carcinogens, radiation, oncogenic viruses, chronic mechanical or thermal trauma, genetic predisposition, chromosomal instability, ineffective DNA repair, and the aging process. All these factors have a common denominator - they affect DNA.[2,4-6] The notion of altered DNA structure and/or function is central to most theories of carcinogenesis since the transformed phenotype is an inherited characteristic of most affected cells - cells of a tumour all show the genetic characteristics of the original transformed cell, ie. tumours are monoclonal. In recent years, the involvement of specific genes has been demonstrated at the molecular level, and advances in molecular biology and tissue culture have allowed the in depth study of specific genes in the pathogenesis of cancer. [1,2,7]

One of the most notable differences between tumour cells and normal cells is the growth rate of these cells. In normal cells constraints exist which enable them to stop dividing as they proliferate within strictly defined patterns. In cancer cells these constraints are either absent or nonexistent and, as a result, cancer cells are characterized by a sudden increase in cell division and invasiveness. The ability of tumour cells to migrate from the original tumour to a distant site in the body is one of the primary causes of death in cancer patients.[2,3] Other distinctive traits characteristic of cancer cells are altered shape, an increased rate of sugar molecule importation and abnormal cells displaying special tumour antigens giving the cells distinctive immunological properties.[1]

The transformation of a normal cell to a cancer cell occurs frequently in the body, however, not all these transformed cells will establish themselves as clinical cancers. The body has control mechanisms to reverse the process of transformation, these include repair enzymes that replace altered DNA and restore its original structure, as well as the immune system which has the ability to eliminate cancer cells. It has been suggested that the selection pressure exerted by the host against transformed cells may be due to certain endogenous substances, and that transformed cells undergo mutations which enable them to escape this selection pressure as well as the body's control mechanisms.[4,6] Prasad and Rama [6]have suggested that even though transformed cells may be able to escape the selection pressure exerted by endogenous substances at physiological concentrations, increasing the levels of these substances to pharmacological concentrations could reduce tumour cell incidences by either inducing a normal phenotype and/or causing cell death. Vitamins A, C and E are considered to be the endogenous substances that exert selection pressure against transformed cells.[8-10]

DIET AND CANCER

The importance of vitamins and other micronutrients in the maintenance of health has been appreciated for some time now as vitamins are essential for growth and development. There is also evidence to suggest a link between dietary levels of these

vitamins and cancer development, with several epidemiological studies having reported the beneficial effect of vitamin E against breast and some gastrointestinal cancers.[11] Several studies have suggested that a lack of antioxidants in the diet contributes to many human malignancies.[12]

CHEMOPREVENTION

The treatment of many solid tumours by established prevention and therapy modalities has proven to be unsuccessful. This can be seen by the high incidence of these tumours and the associated low survival rates. Chemoprevention is one of the most promising new modalities currently being developed and is defined as 'the systematic use of specific natural or synthetic chemical agents to reverse or suppress the progression of a premalignancy to invasive cancer.'[13] Naturally occurring compounds which possess anticarcinogenic and other beneficial properties are referred to as chemopreventers. The majority of chemopreventers, notably vitamins and micronutrients, are available in and consumed from vegetables, fruits and grains.[14]

Vitamin E will now be considered as it has long been recognised as a promising chemopreventative agent.

1.2 VITAMIN E

BACKGROUND

Vitamin E, an essential fat soluble vitamin, was first discovered in 1922 by Evans and Bishop.[14-16] It is a viscous oil at room temperature, with a melting point of 2.5 - 3.5° C, is soluble in aprotic solvents, and absorbs light in the range of 292 - 298nm in ethanol.[17] Deficiency conditions in the normal population are rare and are found only in patients with various genetic or acquired diseases[18]. However, investigations with lab animals and some human studies have shown myopathy, neurological dysfunction and vascular injuries to be associated with vitamin E deficiency conditions.[10] Deficiency symptoms also include haemolytic anaemia in premature newborn babies and decreased erythrocyte stability in patients with cystic fibrosis both due to vitamin E's crucial role in the maintenance of cell membrane integrity and protection of critical biological fluids against oxidant injury.[14,19,20]

NOMENCLATURE AND STRUCTURE

Vitamin E is the generic term used to describe a family of related naturally occurring lipid-soluble antioxidants which exhibit the general biological activity of alleviating the symptoms of vitamin E deficiency.[17,21-23] At present eight different forms of vitamin E are known to exist in nature - four compounds with tocol structure bearing a saturated phytyl C_{16} side chain, ie. the tocopherols and four compounds with tocotrienol structure bearing three double bonds in the phytyl side chain, ie. the tocotrienols. Members of each group are designated α-, β-, γ- and δ[11,20,21,24] according to the position and number of methyl substituents on the aromatic ring. The most biologically active tocopherol is α-tocopherol (figure 1) due to its approximately four fold higher activity than the β-, γ- and δ-forms.[11,20,21,23] Vitamin E and α-tocopherol was used interchangeably for the purposes of this review.

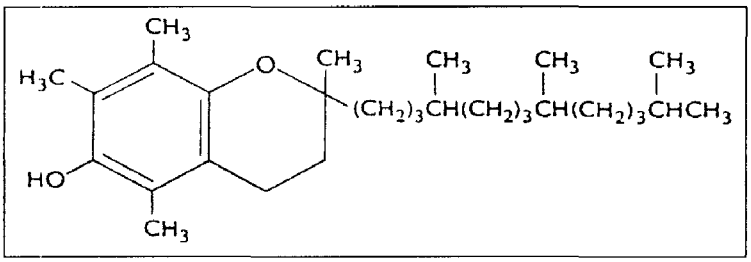

Figure 1 - α-Tocopherol

DIETARY SOURCES

Tocopherols were first isolated from plant oils[17], α-tocopherol being the predominant naturally occurring form.[25] In the diet vitamin E is obtained primarily from vegetable oils, with soya-bean, wheat, cottonseed, corn and safflower oils as rich sources.[18,25] Other plant sources are lettuce, nuts, seeds, fruits and vegetables. Animal tissue and animal products are low-to-poor sources of vitamin E and are influenced by dietary consumption of α-tocopherol by the animal - liver, muscle, heart, kidney, milk, cheese and eggs all contain variable amounts of α-tocopherol. Fish liver oils, although rich in vitamins A and D, are poor sources of α-tocopherol.[21,23,26]

Vitamin E occurs in the diet as a free alcohol,[11,26] but commercial foods and pharmaceutical supplements contain synthetic stable forms of α-tocopherol such as the

non-polar acetate ester - the most common form of vitamin E marketed and the ester primarily used in dietary supplements, as well as the more polar succinate, phosphate and nicotinate esters of α-tocopherol. α-Tocopherol esters are used because they are more resistant to oxidation than α-tocopherol - α-tocopherol being unstable in light and air is easily destroyed under adverse conditions.[11,18]

The required dosage for most vitamins is based on the level at which deficiency becomes clinically evident. This is not the case with vitamin E as deficiency conditions rarely occur and the recommended intake is therefore based on an estimate of how much α-tocopherol the average person consumes.[27] The recommended daily allowance (RDA) is 8mg α-tocopherol or 12 IU for women and 10mg α-tocopherol or 15 IU for men.[18] There are, however, few side effects even at doses as high as 3200mg per day and investigations on the mutagenic, carcinogenic and tetratogenic effects of vitamin E have produced no evidence of harmful effects.[21]

FUNCTIONS

The primary function of vitamin E, and motivation for most research involving vitamin E, is its role as a chain-breaking antioxidant protecting polyunsaturated fats and other lipids against biological oxidation.[21] α-Tocopherol exhibits antioxidant properties both *in vivo* and *in vitro*[6,17], where it functions to trap free radicals and inhibit lipid peroxidation. Both lipid peroxidation and free radicals, if allowed to react uncontrollably, can cause extensive damage to cells and cellular membranes.[10,14,21,28] Figure 2 illustrates the reactions involved in the inhibition of lipid peroxidation by α-tocopherol.

(Antioxidant)	$TOH + ROO^{*}$ --------> $ROOH + TO^{*}$	....(1)	
	$TO^{*} + ROO^{*}$ --------> T(O)OOR	....(2)	[fast]
(Pro-oxidant)	$TO^{*} + ROOH$ --------> $ROO^{*} + TOH$	....(3)	

Figure 2 - Reaction of α-Tocopherol with Lipid Peroxides

In the first step (reaction 1) the chromanol phenolic group of α-tocopherol reacts with lipid peroxyl radicals ($ROO^{\cdot}$) to form a lipid hydroperoxide (ROOH) and an unusually stable tocopheroxyl radical ($TO^{\cdot}$). The $TO^{\cdot}$ radical then reacts rapidly with another lipid peroxyl radical to yield a non-radical product (reaction 2),[14] effectively terminating the chain reaction that might have been propagated by the $ROO^{\cdot}$.[26]

Relatively small amounts of α-tocopherol are needed for effective antioxidant action. In membranes α-tocopherol is present at very low ratios, sometimes only one molecule of α-tocopherol for 2000 - 3000 lipids, yet α-tocopherol is still effective and is the major lipid-soluble, chain-breaking antioxidant.[29]

Under certain *in vitro* conditions α-tocopherol may act as a pro-oxidant (reaction 3).[30] α-Tocopherol is ineffective as an antioxidant when the initial peroxide content is too high. In the presence of vitamin C (ascorbic acid) there is a dramatic reversal from pro-oxidation to antioxidation.[31] α-Tocopherol can be regenerated by vitamin C and glutathione, the major water-soluble intracellular antioxidants,[29] and these compounds therefore have a sparing effect on α-tocopherol.[19]

Additional functions of vitamin E that are biologically important include:

1) Stabilization of membranes through physicochemical interaction with phytyl side chains and the fatty acyl chains of polyunsaturated phospholipids (figure 3);[5,6,14,26,32]
2) Stimulation of the host's humeral and cell mediated immune response[5,6,20,28] and phagocytic functions[33] - an increase in IgG production is observed with pharmacological levels of α-tocopherol;[26]
3) Suppression of inflammation[34] and platelet aggregation;[6]
4) Inhibition of the conversion of nitrates to nitrosamines in the stomach;[5]
5) Regulation of nucleic acid and protein metabolism;[26,34] and
6) The modulation of various enzyme reactions, including Protein Kinase C (PKC)[35-38] and cyclooxygenase which regulates prostaglandin synthesis via the arachidonic acid cascade.[6,29,39]

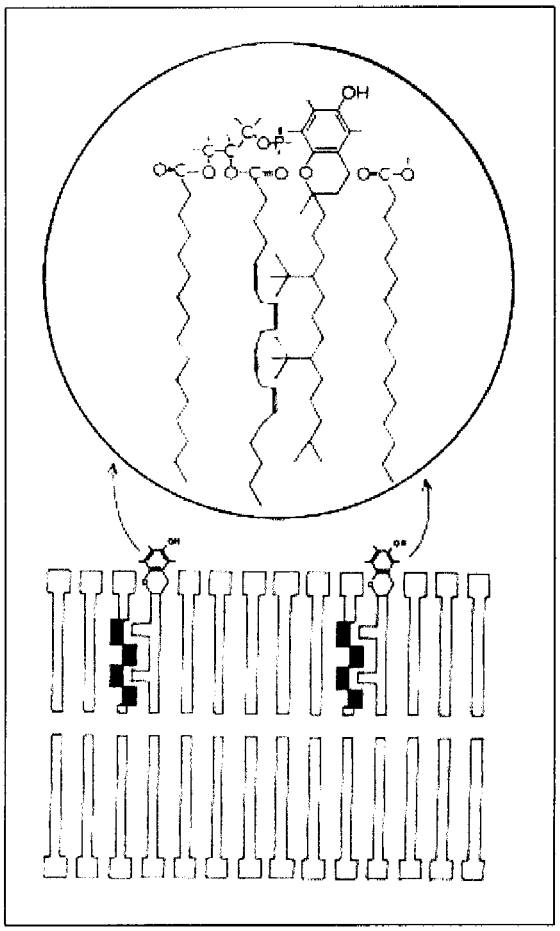

Figure 3 - Diagrammatic Representation of the Proposed Interaction Between α-Tocopherol and PUFAs in a Biological Membrane[17]

(*The isoprenoid chain of α-tocopherol orientates the molecule in the membrane and is responsible for the membrane stabilizing function of α-tocopherol - shortening or elimination of the isoprenoid side chain results in very low activity or no activity. The chromanol nucleus on the other hand lies at the surface of the membrane where the phenolic hydrogen group has access to and is able to quench free radicals, ie. the antioxidant function of vitamin E.*[14,26])

INTRACELLULAR LOCALIZATION

α-Tocopherol is associated with the membranes of cells[5] and is mostly concentrated in the mitochondria and endoplasmic reticulum (ER), with very little α-tocopherol found in the cytosol.[20,25] It appears to be bound with proteins and these vitamin E binding proteins (Mwt 20kDa) are also found in human serum suggesting that they may act as carriers for α-tocopherol.[22,40,41] Radioactive α-tocopherol binds to the crude nuclear and purified chromatin fractions of glioma and neuroblastoma cells, possibly implicating α-tocopherol in the modulation of gene expression in mammalian cells.[6,40,41]

VITAMIN E AND CANCER

More than 80% of human cancer is due to environmental factors and if one could identify the main risk and anti-risk factors it would be possible to prevent most human cancers. Many studies have suggested that the absence of certain dietary compounds, notably antioxidants, contributes to cancer development. The anticancer activity of vitamin E has been well established.[12,42]

Vitamin E is a potent anticancer agent in terms of both cancer prevention and regression. Interest in vitamin E as a chemopreventative agent for cancer began in 1946 following reports by Jaffe that rats fed wheat germ oil exhibited a lower incidence of tumours.[10] These findings were further strengthened by numerous animal experiments, a variety of cell culture studies and epidemiological studies illustrating the effectiveness of vitamin E in cancer chemoprevention,[42,43] which showed inverse relationships between dietary levels of vitamin E and cancer incidence. [12,14,28] It is a growing concept that vitamins in combination with other micronutrients could eventually be used in cancer chemoprevention.[25]

Vitamin E induces multiple effects during the management of tumours including cell death, differentiation, inhibition of cell division, potentiation of the effect of tumour-therapeutic agents, suppression of the toxicity of certain chemotherapeutic drugs and stimulation of the host's immune system. These effects are however tumour type specific and depend on the type of therapeutic agent used.[40,44]

The mechanism(s) by which vitamin E inhibits tumour cell growth is at present unclear although Alfin-Slater and Kritchevsky[10], have proposed the following possible mechanisms:

1) Vitamin E acting as an antioxidant quenching free radicals;
2) Reduction of nitrosamine and nitrosamide formation by competing for nitrates;
3) Acting as a repressor, regulating Coenzyme Q functions as well as specific enzymes and proteins required for tissue differentiation;

4) Maintenance of cellular protein thiols; and

5) Immunostimulatory activity.

These proposed mechanisms have been supported by various other researchers.[5,14,25] In addition to these mechanisms, ElAttar and Lin[42] have suggested that vitamin E may act directly upon the cancer cells, entering the cells and destroying them by altering cell metabolism and perhaps by inactivating oncogenes and oncoproteins that maintain the viability of the abnormal cells.

As vitamin E succinate is used as a vitamin E supplement in our laboratory, this ester derivative of vitamin E will now be reviewed.

1.3 VITAMIN E SUCCINATE

BACKGROUND

Cell culture offers a powerful tool in cancer research. In *in vitro* systems, cells are grown under defined conditions, enabling researchers to study and assess at a cellular level the carcinogenic potential of various physical or chemical agents, as well as identify factors that inhibit transformation and act as cancer preventative agents.[12] For vitamin E administration, ester derivatives of α-tocopherol are used, both for experimental and therapeutic purposes.[45,46] The reason for this is that α-tocopherol is rather unstable and the solvents used as vehicles to allow cell entry and uptake are toxic.[6,22] *In vivo* tocopherol esters are believed to undergo hydrolysis via any number of nonspecific esterases to release α-tocopherol,[47,48] thus providing a stable dosage of α-tocopherol.[45] α-Tocopherol acetate is the most commonly used α-tocopherol derivative,[22] however the succinate ester is the most potent form of vitamin E for antiproliferate effects on tumour cells.[49] Most growth media contain very limited amounts of α-tocopherol and serum does not contain much more, thus cells must obtain α-tocopherol from their environment.[50] This allows for strict regulation of the amount of vitamin E supplementation to the cells and it can be assumed that the resultant effects are indeed a result of the vitamin supplementation.

The succinic ester of vitamin E is widely used in *in vitro* studies for three main reasons:[6,44]

1) Being polar, it is more soluble and stable in growth media;
2) It crosses cellular membranes with ease; and
3 It has a very slow rate of conversion to α-tocopherol so that intracellular levels of tocopherol remain high for longer periods.

STRUCTURE

α-Tocopherol acid succinate (figure 4) is a succinic acid ester of α-tocopherol. It is practically insoluble in water and has a maximum absorption in ethanol of 286nm. The sodium salt, $C_{33}H_{53}NaO_5$,[51] with ethanol as a vehicle, is used as a vitamin E supplement in our laboratory. The acidic nature of α-tocopherol succinate is important for activity, as the cytoprotective properties of this compound are eliminated if the ionic nature of the molecule is reduced or eliminated.[45]

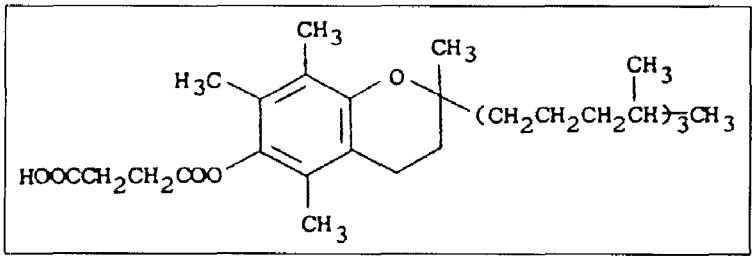

Figure 4 - α-Tocopherol Acid Succinate

FUNCTIONS

α-Tocopherol succinate is the most potent form of vitamin E for inducing morphological changes and growth inhibition in murine B16 melanoma cells in culture[6,43] - the system under investigation in our laboratory - however, the growth and survival of many other cell lines are also inhibited by α-tocopherol succinate.[52] The effect on cell proliferation is cell type dependant, as well as, dose dependant. Inhibition of cellular proliferation is detected at concentrations as low as 0.01μg/ml and the maximum nontoxic concentration that causes growth inhibition is 10μg/ml, higher doses being lethal.[6,43,49]

The mechanism(s) by which α-tocopherol succinate exerts anticarcinogenic effects is

unknown,[52] and there is evidence to both support and contradict an assumption that this compound may have similar functions as α-tocopherol. In terms of antioxidant function, the effects of α-tocopherol succinate on tumour cells are similar to those produced by α-tocopherol and other lipid-soluble antioxidants, however, much higher concentrations of these antioxidants are required.[44,53] For α-tocopherol succinate to exhibit antioxidant function, the succinate group must be cleaved off allowing the phenolic group to interact with free-radicals.[22,49] Fariss *et. al.*, have demonstrated that α-tocopherol succinate cytoprotection results from cellular accumulation of the intact α-tocopherol succinate molecules and is not due to the release of α-tocopherol or succinate.[45,50] Work done in our laboratory supports these findings as supplementation of B16 cells with sodium succinate is ineffective in terms of growth inhibition. This was also shown by Prasad and Rama.[6] This would suggest that α-tocopherol succinate acts as a pro-oxidant rather than an antioxidant[54] and this is further supported by the finding that lipid peroxidation and free radicals are increased after supplementation of α-tocopherol succinate in B16 cells.[55]

On the whole, α-tocopherol succinate inhibition of tumour cell growth appears to be controlled by mechanisms other than the classical role as an antioxidant. Several reports suggest that α-tocopherol succinate administration does stabilise membranes and alter membrane enzyme activity. It has been suggested that the lipophilic tocopherol moiety may interact with the unsaturated fatty acid (UFA) portion of the membrane phospholipids and that the ionic succinate moiety may interact with the polar regions of the phospholipids thus stabilizing the membrane.[45,53] Being less hydrophilic than α-tocopherol, one would expect that α-tocopherol would have a more extensive intracellular distribution as it would be less likely to be entrapped in the outer membrane layers thus allowing distribution to and localization in internal membrane sites.[48]

Another mode of action whereby α-tocopherol succinate regulates cell growth may be at the nuclear level. Rather than inducing tumour cells to differentiate into a nonproliferative state, α-tocopherol succinate appears to block the cell cycle prior to

DNA synthesis, ie. it arrests the cell in the G_0-G_1 phase (*growth and synthesis of proteins)* or early S phase (*growth and DNA synthesis*) - a mechanism similar to that of prostaglandins. Expression of oncogenes in certain cells *in vitro* is also regulated by α-tocopherol succinate.[42,52,53]

VITAMIN E SUCCINATE AND CANCER

α-Tocopherol succinate seems to have a cellular protective function both *in vitro* and *in vivo*. Cellular accumulation of the intact α-tocopherol succinate was found to protect cells from the toxic effects of oxygen intermediates - reactive oxygen intermediates are involved in many disease conditions including cancer[45,46] - and inhibits transformation of mammalian cells treated with carcinogens.[12] The frequency of radiation-induced transformation[52] and tumour development in animals receiving α-tocopherol succinate is significantly inhibited.[42] If these results can be applied to *in vivo* conditions, α-tocopherol succinate may be a useful anticancer agent for melanoma cells and possibly other neoplasms.

The literature has suggested that in addition to its many other functions, vitamin E may be involved in regulation of gene expression in mammalian cells[26,34,42] and it seems probable that vitamin E succinate may have its antiproliferate effect at the nuclear level as well.[42,52,53] This raised the question of whether vitamin E succinate regulates gene expression in murine B16 melanoma cells, and if so at which level of expression this regulation was occurring. In previous studies,[39,54,55] it was found that many enzymes expressed increased activity following vitamin E succinate supplementation, among them, cyclooxygenase, the rate limiting enzyme in prostaglandin synthesis. This enzyme will now be considered.

1.4 PROSTAGLANDIN G/H SYNTHASE

(PGHS, Cyclooxygenase, COX: EC 1.14.99.1)

BACKGROUND

The relationship between prostaglandins (PGs) and cancer has been of interest to many researchers because they affect mitogenesis, cellular adhesion, immune

surveillance and apoptosis. Cancers of the heart, neck, breast, lung and colon form more PGs than normal tissues and PGs of the E series, mostly PGE_2, are often over expressed in carcinomas and sarcomas, as well as in transformed cell lines. In support of the role of PGs in carcinogenesis, it was found that inhibitors of PG formation protect against colon, mammary, esophageal, lung and oral cancers in animals and humans.[56,57]

PGs are vital in normal cellular functioning, as they play a role in normal homeostasis. These prostanoids are potent biological mediators with diverse normal physiological effects modulating kidney functions, respiratory functions, reproduction, the immune response and gastrointestinal functions, and they play a role in platelet aggregation. PGs are also important in a variety of pathological conditions, including neoplastic transformation and inflammation. When released at the site of trauma, they directly mediate pain and induce fever.[57-59] Increased PG synthesis in tumour cells is attributed to an increase in PGHS synthesis and *in vitro* studies have shown that this enzyme is induced by mitogenic stimulation and transformation.[56]

PGHS activity can either be stimulated or inhibited by antioxidants. Vitamin E's role in PGHS reactions has been disputed, but it was suggested that vitamin E stimulates PGHS activity by protecting the enzyme from inactivation.[39,60] Fujimoto, *et. al.* have shown that very low concentrations of hydroperoxides are required to activate PGHS, and at high concentrations of hydroperoxide the enzyme is inhibited. Vitamin E therefore seems to play an important role in regulation of PG metabolism and this is further substantiated by the fact that it was found to regulate platelet aggregation via inhibition of PGHS activity thereby causing a decline in PG production.[31]

ENZYME REACTION

PGHS is the key enzyme in the biosynthesis of PGs. PGs are products of arachidonic acid (AA), an essential 20-carbon polyunsaturated fatty acid (PUFA) found in membrane lipids. AA is consumed in the diet or derived from elongation and desaturation of ingested linoleic acid. The common intermediate in the synthesis of

PGs is PGG_2, a cyclic hydroperoxide formed by the addition of two molecules of O_2 to AA. PGG_2 is an unstable intermediate and is reduced to PGH_2, an alcohol. PGH_2 is the common precursor for all prostanoids, and is further metabolized by different but specific synthases and isomerases to produce a variety of eicosanoid products (PGs, thromboxanes and prostacyclins) depending on the type of cell or tissue.[57,59,61-64]

PGHS carries out two distinct, sequential biochemical reactions. The cyclooxygenase activity cyclizes and oxygenates AA to PGG_2, and the hydroperoxidase activity reduces PGG_2 to PGH_2. PGHS is often referred to as COX, for cyclooxygenase, but it is important to note that the two activities of PGHS can be separately involved in other reactions and are inhibited by different substances. For the purposes of this review, COX is used interchangeably with PGHS and does not specifically refer to the cyclooxygenase activity.[57,58,64]

The rate-limiting reaction in PG synthesis is the activity of PGHS because this enzyme is 'suicide' inactivated, physiologically, during catalysis.[62,65,66] Two different inactivation processes occur for PGHS. The first is the autoinactivation or 'suicide' reaction of cyclooxygenase, which occurs when PGHS is incubated with AA. The rate of this 'suicide' inactivation is related to the rate of cyclooxygenase catalysis and does not depend on product formation. The second reaction is peroxidase-dependant inactivation of the cyclooxygenase and peroxidase activities, which is due to modification of the heme group when the enzyme is incubated with hydroperoxides.[67]

The enzyme is an integral membrane protein, with monotopic association to the membrane as it does not have a transmembrane domain. There is a single heme binding site per monomer that is essential for activity.[57,58,68]

ISOZYMES

Two different isoforms of PGHS, which are approximately 60% homologous within a species, have been identified. The isoforms are almost identical at the protein level, but differ significantly in terms of gene and promoter structures, stability of mRNA and

intracellular localization of the gene products, which accounts for their separate physiological functions.[57,68,69] A concise summary outlining the similarities and differences between the two isoforms of PGHS can be found in table 1.

Table 1 - A comparison of COX-1 and COX-2 (adapted from Subbaramaiah *et. al., 1997*)[57]

	COX-1	COX-2
Regulation	Constitutive	Inducible
Expression range	Can ↑ 2 to 4-fold	Can ↑ 10 to 80-fold
Protein size	Single band ± 72kDa on SDS-PAGE	Doublet on SDS-PAGE with Mwt 72 and 74kDa
Kinetic parameters	Similar Km and Vmax for AA	Similar Km and Vmax for AA, more effective with alternate substrates
C-terminus	18-amino acid cassette absent	18-amino acid cassette present
Prosthetic group	Heme	Heme
Gene size	>22kb, 11 exons and 10 introns	8-9kb, 10 exons and 9 introns
Human chromosome	9	1
mRNA size	2.7 to 3kb	4 to 4.5kb, numerous Shaw-Kamen sequences
Localization	ER	ER and nuclear membrane
Effect of glucocorticoids	Little or none	Inhibit expression
Cell and tissue expression	Most tissues	Parts of brain (rat), during inflammation and in tumour cells

i) COX-1

COX-1 was purified in 1976 (Miyamoto, et. al.).[cited in 70] This isoform is constitutively expressed, ie. the mRNA is constantly being transcribed from the gene and the protein synthesized at a steady rate independent of extracellular signals. It is expressed in most tissues, and is involved in physiological production of PGs for maintenance of

normal homeostasis, ie. gastric cytoprotection, vascular homeostasis and kidney function.[57,59,61,62,65,66,68,71] The COX-1 gene is located on chromosome 2 (human) and chromosome 9 (mouse) and is >22kb in size.[57,62,68,70] cDNA clones have been characterized in various mammals, including mouse, sheep and human, and the mRNA transcript was found to be 2.7kb - 3kb in size.[57,62,66,69,70] COX-1 protein migrates as a single band of 72kDa on SDS-PAGE and the deduced amino acid sequence is 600-602 residues.[57,62,66]

ii) COX-2

COX-2, first identified in 1989 (Simmons *et. al.*),[cited in 69] was more recently described. This isoform is tightly regulated, is sensitive to a wide range of extracellular and intracellular stimuli, and expression may be enhanced by activation of cell surface receptors.[68-70] COX-2 is characterized by rapid induction by a number of agents, including mitogens, cytokines, hormones, growth factors, oncogenes, serum and inflammatory mediators, and is proposed to play a role in physiological and pathological processes characterized by increased production of PGs, including cell growth and inflammation.[57,59,61,65,68] It is not detectable in most normal tissues under basal conditions and rat brain is the only organ with COX-2 as the dominant isoform. Increased production of PGs in transformed cells and tumours is associated with upregulation of COX-2.[57,62,66,68] The COX-2 gene is located on chromosome 1 (mouse and human) and is 8kb in size.[57,62,68,69] COX-2 is encoded by a 4kb - 4.5kb mRNA transcript, which has been cloned as an inducible, immediate-early response gene from human, mouse, rat and chicken.[57,62,66,69,70] The amino acid sequence of COX-2 is 603/604 residues, and the protein appears as a doublet on SDS-PAGE with molecular weights of 72kDa and 74kDa.[57,62,66]

PROTEIN STRUCTURE

The protein and crystal structures, as well as enzymatic activities of the two COX isoforms are very similar, sharing >60% homology in amino acid sequence.[66,68] Cyclooxygenase and hydroperoxidase regions are conserved between the two proteins, and functionally relevant sites, ie. the heme-coordinating histidine, catalytic site tyrosine and serine residue acetylated by aspirin, are also conserved.[57,62,68,70] With AA, the isoforms have similar Km and Vmax values, however, COX-2 is more effective with alternative substrates. Mutational studies suggest that COX-2 may have a larger active

site pocket than COX-1, so although structurally very alike, the isoforms have different drug sensitivities.[58,62,68]

COX-2 differs from COX-1 in that it has a slightly truncated N-terminal signal peptide and a unique 18 residue insert near the C-terminal which allows for the generation of specific peptide antibodies against COX-2.[57,62,66,68,70] The isoforms also differ with regard to post-transcriptional glycosylation. COX-1 has 3 N-linked glycosylation sites that are conserved in COX-2, however COX-2 has an additional partially used N-linked glycosylation site located in the 18 amino acid C-terminal insertion. This additional N-linked glycosylation site in COX-2 explains why two bands are observed with SDS-PAGE.[57,68,70] COX-2 has been found to form large aggregates under conditions where COX-1 is a dimer.[58]

GENE STRUCTURE

COX-1 and COX-2 are single copy genes, located on different chromosomes. The intron/exon arrangements are almost identical for the two isoforms, except that exons 1 and 2 of COX-1 are condensed into a single exon in COX-2, (figure 5) - these exons contain the translational start site and signal peptide. COX-1 has 11 exons and 10 introns. The exons of COX-2 are similar in size to those of COX-1, the introns are however much smaller. COX-2 has 10 exons and 9 introns.[57,62,68,70]

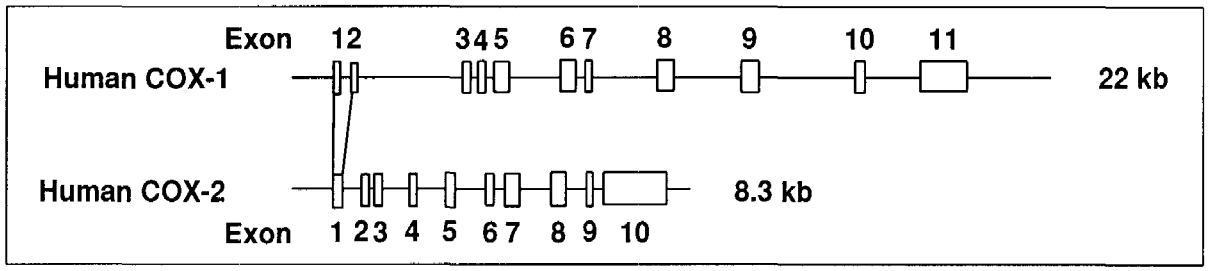

Figure 5 - COX-1 and COX-2 Genomic Structure.[70]

The two isoforms of PGHS differ with regard to structure and regulation at the level of RNA and DNA. The different gene structures afford a molecular basis for COX-1 as a continuously transcribed stable message providing relatively constant level of enzyme in most cells, and this isoform is characterized as a housekeeping gene. COX-2 as a different gene product, is regulated at the level of transcription and has features consistent with rapid mRNA processing. It is an immediate-early gene with reduced

mRNA stability similar to many cytokine and protooncogene mRNAs.[66,68,72]

COX-2 has a long 2.1kb 3'-untranslated region, rich in 5'-AUUU$_n$A-3' motifs. These motifs are known as Shaw-Kamen sequences and confer enhanced mRNA degradation. (In humans 17 repeats have been identified, and there are 18 in mouse.) This untranslated region also contains several polyadenylation signals. These elements are common to highly regulated genes. The 5'-flanking region of COX-2 contains a TATA motif. COX-1 has greater mRNA stability than COX-2, attributed to the lack of Shaw-Kamen sequences, and COX-1 has a TATA-less promoter and has not been found to have an inducible transcription.[57,62,66,70,72]

REGULATION

COX-1 expression is increased in amnion cells at the onset of labour, but generally this isoform has a very limited range of expression.[69,70] Rapid turnover of COX-2 mRNA however allows for tightly controlled responses to multiple stimuli, including inhibition by glucocorticoids and stimulation by interleukin 1β, growth factors and oncogene activators. Regulation of mRNA has potential to occur at several levels including transcription, stability and possibly alternative polyadenylation, but dynamic modulation of mRNA levels may not fully explain the rapid decline of COX-2 protein levels in cells exposed to certain inhibitors, which suggests that translational regulation may also occur.[72]

INTRACELLULAR LOCALIZATION

The two isoforms of PGHS differ with regard to their subcellular localization, and there is evidence to suggest that they function as separate enzyme systems. COX-1 functions primarily in the endoplasmic reticulum (ER) and COX-2 is located both in the ER and nuclear membrane. Consequently, it has been suggested that the metabolites may be compartmentalized and that this leads to a partitioning of their functions. The COX-2 metabolites, colocalized with the nuclear material, may have important effects in the nucleus, modulating expression of target genes.[57,61,70] Nuclear receptors with eicosanoids as natural ligands that act as transcriptional factors have been identified.[68] It has also been suggested that COX-1 and COX-2 use different intracellular pools of AA, and this is expected to have functional consequences.[57]

INHIBITION

The COX isoforms may also be distinguished on a pharmacological basis since differences in their inhibition by non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids have been detected. COX-1 is 100 fold more sensitive to aspirin (a NSAID) than COX-2 and dexamethasone (a synthetic glucocorticoid) selectively inhibits COX-2, having little or no effect on COX-1 levels. The effect of NSAIDs is only at a translational level, ie. protein levels are affected, while the effect of dexamethasone (DEX) is both at the level of transcription and translation, ie. mRNA levels are affected as well.[61,62,65,72,73]

It has been suggested (Sabbaramaiah *et. al.*[57] and Crofford[68]) that selective inhibition of COX-2 with preservation of COX-1 function would maintain PGs that are important for protection of normal gastrointestinal, kidney and platelet function as COX-2 is the isoform upregulated during inflammation and other pathological processes. Peptic ulcers and reduced kidney function are often complications of treatment with NSAIDs because these compounds indiscriminantly inhibit PGs synthesis. There is also much evidence to link increased levels of COX-2 and tumorigenesis, thus the availability of selective inhibitors of COX-2 would be advantageous.

COX AND CANCER

There is much evidence to suggest a role for PGHS in the pathogenesis of malignancy. Human colon cancer has increased expression of COX-2 mRNA compared with normal colon specimens from the same patient.[63] Breast, head and neck cancers produce higher levels of PGs most likely due to COX-2 and it has been suggested that raised PG production due to expression of this isozyme may be a common mechanism by which cellular phenotype is altered to allow dysregulated growth. Transcription of COX-2 in transformed mammary epithelial cells was also found to be enhanced.[64,68]

PROJECT OBJECTIVES

As indicated previously, vitamin E succinate is the most potent form of vitamin E for inducing morphological changes and growth inhibition in B16 murine melanoma cells *in vitro*. Although the mechanism(s) by which vitamin E succinate exerts these effects is unclear, certain mechanisms have been suggested. The dominant hypothesis is that vitamin E succinate acts as an antioxidant,[44,53] but this molecule is not a physiological antioxidant in that cleavage of the ester bond is required for vitamin E succinate to exhibit antioxidant properties.[22,49]

Studies in our laboratory have suggested that, since an increase in lipid peroxidation and free radicals was observed after supplementation of vitamin E succinate to B16 murine melanoma cells, it was unlikely that this molecule was acting as an antioxidant in this cell line.[39,55] However, whether cleavage of vitamin E succinate to vitamin E was occurring in these cells still needed to be determined. This issue was addressed in the present study using HPLC to determine whether vitamin E succinate is metabolised to vitamin E in this cell line.

It has also been suggested that vitamin E succinate may regulate various metabolic pathways, and in this regard the AA cascade pathway was studied in our laboratory. A significant increase in the activity of various enzymes, including cyclooxygenase, which is the rate-limiting enzyme in prostaglandin synthesis, was found to occur with supplementation of 7 and 10μg/ml of α-tocopherol.[39] In this study, cyclooxygenase was further investigated, but in this case in terms of regulation of expression of the enzyme at the molecular level as this is another mechanism by which vitamin E succinate is believed to exert its anticarcinogenic effects.

In brief, the objectives of this study were:

1) to determine the effect of vitamin E succinate supplementation on the *in vitro* growth of B16 murine melanoma cells;
2) to establish whether these effects were due to the intact vitamin E succinate or to the vitamin E component of the molecule; and
3) to evaluate the effect of this supplementation at a molecular level by examining cyclooxygenase activity and expression.

CHAPTER 2

GROWTH STUDIES

2.1 INTRODUCTION

It seems unlikely that a cure for many types of cancer will be found in the foreseeable future and the lack of success of established preventative and therapeutic strategies in controlling the high incidence and low survival rates of patients with solid tumours is therefore a powerful motivator to put considerable effort into cancer prevention. Judging by the frequency of occurrence of cancers in different countries, environmental factors have more influence on the incidence of cancers than genetic factors do - a conclusion reinforced by studies of migrant populations - with diet being the most likely cause of cancer.[13,74] Thus, chemoprevention seems to be a promising means of reducing cancer incidence and the effectiveness of vitamin E as a chemopreventer has been established in numerous studies.[12,14,28,42,43] It has been demonstrated both *in vivo* and *in vitro* that vitamin E succinate has a protective function in mammalian cells, and this form of vitamin E is also the most potent for inducing morphological changes and growth inhibition of B16 cells *in vitro*.[6,43]

Because many properties of transformed cells grown in tissue culture resemble those of cancer cells, it is possible to study the factors that lead to uncontrolled growth or an inhibition of growth *in vivo* by the effects they have on tissue culture cells under *in vitro* conditions.[74] Tissue culture also has the added advantage of allowing one to grow the cells under defined conditions thus eliminating unwanted variables which complicate matters *in vivo*.[12]

Growth studies were undertaken to determine the effect of vitamin E acid succinate supplementation at varying levels (1-10µg/ml) on the growth of B16-F10 (highly metastatic murine melanoma) cells in culture. A non-malignant LLCMK (monkey kidney) cell line was also supplemented with varying concentrations (1-10µg/ml) of vitamin E succinate.

2.2 MATERIALS AND METHODS

MATERIALS

Murine B16-F10 (B16) melanoma cells and non-malignant monkey kidney (LLCMK) were obtained from Stellenbosch University, South Africa. Basal minimum essential medium (MEM) and McCoys 5A modified medium, L-serine, and (+)-α-tocopherol acid succinate (vitamin E succinate) were purchased from Sigma Chemical Co., USA. Foetal calf serum (FCS) was obtained from Delta Bioproducts South Africa, while sterile disposable tissue culture flasks ($25cm^2$ and $75cm^2$) were purchased from Corning, USA. Streptomycin sulphate (novo-Strep) and sodium benzylpenicillin (novo-Pen) were purchased from Novo Industries (Pharmaceuticals Ltd), South Africa. Glycine, D-glucose, phenol red, dimethylsulphoxide (DMSO) were purchased from BDH Chemicals Ltd, England. Trypsin was purchased from Boehringer Mannheim, Germany, while ethylenediaminetetraacetic acid (EDTA) and potassium chloride (KCl) were purchased from Holpro Chemicals, South Africa. Sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), potassium dihydrogen orthophosphate (KH_2PO_4), and sodium dihydrogen orthophosphate (NaH_2PO_4) were purchased from SAARCHEM (Pty) Ltd, South Africa. A haemocytometer was purchased from Neubauer, Germany (bright-line, double ruling).

METHODS

2.2.1 Preparation of Culture Reagents

i) *McCoys Medium (B16 Cells)*

The following compounds were added to McCoys 5A modified medium, which contained modified Hanks salts and glutamine:

2.2g/l	$NaHCO_3$
0.01g/l	serine
0.006g/l	glycine
5ml/l	novo-strep and novo-pen mixture

ii) *Hanks or Eagles Basal Medium (LLCMK Cells)*

The following compounds were added to basal MEM, which contained Hanks salts and

glutamine:

1.68g/l	$NaHCO_3$
0.01g/l	serine
0.006g/l	glycine
5ml/l	novo-strep and novo-pen mixture

iii) *Novo-strep and Novo-Pen Mixture*

This was prepared by adding 1 vial of novo-strep (1g/ml) and 600mg of novo-pen (5 million U) to 100ml of milli Q water.

iv) *Medium Filtration*

The medium was filtered using a millipore filtration unit (Millipore Corporation, USA), and the following filters: a prefilter type AW "Membr filter" 50k (size 130); a 0.45µm type HA filter (HAWP 14250), and a 0.22µm type GS filter (GSWP 14250). The filtration unit was rinsed using 500ml of milli Q water, after which the medium was pumped through. The first 200ml of medium was discarded, while the remaining medium was filtered into autoclaved bottles. The filtration unit was then rinsed with 500ml of milli Q water. The bottles of medium were stored at 4°C until required. Before use, the bottles of medium were incubated at 37°C for 72 hours to test for contamination.

v) *Growth and Freezing Medium*

Growth medium was prepared by filtering FCS through a 0.45µm filter using a Swinnex-25 holder (Millipore corporation, USA), until the medium contained 10% (v/v) FCS. After the addition of FCS, the medium was incubated at 37°C for 72 hours to test for contamination. Freezing medium was prepared in the same manner, except that 20% FCS was added and 10% DMSO, after which the media was frozen till required. If the pH of the growth medium was too acidic, a few drops of sterile 1M NaOH solution were added to return it to physiological pH. If the pH of the growth medium was too basic, a few drops of sterile HCl solution were added.

vi) *Trypsin Solution*

A 0.05% solution of trypsin was required, hence a 500U/ml solution was prepared. The trypsin solution contained the following:

33.33mg/l	trypsin
8.0g/l	NaCl
0.4g/l	KCl
1.0g/l	D-glucose
0.58g/l	$NaHCO_3$
0.2g/l	EDTA
0.02g/l	phenol red
10ml/l	novo-strep and novo-pen mixture

Trypsin was stored at -20°C until required. All trypsin was filtered directly into the culture flasks, through a 0.45µm Millipore filter using a Swinnex-25 holder.

vii) *Phosphate Buffered Saline Solution*

Phosphate buffered saline (PBS), pH 6.6 was prepared in milli Q water, as follows:

8.0g/l	NaCl
0.2g/l	KH_2PO_4
0.2g/l	KCl
0.15g/l	$NaHPO_4.2H_2O$

2.2.2 Cell Culture

i) *Routine Procedures*

All work was done using sterile conditions in a laminar flow bench, which was sterilized by ultra-violet light exposure for a minimum duration of 30 minutes, and all equipment used was sterile having been purchased sterile or autoclaved for 45 minutes prior to use. Before the laminar flow bench was used it was swabbed with 70% alcohol. Non-malignant LLCMK (monkey kidney) and malignant B16 (murine melanoma) cells were used. The cells were maintained at 37°C in 75cm^2 flasks containing 30ml of 10% (v/v) FCS medium, when not used for experimental purposes. The medium was changed

as required. Once the cells reached confluency, they were passaged into 3 or 4 flasks. The growth medium was discarded and 10ml of sterile trypsin was added to the flask. The cells were incubated with trypsin at 37°C until the cells detached from the flask surface. Once all the cells had lifted, the cell suspension was equally divided into 3 or 4 flasks and 30ml of growth medium was added to each flask. Cells were frozen down for storage once they were no longer required for experimental purposes. This involved harvesting of the cells by addition of 10ml sterile trypsin, centrifugation of the cells at 480g for 4 minutes (Eppendorf centrifuge 5403) to obtain a pellet, reconstitution of the pellet with 2ml of freezing medium and the transfer of the cell suspension into a cryogenic vial. The vials were then stored in liquid nitrogen (or at -70°C) until they were required.

On reaching confluency LLCMK or B16 cells were trypsinized with 10ml of trypsin and centrifuged at 480g for 4 minutes to pellet cells. The resulting suspension (trypsin solution) was discarded and the cell pellet resuspended in 2ml of growth medium. Cells were counted using a haemocytometer and the volume of the cell suspension required to seed 500 000 cells into 75cm^2 flasks, or 300 000 cells into 25cm^2 flasks, was calculated.

2.2.3 Effect of Vitamin E Succinate Supplementation on Malignant B16 (Murine Melanoma) Cell Growth

***- Experimental procedures*:**

i) *Routine cell culture procedures*

On reaching confluency B16 or LLCMK cells were trypsinized with 10ml of trypsin and centrifuged at 480g for 4 minutes to pellet cells. The resulting suspension (trypsin solution) was discarded and the cell pellet resuspended in 2ml of growth medium. Cells were counted using a haemocytometer and the volume of the cell suspension required to seed 500 000 cells into 75cm^2 flasks, or 300 000 cells into 25cm^2 flasks, was calculated.

ii) *Preparation of experimental medium*

Stock solutions of vitamin E succinate (1, 3, 5, 7 and 10mg/ml) were prepared in 1ml of absolute ethanol, and 100µl of each stock solution was added to 100ml of medium containing 10% (v/v) FCS. This brought the final concentration of vitamin E succinate being supplemented to the cells to 1, 3, 5, 7 and 10µg/ml respectively. Controls containing 0.1% final volume absolute ethanol labelled OE, were prepared by adding 100µl of absolute ethanol to 100ml of medium containing 10% (v/v) FCS.

iii) *Growth of cell cultures*

Six sets of 3 75cm^2 or 5 25cm^2 flasks were seeded with 500 000 or 300 000 (B16 or LLCMK) cells, respectively. Five sets of these flasks were supplemented with 30ml (75cm^2) or 10ml (25cm^2) of MEM media containing varying levels (1-10µg/ml) of vitamin E succinate. The sixth set of flasks received 30ml (75cm^2) or 10ml (25cm^2) MEM media containing 0.1% final volume of ethanol and was referred to as a control culture (OE). All flasks were incubated at 37°C for 5 to 7 days, with a media change performed one day prior to confluency. The flasks were again incubated at 37°C and the cells were harvested the following day.

iv) *Harvesting of cells*

On first flask reaching confluency, the cells were harvested under non-sterile conditions. The cells were incubated with 10ml (75cm^2) or 5ml (25cm^2) of trypsin at 37°C until they detached from the flask surfaces. Resulting cell suspensions were then poured into centrifuge tubes and centrifuged at 480g for 4 minutes. The trypsin solution was discarded and cell pellets were resuspended in 1ml of PBS (pH6.6). The cells were counted using a haemocytometer and the samples were stored at 4°C until required. The cell counts were used as an indicator of cell growth.

2.2.4 Effect of Vitamin E Succinate Supplementation on Non-Malignant LLCMK (Monkey Kidney) Cell Growth

***- Experimental procedures*:**

Refer to section 2.2.3 (i) to (iv) for the method used.

2.2.5 Statistical Analysis

The results obtained were analyzed using a One Way Analysis of Variance (ANOVA) followed by a Student-Newman-Kuels Multiple Range test.

2.3 RESULTS

2.3.1 Effect of Vitamin E Succinate Supplementation on Malignant B16 (Murine Melanoma) Cell Growth

Supplementation of B16 murine melanoma cells with varying concentrations of vitamin E succinate (1-10µg/ml) had an inhibitory effect on growth of this cell line, as is illustrated in figure 6. Results shown in this figure represent the mean values of five experiments each performed in triplicate. These results are partially relevant to the cleavage studies described in chapter 3 and will be discussed relative to these studies at a later stage.

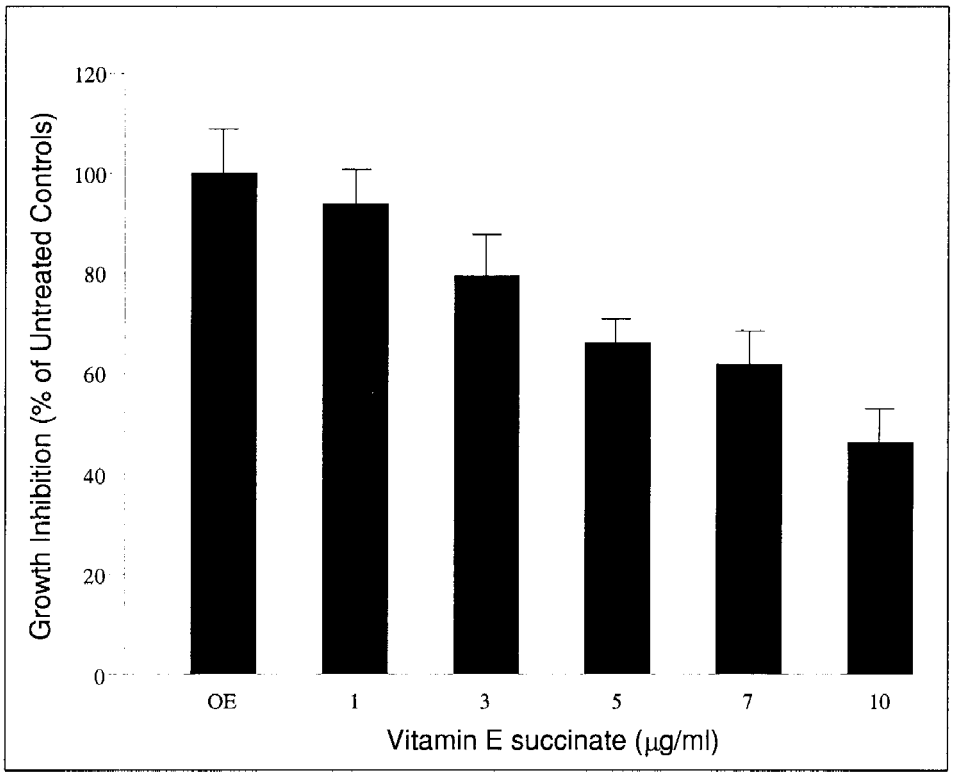

Figure 6 - The Effect of Varying Concentrations of Vitamin E Succinate (1-10µg/ml) on Malignant B16 Melanoma Cell Growth.

(*Each Bar Represents the Mean Of 5 Sets of 3 Determinations ±SEM.*)

The overall mean growth inhibitory effects of vitamin E succinate supplementation on B16 cell growth were a significant decrease in growth of this cell line at concentrations of 5 ($p \leq 0.01$), 7 and 10µg/ml ($p \leq 0.001$), while concentrations of 1 and 3µg/ml resulted in no significant change in growth, as compared to control cultures OE.

2.3.2 Effect of Vitamin E Succinate Supplementation on Non-Malignant LLCMK (Monkey Kidney) Cell Growth

Supplementation of LLCMK cells with varying concentrations of vitamin E succinate also had an inhibitory effect on growth. These results are illustrated in figure 7. Results shown in this figure are relevant to the experiments described in chapter 3, and will be discussed relative to these studies at a later stage.

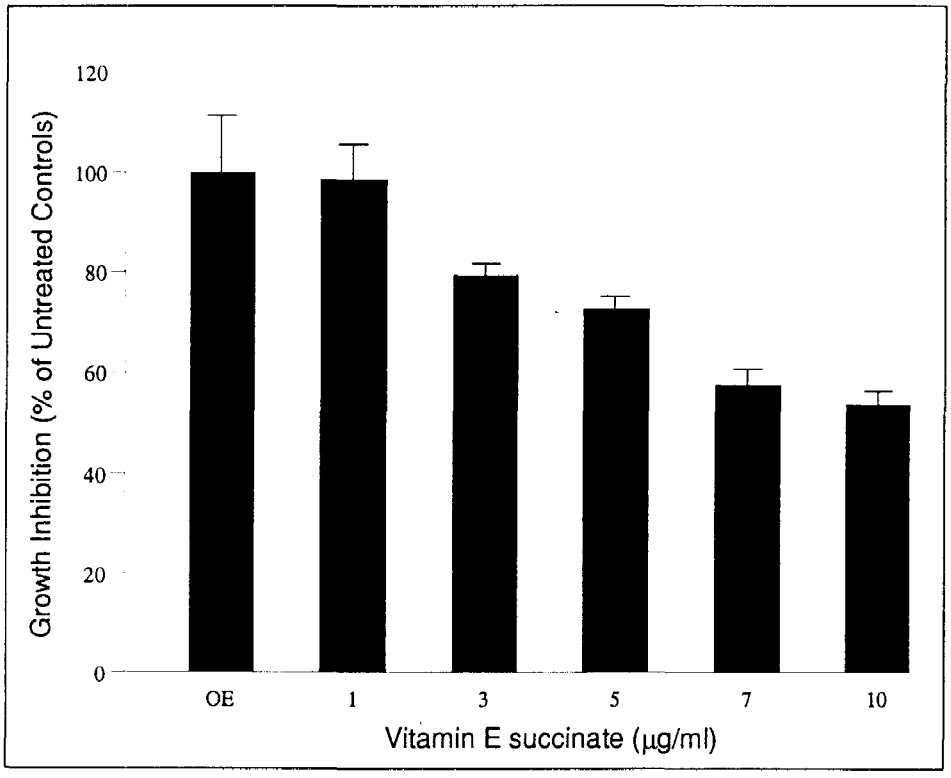

Figure 7 - The Effect of Varying Concentrations of Vitamin E Succinate (1-10µg/ml) on Non-Malignant LLCMK Cell Growth.

(Each Bar Represents the Mean Of 3 Sets of 3 Determinations ±SEM.)

LLCMK cell growth was significantly inhibited by vitamin E succinate supplementation only at higher the concentrations with 5 ($p \leq 0.05$), 7 and 10µg/ml ($p \leq 0.001$) causing

the most significant decrease in growth of this cell line compared to control cultures OE.

2.4 DISCUSSION

Vitamin E succinate is the most potent form of vitamin E for inducing morphological changes and growth inhibition in B16 cells *in vitro*. This has been established in various studies.[6,43,44] The effects of this vitamin E ester are however not only restricted to B16 cells as similar effects have been reported in C4#1 (an avian retrovirus-transformed lymphoid cell line)[49], murine and human neuroblastoma, rat glioma, and human prostate carcinoma cells *in vitro*[52]. In this study supplementation of B16 cells with varying concentrations of vitamin E succinate (1-10µg/ml) resulted in a significant inhibition of cell growth at concentrations of 5, 7 and 10µg/ml, compared to control cultures OE. This effect is confirmed by a number of other studies.[6,44,52]

Few studies have investigated the effects of vitamin E succinate supplementation of normal or non-transformed cells. However, Kline *et. al.*[49] showed that concentrations of 5 and 10µg/ml of vitamin E succinate were cytotoxic to lymphocytes in chickens and that lower concentrations (0.1-1µg/ml) of vitamin E succinate enhanced cell growth. In this study supplementation of LLCMK cells with varying concentrations of vitamin E succinate (1-10µg/ml) resulted in an inhibition of cell growth, with significant inhibition occurring at the highest concentrations.

Control experiments were conducted in our laboratory to confirm that the growth inhibitory effects observed in these cell lines were indeed due to vitamin E succinate and not due to vehicle treatment (ethanol) or the succinic acid part of the vitamin E molecule. These results showed that with ethanol and succinic acid treatment, there was neither a significant increase or decrease in LLCMK cell growth, while in B16 cells there was a non-significant increase in growth.[39] It is therefore reasonable to assume that the resultant growth effect observed with vitamin E succinate supplementation were not as a result of ethanol or succinic acid. The possibility that these growth effects were due to the vitamin E part of the vitamin E succinate molecule could however not

be ruled out.

As it was still unclear whether the growth effects observed with vitamin E succinate supplementation were due to the intact vitamin E succinate or to the vitamin E part of the molecule, cleavage studies were done. These studies will be discussed in the following chapter.

CHAPTER 3

CLEAVAGE STUDIES

3.1 INTRODUCTION

Vitamin E succinate is a powerful inhibitor of B16 cell growth as is evident from growth studies in section 2.3.1. The mechanism(s) of inhibition by vitamin E succinate is however unknown.[52] Some researchers have suggested that vitamin E succinate acts as a carrier for vitamin E, which then acts via antioxidant mechanisms.[48,75] However, vitamin E succinate is a non-physiological antioxidant which can only exhibit antioxidant properties if the succinate group is cleaved off allowing the phenolic group of the vitamin E to quench free radicals.[22,49,76]

In vivo vitamin esters undergo hydrolysis to free vitamin E via nonspecific esterases which are associated with membranes in most cells.[47,48] No species specific cleavage of vitamin E succinate has however been demonstrated in B16 cells. Fariss *et. al.*[45,46] have demonstrated that vitamin E succinate cytoprotection is as a result of accumulation of the intact vitamin E succinate molecule and not due to cleavage products, and there is evidence to suggest that vitamin E succinate may act via mechanisms other that of an antioxidant.[45,52,53] Thus cleavage studies were undertaken to determine whether vitamin E succinate was being metabolised to vitamin E in B16 cells.

3.2 MATERIALS AND METHODS

MATERIALS

Iminodiacetic acid, Neostigmine bromide, L-ascorbic acid and (±) α-tocopherol (95%) were purchased from Sigma Chemical Co., USA. Nitric acid (HNO_3) was purchased from SAARCHEM (Pty) Ltd, South Africa. High Performance Liquid Chromatography (HPLC) grade methanol, acetonitrile, hexane and ethanol were purchased from BDH Chemicals Ltd, England. A reverse phase Bondclone 10 C^{18} HPLC column (300mm x 3.9mm) was purchased from Phenomex, USA. Guard-Pak precolumns (5µm, ODS) were obtained from Metachemicals, USA.

METHODS

3.2.1 Cleavage of Vitamin E Succinate to Vitamin E in Malignant B16 Cells and Non-Malignant LLCMK Cells

***- Experimental procedures*:**

i) *Glassware preparation*

All glassware was prepared using a modified method of Kelley *et. al.*[50] The glassware was cleaned and then soaked for 48 hours in 25% (v/v) HNO_3 to remove divalent cations which cause oxidation of the α-tocopherol. Glassware was then rinsed in milli Q water and soaked for a further 48 hours in 0.5% (w/v) iminodiacetic acid - a chelating agent. A final rinse in milli Q water followed.

ii) *Preparation of experimental medium*

Refer to section 2.2.3 (ii) for the method used.

iii) *Growth of cell cultures*

Six sets of 3 75cm^2 flasks were seeded with 500 000 (B16 or LLCMK) cells. Five sets of flasks were supplemented with 30ml of MEM media containing varying levels (1-10µg/ml) of vitamin E succinate. The sixth set of flasks received 30ml MEM media containing 0.1% final volume of ethanol and was referred to as a control culture (OE). All flasks were incubated at 37°C for 5 to 7 days with one media change during this period. Upon the first set of flasks reaching confluency, the cells were harvested.

iv) *Harvesting of cells*

On reaching confluency all the cells were harvested under non-sterile conditions. The cells were incubated with 10ml of trypsin at 37°C until they detached from the flask surface. Cell suspensions were poured into centrifuge tubes and centrifuged at 480g for 4 minutes. The trypsin solution was discarded and the pellets were resuspended in 1ml of PBS (pH7.4, which contained 0.5mg/ml EDTA - a chelating agent - and 10nM neostigmine bromide - an esterase inhibitor). The cells were counted using a haemocytometer and kept on ice. Once the counts were completed, the samples were stored at -40°C until extraction of the α-tocopherol.

v) *Extraction procedure*

A modified method of Kelley *et. al.*[50] was used to extract the α-tocopherol from the cells. To the cell pellets was added 20µl of 200mM ascorbic acid (functions to protect the α-tocopherol from oxidation). The cell suspensions were centrifuged at 750g for 10 minutes, the supernatant discarded, and the wash repeated with the addition of the same amount of ascorbic acid. To the final wash pellet was added 5µl of 200mM ascorbic acid and 500µl of ice-cold 95% ethanol. The sample was vortexed and placed on ice for 2 minutes. The sample was then homogenized 30 times with a tight plunger. The homogenized samples were transferred back into centrifuge tubes and 500µl of ice-cold hexane was added. The samples were vortexed for 1 minute and centrifuged at 300g for 3 minutes to separate the phases. The top (hexane) phase was removed using a Pasteur pipette and was placed into a Durham tube. The head space was replaced with N_2 and samples were stored at -70°C until required.

vi) *HPLC analysis*

The hexane was evaporated under a stream of N_2 and the samples were resuspended in 150µl of acetonitrile (HPLC grade), vortexed and transferred to Eppendorf tubes. The head space was replaced with N_2 and the tubes were kept on ice. The samples were analysed by reverse-phase HPLC. Reconstituted samples were injected (50µl injection volume) into HPLC column, using a Hamilton syringe. A Bondclone C^{18} column with a Guardpack precolumn was used with a mobile phase of Methanol:Acetonitrile (60:40, v/v) at a flow rate of 1.5ml/min. α-Tocopherol and α-tocopherol succinate levels were determined using a UV/VIS detector at 294nm (Beckman, System Gold programmable detector module, mode 166, USA). Co-chromatography with authentic standards ((±) vitamin E and vitamin E succinate) was used to identify the separate compounds.

3.2.2 Statistical Analysis

Refer to section 2.2.5 for method used.

3.3 RESULTS

Levels of vitamin E and vitamin E succinate found in B16 and LLCMK cells supplemented with varying levels (1-10µg/ml) of vitamin E succinate are compared in table 2.

Table 2 - Effect of Vitamin E Succinate Supplementation on Vitamin E and Vitamin E Succinate Levels in B16 and LLCMK Cells

(Each value represents the mean of 3 determinations ±SEM.)

Vitamin E Succinate (µg/ml)	Vitamin E Succinate (nmoles/10^6 cells)		Vitamin E (nmoles/10^6 cells)	
	B16	LLCMK	B16	LLCMK
OE	ND	ND	ND	ND
1	0.294 *±0.069*	0.193[e] *±0.026*	0.075 *±0.018*	0.015 *±0.002*
3	1.027[bc] *±0.193*	0.054 *±0.004*	0.235 *±0.121*	0.073[f] *±0.031*
5	0.921[bd] *±0.104*	0.229 *±0.075*	0.138 *±0.060*	0.091 *±0.032*
7	2.152[ac] *±0.234*	0.453[e] *±0.233*	0.056 *±0.005*	0.108 *±0.010*
10	4.084[ac] *±0.278*	0.167 *±0.032*	0.160 *±0.037*	0.151[g] *±0.034*

ND: Not Detected
a - $p \leq 0.001$: Relative to 1µg/ml vitamin E succinate supplemented B16 cells.
b - $p \leq 0.05$: Relative to 1µg/ml vitamin E succinate supplemented B16 cells.
c - $p \leq 0.001$: Relative to vitamin E levels in B16 cells in the relevant groups.
d - $p \leq 0.01$: Relative to vitamin E levels in 5µg/ml vitamin E succinate supplemented B16 cells.
e - $p \leq 0.01$: Relative to vitamin E levels in LLCMK cells in relevant groups.
f - $p \leq 0.05$: Relative to vitamin E succinate levels in 3µg/ml supplemented LLCMK cells.
g - $p \leq 0.01$: Relative to 1µg/ml vitamin E succinate supplemented B16 cells.

It was found that in B16 cells the levels of vitamin E succinate increased significantly with increasing levels of vitamin supplementation with significant increases occurring at 3, 5 ($p \leq 0.05$), 7 and 10µg/ml ($p \leq 0.001$) respectively, compared with 1µg/ml vitamin E succinate supplemented cultures. However, levels of vitamin E in B16 cells showed no significant trend with increasing levels of vitamin E succinate supplementation

compared with 1µg/ml samples. In LLCMK cells, the levels of vitamin E succinate present in the cells followed no trend as the level of vitamin E succinate supplementation was increased, however, the vitamin E levels increased with increasing levels of supplementation. A significant increase in vitamin E relative to the 1µg/ml supplemented culture was noted at 10µg/ml vitamin E succinate supplementation in LLCMK cells.

Comparison of the relative vitamin E succinate and vitamin E levels in B16 cells revealed that the levels of vitamin E succinate were generally higher, significantly so at 7 and 10µg/ml. A similar trend was noted in LLCMK cells, with significantly higher levels of vitamin E succinate ($p \leq 0.01$) found at 1 and 7µg/ml relative to vitamin E levels in the relevant groups. The exception was the 3µg/ml supplemented cultures of LLCMK cells, the vitamin E levels being significantly higher ($p \leq 0.05$) relative to the vitamin E succinate levels in this group. The levels of vitamin E succinate present in B16 cells were found to be between 1.5 and 25 fold higher than that in LLCMK cells.

3.4 DISCUSSION

Vitamin E ester derivatives are commonly used as vitamin E supplements both *in vivo* and *in vitro* as they are more resistant to oxidation and are less likely to be destroyed under adverse conditions.[11,18,45] These esters are believed to undergo hydrolysis via nonspecific esterases to release vitamin E, as is the case *in vivo*.[47,48] Under *in vitro* conditions hydrolysis of vitamin E succinate to vitamin E does occur in some cells,[47,50] however, it is not the case with others[45,46] and no species specific hydrolysis of vitamin E succinate has been demonstrated in B16 or LLCMK cells.

Cleavage studies were performed to determine whether the effects on B16 cell growth were due to the intact molecule of vitamin E succinate or if the effects were due to vitamin E. Results from these studies showed that the levels of vitamin E succinate were greater than levels of vitamin E in B16 cells and that no significant cleavage of the vitamin occurred. In LLCMK cells, the intracellular levels of vitamin E succinate were found to be greater than those of vitamin E, although the levels of vitamin E did

increase with increasing levels of vitamin E succinate supplementation. However, it appears that cleavage did occur in these cells at 3µg/ml as the amount of vitamin E present was significantly higher than the amount of vitamin E succinate at this concentration. It has been previously demonstrated that non-transformed cells can be enriched with vitamin E by addition of vitamin E succinate to the growth media.[50]

These results suggested that no specific cleavage of vitamin E succinate to vitamin E occurres in B16 cells, whereas in LLCMK cells esterases may be present which allow cleavage of the esterase bond of vitamin E succinate liberating succinic acid and vitamin E. These enzymes are either inactive or absent in B16 cells. It can thus be assumed that in B16 cells the growth inhibitory effects observed are not due to vitamin E, but rather due to the accumulation of the intact vitamin E succinate molecule in the cells. Further studies were carried out using the transformed B16 cells only as the effects of vitamin E succinate supplementation on tumour cells, rather than its effects on untransformed cells, was the topic of interest in this study.

It is evident that vitamin E succinate, as an intact molecule, is a potent inhibitor of B16 murine melanoma cell growth, however, these studies do not shed any light on the mechanism(s) by which vitamin E succinate is having this effect. This issue will be addressed in the following chapters.

CHAPTER 4

TIME COURSE ASSAYS OF CYCLOOXYGENASE ACTIVITY

4.1 INTRODUCTION

Prostaglandin H synthase, an integral membrane protein that converts AA to various eicosanoid products, is the rate-limiting enzyme in prostaglandin synthesis.[57,62] Vitamin E is believed to modulate PG synthesis at various points and it has been suggested that COX activity is either inhibited, stimulated or not effected in any way by antioxidant stimulation. These effects are dependent on the type and concentration of the antioxidant used as well as the system under investigation.[39,60]

In this laboratory, vitamin E succinate supplementation was shown to cause a significant increase in COX activity in B16 murine melanoma cells at concentrations of 7 and 10μg/ml with supplementation over a period of 4 to 6 days.[39] However, to date it had not been determined at which time after supplementation the vitamin E succinate exerted its effect on COX activity in B16 cells and subsequently, this issue was addressed in this chapter.

It is possible to distinguished between the two COX isoforms on the basis of pharmacological inhibition as they exhibit differences in their sensitivity to non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids. It is also possible to determine at which level of expression an enzyme is being regulated due to its response to different inhibitors, used alone and in combination.[61,62,65,72] In this study the possible effect of two inhibitors on COX activity and expression in B16 cells was examined. The inhibitors used were dexamethasone, a synthetic glucocorticoid which selectively inhibits COX-2, and cycloheximide, which inhibits the ribosomes and causes translation to come to a halt.[72] Dexamethasone is known to inhibit COX both at the level of transcription and translation.[73] Therefore, to determine at which level of expression vitamin E succinate was having its effect on COX enzyme activity in B16 cells, cycloheximide was used in conjunction with dexamethasone as it is possible to conclude that modulation of COX enzyme activity by dexamethasone in the presence of cycloheximide would suggest transcriptional regulation.[72]

4.2 MATERIALS AND METHODS

MATERIALS

Arachidonic acid (5,8,11,14-Eicosatetraenoic acid) sodium salt, cycloheximide, dexamethasone and N,N,N',N'-tetramethylphenylenediamine (TMPD) were purchased from Sigma Chemical Co., USA. Tris-(hydroxymethyl)aminomethane (tris, free base) was purchased from B and M Scientific cc, USA.

METHODS

4.2.1 Preparation of Culture Reagents

i) *Tris-HCl Solution*

0.1M Tris-HCl solution was prepared by the addition of 12.11g tris base to 800ml of milli Q water, the pH being adjusted to 8.5 using HCl. After allowing the solution to cool to room temperature, final pH adjustments were made and the volume was adjusted to one litre. The solution was then sterilized by autoclaving.

4.2.2 Effect of Vitamin E Succinate Supplementation on Cyclooxygenase Activity Over a Time Course of 2 to 12 Hours

- Experimental Procedures:

i) *Preparation of Experimental Medium*

As the quantity of flasks to be assayed became very cumbersome, it was decided that only the most significant concentration of vitamin E succinate in terms of growth inhibition and cyclooxygenase activity would be studied. The 10µg/ml vitamin E succinate concentration was selected. This concentration was prepared as described in section 2.2.3 (ii).

ii) *Growth of Cell Cultures*

Two sets of 20 and 1 set of 5 25cm^2 flasks were seeded with 300 000 B16 cells. These cells were grown up for 3 to 5 days at 37°C in growth medium containing 10% (v/v) FCS. One day prior to confluency a media change was performed. Media was prepared as mentioned above. One set was supplemented with 10ml of medium

containing 10µg/ml of vitamin E succinate. The other set was supplemented with medium containing 0.1% final volume of ethanol and was referred to as control culture OE. The third set was referred to as control culture 0, and did not receive a medium change but was harvested immediately. The remaining two sets of flasks were incubated at 37°C and were harvested, 5 flasks at a time, at intervals of 2, 4, 8, and 12 hours.

iii) *Harvesting of Cells*

All cells were harvested under non-sterile conditions. The cells were incubated with 5ml of trypsin at 37°C until they detached from the flask surfaces. Cell suspensions were poured into centrifuge tubes and centrifuged at 480g for 5 minutes. The trypsin solution was discarded and the pellets were resuspended in 2ml PBS (pH6.6). Cells were counted using a haemocytometer. The cells were again centrifuged at 480g for 5 minutes and the PBS was discarded. The pellets were resuspended in 1ml Tris-HCl (pH8.5, sterilized by autoclaving) and the samples were stored at 4°C until required.

iv) *Homogenization and Extraction of Membrane Fractions*

The cells were homogenized 30 times with a tight plunger. The suspension was then poured into centrifuge tubes (JA-21 rotor tubes) and centrifuged at 4000g for 20 minutes (Beckman centrifuge). The supernatant was retained and further centrifuged at 20 000g for 30 minutes. The supernatant (stroma fraction) was discarded and the pellets (membrane fraction) were reconstituted in 1ml Tris-HCl (pH8.5), and were transferred into 1.5ml eppendorf tubes. The samples were stored at -20°C until required.

v) *Cyclooxygenase Assay*

A modified method of Kulmacz and Lands[77] was used to determine COX activity. The principle of this colorimetric assay is based on the sequential reactions of the cyclooxygenase (generating PGG_2) and the peroxidase (converting PGG_2 to PGH_2) activities contained in PGHS, with the peroxidase activity being the one actually measured. The peroxidase co-substrate (TMPD) is converted to a relatively stable

chromophore in a predictable stoichiometric relationship to the reduction of PGG_2. 20µl of 4mg/ml TMPD was added to the crude enzyme extract (membrane fraction), and the change in absorbance at 611nm was recorded as a function of time for approximately one minute to establish the background rate of TMPD oxidation. The substrate, 5µl 20mM AA, was then added and the change in absorbance was monitored for a further three to four minutes. The rate of absorbance before addition of AA was subtracted from the optimal velocity value. Conversion from units of $Abs_{611nm}/time$ to *[Peroxide reduced]/time* was accomplished by using an extinction coefficient of 13.5 (mM TMPD oxidized)$^{-1}$cm^{-1} and a stoichiometry of 2 mol TMPD oxidized per mol PGG_2 reduced.

4.2.3 Effect of Combined Vitamin E Succinate and Dexamethasone Supplementation on Cyclooxygenase Activity Over a Time Course of 2 to 12 Hours

- Experimental Procedures:

i) *Preparation of Experimental Medium*

The method described above was used, with the exception that 1mM of dexamethasone (dissolved in 0.1M Tris-HCl, pH8.5) was added to the growth medium. The OE control was now designated as OE-DEX, and an additional control was set up containing only 1mM dexamethasone which was referred to as DEX.

ii) *Growth of Cell Cultures*

The method described above was used with the following exceptions.

- Three sets of 20 and 1 set of 5 25cm^2 flasks were seeded with 300 000 B16 cells.
- The first set was supplemented with 10ml of medium containing 10µg/ml of vitamin E succinate and 1mM dexamethasone.
-The second set was supplemented with medium containing 0.1% final volume of ethanol and 1mM dexamethasone and was referred to as control culture OE-DEX.
- The third set was referred to as control culture DEX, and contained growth media supplemented with 1mM dexamethasone.

iii) *Harvesting of Cells*

The method described above was used.

iv) *Homogenization and Extraction of Membrane Fractions*

The method described above was used.

v) *Cyclooxygenase Assay*

The method described above was used.

4.2.4 Effect of Combined Vitamin E Succinate and Cycloheximide Supplementation on Cyclooxygenase Activity Over a Time Course of 2 to 12 Hours

- Experimental Procedures:

i) *Preparation of Experimental Medium*

The method described above was used, with the exception that 25mM of cycloheximide (dissolved in 0.1M Tris-HCl, pH8.5) was added to the growth media. The OE control was now designated as OE-CYC, and an additional control was set up containing only 25mM cycloheximide which was referred to as CYC.

ii) *Growth of Cell Cultures*

The method described above was used with the following exceptions.

- Three sets of 20 and 1 set of 5 25cm^2 flasks were seeded with 300 000 B16 cells.
- The first set was supplemented with 10ml of medium containing 10µg/ml of vitamin E succinate and 25mM cycloheximide.
- The second set was supplemented with medium containing 0.1% final volume of ethanol and 25mM cycloheximide and was referred to as control culture OE-CYC.
- The third set, referred to as control culture CYC, contained growth media supplemented with 25mM cycloheximide.

iii) *Harvesting of Cells*

The method described above was used.

iv) *Homogenization and Extraction of Membrane Fractions*

The method described above was used.

v) *Cyclooxygenase Assay*

The method described above was used.

4.2.5 Effect of Combined Vitamin E Succinate, Dexamethasone and Cycloheximide Supplementation on Cyclooxygenase Activity Over a Time Course of 2 to 12 Hours

- Experimental Procedures:

i) *Preparation of Experimental Medium*

The method described above was used, with the exception that 1mM of dexamethasone and 25mM of cycloheximide were added to the growth media. The OE control was now designated as OE-DEX-CYC, and an additional control was set up containing 1mM of dexamethasone and 25mM cycloheximide which was referred to as DEX-CYC.

ii) *Growth of Cell Cultures*

The method described above was used with the following exceptions.

- Three sets of 20 and 1 set of 5 25cm^2 flasks were seeded with 300 000 B16 cells.
- The first set was supplemented with 10ml of medium containing 10µg/ml of vitamin E succinate with 1mM dexamethasone and 25mM cycloheximide.
- The second set was supplemented with medium containing 0.1% final volume of ethanol with 1mM dexamethasone and 25mM cycloheximide and was referred to as control culture OE-DEX-CYC.
- The third set, referred to as control culture DEX-CYC, contained growth media supplemented with 1mM dexamethasone and 25mM cycloheximide.

iii) *Harvesting of Cells*

The method described above was used.

iv) *Homogenization and Extraction of Membrane Fractions*

The method described above was used.

v) *Cyclooxygenase Assay*

The method described above was used.

4.2.6 Effect of Vitamin E Succinate, Dexamethasone and Cycloheximide Supplementation, Alone and in Combination, on Cyclooxygenase Activity Over a Period of 4 to 6 Days

- Experimental Procedures:

i) *Preparation of Experimental Medium*

The methods described above were used.

ii) *Growth of Cell Cultures*

The method described in section 2.2.3 (iii) was used with the following exceptions.

- Twelve sets of 5 25cm^2 flasks were seeded with 300 000 B16 cells.
- Only the 10µg/ml vitamin E succinate concentration was used for vitamin supplemented samples.
- The inhibitors dexamethasone (1mM) and cycloheximide (25mM) were supplemented to the growth medium, alone and in combination, and were supplemented to vitamin, OE and 0 control samples.

iii) *Harvesting of Cells*

The method described above was used.

iv) *Homogenization and Extraction of Membrane Fractions*

The method described above was used.

v) *Cyclooxygenase Assay*

The method described above was used.

4.2.7 Statistical analysis

Refer to section 2.2.5 for method used. As a result of much variability between samples, the SEM's were very large.

4.3 RESULTS

COX activity was determined at intervals of 0, 2, 4, 8 and 12 hours after supplementation with experimental medium and was expressed as a percentage of the 0 hour control samples. Table 3 summarizes these results.

Table 3 - The Effect of Vitamin E Succinate on Cyclooxygenase Activity in B16 Murine Melanoma Cells Represented as a % of the 0 Hour Controls

(Each value represents the mean of 15 determinations ± SEM.)

Time (hours)	OE Controls	Vitamin E Succinate (10µg/ml)
0	100 ± *10.737*	100 ± *10.737*
2	132.5 ± *13.144*	142.5 ± *33.369*
4	72.5 ± *7.583*	67.5 ± *22.298*
8	67.5 ± *19.210*	37.5 ± *11.759*
12	7.5 ± *5.26*	85 ± *17.185*

COX enzyme activity at time 0 was taken to represent a baseline activity and was calculated as 100%. At 2 hours after vitamin E succinate supplementation of 10µg/ml, a nonsignificant increase in COX activity above baseline activity was observed. This nonsignificant increase was also observed in the OE control cultures. COX activity decreased below the baseline activity from 4 to 12 hours in the OE control cultures and the 10 µg/ml supplemented vitamin E succinate samples follow the same trend, with

the exception of the 12 hour samples which showed an increased COX activity. Due to the nature of the assay, much variability between samples was found to occur and the SEM values were very large. These results were therefore nonsignificant.

Studies using dexamethasone and cycloheximide, alone and in combination, revealed no significant effect on COX activity over the 12 hour assay period, and determining the effects on COX activity in B16 cells over a period of 4 to 6 days was not possible as these inhibitors affect cell adhesion to the culture flasks and appear to be lethal when added prior to the cells becoming established in the culture flasks.

4.4 DISCUSSION

In our laboratory it was observed that vitamin E succinate significantly inhibits cell growth at a concentration of 10µg/ml, while at the same concentration a significant increase in cyclooxygenase activity occurs in B16 cells with supplementation of this vitamin over a period of 4 to 6 days.[39,55] However, most studies on the role of cyclooxygenase in carcinogenesis have demonstrated that increased levels of COX-2 are associated with transformed cells and tumours, and have also shown that inhibition of COX-2 leads to growth inhibition of these cells.[63,64,68]

In this study, a nonsignificant increase in COX activity was observed both in the experimental and OE control cultures 2 hours after supplementation, followed by a decrease in activity up to 8 and 12 hours in vitamin E succinate supplemented and OE samples respectively. A transient induction of COX activity has been known to occur after 2 hours as a result of 10% FCS supplementation to the cells, and the increased COX activity observed in B16 cells was probably due to this FCS effect.[66] At 12 hours after supplementation with vitamin E succinate a nonsignificant increase in COX activity was observed.

A comparison of vitamin E succinate supplemented samples with the OE controls revealed that no significant effect was exerted by vitamin E succinate on COX activity in B16 cells. However, unpublished studies currently being conducted in this laboratory

have revealed that vitamin E, rather than vitamin E succinate, causes a significant increase in COX activity over a period of 2 to 12 hours. The observed effect on COX activity may therefore be an antioxidant effect.

It was not possible to make any conclusions about vitamin E succinate with regard to expression and regulation of COX in B16 cells as inhibitor studies were inconclusive and over a time course of 12 hours no significant effect on COX activity was observed.

In the light of this evidence it may be suggested that other factors still to be identified, are involved in the mechanism of growth inhibition by vitamin E succinate.

As COX activity started to increase after 12 hours in vitamin E succinate supplemented samples, and because previous reports indicated significant increases in COX activity over a period of 4 to 6 days, it was decided to use both a time course of 2 to 12 hours and 6 days in molecular studies. These studies will be discussed in chapter 5.

CHAPTER 5

REGULATION OF CYCLOOXYGENASE AT A MOLECULAR LEVEL

5.1 INTRODUCTION

The isoforms of Prostaglandin H synthase differ regarding gene structure, stability of mRNA and intracellular localization of gene products.[57,68,69] They also differ with regard to size of the mRNA. COX-1 is constitutively expressed, functions primarily in the ER, and is believed to be involved in physiological production of PGs for maintenance of normal homeostasis. The murine mRNA has a transcript of 3.0kb in size.[57,68-71] COX-2 on the other hand, is inducible and is sensitive to a wide range of extracellular and intracellular stimuli. This isoform is believed to play a role in physiological and pathological processes characterized by increased production of PGs and is found both in the ER and nuclear membrane. Murine COX-2 mRNA is 4.1kb in size.[57,68-71]

COX-2 may have important effects in the nucleus, modulating expression of target genes,[68] and vitamin E succinate was shown to act at a nuclear level.[42,52,53] Since it was found that vitamin E succinate increased COX activity at concentrations of 7 and 10μg/ml supplementation,[39,55] it raised the question of whether vitamin E succinate exerts its anticarcinogenic effects in B16 cells by modulation of COX-2 mRNA. Increased COX-2 mRNA expression was found to occur in human colon cancer,[63] and breast, head and neck cancers produce higher levels of this isozyme of COX suggesting that COX-2 may have an important role to play in cancer.[64,68]

Regulation of mRNA has the potential to occur at several levels including transcription, stability, alternative polyadenylation and translation. In chapter 4, the effect of vitamin E succinate on PGHS levels over 2-12 hours was studied using enzyme assays and inhibition with dexamethasone and cycloheximide, to determine the level at which regulation of expression was occurring. These studies were also used to give an indication of when the genes were being "switched on." In this chapter, the regulation of PGHS expression in B16 cells by vitamin E succinate was investigated at the level

of RNA and COX-1 and COX-2 cDNA clones were used as probes to determine which isoform of PGHS was being affected by vitamin E succinate supplementation. These effects were examined over 4 to 6 day and 2 to 12 hour periods as there was evidence for an increase in COX activity both 12 hours after vitamin E succinate supplementation and, from previous studies, over a period of 4 to 6 days.

5.2 MATERIALS AND METHODS

MATERIALS

Oligonucleotide: AVDM1 (COX-1 and COX-2 specific) was synthesised by "The Great American Gene Company", (E-mail geneco@ix.netcom.com). Prostaglandin H synthase 1 (Murine) cDNA probe (COX-1) and prostaglandin H synthase 2 (Murine) cDNA probe (COX-2) were purchased from Cayman Chemical Company, USA, and a Prime-a-Gene® labelling system was purchased from Promega Corporation, USA. An RNeasy mini kit and Oligotex™ mRNA mini kit were purchased from Qiagen, Germany. Diethyl pyrocarbonate (DEPC), MOPS (3-[N-morpholineno]propanesulfonic acid) sodium salt, dextran sulfate sodium salt, ficoll® (Type 400-DL), polyvinylpyrrolidone (PVP), phenol (Molecular biology grade) and formaldehyde (Molecular biology grade) were purchased from Sigma Chemical Co., USA. Sodium lauryl sulfate (SDS), sodium acetate anhydrous ($NaC_2H_3O_2$), sodium citrate ($Na_3C_6H_5O_7.2H_2O$) and bromophenol blue were purchased from SAARCHEM, South Africa. Agarose (Genetic technology grade) was purchased from FMC Bioproducts, USA and RNase Away® was purchased from Molecular Bioproducts, USA. Formamide, ethidium bromide, BSA (Albumin - Fraction V), DNA from herring sperm - lyophilized sodium salt, and RNA molecular marker - weight marker II, were purchased from Boehringer Mannheim, Germany. Glacial acetic acid was purchased from BDH Laboratory Suppliers, England. T4 polynucleotide kinase (PNK), Hybond-N+; positively charged nylon membrane, Redivue™ α-^{32}P-dCTP (3000Ci/mmol and 6000Ci/mmol), hypercassette with a Kodak Biomax TranScreen - HE (High Energy) intensifying screen and Biomax MS - Kodak scientific imaging film (High Performance) were purchased from Amersham Life Sciences, Inc., USA. Photographic fixative, developer and starter were purchased from Protea Medical Services, South Africa.

METHODS

5.2.1 Preparation of Stock Solutions[78]

i) *1M Tris*

121.1g of tris base was dissolved in 800ml of water. The pH was then adjusted to the desired value (pH7.4, 7.6 or 8.0) by the addition of concentrated HCl. After allowing the solution to cool to room temperature, the final pH adjustments were made and the volume of the solution was adjusted to one litre. Aliquots of the solution were sterilized by autoclaving.

ii) *0.5M EDTA (pH8.0)*

EDTA was prepared by addition of 18.61g disodium EDTA.$2H_2O$ to 80ml of water. The pH was adjusted to 8.0 using NaOH (the disodium salt of EDTA only goes into solution at pH~8.0). The final volume of the solution was adjusted to 100ml and aliquots were sterilized by autoclaving.

iii) *5M NaCl*

29.22g of NaCl was dissolved in 80ml of water. The volume was adjusted to one litre and aliquots were sterilized by autoclaving.

iv) *3M Sodium Acetate (pH5.2)*

40.81g of anhydrous sodium acetate was dissolved in 80ml of water. The pH was adjusted to 5.2 using glacial acetic acid. The volume was adjusted to 100ml and aliquots were sterilized by autoclaving.

v) *10% Sodium Dodecyl Sulfate (SDS) (also called Sodium Lauryl Sulfate)*

10g of SDS was added to 90ml of water and this was then heated to 68°C to assist dissolution. The solution was allowed to cool and the pH was adjusted to 7.2 using concentrated HCl. The volume was adjusted to 100ml. (Caution should be used when weighing SDS. There is no need to sterilize 10% SDS.)

vi) *20x SSC*

SSC buffer was prepared by dissolving 175.3g of NaCl and 88.2g of sodium citrate in 800ml of water. The pH was adjusted to 7 using 10N NaOH, and the final volume was brought to one litre using water. The solution was sterilized by autoclaving.

vii) *Ethidium Bromide*

1g of ethidium bromide was added to 100ml of water. The solution was stirred for several hours to ensure that the dye had dissolved and was then stored in a dark bottle at 4°C. (Ethidium bromide is a powerful mutagen and extreme caution must be used when working with this solution.)

viii) *TE Buffer*

TE buffer was prepared by combining of the following solutions:

pH7.6	10mM Tris-HCl (pH7.6)
	1mM EDTA (pH8.0)
pH8.0	10mM Tris-HCl (pH8.0)
	1mM EDTA (pH8.0)

Note: All solutions that were used for RNA work were made up using DEPC-treated milli Q water. (Refer to section 5.2.2 (iv) below.)

5.2.2 Experimental Procedures Observed when Working with RNA[79,80]

- Handling RNA

Ribonucleases (RNases) are very resilient enzymes which degrade RNA molecules both through endo- and exonucleolytic activity. It is very important to eliminate RNase contamination from plasticware, glassware, reagents and samples as even a small amount of RNase will destroy RNA. The following precautions were taken:

I) *General Handling*

When working with RNA, extreme caution was exercised. Microbial aseptic techniques were used and latex or vinyl gloves were worn at all times while handling reagents and RNA samples to prevent contamination by RNase from the skin or dust particles. Work surfaces were wiped down with RNase Away, gloves were changed frequently and samples and reagents were kept closed when not in use.

ii) *Preparation of nondisposable plasticware and glassware*

Sterile, disposable polypropylene tubes were used whenever possible as these tubes are generally RNase-free and do not require pretreatment to inactivate RNases. Nondisposable plasticware and glassware was treated as follows before use to inactivate RNases:

-Nondisposable plasticware

Nondisposable plasticware was rinsed with 0.1M NaOH, 1mM EDTA followed by RNase-free water.

- Glassware

Glassware was cleansed with detergent, rinsed and then treated with DEPC (0.1% in water). This was allowed to stand overnight at 37°C, and was then autoclaved to remove residual DEPC. (DEPC is a strong RNase inhibitor which inactivates RNases by covalent modification. It is extremely toxic and caution should be exercised when working with this chemical.)

iii) *Electrophoresis tanks*

Electrophoresis tanks were wiped down with RNase Away, rinsed with DEPC-treated water, soaked in 0.1M NaOH, 1mM EDTA and were again rinsed with DEPC-treated water before use to ensure that no unwanted RNase contamination was present.

iv) *Solutions*

Solutions (ie. water and other solutions) were treated with 0.1% DEPC to inactive RNases. After the solutions were prepared, 0.1% DEPC was added and solutions were shaken vigorously to bring the DEPC into solution. The solutions were allowed to stand

overnight and were then autoclaved to remove any traces of DEPC. (Note that Tris buffers cannot be treated with DEPC directly, subsequently these buffers must be prepared in water pretreated with DEPC, and then re-autoclaved to sterilize.)

5.2.3 Effect of Vitamin E Succinate Supplementation on COX-1 and COX-2 mRNA Expression in B16 Cells

SECTION A

- *Experimental procedures*

i) *Preparation of experimental medium*

The method described in section 2.2.3 (ii) was used.

ii) *Growth of cell cultures*

Refer to section 2.2.3 (iii) for the method used. In addition to this, six sets of ten 25cm^2 flasks were also used in some experiments in an attempt to increase the amount of available RNA.

iii) *Harvesting of cells*

Refer to section 2.2.3 (iv) for the method used.

iv) *RNA isolation*

- Total RNA isolation

Total RNA was isolated using an RNeasy mini kit and the protocol for isolation of total RNA from animal cells was used[80].

- 600µl of Buffer RLT was added to the cells and the mixture was vortexed to mix.
- The lysate was then passed through a 20-G needle fitted to a syringe, at least 5 times, to homogenise the sample.
- 1 volume of 70% ethanol was added to the homogenized lysate and this was then mixed by pipetting.
- The sample was then applied to an RNeasy mini spin column contained in a 2ml collection tube, and was centrifuged at 8000g for 15 seconds in

a desktop microfuge.

- 700µl of Buffer RW1 was pipetted onto the RNeasy column and the sample was again centrifuged at 8000g for 15 seconds.
- The RNeasy column was transferred to a new 2ml collection tube, 500µl of Buffer RPE was pipetted onto the column and the column was centrifuged at 8000g for 15 seconds.
- 500µl of Buffer RPE was again pipetted onto the RNeasy column and this was centrifuged for 2 minutes at maximum speed to dry the RNeasy membrane.
- The RNeasy column was placed into a 1.5ml collection tube, 30µl of RNase-free water was pipetted onto the membrane and this was centrifuged at 8000g for 1 minute to elute the RNA .
- A further 30µl of RNase-free water was pipetted onto the membrane and this was again centrifuged at 8000g for 1 minute to elute any residual RNA (the elute was collected in the same collection tube).
- The RNA was quantitated by measuring the absorbance of a 200 fold diluted sample at 260nm and 280nm in a spectrophotometer (an absorbance unit of 1 at 260nm corresponds to 44.19µg of RNA/ml).

- mRNA isolation

mRNA was isolated using an Oligotex mRNA mini kit and the Oligotex spin column protocol was used[81].

- Total RNA was mixed with DEPC-treated water (up to a volume of 250µl), 2x-Binding Buffer (250µl), and Oligotex Suspension(15µl) and the mixture was incubated at 65°C for 3 minutes to disrupt the RNA secondary structure.
- The sample was then left to stand at room temperature for 10 minutes allowing hybridization between the oligo dT_{30} linked to the latex particles and the poly A tail of the mRNA.
- The sample was centrifuged at full speed for 2 minutes and the supernatant was carefully removed using a pipette so as not to disturb

the Oligotex resin.

- The pellet was resuspended in 400µl of Wash Buffer OW2, transferred to the spin column contained in a 1.5µl eppendorf tube and was then centrifuged at full speed for 30 seconds.
- The spin column was washed in another 400µl of Wash Buffer OW2 and was again centrifuged at full speed for 30 seconds.
- The mRNA was then eluted from the spin column by pipetting 2 volumes of 20µl preheated (-70°C) Elution Buffer through the column and centrifuging at full speed for 30 seconds.

Samples were concentrated by addition of 2.5 volumes of ethanol and 3M sodium acetate to a final concentration of 0.3M. The samples were kept at -40°C for 1 hour (or can be stored indefinitely at this stage) followed by centrifugation at 14 000g for 5 minutes to pellet the RNA. The samples were dried until all the ethanol was removed and were then resuspended in a volume of 10-30µl of TE buffer (pH8.0).

v) *Formaldehyde-Agarose gel electrophoresis*

The electrophoresis protocol used to analyse the RNA samples was adapted from Farrell.[79] Denaturing gels are frequently used as a diagnostic tool to assess the quality of the RNA as intact, biologically competent RNA always bands in a reproducible fashion under conditions that characterise denaturing gel electrophoresis. A 1% agarose gel was made in 1x MOPS buffer. The agarose was melted by microwaving and after allowing the agarose to cool to 65°C it was poured onto a gel support. 1-5µg of RNA was combined with 5x Loading buffer (ratio of 4:1), it was heated to 60°C for 10 minutes and then chilled on ice before loading onto the gel. (1µl of size marker was loaded and 1µg of the oligonucleotide AVDM1 was used as a positive control - they were treated in the same manner as the RNA samples). The samples were electrophoresed at 75-100V for approximately 1-1½ hours after which the gel was stained for 10 minutes in ethidium bromide (0.5µg/ml in water) to visualize the RNA.

10x MOPS: 200mM MOPS
50mM sodium acetate
10mM EDTA
Adjust to pH 7.0 with NaOH

5x Loading Buffer: 0.25% Bromophenol Blue
80µl 0.5M EDTA, pH 8.0
720µl 37% formaldehyde
2ml 100% glycerol
3084µl formamide
4ml 10x MOPS buffer
Add RNase free water to 10ml

vi) *Northern blot*

- *Capillary transfer*

The formaldehyde gel was destained by soaking the gel in 1x MOPS buffer, 20x SSC or DEPC-treated water for 5 minutes to remove the formaldehyde. This was repeated 3 times. Gel transfer for the Northern blot was then done as described in Farrell.[79] This is illustrated in figure 8.

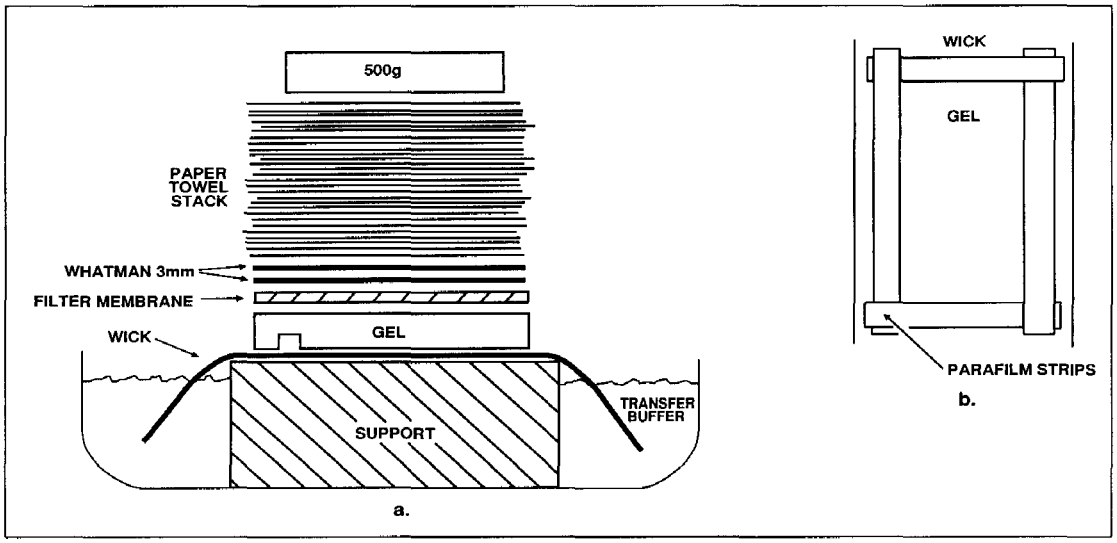

Figure 8 - A Typical Setup for Capillary Transfer of RNA from Denaturing Agarose Gels to a Membrane.[79]

- The left and right edges of the gel were trimmed allowing a 1cm margin bordering the lanes of interest and the membrane was cut to the size of the gel.
- The nylon membrane was floated on the surface of DEPC-treated water and was then submerged for 5 minutes, this was followed by equilibration in transfer buffer (10x SSC is preferred for nylon membranes) until the membrane was required.
- A sheet of Whatman 3MM paper was cut approximately 1 inch wider and approximately 8 inches longer than the gel to serve as a wick, this was saturated with transfer buffer and was draped over a plexiglass sheet supported by a baking dish. The dish was filled with transfer buffer so that a minimum of 2-3cm of each end of the wick was submerged and air bubbles were removed from the wick using a sterile pipette to smooth the surface.
- The gel was placed, with wells facing down, in the middle of the wick and air bubbles were removed as before. The area surrounding the gel was masked with strips of parafilm to prevent short circuiting the system and the membrane was then placed on top of the gel and any air bubbles were once again removed. Two to three sheets of Whatman 3MM paper were cut to the same size as the gel, with at least one of the sheets being pre-wet in transfer buffer. The wet sheet of Whatman paper was positioned on top of the filter membrane and air bubbles were removed. The dry pieces of paper were then placed on top followed by a stack of paper towel (2-3inches when compressed) cut to the size of the gel. The entire stack was then covered with a 500g weight.
- Transfer of the RNA from the gel to the membrane was allowed to proceed overnight.
- The membrane was peeled away from gel with forceps and was marked asymmetrically for orientation. Post-transfer washes in 5x SSC for approximately 30 seconds were carried out and the membrane was then air dried on a piece of Whatman paper.

- The RNA was crosslinked to the membrane by exposure to shortwave UV irradiation for 3 minutes and was then stored between Whatman paper in a dry place until required.

- Prehybridization

The membrane was pre-wet in 5x SSC for 20-30 minutes and was then prehybridized for 3-4 hours at 42°C in prehybridization buffer (250µl - 1000µl/cm^2). Prehybridization buffer was prepared as follows:

- Prewarm formamide to 45°C (50% deionized formamide).
- Mix the hybridization buffer:

  5x SSC

  1x Denhardt's solution: 0.02g each of Ficoll, BSA, PVP

  31mM KH_2PO_4

  0.25% SDS
- Mix herring sperm DNA (100µg/ml - prepared as follows: 10mg/ml stock was sonicated for 30 seconds and chilled on ice for 30 seconds, this was repeated 6 times and the DNA was then autoclaved) in water (volume required to bring prehybridization buffer to a final volume), and boil for 8-10 minutes. Add the herring sperm DNA to the SSC mix and then add the mixture to the formamide.
- Store at 42°C until used.

- Probe preparation

a) Radioactive labelling

The oligonucleotide used as a probe was synthesised using a sequence common to both COX-1 and COX-2, it was 34 base pairs long, rich in G/C bases, and had the following sequence: GGT CCC GGG AAG GAG GGC ATG CTC TAC TGA CGG G. The radioactive probe was prepared by a modified method taken from Maniatis *et. al.*

- The following were added together and incubated at 37°C for 1 hour:

  - 2µg DNA (oligonucleotide AVDM1) [DNA] = 1mg/ml 2µl

  - α-^{32}P dCTP (30µCi) 3µl

- 1U PNK 3µl
- DEPC-treated water 22µl

- Ethanol washes to remove unreacted α-^{32}P dCTP were performed as follows:
  - Add 3M sodium acetate to a final concentration of 0.3M, 2.5 volumes of 70% ethanol and incubate at -40°C for 1 hour
  - Centrifuge: 14 000g for 10 minutes (to pellet DNA), followed by
    14 000g for 5 minutes (wash step - to be repeated)
  - Dry for 15 minutes in a desiccator
  - Redissolve in 50-100µl TE buffer (pH 8.0)
  - Store @ -20°C until used
- Approximately 10^6cpm was used per blot.

b) *Probe denaturation*

Before the probe was used in the hybridization reaction 0.1 volume 1N NaOH was added to the probe and this was then incubated at 37°C for 10 minutes. The probe was now ready to be added to the hybridization buffer.

- Hybridization

The prehybridization buffer was poured off and the hybridization buffer (100µl/cm^2) (made up as for prehybridization buffer with the exception that no herring sperm DNA was added) was added to the membrane. The probe was then added to the hybridization buffer and was allowed to hybridize overnight at 42°C. (1-5 x10^6cpm/ml probe was used per blot).

- Post hybridization washes

The hybridization buffer was decanted and the membrane was washed to remove all the unreacted probe molecules. The washes were performed as follows:

- The membrane was washed for 30 seconds in 2x SSC, 0.1% SDS and was then drained.
- The membrane was transferred to 2x SSC, 0.1% SDS and was washed

for 15 minutes at room temperature - this wash was repeated.

- Next the membrane was washed in 0.1x SSC, 0.1% SDS for 15 minutes at 37°C - this wash was repeated.
- If the background was still too high, a final wash in 0.1x SSC, 0.1% SDS for 30 minutes at 42°C was done.

The membrane was rinsed briefly in 2x SSC and was dried between sheets of Whatman 3MM paper. Next it was wrapped in plastic wrap and the autoradiograph was set up.

vii) *Autoradiography*

The membrane was taped to the X-ray cassette and was placed under film in the dark room. The film was exposed at -40°C overnight and was then developed. The membrane was put under film again and was exposed for a further 3 days. To develop the film it was removed from the cassette in the dark room and was then processed by soaking 2 minutes in X-ray developer, 1 minute in 3% acetic acid to stop the reaction, 2 minutes in fixer and finally it was rinsed in running water. The film was then dried and analysed.

SECTION B

- *Experimental procedures*

i) *Preparation of experimental medium*

Refer to section 4.2.2 (i) for the method used, control cultures OE (supplemented with medium containing 0.1% final volume of ethanol) and 0 (supplemented with growth medium containing only 10% (v/v) FCS) were also used .

ii) *Growth of cell cultures*

Refer to section 4.2.2 (ii) for the method used.

iii) *Harvesting of cells*

Refer to section 2.2.3 (iv) for the method used.

iv) *RNA isolation*

Total RNA was isolated using a modified method of Farrell.[79] This method facilitates the simultaneous isolation of RNA and DNA from mammalian cells and relies entirely on the fact that only RNA will remain in the aqueous phase following phenol extraction if the pH is acidic. An added advantage of this protocol is that the lysis buffer contains SDS which disrupts RNase tertiary structure and a phenol extraction step is used, phenol being able to inactivate RNases, therefore one need not take additional measures to combat RNase activity. All the work was conducted on ice, except were specifically stated to the contrary.

- The cells were washed twice in 2ml of ice-cold PBS (pH6.6) and were then pelleted by centrifugation at 750g for 5 minutes. The PBS was decanted and 1ml of lysis buffer (10mM EDTA (pH 8.0), 0.5% SDS) was added.
- The cells were then homogenized using 5 passes through a 20G needle attached to a syringe.
- 1ml 0.1M sodium acetate (pH 5.2), 10mM EDTA (pH 8.0) was added and the samples were mixed by vortexing.
- 2ml of phenol (equilibrated in water, pH4.0) was added and the samples were mixed by gently inverting the tubes for 2 minutes (at room temperature). The phases were then separated by centrifugation at 750g for 10 minutes at 4°C. This phenol extraction step was repeated.
- The upper (aqueous) phase was transferred to a tube containing 220µl of ice-cold 1M Tris-Cl (pH 8.0) and 90µl of 5M NaCl. 2 volumes of ice-cold 70% ethanol were added and the samples were then mixed and stored on ice for 30 minutes.
- RNA was collected by centrifugation at 750g for 10 minutes at 4°C. The ethanol was removed and the tubes were stored in an inverted position until all the ethanol had evaporated.
- The RNA was redissolved in 200µl ice-cold TE buffer (pH 8.0) for approximately 30 minutes and was then transferred to a sterile eppendorf, containing 4µl 5M NaCl and 500µl ice-cold 70% ethanol.
- The RNA precipitate was collected by centrifugation at 12 000g for 5 minutes.

The ethanol was evaporated and the RNA redissolved in TE buffer (pH 8.0).

- RNA was either used, or stored at -40°C in 2.5 volumes ethanol and sodium acetate added to a final concentration of 0.3M.
- To recover the RNA after storage, the samples were centrifuged at 12 000g for 5 minutes and the ethanol was allowed to evaporate. The RNA pellet was then redissolved in TE buffer (pH 8.0) for approximately 30 minutes.

v) *Formaldehyde-agarose gel electrophoresis*

Very little success was achieved using the method described in section A (v) above, therefore the method was modified. A 1% agarose gel was made in DEPC-treated water, the agarose was melted by microwaving at 50% for 5 minutes and was then allowed to cool to 65°C. 10x MOPS was added to a give final concentration of 1x MOPS, and formaldehyde was added to a final concentration of 16.2% (v/v). The gel was then poured onto a gel support. 20µl of sample was combined with 10µl of loading buffer and this was heated at 65°C for 15 minutes together with 1µl of COX-1 and COX-2, respectively, made up to 5µl with DEPC-treated water combined with 10µl of loading buffer (used as positive controls). The samples were chilled on ice before loading onto the gel and the gel was then run at 75-100V for approximately 2 hours. (There was no need to stain the gel as ethidium bromide stain was included in the loading buffer and allowed for visualization of the RNA bands.)

Formaldehyde Gel Loading Buffer:	formamide	50µl
	formaldehyde	20µl
	10 x MOPS	10µl
	ethidium bromide (1mg/ml)	10µl

vi) *Northern blot*

A modified northern blot protocol was used This was adapted from the protocol generally used with the Zeta Probe System.

- *Capillary transfer*

Refer to section A (vi) above, for the method used.

- *Prehybridization*

The nylon membrane was soaked in hybridization solution and herring sperm DNA (100µg/ml), preboiled for 8-10 minutes, was then added. The membrane was allowed to prehybridize for 2-3 hrs at 42°C with shaking.

Hybridization solution: 1mM EDTA (pH 8.0)
0.5M NaH_2PO_4 (pH 7.2)
7% SDS

- *Probe preparation*

a) *Radioactive labelling*

The probes were made using COX-1 and COX-2, respectively. These cDNA clones are specific for murine prostaglandin H synthase 1 and 2. The radioactive probe was prepared using the Prime-a-gene® Labelling System protocol.[82] All work was conducted on ice unless specifically stated to the contrary.

- The kit was thawed on ice except for the Klenow which was kept at -20°C, the DNA was denatured by heating at 95-100°C for 2 minutes and was then chilled on ice.
- The following were then assembled:

- DEPC-H_2O	28.75µl	or	25µl
- 5x buffer	10µl		
- dNTP's (mix)	2µl		
- DNA (2µg/100ml)	1.25µl	or	5µl
- BSA	2µl		
- α-^{32}P dNTP	5µl		(3000Ci/mmol or 6000Ci/mmol)
- Klenow (5U/µl)	1µl		
(Total volume = 50µl)			

- The reaction mixture was incubated at room temperature for 1 hour.
- The reaction was terminated by heating at 95-100°C for 2 minutes. The probe was then chilled on ice and EDTA to 20mM final concentration (0.5M stock) was added.
- Ethanol washes to remove unreacted α-^{32}P dCTP were performed as follows:
  - Add 2.5 volumes of ethanol and 3M sodium acetate to a final concentration of 0.3M to the probe mixture and incubated at -40°C for 1 hour
  - Centrifuge: 14 000g for 10 minutes (to pellet DNA)
    14 000g for 5 minutes (wash step - to be repeated)
  - Dry for 15 minutes in a desiccator
  - Redissolve in 50-100µl TE buffer (pH 8.0)
  - Store @ -20°C until used
- Approximately 10^6cpm was used per blot

b) *Probe denaturation*

Refer to section A (vi) above, for the method used.

- Hybridization

The method described in section A (vi) above was used with the exception that the hybridization buffer was made up as outlined in section B (vi) above.

- Post hybridization washes

The hybridization buffer was decanted and the membrane was washed to remove all the unreacted probe molecules. The washes were performed as follows:

- the membrane was washed twice for 15-30 minutes in hybridization wash 1, followed by two washes for 15-30 minutes in hybridization wash 2.

Hybridization - Wash 1: 1mM EDTA
40mM NaH_2PO_4 (pH 7.2)
5% SDS

Hybridization - Wash 2: 1mM EDTA
40mM NaH_2PO_4 (pH 7.2)
1% SDS

The membrane was dried between sheets of Whatman 3MM paper and was wrapped in plastic wrap. The autoradiograph was then set up.

vii) *Autoradiography*

Refer to section A (vii) for the method used. In this section, high performance film and a high energy intensifying screen were used.

viii) *Membrane stripping*

Removal of the probes for reprobing of membranes was done using the method outlined in the Hybond^{TM}-N^{+} booklet.[82] Successful removal of probes depends on the membranes not being allowed to dry during hybridization or after hybridization washing. Membrane stripping was achieved by pouring a boiling solution of 0.5% (w/v) SDS on the membrane and allowing this to cool to room temperature. The membrane was then prehybridized and hybridized with another probe.

5.3 RESULTS

Using mRNA technology to study the effect of vitamin E succinate supplementation on COX-1 and COX-2 expression in B16 cells produced no results initially and this gave rise to a number of questions. Issues that were address were the following:

1) In terms of the gels, no RNA bands were visualized and the questions of technique and sterility - specifically RNase-sterility - were addressed. Strict measures were then taken to prevent RNase contamination and these were rigidly adhered to.
2) When there were still no RNA bands visualized on the gels, different denaturing gel protocols were tried and the quantity of RNA loaded onto the gels was increased. In this regard different set up procedures were used to increase the amount of starting material and different RNA isolation protocols were tried.

Small amounts of RNA were now visible on the gels and the gels were used in Northern blot analysis.

3) Using the oligonucleotide probe (AVDM1), which was specific for both COX-1 and COX-2, no results were obtained. A different Northern blot protocol was tried, still giving no results. This suggested that cyclooxygenase may be a rare mRNA or that it may have a very low copy number resulting in insufficient mRNA availability. It was decided that specific clones should be ordered as a specific cDNA clone would be more likely to hybridize to the respective mRNAs.

4) Using COX-1 and COX-2 as probes still produced no results. At this point, a Southern blot was done to test the viability of the clones. The clones were found to hybridize with the probes. The autoradiographic technique was improved by using film with a higher specificity, an intensifying screen was used to try and amplify the signal and various quantities of the clones were added in the labelling protocol to try and make the probes hotter.

5) The issue of mRNA stability was considered and it was decided that time course assays should be conducted to determine when the genes were being "switched on" in B16 cells after supplementation with vitamin E succinate. In light of the results obtained during preliminary time course assays of cyclooxygenase activity, it was decided to change the experimental protocol and rather than supplementation over a week, the cells were supplemented for 2, 4, 8 and 12 hours only. RNA was again isolated and this time there appeared to be significantly greater amounts of RNA on the gels.

6) Hybridization between the COX-1 and COX-2 positive controls on the Northern blot indicated that the probes were able to detect COX-1 and COX-2 at a concentration of 20ng. However, the isolated COX mRNA was still not detected. Radioactivity of a higher specific activity was used, which finally resulted in a successful Northern blot.

Figure 9 illustrates the expression of COX-1 and COX-2 in B16 cells after supplementation with 10μg/ml of vitamin E succinate. COX-1 and COX-2 were probed simultaneously as COX-1 is specific for COX-1 and COX-2 is specific for COX-2, and

no cross-reactivity was found to occur. COX-1 and COX-2 positive controls (20ng each) were visualized in lane 2 and 3, respectively. Lanes 5 to 8 contained the OE control samples for 2, 4, 8, and 12 hour intervals after ethanol supplementation, and lanes 10 to 13 contained the 10µg/ml vitamin E succinate supplemented samples for 2, 4, 8, and 12 hour intervals after vitamin E succinate supplementation. Lane 15 contained the 0 control sample supplemented with only 10% FCS and which was harvested at time 0 hours - neither COX-1 or COX-2 RNA could be seen in this lane.

Total RNA was loaded onto the gel, therefore comparison between lanes is restricted to whether the COX-1 and COX-2 were present in each lane.

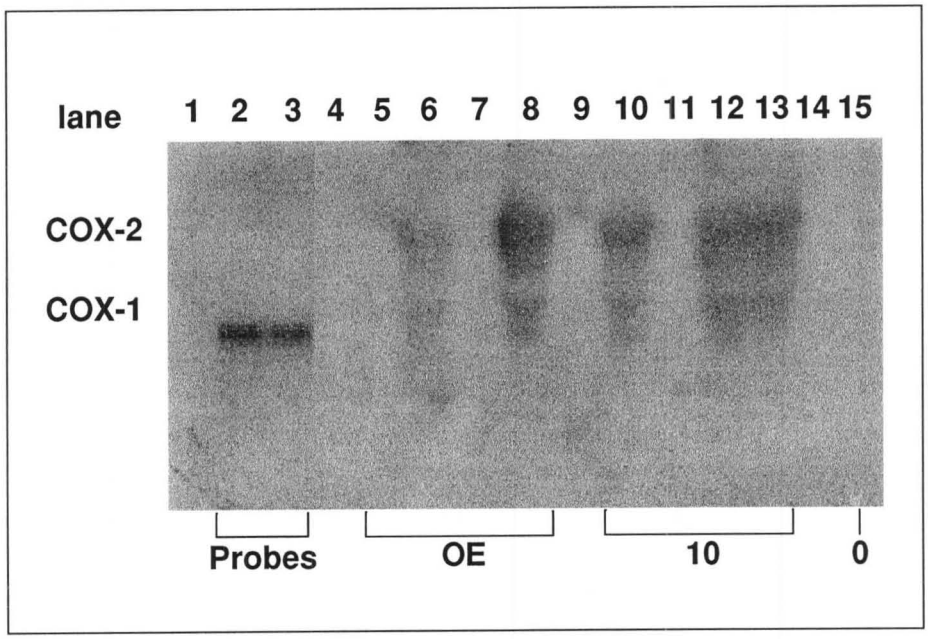

Figure 9 - Time Course for Levels of mRNA for COX-1 and COX-2 in B16 Cells Supplemented with Vitamin E Succinate as Analysed by Northern Blot.

From this blot, it was evident that in the OE supplemented cultures, COX was produced 12 hours after supplementation with ethanol. Faint bands were visible after 4 hours, but these were no longer present at 8 hours. For the 10µg/ml vitamin E succinate supplemented samples, COX appears to be produced 8 hours after supplementation, with no further increase or decrease after 12 hours. Faint bands were visible after 2 hours.

In terms of relative quantities of COX-1 and COX-2, the COX-2 band was always much larger and more prominent than the COX-1 band.

5.4 DISCUSSION

The isoform of COX can be distinguished on the basis of mRNA size, as COX-1 mRNA is 2.7 to 3kb in size, whereas COX-2 is 4 to 4.5kb in size,[57] therefore it should be possible to determine the effects of vitamin E succinate supplementation on this enzyme using cDNA clones specific for the respective isozymes.

Initially no results were obtained and optimization of the mRNA techniques used in this chapter resulted in a number of questions which had to be dealt with before results could be obtained. Issues addressed included, a) RNase-sterility, b) experimental protocols used, c) abundance of COX mRNA in the cell, and d) stability of COX mRNA. The question of COX mRNA stability was dealt with by using supplementation over a time course of 2 to 12 hours as opposed to supplementation over a period of 4 to 6 days and it would appear that the question of mRNA stability was indeed important as results were obtained using this revision in protocol.

Using the modifications indicated in section B, after 2 hours both COX-1 and COX-2 were detected in the 10µg/ml supplemented vitamin E succinate samples, however, this effect was lost after 4 hours. This may be explained by the fact that 10% FCS supplementation has been shown to cause transient induction of COX in cells with limited FCS.[66] This may also account for the transient presence of COX-1 and COX-2 at 4 hours in the ethanol supplemented OE control.

Vitamin E succinate seems to induce COX mRNA at 8 hours after supplementation, an effect which continues up to 12 hours after supplementation. OE control cultures supplemented with ethanol (vehicle treatment) induced COX mRNA 12 hours after supplementation.

Comparison of the relative quantities of COX isoforms revealed that the COX-2 band

was much larger and more prominent than the COX-1 band. The reason why there appears to be two bands in the COX-2 position may be explained by an observation made by Kutchera *et. al.*,[63] who have reported some reactivity of COX-2 with 18S RNA (located just below the COX-2 band), therefore this result may be slightly exaggerated. There does, however, appear to be greater induction of COX-2 than COX-1. This will have to be confirmed with further studies using equal amounts of poly A mRNA.

These results will now be discussed in terms of the observations made in chapters 2 to 4.

CHAPTER 6

CONCLUSION

Vitamin E succinate is reported to reduce the frequency of radiation-induced transformation,[52] inhibit transformation of mammalian cells treated with carcinogens,[12] and reduce tumour incidence in animals receiving vitamin E succinate supplementation.[42] Vitamin E succinate is also reported to be the most potent form of vitamin E for inducing morphological changes and growth inhibition of B16 murine melanoma cells *in vitro*,[43] and this has been confirmed by numerous researchers.[6,44,52] In this laboratory supplementation of B16 cells with vitamin E succinate at concentrations of 5-10μg/ml resulted in significant inhibition of cell growth of B16 murine melanoma cells. Vitamin E succinate supplementation at concentrations of 7 and 10μg/ml was also found to inhibit growth of non-malignant LLCMK cells.

Ester derivatives of vitamin E are commonly used as vitamin E supplements *in vivo* and *in vitro* as they are more resistant to oxidation and are less likely to be destroyed under adverse conditions.[11,18,45] These esters are believed to undergo hydrolysis via nonspecific esterases to release vitamin E, as is the case *in vivo*.[47,48] *In vitro* hydrolysis of vitamin E succinate to vitamin E occurs in some,[47,50] but not other cell lines.[45,46] Cleavage studies were performed to determine whether the growth inhibitory effects of vitamin E succinate supplementation on B16 cell growth were due to the intact molecule of vitamin E succinate or rather due to vitamin E.

Levels of vitamin E succinate in B16 cells were greater than the levels of vitamin E in these cells and no significant cleavage of the vitamin was found to occur. In LLCMK cells, levels of vitamin E succinate were greater than those of vitamin E in the cells, however, vitamin E levels increased with increasing vitamin E succinate supplementation. There was significant cleavage of vitamin E succinate in LLCMK cells at 3μg/ml supplementation with the level of vitamin E present at this concentration being significantly greater than the level of vitamin E succinate. The levels of vitamin E succinate present in LLCMK cells were lower than those observed in the B16 cells,

but cleavage of vitamin E succinate to vitamin E was greater than in B16 cells. This suggests that an esterase enzyme may be present in the LLCMK cells which is able to cleave the ester bond of vitamin E succinate causing the liberation of vitamin E, and that this enzyme is either absent or non-functional in B16 cells.

The observed cell growth effects in B16 cells can thus be credited to the intact form of vitamin E succinate, as with increasing levels of vitamin E succinate an inhibition of cell growth was observed. In LLCMK cells, the observed growth effects were not due to vitamin E succinate, but rather due to the vitamin E as in this case a slight decrease in LLCMK cell growth was observed which corresponded to increasing levels of vitamin E.

Prostaglandin H synthase is a membrane protein that converts arachidonic acid to various eicosanoid products[57,62] and two isozymes, the constitutively produced COX-1 and the inducible COX-2 forms, have been identified.[68-71] Vitamin E was shown to regulate the synthesis of prostaglandins via this enzyme[39,60] and studies previously conducted in this laboratory have shown vitamin E succinate to affect COX enzyme activity and thereby prostaglandin synthesis and cell growth.[39]

Further studies using B16 cells were conducted to determine when the COX enzyme was being 'switched on' so that COX mRNA could be detected using Northern blot analysis. The results obtained suggested that extraction of mRNA from B16 cells after a time course of 2 to 12 hours, rather than a period of supplementation over 4 to 6 days, would meet with greater success in terms of COX mRNA isolation and detection. An increase in COX enzyme activity above the baseline was observed at an interval of 2 hours after vitamin E succinate supplementation in B16 cells and this was followed by decreased COX activity below baseline levels up to 8 hours. The activity then increased at 12 hours after vitamin E succinate supplementation. These results were all non-significant compared to the OE control samples and factors that needed to be considered were a) 'suicide' inactivation of the COX enzyme when incubated with arachidonic acid which is related to the rate of COX catalysis,[67] and b) the enzyme

assay used crude enzyme extracts and indirect detection of COX enzyme activity and relied on the conversion of a co-substrate to a coloured intermediate by the peroxidase activity of the enzyme.[77] Due to the nature of the assay used, the trend followed by the COX enzyme activity rather than the actual activity was of importance.

The isoforms of COX can be distinguished on the basis of mRNA size, as well as on the basis of pharmacological inhibition by various nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids.[57] An attempt was made to use both methods to determine the effect of vitamin E succinate on COX expression and regulation. Inhibition studies using dexamethasone and cycloheximide, alone and in combination, were inconclusive, however, use of Northern blots to detect COX mRNA, was more successful.

COX mRNA was detected 2 hours after vitamin E succinate supplementation and this coincides with the detection of COX activity as was found to occur in the enzyme assays. There is, however, a strong possibility that this transient induction of COX was due to the supplementation of 10% FCS to the cells.[66] COX mRNA was again detected 8 to 12 hours after vitamin E succinate supplementation and COX enzyme activity was found to increase at 12 hours. These results suggest that the COX gene is 'switched on' at an early stage after vitamin E succinate supplementation and would explain why no COX mRNA was detected by Northern blot analysis of samples supplemented with vitamin E succinate over a period of 4 to 6 days.

It appears that vitamin E succinate does induce COX, with COX-2 being induced to a greater extent than COX-1. Kutchera *et. al.*[63] reported that some cross reactivity occurred between COX-2 probes and the 18S rRNA which is located directly below the COX-2 band. These results may therefore be obscured due to cross reactivity of the COX-2 probe with the 18S rRNA, a problem that may be avoided by the use of poly A mRNA only as rRNA contamination would effectively be eliminated.

In this laboratory it was thus demonstrated that in B16 cells, vitamin E succinate

significantly inhibited cell growth at a concentration of 10µg/ml, while at the same concentration a significant increase in cyclooxygenase activity has previoulsy been found to occur.[39,55] Most studies on the role of cyclooxygenase in carcinogenesis have, however, shown that increased levels of COX-2 are associated with transformed cells and tumours, and have also shown that inhibition of COX-2 leads to growth inhibition of these cells.[63,64,68] The studies discussed in this thesis were not conclusively able to shed any light on the involvement of COX as a mechanism of vitamin E succinate growth inhibition in B16 cells. However, at this stage, it would seem appropriate to suggested that other factors may be involved in the actual mechanism of growth inhibition by vitamin E succinate as contradictory evidence in terms of the involvement of COX in growth inhibition in cancer cells has been found.

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