THE EFFECTS OF PGA_2 ON CELL CYCLE PROGRESSION IN OESOPHAGEAL CARCINOMA CELLS

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By

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PROMOTER: DR MARYNA DE KOCK

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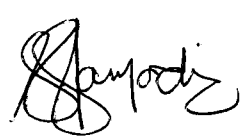
This thesis is dedicated to my parents for giving me a quality education

DECLARATION

I declare that *The effects of PGA_2 on cell cycle progression in oesophageal carcinoma cells* is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Sedicka Samodien January 2003

Signed: 

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KEYWORDS

Unsaturated fatty acids

Prostaglandin A_2

Tumor cells

Inhibition

Cytotoxic

Cell cycle

Mitosis

Spindle assembly checkpoint

Apoptosis

Cytoskeleton

SUMMARY

The molecular mechanisms causing anti-mitotic and cytotoxic effects of prostaglandin A_2 [PGA_2 ($C_{20}H_{30}O_4$)] treatment of human oesophageal carcinoma (WHCO3) cells were investigated.

WHCO3 cells, incubated with cytotoxic doses of PGA_2 (20 and 50µg/ml) for 3, 24 and 48 hrs showed characteristic morphological features of apoptosis such as chromatin condensation, nuclear fragmentation, as well the formation of apoptotic bodies. Chromatin condensation was more prevalent in cells exposed to 20µg/ml PGA_2 for 24 hrs. Apoptosis induction was found at both concentrations and cells exposed to 50µg/ml showed apoptosis at 3 hrs already. An increase in abnormal metaphases was observed in cells exposed to 20µg/ml PGA_2 for 24 hrs. This outspoken increase in metaphases could be due to the activation of the spindle assembly checkpoint. Activation of this checkpoint blocks the onset of anaphase and arrests cells in metaphase. The flow cytometry studies also showed an increase of cells in metaphase. Metaphase arrest occurs when any of the following situations have effect: unattachment of a single kinetochore to the microtubules, unbalanced tension on chromosomes or if there is abnormal dynamic behaviour of the microtubules attached to the kinetochore (Bunz *et al.*, 1998; Gorbsky, 1997; Gorbsky, 2001; Murray, 1994). WHCO3 cells exposed to 20µg/ml PGA_2 for 24 hrs showed an accumulation of cells with condensed chromosomes and according to Cahill *et al.*, 1998, this accumulation is characteristic of a mitotic block. After 48 hrs, cells exposed to 20µg/ml PGA_2 showed a decrease in the number of apoptotic cells and it appeared that the cells were able to overcome the arrest. Indirect immunofluorescent studies of α-tubulin showed that PGA_2 had varied effects on the cytoskeleton. Fewer but intact microtubules were observed when cells were exposed to 20µg/ml PGA_2 for 24 hrs, whereas the results obtained from cells exposed to 20µg/ml PGA_2 for 48 hrs showed some normal spindles, a few abnormal arranged spindles and others disrupted microtubules. Cotter *et al.*, 1992 showed that disrupted microtubules prevent the formation of apoptosis. The results of the 20µg/ml PGA_2 studies for 24 and 48 hrs can possibly be explained by this phenomenon. The cells exposed to 20µg/ml PGA_2 for 24 hrs showed increased chromatin condensation and apoptosis formation, whilst the cells exposed to the same concentration for 48 hrs showed a

decrease in apoptosis formation. This result can possibly be ascribed to either the ability of the cell to overcome the spindle assembly checkpoint or an increase in the disruption of the microtubule arrangement, thus preventing the induction of apoptosis. In cells exposed to 50µg/ml PGA_2 total rearrangement of α-tubulin was very much evident and this rearrangement could be a cause of apoptosis induction as indicated by the large amount of apoptotic cells (28.93% and 51.63% during 24 and 48 hrs respectively) with flow cytometry. Silver staining of PGA_2 treated WHCO3 cells showed segregation of granular and fibrillar components. This segregation is usually associated with non-functional nucleoli and indicative of an inhibition in protein synthesis (Hadjiolov, 1985).

DNA fragmentation was also observed with the two higher concentrations of PGA_2. On agarose gel electrophoresis, DNA laddering was evident in the cells exposed to 50µg/ml for 3 hrs and 20µg/ml for 24 hrs. It was furthermore found that caspase 3 could possibly play a role in the induction of apoptosis, internucleosomal DNA degradation, chromatin condensation, membrane blebbing and disassembly into membrane bound vesicles at the higher concentrations.

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ABBREVIATIONS

AA (20:4n-6)	:	arachidonic acid
AC	:	adenylate cyclase
$AgNO_3$	:	silver nitrate
AIF	:	apoptosis inducing factor
ALA (18:3n-3)	:	alpha-linolenic acid
ANOVA	:	one way analysis of variance
Apaf-1	:	apoptotic protease activating factor-1
APC/C	:	anaphase promoting complex/cyclosome
APS	:	ammonium persulfate
ATM	:	ataxia telangiectasia mutated
ATP	:	adenosine triphosphate
ATR	:	ataxia-and-Rad related
BSA	:	bovine serum albumin
BUB	:	budding uninhibited by benzimidazoles
CAK	:	cdc2 activating kinase
cAMP	:	cyclic AMP
Cdc	:	cell division cycle genes
Cdks	:	cyclin-dependent kinases
CDKIs	:	cyclin dependent kinase inhibitors
CENP	:	centromere protein
CoA	:	coenzyme A
$cPLA_2$	:	cytoplasmic phospholipase A_2
CCP32	:	caspase 3
DAG	:	diacylglycerol
D6D	:	Δ6 desaturase
DFF	:	DNA fragmentation factor
DGLA (20:3n-6)	:	dihomogamma-linolenic acid
DHA (22:6n-3)	:	docosahexaenoic acid
DMEM	:	Dulbecco's minimum essential medium

DNA-PK	:	DNA dependent protein kinase
DR3	:	death receptor 3
DR4	:	death receptor 4
DR5	:	death receptor 5
EDTA	:	ethylene diaminetetra-acetate
EFAs	:	essential fatty acids
EGF	:	epidermal growth factor
EPA	:	eicosapentaenoic acid
ER	:	endoplasmic reticulum
EtBR	:	ethidium bromide
FAs	:	fatty acids
FasL	:	Fas ligand
FB_1	:	fumonisin B_1
FCS	:	fetal calf serum
FITC	:	fluorescein isothiocyanate
FSC	:	forward scatter
GFs	:	growth factors
GLA (18:3n-6)	:	gamma-linolenic acid
HCl	:	hydrochloric acid
HDL	:	high density lipoprotein
H & E	:	haematoxylin and eosin
HETE	:	hydroxyeicosatetraenoic acid
HODEs	:	9- or 13- hydroxyoctadecadienoic acids
HPETEs	:	hydroperoxyeicosatetraenoic acids
HPV	:	human papilloma virus
hrs	:	hours
HU	:	hydroxyurea
ICE	:	interleukin- 1β converting enzyme
IgG	:	immunoglobulin
IP_3	:	inositol-1,4,5-triphosphate
LA (18:2n-6)	:	linoleic acid

LDL	:	low density lipoprotein
LTs	:	leukotrienes
mA	:	milli ampere
MAD	:	mitotic arrest deficient
MAPK	:	mitogen-activated protein kinase
MAPKK	:	mitogen-activated protein kinase kinase
MI	:	mitotic indices
mRNA	:	messenger RNA
NaCl	:	sodium cloride
Na_2EDTA	:	disodium ethylene diaminetetra-acetate
NOR	:	nucleolar organizer region
OC	:	oesophageal cancer
OH	:	hydroxyl group
PARP	:	poly(ADP-ribose) polymerase
PBS	:	phosphate buffered saline
PCD	:	programmed cell death
PGs	:	prostaglandins
PGA_2	:	prostaglandin A_2
PGE_1	:	prostaglandin E_1
PGI_2	:	prostacyclin I_2
PGG/H	:	prostaglandin G/H
PI	:	propidium iodide
PI-3	:	phosphatidylinositol 3-kinase
PKA	:	protein kinase A
PKC	:	protein kinase
pRb	:	retinoblastoma tumour suppressor protein
PTKs	:	protein tyrosine kinases
PUFAs	:	polyunsaturated fatty acids
RasGAP	:	Ras activating protein
RDA	:	recommended dietary allowance
rDNA	:	ribosomal DNA

rRNA	:	ribosomal RNA
rpm	:	revolutions per minute
RTK	:	tyrosine kinase receptors
SCC	:	side scatter
SD	:	standard deviations
SDS	:	sodium dodecyl sulphate
SDA PAGE	:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	:	standard error of the mean
TBE	:	Tris boric acid (Na_2EDTA)
Tblsp	:	tablespoon
TBS	:	Tris-buffered saline
TBS-T	:	TBS and Tween 20
TBXs	:	thromboxanes
TEMED	:	N,N,N',N'-tetramethylethylenediamine
Thr 161	:	threonine 161
TNF	:	tumor necrosis factor
TNFR1	:	tumor necrosis factor receptor
TRADD	:	tumor necrosis factor receptor associated death domain protein
TRAF1	:	tumor necrosis factor receptor associated factor 1
Tsp	:	teaspoon
Tyr	:	tyrosine
TXA_2	:	thromboxane A_2
UFA	:	unsaturated fatty acid
V	:	voltage
WHCO3	:	oesophageal carcinoma cell line
w/v	:	weight per volume

CHAPTER 1
INTRODUCTION
LITERATURE REVIEW AND MOTIVATION FOR THIS STUDY

1.1 Essential fatty acids

The role of essential fatty acids (EFAs) in the diet was discovered by George and Mildred Burr in 1929 at the University of Minnesota (Horrobin, 1982). They proved that rats subjected to a fat free diet resulted in stunted growth. The growth could be restored to normal by the administration of modest amounts of linoleic acid [18:2n-6 (LA)]. This restoration of growth could also be seen when they added arachidonic acid [20:4n-6 (AA)] or α-linolenic acid [18:3n-3 (ALA)] to the diet. Due to this key role of defining the growth of the individual, these three fatty acids (FAs) were regarded as essential and termed EFAs (Pandian *et al.*, 1999).

EFAs, like vitamins cannot be synthesized by the body and must be acquired from ingested food (Horrobin, 1983; Purasiri *et al.*, 1994). LA and ALA are the precursors of the n-6 and n-3 series of polyunsaturated fatty acids (PUFAs), which are necessary for the body to function properly (Horrobin, 1982; Horrobin, 1993; Horrobin and Manku, 1990; Ramesh *et al.*, 1992; Simopoulos, 1991). Many PUFAs are termed EFAs but LA and ALA are the only 2 FAs that are truly essential. Their derivatives can be formed by desaturation or elongation reactions (Fig 1.1). LA and ALA are oxidized to provide energy and have no biological activity on their own. They require specific biochemical transformation in the body if they are to function as EFAs (Pandian *et al.*, 1999; Simopoulos, 1991). The n-6 and the n-3 series of EFAs are not interchangeable in animals, but the enzymes that metabolize them seem to be identical (Horrobin, 1982).

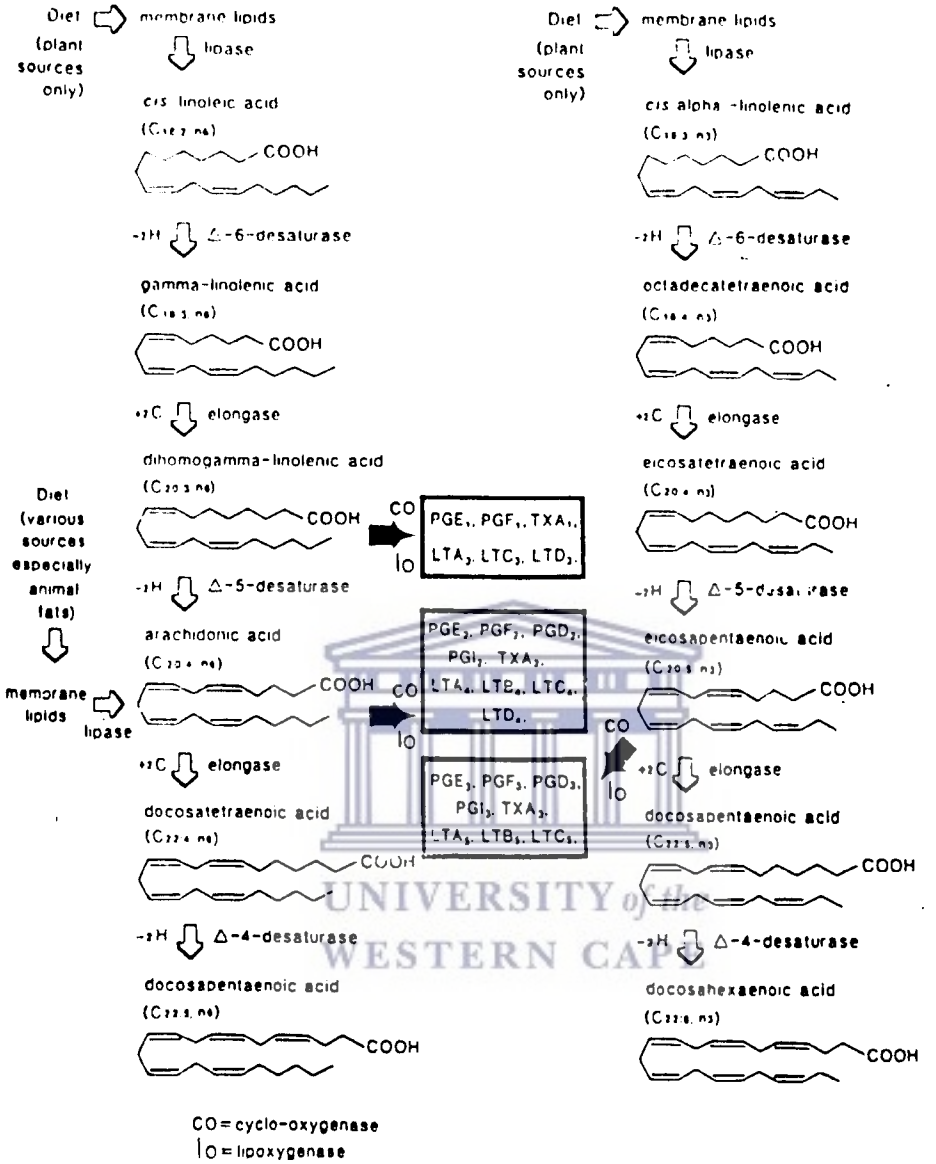


Figure 1.1 The pathways of metabolism of the n-6 and n-3 EFAs (Dippenaar, 1986; Horrobin, 1983). LA is an 18-carbon chain fatty acid with 2 double bonds in the middle. The first double bond (from the omega end) occurs after the sixth carbon atom. ALA is an 18-carbon fatty acid with three double bonds (from the omega end) at the 3rd, 6th and 9th carbon positions. EFAs are converted by a series of alternating desaturations and elongations to their metabolites. The alternating desaturations add on a double bond and the elongations add on 2 carbon atoms (Horrobin, 1983). The 1st step is Δ6 desaturation, then an elongation step, followed by Δ5 desaturation, then another elongation and then Δ4 desaturation. The desaturation steps are slow and the elongation steps are rapid, which are particularly true of the 1st desaturation and elongation steps. This is why gamma linolenic acid (18:3n-6 GLA), (a derivative of LA) of the n-6 series and stearidonic acid (18:4n-3) of the n-3 series are found only in very small quantities in the body (Horrobin and Manku, 1990).

Another sequence of unsaturated fatty acids (UFAs) is present in which oleic acid (18:1n-9) corresponds to LA and ALA of the EFA series. These series of FAs are not essential, since both can readily be produced by most of the cells from simple precursors (Pandian *et al.*, 1999; Simopoulos, 1991). The n-3, n-6 and n-9 series of UFAs are metabolized by the same sequence of enzymes. The n-3 series is first metabolized by the enzymes, then the n-6 and thirdly the n-9 series. This results in competition amongst the 3 series with n-3 EFAs inhibiting the metabolism of n-6 and n-9 FAs and n-6 EFAs inhibiting metabolism of n-9 FAs. Therefore metabolites of oleic acid are normally found only in trivial amounts (Horrobin, 1983).

1.1.1 EFA requirements

The amount of EFAs needed depends on a person's level of activity and stress, nutritional state, body weight, season and latitude.

In 1990, the Canadian Ministry of National Health and Welfare released a report of its Scientific Review Committee, which stated the recommended nutrient intake of EFAs (Table 1).

Table 1: Recommended dietary allowance (RDA) for LA and ALA according to the Canadian Ministry of National Welfare (http://www.multidiet.com).

EFAs	Source	Amount	Calories	RDA
LA	1 Tblsp (14 g) Safflower Oil	10 grams	120	7-11 grams
ALA	1 Tsp (4.6 g) Flaxseed Oil	2.3 grams	40	1.1-1.8 grams

A tablespoon (14g) of safflower oil contains about 10g of LA and 120 Calories, which is almost equal to the RDA. A teaspoon (4.6g) of flaxseed oil contains about 2.3g of ALA and 40 Calories. This is slightly more than the RDA.

Nutrients essential for LA and ALA functions include magnesium, selenium, zinc and vitamins A, carotene B3, B6, C and E.

1.1.2 Dietary sources of EFAs

Dietary sources containing EFAs include plants and any food with animal cell membranes. The main dietary sources of the various EFAs are as follows:

Linoleic acid (18:2n-6 LA):

Certain seed oil such as sunflower, safflower, corn, soy, and evening primrose oil are very rich in LA (Crawford, 1983). Substantial amounts are found in dairy products and organ meats (liver and human milk). Peanut oil has a moderate amount and olive oil a small amount. The most important sources of LA in the modern diet are milk products and vegetable seed oils together with foods derived from them (Horrobin, 1983).

Gamma-linolenic acid (18:3n-6 GLA):

The most studied sources of GLA are the Efamol (Epogam) variety of seed oil of the evening primrose oil, *Oenothera spp*. Human milk (0.3-1.0%) and evening primrose oil (Efamol) are the only readily available sources (Gibson and Kneebone, 1981; Horrobin, 1982) and a small amount is found in oats (Pandian *et al*., 1999; Purasiri *et al*., 1994).

Dihomogammalinolenic acid (20:3n-6 DGLA):

Human milk contains some DGLA (Gibson and Kneebone, 1981) and small amounts are found in some organ meats such as the adrenals, spleen, and kidney (Horrobin, 1983).

Arachidonic acid (20:4n-6 AA):

Moderate amounts are found in human milk. Substantial amounts are found in meat, dairy products, some in seaweeds, shrimps and prawns (Horrobin, 1983).

α-linolenic acid (18:3n-3 ALA):

The lipids of green leafy vegetables contain ALA (Crawford, 1983). It is found in a variety of grains, small amounts in soy oil and large amounts in linseed oil. Flaxseed oil is a good source

of n-3 EFAs. It is difficult for the digestive system to break down flaxseeds sufficiently to obtain enough EFAs, so flaxseed oil or grinding the seeds is preferable. The traditional diet of Eskimo's include a very high intake of these n-3 EFAs and this may contribute to their remarkable absence of cardiovascular disease and possibly cancer (Horrobin, 1983).

Eicosapentaenoic acid (20:5n-3 EPA):

The long chain n-3 FA (EPA) is mainly found in marine oils or in oils of migratory fish and shellfish. Herring, menhaden and salmon oil all contain small amounts. Large amounts are present in the meat of seals and whales (Pandian *et al*, 1999; Purasiri *et al*., 1994).

1.1.3 Horrobin Hypothesis

In 1980 Horrobin made the suggestion that cancer can be treated by the normalization of cancer cells using techniques harmless to normal tissues. He proposed that the majority of malignant cells have six common features, which do not appear in normal cells although each malignant cell type has its own individual features (Horrobin, 1980). The common features are:

- Aerobic glycolysis – the ability to switch off excess lactate production in the presence of excess oxygen
- Loss of feedback regulation of cholesterol biosynthesis
- Loss of the activity of Δ6 desaturase, the enzyme which converts LA to GLA
- Excess production of 2 – series prostaglandins (PGs)
- Loss of the ability to maintain intracellular calcium homeostasis
- A decrease in cAMP synthesis

He reasoned that the local deficiency of prostaglandin E_1 (PGE_1) or thromboxane A_2 (TXA_2) might give rise to the alterations in metabolism present in malignant cells and that by providing GLA and DGLA restoring the normal PGE_1 synthesis will enable the cell to bypass the biochemical block. Horrobin suggested that this procedure would normalize malignant cells and reverse cancer cell growth.

1.1.4 Importance of Δ6 desaturase (D6D)

Most functions of LA are performed by its D6D metabolites (Horrobin and Manku, 1990). D6D is the key first step in the metabolism of both LA and ALA. It converts LA to GLA and ALA to octadecatetraenoic acid (18:4n-3) (Horrobin, 1983; Horrobin, 1993). These EFAs can themselves be metabolized further to produce a variety of essential intermediate compounds (Davidson *et al.*, 1991). In order for the EFAs in the diet to fulfill their regulatory functions, they must first be converted into a number of intermediates, which can then mediate particular cellular events (Horrobin, 1993). Hence, the crucial function of D6D cannot be over emphasized (Pandian *et al*, 1999).

D6D acts in 2 steps, namely the formation of linoleyl-CoA and the actual desaturation. The CoA derivative formation is rapid and the desaturation is the rate-limiting step (Davidson *et al.*, 1991). If the consumption of 6 desaturated metabolites are increased, then the slow nature of even normal 6 desaturation may lead to a fall in the levels of the derived EFAs, as they cannot be replaced at the rate at which they are consumed (Horrobin and Manku, 1990).

The action of D6D has been shown to be modulated by a variety of hormones. Glucagon, glucocortocoids and thyroxine all inhibit the enzyme (Horrobin, 1982; Horrobin, 1983). Dietary manipulation also modifies the activity of the enzyme. Total fasting and a protein deficiency reduce its activity whilst partial caloric restriction increases D6D activity more than 3 fold (Horrobin, 1983). Inhibition of D6D may also be viewed as comparable to an EFA deficiency (Pandian *et al.*, 1999). The enzyme is inhibited by saturated fats, trans fatty acids, excessive alcohol consumption, oncogenic viruses, ionizing radiation, diabetes, premenstrual syndrome and mastalgia, hepatic disorders, aging, some immunological abnormalities and inflammatory disorders including arthritis, atopic eczema and multiple sclerosis, zinc and pyridoxine deficiency and cancer (Pandian *et al.*, 1999; Horrobin and Manku, 1990; Horrobin, 1983).

A number of malignant cells have been reported to have a reduced activity of D6D (Sagar *et al.*, 1992). The loss of the activity of this enzyme has been suggested to be a characteristic feature of malignant transformation, thus being unable to synthesize GLA, DGLA, AA and

other long chain EFAs from the precursor LA and therefore their subsequent metabolites (such as PGs) (Gardiner and Duncan 1991).

1.1.5 Functions of essential fatty acids

Since EFAs give rise to PGs it is important to look at some aspects of EFAs. The EFAs play major roles in our bodies with a number of derivative functions arising as a consequence of these major roles (Horrobin and Manku, 1990). They are important in the structure of all animal cellular membranes as part of phospholipids. EFAs give membranes the properties of fluidity and flexibility (Purasiri *et al.*, 1994), which are key determinants of permeability (Manku *et al.*, 1983). EFAs regulate many of the steps that are involved in signal transduction either positively or negatively (Sumida *et al.*, 1993). They act directly on the cell membrane as first messengers if they are added to the cell from extracellular sources or in response to other signals are incorporated into the membrane phospholipids from which they can be released by cellular phospholipase. Acting as second messengers, they can propagate messages, or they can modulate signals from other pathways such as the binding of steroid hormones to intracellular receptors (Sumida *et al.*, 1993). Once EFAs and their metabolites are produced, they may contribute to the onset of several morphological changes that occur during mitogenesis (Lysz *et al.*, 1994). Mitogen-activated protein kinase (MAPK) is activated by AA and 15-(hydroxyeicosatetraenoic acid (HETE) partly through protein kinase C (PKC) and thus effects a positive feedback regulation on their own production in vascular smooth muscle cells (Rao and Reddy, 1994). EFAs are further more the precursors of a group of highly reactive, short lived molecules, the prostaglandins (PGs) and leukotrienes (LTs) (eicosanoids) (Davidson *et al.*, 1991) (Fig 1.1). Generally a FA must have at least 20 carbon atoms and 3 double bonds before it can be metabolized to PGs and LTs. In AA where the initial fatty acid structure is appropriate, the hydroxy acids can further be metabolized to the highly biologically active LTs (Horrobin, 1983). The cyclo-oxygenase system can only metabolize some FAs to endoperoxides, which are the first steps in the formation of PGs and thromboxanes (TBXs). The best-known precursors of PGs and LTs are DGLA, AA and EPA (Sprecher *et al.*, 1982).

2.1 PROSTAGLANDINS

All mammalian cells, with red blood cells as an exception, produce PGs and their related compounds, the prostacyclins, TBXs and also LTs - collectively known as eicosanoids (Voet and Voet, 1995). PGs are short-lived highly active hormone like chemicals, which are required for the second to second regulations of most tissues in the body. They are membrane-associated cell to cell messengers often produced in response to extracellular stimuli (Polgar *et al.*, 1980). They are released into the blood in minute quantities and are potent in their action. PGs are also called local or tissue hormones because they act in the immediate area in which they are produced (Tortora and Anagnostakos, 1990). There are three series of PGs that are produced by EFAs: PG_1, PG_2 and PG_3. The one series PGs are synthesized from DGLA (20:3n-6), whereas series-2 PGs are synthesized from AA (20:4n-6). EPA gives rise to the series-3 PGs (Pandian *et al.*, 1999; Ramesh *et al.*, 1992; Voet and Voet, 1995).

2.1.1 The chemistry of prostaglandins

PGs are derivatives of prostanoic acid (C20 FAs). It is a long chain FA in which carbons 8-12 comprise a cyclopentane (five membered) ring (Voet and Voet, 1995) (Fig. 1.2*a*). PGs differ from one another in the nature of their cyclopentane rings (with PGG and PGI as an exception) and in the number of double bonds in the open chain part of their molecules. The ring structure is identified by the letter (E, F, A, B, C, D or H) (Fig. 1.2*b*). The numerical subscript ($_1$, $_2$ or $_3$) indicates the number of double bonds, which is contained on the side chain of the cyclopentane ring (Fig. 1.2*c*).

The PG molecule has a completely uniform position of double bonds irrespective of the series. All PGs have a double bond in the C13-C14 position. Those with no other double bond in the chains carry the suffix. An additional double bond at position C5-C6 is found in those with the suffix 2. Those labeled three have a further double bond linking carbon atoms 17 and 18. Free rotation of the groups they connect is restricted by the double bonds. PGs of the G and H series are known as the PG endoperoxides because they bear the –OOH group instead of the hydroxyl (OH) group at position 15 (Fig. 1.2*b*).

If the length of the open chain is reduced by one, two, three or more methylene groups, the prefix nor, dinor, trinor, etc. is attached to the name of the corresponding PG. A further prefix, α or ω is attached to indicate the shortening to the carboxyl containing (α) or to the hydroxyl carrying (ω) chain. (Voet and Voet, 1995).

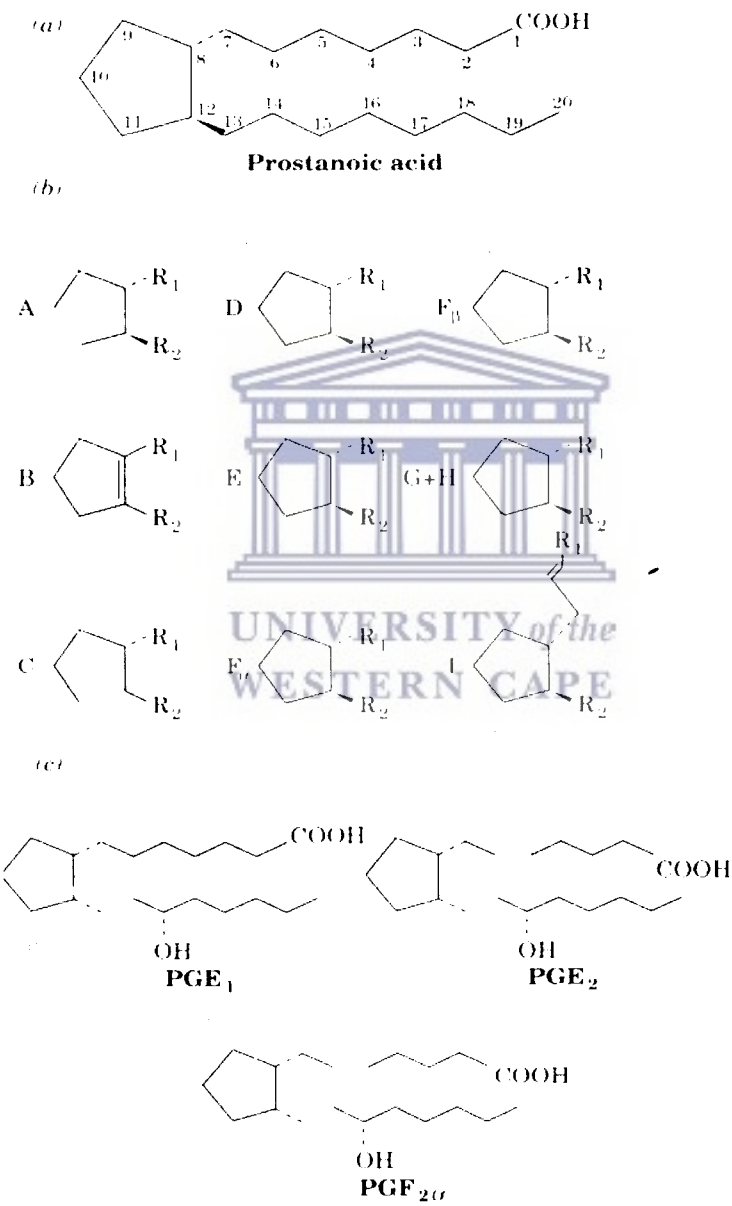


Fig.1. 2 Prostaglandin structures: *(a)* the carbon skeleton of prostanoic acid, the PG parent compound. *(b)* Structures of PGA to I. *(c)* Structures of PGE_1, E_2, and $E_{2\alpha}$ (Voet and Voet, 1995).

2.1.2 Prostaglandin synthesis

In most mammals the major precursor for PGs is the most abundant C20 polyunsaturate AA. PGs are synthesized on a continuous basis in membranes from precursors cleaved from membrane phospholipids by phospholipase A_2 (Smith, 1989) (Fig. 1.3).

There are two main pathways of arachidonate metabolism. In the cyclic pathway cyclo-oxygenase and related systems are the main enzymes giving rise to PGs and thromboxanes. The cyclic pathway forms PGs' characteristic cyclopentane ring. In the linear pathway the 5-, 12-and 15 lipoxygenase give rise to a variety of oxygenated metabolites, which include the LTs and hydroperoxyeicosatetraenoic acids (HPETEs) (Horrobin and Manku, 1990; Voet and Voet, 1995).

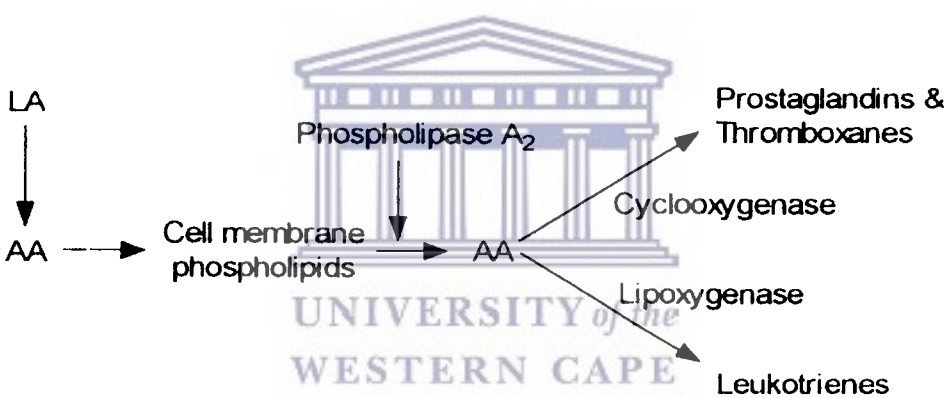


Fig. 1.3 Production of eicosanoids from EFAs

The prominent features of the cyclo oxygenase biosynthetic pathway include three stages:

a) the release of AA from precursor glycerophospholipids (Crawford, 1983)

b) oxygenation of free AA by PG endoperoxide G/H (PGG/H) synthase (cyclo-oxygenase)

c) metabolism of PGH_2 to a specific biologically active end product PGE_2, $PGE_{2\alpha}$, PGD_2, PGI_2 (prostacyclin), or thromboxane A_2 (TXA_2).

a) AA release

Prostanoids are synthesized in response to cell specific proteolytic or hormonal stimuli. The increase in the concentration of free arachidonate in the proximity of PGG/H synthase is an immediate effect of these stimuli. An increase in the prostanoid production is briefly related with arachidonate release; AA release is receptor mediated. Hormones, autocoids, growth factors and tumor promoters can cause the release of arachidonate. Prostanoid formation can also be derived from mechanical stresses on cells, which may not be receptor mediated. Arachidonate may also be elicited from the sequential hydrolysis of phosphoinositides by phophoslipase C and diacylglycerol lipase where the major sources are probably glycerophospholipids, phosphaditylcholine and phosphotidylethanolamine. The key enzyme is phospholipase A_2 (Smith, 1989; Voet and Voet, 1995). This generation of inositol phosphates is induced by dietary FAs (Simopoulos, 1991).

b) PG endoperoxide synthesis

The integral membrane protein PGG/H synthase mediates the conversion of AA to PG endoperoxide PGH_2. This protein is found in greatest abundance in the endoplasmic reticulum (ER) of prostanoid forming cells. PGG/H synthase contains 2 distinct catalytic activities:

- a cyclo oxygenase activity involved in PGG_2 formation by catalyzing the addition of 2 O_2 molecules to AA to form PGG_2 and
- a peroxidase activity which converts the hydroperoxy function of PGG_2 to an OH group (PGH_2) by mediating a net two electron reduction of the 15 hydroperoxyl group of PGG_2. PGH_2 is the immediate precursor of the series-2 PGs, prostacyclin and thromboxanes (Smith, 1989; Voet and Voet, 1995).

c) endoperoxide metabolism

The synthesis of the biologically active prostanoids (PGD_2, PGA_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2) occurs in a cell-specific manner from PGH_2. Any cell that forms prostanoids tends to form only one of these compounds as its major product. PGG/H synthase catalyzes the metabolism of PGH_2. PGI_2 synthase and TXA_2 are membrane bounded related haemoproteins with a cytochrome P-450 chromophore. PGE_2 formation requires reduced glutathione and is

catalyzed by membrane bound PGH-PGE isomerases, which lack gluthathione s-transferase activity (Smith, 1989).

All animal and human originated cancers so far studied produce excessive amounts of 2-series PGs when compared to the normal tissues from which the cancers are derived. A universal characteristic of human cancers is therefore the loss of the normal regulation of 2-series PG formation. PGE_1 and TXA_2 may play an important role in inhibiting the mobilization of AA. PGE_1 is able to inhibit the release of AA by increasing the production of cyclic AMP. TXA_2 may also control 2-series PG pathway. This is achieved by a feedback mechanism, which blocks AA release. Thus, loss of the ability to form PGE_1 and/or TXA_2 will lead to excessive mobilization of AA and formation of 2-series PGs (Smith, 1989).

2.1.3 Functions of PGs

Each cell type or organ produces its own form of PG to carry its function. PGs bind to plasma proteins and cell membranes in a manner analogous to free fatty acids. They possess a variety of biological effects and play a regulatory role in a number of systems. Different types of PGs can have opposing effects on the same systems, and differing concentrations of the same PG may have opposing effects in a single system, for example, low concentrations of PGE_1 stimulate inflammatory responses while high concentrations inhibit them (Easty and Easty, 1976).

When trauma or reception of a hormone stimulates a cell, a series of synthetic reactions take place in the plasma membrane. PG is released into the cytoplasm and secreted from cell to cell. Many different tissues produce many different types of PGs. Certain PGs cause muscle contraction in the uterus; therefore some women experience pain and discomfort during menstruation (Mader, 1993). Certain PGs are also used in the treatment of ulcers because they reduce gastric secretion; to treat hypertension because they lower blood pressure; and to prevent thrombosis because they inhibit platelet aggregation (Mader, 1993). They stimulate uterine contraction, induce labor and abortion, transmit nerve impulses, regulate metabolism, inhibit lipid breakdown, regulate muscular contraction of the gastrointestinal tract and stimulate the migration of phagocytes through capillary walls. PGs of the E series are released

by damaged cells and intensify the effects of histamine and kinins (Tortora and Anagnostakos, 1990). Series 1 and 3 PGs raise cAMP levels (Horrobin and Manku, 1990), dilate blood vessels, reduce clotting (Voet and Voet, 1995), lower harmful low-density lipoprotein (LDL) cholesterol levels, raise beneficial high-density lipoprotein (HDL) cholesterol and have anti inflammatory actions. Series-2 PGs are produced for example if we twist our knee. The tissues in the joint produce series-2 PGs and LTs (another inflammatory substance) and this is part of the healing response (Voet and Voet, 1995). After a while, the production of series-2 PGs are turned off and the production of anti-inflammatory series-1 and 3 PGs are increased. The swelling and pain disappear. PGs also mediate the regulation of sleep/wake cycle (Voet and Voet, 1995).

AA and eicosanoids act as intermediates in growth factor (GF) transmembrane signaling pathways. The binding of GFs to their tyrosine kinase receptors (RTK) produces the autophosphorylation of the latter as well as of inter alia phospholipase C-γ2 and Ras activating protein (RasGAP). The subsequent binding of these two proteins to RTK leads eventually to the liberation of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) on the one side and of active Ras on the other. DAG and IP_3 stimulate protein kinase C (PKC) and Ca^{2+} liberation from the endoplasmic reticulum (ER). RTK may directly activate PKC by catalyzing its phosphorylation. Active Ras stimulates a mitogen-activated protein kinase (MAPK) through the indirect activation of MAPK kinase (MAPKK). $MAPK_1$, PKC and Ca^{2+} then synergyze by inducing the translocation/activation of cytoplasmic phospholipase A_2 ($cPLA_2$) with subsequent release of AA and eicosanoids. PKC can also activate the synthesis (expression) of $cPLA_2$ mRNA. AA may induce activation of MAP kinase and production of Ras or suppress RTK autophosphorylation, thus operating respectively positive and negative feedback regulation. HETEs and 9- or 13- hydroxyoctadecadienoic acids (HODEs) as well as PGE_2 play a dual role in the regulation of mitogenesis and DNA synthesis. The former induce cytoskeletal alteration via PKC stimulation and induce gene expression, while PGE_2 probably through adenylate cyclase (AC) coupled receptor EP_2, cAMP accumulation and protein kinase A (PKA) stimulation, inhibits DNA synthesis and counteracts MAPK/MAPKK action. Therefore, AA and eicosanoids produce upon RTK stimulation and the sequence of

phosphorylation steps described above, may affect both a negative and a positive regulation, either upstream or downstream in GF intracellular signalling (Di Marzio, 1995).

A specific carrier on the cell surface actively transports PGs into the cell where they accumulate in the nuclei. This uptake and accumulation are closely related to their growth inhibitory activity (Kim, *et al.*, 1993).

2.1.4 Effects of PGA_2 in various cancers

Several *in vitro* studies have shown that EFAs and their metabolites have selective cytotoxic activity against human cancer cells (Chow *et al.*, 1989; Das, 1990; Ellatar and Lin, 1989; Sagar *et al.*, 1992) without harming normal cells (Cantrill *et al.*, 1993; Das, 1999; Ramesh *et al.*, 1992; Sagar and Das, 1995; Seegers *et al.*, 1997). An extension of these studies indicated that these fatty acids induce cytotoxic actions by augmenting free-radical generation and lipid peroxidation in the tumour cells (Ramesh *et al.*, 1992). A more complex study done in 1995 investigated the effects of a variety of EFAs on different cell lines and found that, in general, malignant cells are killed by the addition of EFAs *in vitro*. However, the efficacy of the different EFAs varied depending on the type of cancer cell line and the length of exposure of the EFA (Pandian *et al.*, 1999). In all these studies the effects were ascribed to the EFAs, but could just as well have been due to metabolites of the EFAs such as PGs.

A number of studies have shown that PGs affect cell proliferation (Hori *et al.*, 1990). PGs are also said to modulate tumor metastasis, anti tumor response and dissemination (Elattar and Lin, 1989; Pandian *et al.*, 1999). Various transformed cell lines treated with PGs showed inhibition of cell growth or cytotoxicity. Colquhoum *et al.*, 1998, found that both PGE_1 and PGE_2 caused inhibition of human larynx tumor cells and rat Walker tumor cells within 12 hrs of exposure. PGE_2 was slightly but not significantly less inhibitory that PGE_1. PGE_2 also show anti-mitotic effects in oesophageal carcinoma cells (Joubert *et al.*, 1999; Seegers *et al.*, 2000). Santora *et al.*, 1986, analyzed the effect of several PGs on the growth of human erythroleukemic cell lines. They found that at a dose of 4μg/ml after 48hrs PGA_1 and PGD_2 totally blocked cell division, while PGA_2 and PGE_2 (both 4μg/ml) partially inhibited it. Previously they have demonstrated that PGs of the E and A series are potent inhibitors of the

melanoma and the Friend virus-induced erythroleukemia both *in vitro* and *in vivo* (Santora *et al.*, 1986). Narumiya and Fukushima, 1986, described a carrier-mediated active transport system specific for cyclopentenone PGs such as PGA_2 and Δ^{12} -PGJ_2 in L-1210 murine leukemia cells and human erythrocytes and demonstrated that this transport was correlated with induction of growth inhibition by these PGs. Kim *et al.*, 1993, treated murine leukemia cells with PGA_2 and Δ^{12} -PGJ_2 and found a significant G_2/M arrest and DNA fragmentation at concentrations of 10ug/ml and 2ug/ml respectively. Examination of the morphology of the cells by electron microscopy, incubated with the above mentioned dose of PGA_2 or Δ^{12} -PGJ_2 for 24 hrs showed characteristic morphological features of apoptosis such as chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (Kim *et al.*, 1993). Joubert *et al.*, 1999, evaluated the effect of PGA_2 in two squamous oesophageal carcinoma cell lines and compared it to normal monkey kidney cells. They found that PGA_2 inhibited cell growth significantly in the cancer cell lines with no significant effects in the normal cell line. PGA_2 has been shown to be a potent inhibitor of tumor growth *in vivo* and a potent anti-mitotic agent *in vitro* (Seegers *et al.*, 1997). PGA_2 and Δ^{12}-PGJ_2 have potent anti-proliferative activity on various tumor cell growth *in vitro*. Ahn *et al.*, 1998, investigated the mechanism of PGA_2 and Δ^{12}-PGJ_2 mediated apoptosis in human hepatocarcinoma Hep3B cells and found a time-dependent DNA fragmentation characterized by marked apoptosis.

PGs that have anti-proliferative effects on tumor cells are PGD_2, PGJ_2, Δ^{12}-PGJ_2, PGA_1, Δ^7-PGA_1 and PGA_2 (Sasaki and Fukushima, 1994). The anti-proliferative activity seems to correlate with the PGs with the cyclopentane ring containing an α, β -unsaturated ketone (Bregman *et al.*, 1986; Fukushima and Kato, 1984; Honn and Marnett, 1985; Narumiya and Fukushima, 1986; Santoro *et al.*, 1986; Sasaki and Fukushima, 1994). PGs are known to be involved in the regulation and proliferation in a large number of cell lines as mentioned and their action varies based on their molecular structures, the dose and the animal model in which their role is analyzed (Santoro *et al.*, 1986).

3. OESOPHAGEAL CANCER

Oesophageal cancer (OC) is the third most common cause of cancer of the gastrointestinal tract (Tanaka *et al.*, 1999). It is endemic to South Africa (Sammon, 1999), China, Iran, the Caspian littoral region and is particularly common to these areas (Sammon and Alderson, 1998). In South Africa, particularly Transkei, OC is the most common cancer in black males and this area is one of the highest incidence areas of OC in the world. It is known as one of the major causes of death in certain areas of Asia (Ohashi *et al.*, 1999).

Many factors have been shown to be associated with OC. Common factors include a diet either based on maize (Sammon, 1999) or wheat (Sammon and Alderson, 1998), deficiency of minerals and vitamins and micronutrients such as zinc, magnesium, riboflavin and nicotinic acid. It has also been ascribed to a diet that does not include enough fresh fruit and vegetables. An increase of tobacco use also has a clear association with OC in Africa. Other ingested carcinogens that have been postulated include fungal contamination and/or the preservatives used in storing food (nitrosamine), alcoholic concoctions in South Africa and opium in Iran (Sammon and Alderson, 1998). Wherever maize meal is the staple, a strong association has been found with OC (Sammon, 1999). The high incidence of oesophageal cancer in South Africa is associated with Fumonisin B_1 (FB_1), which is a common contaminant of grain. FB_1 is a mutagen and may be a tumor initiator and promoter. OC is prevalent in the Transkei area in South Africa with a diet high in maize content, which is often contaminated with FB_1 (Seegers *et al.*, 2000).

As this study investigates the effects of an endogenous metabolite (PGA_2) on cell growth control it is necessary to firstly look at the cell cycle itself.

4. THE CELL CYCLE

The cell cycle involves the general sequence of events that occur during the lifetime of a eukaryotic cell (Voet and Voet, 1995). It is a set of events that is responsible for DNA duplication once during S phase and the equal distribution of identical chromosomal copies to two daughter cells (Sherr, 1996). The events (cell growth, DNA synthesis, chromosome condensation, nuclear breakdown, segregation of chromosomes and cytokinesis) must be

executed in an orderly fashion (Motokura and Arnold, 1993). The following are the 4 major events in the lives of actively growing cells (Amon *et al.*, 1993; Planas-Silva and Weinberg, 1997; Voet and Voet, 1995):

- ❖ M phase or mitosis, the period of cell division
- ❖ G_1 phase (for gap 1), a period of growth before DNA replication
- ❖ S phase (for synthesis) which is the only period in the cell cycle when DNA is replicated
- ❖ G_2 phase, (for gap 2), the period of growth where the now tetraploid cells prepare for mitosis, enters M phase once again and thereby commences a new round of the cell cycle. The restriction (R) point occurs in mid-late G_1 phase, subdividing G_1 into 2 phases- early/mid G_1 phase and late G_1 phase (Pardee, 1989; Planas-Silva and Weinberg, 1997). Following mitosis, the daughter cells may re enter the G_1 phase or proceed to a G_0 phase where growth and replication stops. Cells that are in G_0 are said to be quiescent. G_0 cells may eventually re enter G_1 or perhaps die.

Cell cycle progression occurs via control mechanisms. Some of these integrate cell proliferation with cell growth and differentiation (Hinds and Weinberg, 1994) such as extracellular factors, which determine whether a quiescent cell will begin to proliferate, and whether a normal proliferating cell in G_1 will continue to enter the cycle or maybe revert to quiescence (Pardee, 1989).

4.1 Cell cycle progression

Progression through each of the distinct phases (G_1, S, G_2 and M) is carefully controlled by the sequential formation, activation and subsequent degradation or modification of a series of cyclins and their partners, the cyclin–dependent kinases (cdks) (Pines, 1994; Reed, 1992; Sherr, 1993) (Fig. 1.4). In addition, a further group of proteins, the cyclin dependent kinase inhibitors (CDKIs) are important for coordination of each stage. This transition from one stage to the next is regulated by a number of checkpoints, which prevent premature entry into the next phase of the cell cycle (Hartwell and Weinert, 1989; Murray, 1994). The degradation of various cyclins occurs at each checkpoint and it is the mechanism together with interaction of the CDKIs, which allows the cell to enter the next phase.

4.2 Cell cycle control

The DNA of a cell must be intact and the cells must meet the criteria of each cell cycle in order to progress from $G_0 \rightarrow G_1 \rightarrow S \rightarrow G_2 \rightarrow M$. These events occur only after major commitments by the cell that occur at a discrete point in time known as a checkpoint. Checkpoints are essentially proofreading functions that integrate extracellular and intracellular signals to ensure that the late events are not initiated before early events are completed, thus protecting the integrity of the genetic material (Murray, 1994). In humans, the loss of the genetic integrity can cause cancer and genetic disease (Russell, 1998). Aneuploid, dead or mutant cells may be produced if there is a failure to repair DNA damage and entering mitosis with faulty DNA (Morgan, 1992; Woollard and Nurse, 1995). Cancer can arise if aneuploidy and mutation produce uncontrolled cell proliferation (Murray, 1994).

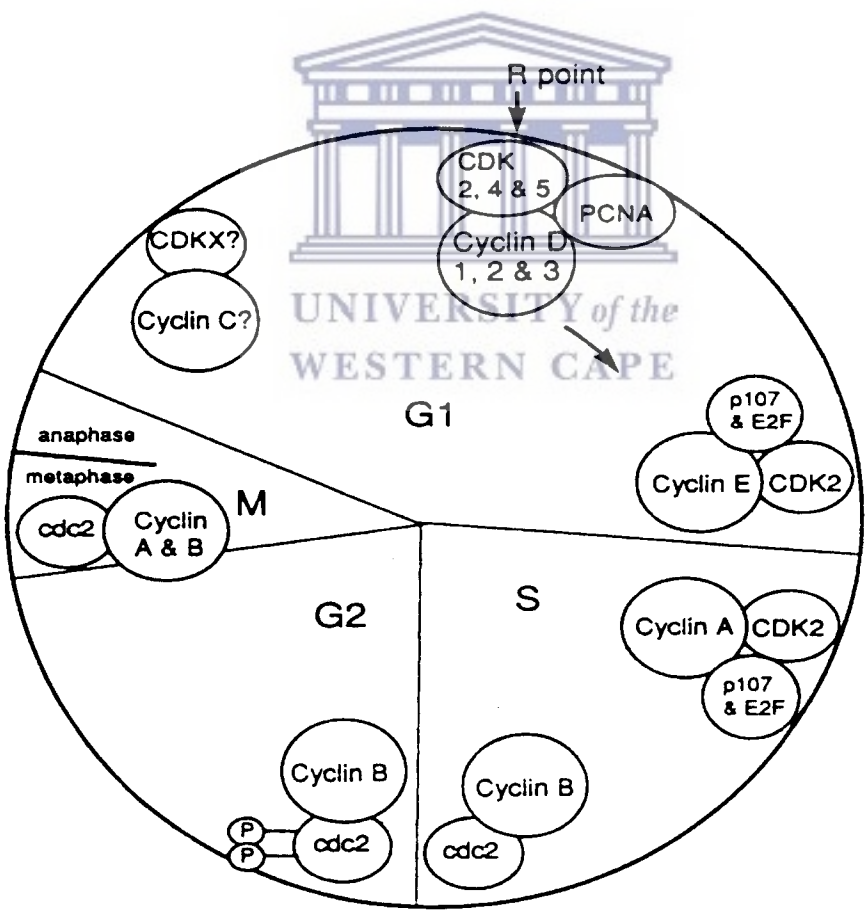


Fig. 1.4 Cyclins and cyclin dependent kinase subunits in vertebrates at different stages of the cell cycle (Pines, 1993).

There are three mechanisms (Murray, 1994; O'Connell and Nurse, 1994) cells use to ensure accurate transmission of their genetic information:

- ❖ spontaneous or environmentally induced errors in DNA replication and chromosome alignment are corrected by a repair mechanism
- ❖ detection of errors and arrest of the cell cycle until repairs are completed by a delay mechanism and
- ❖ inducing the death of damaged cells as a way of preventing them from giving rise to mutant progeny

The most evident checkpoints in the cell cycle occur in G_1, G_2 and M phases. A point in G_1 termed START, at which cells become committed to DNA synthesis and governing entry into S phase (Hunter and Pines, 1991). S phase DNA damage checkpoint prevents initiation at DNA replicas following DNA damage, without affecting DNA synthesis, which has already started. G_2 governs entry into M phase before DNA has been checked for replication faults and exits M phase, which is the mitotic spindle assembly checkpoint that prevent chromosome segregation before spindle formation and chromosomal attachment have been accomplished (Weinert, 1997). Many checkpoint signals are DNA based, monitoring the completion of chromosome replication, the presence of DNA damage, or the alignment of chromosomes on the metaphase spindle (Elledge, 1996).

Normal cell division is found in some embryonic cells without a tight checkpoint control (Hoyt *et al.*, 1991), but cancer cells are typical of uncontrolled cell proliferation, usually due to damage of the genes that regulate their cell cycle (Sherr, 1996). The activation of cell cycle checkpoints and inhibition of cell cycle progression might be used to exploit the differences between normal and malignant cells and thus improve the selectivity of chemotherapy (Cahill *et al.*, 1998).

4.2.1 The G_1 checkpoint

The D-type cyclins are linked to the regulation of the first checkpoint at G_1 in mammalian cells (Hunter and Pines, 1991). During a normal cell cycle, D-type cyclins are synthesized in response to external cues such as growth factors, which lead to the activation of DNA

prereplication complexes. Activation of the cdks by complexing with cyclin D and phosphorylation on threonine 161 (Thr 161) drive cells through this checkpoint by phosphorylation and inactivation of the retinoblastoma protein (pRb) which releases the E2F transcription factor allowing it to activate genes necessary for DNA synthesis (Jacks and Weinberg, 1998). G_1 cdk activity enables mitosis by causing inactivation of the anaphase promoting complex (APC) and other proteolytic enzymes (cyclosomes). APC triggers the events leading to destruction of the cohesions and thus allowing sister chromatids to separate. It also allows mitotic cyclins (cyclin B/cdc, also known as cdc2 kinase) to accumulate (King *et al.*, 1996).

Mammalian cells that undergo DNA damage in G_1 are prevented from entering the S phase and cannot begin DNA replication (Murray, 1994). DNA-dependent protein kinases (DNA-PK) activate p53 by phosphorylation at serine 15 and 37 (Woo *et al.*, 1998) and then bind to specific DNA sequences. The G_1/S arrest results from p53 regulated synthesis of p21 (a CDKI) (Elledge and Harper, 1994). Activation of p53 by DNA damage results in increased levels of p21. It can then bind to a number of cdk complexes thereby preventing phosphorylation of pRb causing the cell cycle to arrest in G_1 (Bunz *et al.*, 1998; Shim *et al.*, 1996). However, if the damage is beyond repair, p53 triggers apoptosis (Donehower, 1994; Kastan *et al.*, 1991).

4.2.2 S phase of the cell cycle

Once cells enter the S phase, their entire DNA content must be replicated completely and precisely and this happens in a period of a few hours. In order to replicate their DNA, S phase cytoplasm induces nuclei from non-proliferating cells. Progression into S phase requires the activity of cyclin-cdk complexes and exit from the cycle requires down regulation of these complexes (O'Connell and Nurse, 1994). Firstly, cyclin E-cdk2 is activated and later the cyclin A-cdk2 complexes. Cyclin A is expressed in the S phase through to G_2 and M. The cyclin A-cdk2 complex dissociates when the S phase is completed. If cells fail to complete the S phase, a signal is sent to block mitosis. A direct link between the checkpoint controls monitoring the completion of S phase and its coupling to mitosis is provided by $p34^{cdc2}$ protein kinase (Woollard and Nurse, 1995).

4.2.3 The G_2 checkpoint

The onset of M phase is controlled by the activation of CDK1 also known as serine-threonine kinase $p34^{cdc2}$ and cyclin B (Dunphy, 1994; Murray, 1994; Solomon, 1994). It forms a complex with cyclin B (cyclin B/cdc2) before DNA synthesis is stopped and mitosis can occur (Gottesfeld and Forbes, 1997; King *et al.*, 1996; Shi *et al.*, 1994) (Fig. 1.5). During interphase, newly synthesized cyclin B binds to $p34^{cdc2}$ inducing phosphorylation of the three residues (threonine161, threonine 14 and tyrosine 15). CAK (cdc2 activating kinase) or cyclin H/cdk7 activates $p34^{cdc2}$ by phosphorylating the Thr 161 site. However, cyclin B/cdc2 is kept inactive by phosphorylation of tyrosine 15 (Tyr 15) and threonine 14 (Thr 14) residues (Voet and Voet, 1995), a process regulated by the weeI/mikI kinases (Boddy and Russell, 2001; Coleman and Dunphy, 1994). At the end of G_2, cdc25 phosphatase dephosphorylates the inhibitory phosphates Thr 14 and Tyr 15 thereby activating cdc2. This allows the catalysis of ATP within an ATP binding pocket (Nurse, 1990; Peng *et al.*, 1997; Shen *et al.*, 1998). Exit from mitosis occurs with degradation of cyclin B and dephosphorylation of Thr 161 on $p34^{cdc2}$ (Coleman and Dunphy, 1994; Voet and Voet, 1995).

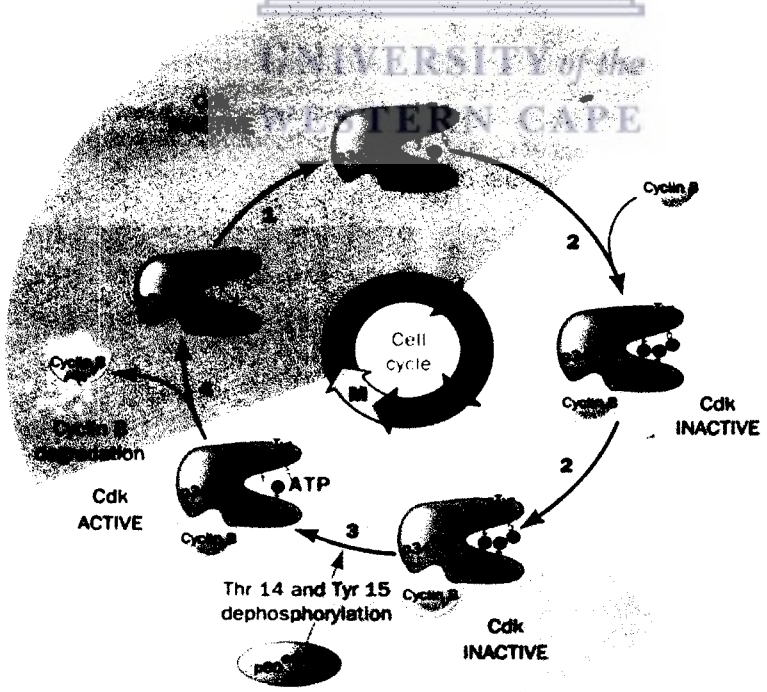


Fig. 1.5 The regulation of cyclin B-dependent protein kinase activity of $p34^{cdc2}$ (Voet and Voet, 1995).

The G_2/M checkpoint is also activated in response to DNA damage and leads to the production of the DNA replication checkpoint gene products Rad 3 (in fission yeast) and probably ATM (ataxia telangiectasia mutated) and ATR (ataxia-and-Rad related) kinases (Fig. 1.6). ATM and ATR are two large phosphatidylinositol 3-kinase (PI-3) kinases that are central to the DNA damage checkpoints (Caspari and Carr, 2002; Melo and Toczyski, 2002). They are able to phosphorylate and activate the Chk1/CHK1 protein kinases respectively (Bunz *et al.*, 1998; Matsuoka *et al.*, 1998; Russell, 1998; Weinert, 1997). Chk1 is essential for repair checkpoints and is an activator kinase (Boddy and Russell, 2001; Caspari and Car, 2002). DNA damage in mammalian cells also lead to the rapid phosphorylation and activation of the protein kinase Chk2 (a homologue of the budding yeast Rad53 and the fission yeast Cds1) (Boddy and Russell, 2001) in an ATM dependent manner (Melo and Toczyski, 2002). When Chk1 is activated, it phosphorylates human Cdc25C on serine 216 creating a recognition motif for the 14-3-3 proteins (Matsuoka *et al.*, 1998; Russell, 1998). In the cytoplasm, 14-3-3 proteins sequester Cdc25 and cannot activate cdc2 (in the nucleus) by phosphorylation. Entry into mitosis is not possible if cdc2 is not activated (Boddy and Russell, 2001) and therefore a G_2 arrest occurs in response to DNA damage (Bunz *et al.*, 1998; Michael and Newport, 1998; Russell, 1998).

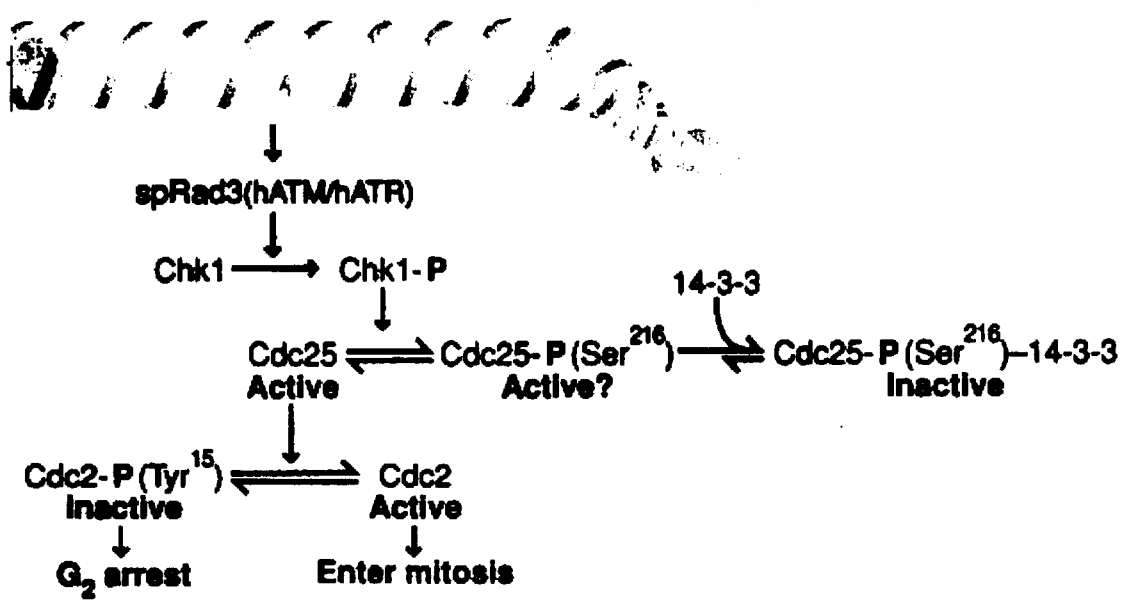


Figure 1.6 DNA damage leading to G_2 arrest (Weinert, 1997). See text for detail.

4.2.4 Mitosis

Entry into M phase is brought about by $p34^{cdc2}$ protein kinase activation (Andreassen and Margolis, 1991; Gottesfeld and Forbes, 1997; Nurse, 1990). Cellular processes necessary for chromosomal condensation, cytoskeletal reorganization, nuclear envelope breakdown and cell shape changes are induced by $p34^{cdc2}$ protein kinase (Nurse, 1990). These cellular processes include phosphorylation of histone H1 for chromatin condensation (Andreassen and Margolis, 1991; Gottesfeld and Forbes, 1997; Nurse, 1990), a 13S condensin (a five-subunit protein complex essential for mitotic chromosome condensation) complex for DNA supercoiling (Kimuri *et al.*, 1998 and Murray, 1998), phosphorylation of lamin B for nuclear envelope breakdown, phosphorylation of nucleolin and NO38 for nucleolar disappearance (Andreassen and Margolis, 1991), and microtubule-associated proteins to prevent premature cytokinesis (Holloway *et al.*, 1993). After the disappearance of the nuclear membrane, chromatin condensation is induced by cdc2 kinase and the chromosomes attach to the opposite poles of the mitotic spindle and align on the metaphase plate. Only when this has happened can separation of daughter chromosomes occur.

Some $p34^{cdc2}$ is associated with centrosomes during G_2 and mitotic onset. When $p34^{cdc2}$ kinase phosphorylates centrosomal proteins, it could play a role in the reorganization of the microtubular cytoskeleton at mitosis (Nurse, 1990). An important feature of the cytoskeleton is its dramatic change in architecture or shape during mitosis (M phase).

4.2.4.1 Spindle assembly checkpoint

There is one final checkpoint that occurs at the end of metaphase. This checkpoint, known as the spindle assembly checkpoint consists of a group of mechanisms, which prevent the onset of anaphase and progress into the next cell cycle unless a bipolar spindle is present and all the chromosomes are attached to the spindle and are aligned on the metaphase plate (Wells, 1996). Normal cells arrest at this point if there are defects, whereas in tumor cells abnormalities of spindle formation can occur, suggesting that the checkpoint control is lost.

Cdc2-cyclin B kinase is activated at the onset of M phase and promotes chromosome condensation and spindle apparatus formation. During metaphase or the mid-mitotic stage,

chromosomes are condensed and aligned on the equatorial plane of the spindle to form a metaphase plate, thereby delaying anaphase until this has occurred. Accurate chromosome segregation requires that sister chromatids attach to microtubules that come from opposite poles of the spindle (Murray, 1994; Yanagida, 1995). If the microtubules and chromosomes connect, there will be no unattached kinetochores and all chromosomes will be under mechanical tension between the two poles of the spindle. Kinetochores are organelles that are formed on each chromosome during chromatin condensation. They interact with microtubules of the mitotic spindle and this is the major force for chromosome movement. Together with chromosome motility, the kinetochore plays a vital role in signaling the spindle checkpoint (Gorbsky, 2001). They assemble on the centromere carrying the responsibility for chromosome attachment to the spindle (Wells, 1996). The mitotic spindle checkpoint is activated and blocks anaphase (Gorbsky, 2001) when either unattached kinetochores are present, or if the tension on chromosomes is unbalanced (Glotzer, 1996). Cdc2 kinase inactivation and cyclin degradation occur simultaneously with metaphase/anaphase progression (Yanagida, 1995).

Rieder *et al.*, 1994, found that the presence of a single unattached kinetochore to microtubules in cultured cells can delay the onset of anaphase. They have proposed three features of abnormal spindles to generate the signal leading to mitotic arrest. They include unoccupied microtubule-binding sites at the kinetochore, the absence of tension at the kinetochore, and abnormal dynamic behavior of the microtubules attached to the kinetochore (Gorbsky, 2001; Rieder *et al.*, 1994).

4.2.4.1.1 Unattached kinetochores

Mad (mitotic arrest deficient) and *bub* (budding uninhibited by benzimidazoles) genes in yeast are potentially involved in the spindle checkpoint (Wells, 1996). MAD2 and BUB1 are human homologues and mutations of MAD and BUB genes lead to a failure in arresting the spindle checkpoint when the spindle is disrupted by microtubule-depolymerizing drugs (Wells, 1996). The regulation of the mitotic ubiquitin ligase termed the anaphase-promoting complex or cyclosome (APC/C) is a critical feature in the timing of anaphase onset (Gorbsky, 2001). A collar is formed around the kinetochore by the centromere protein E (CENP-E) -a structural

component of the kinetochore). This serves to promote chromosomal capture by the microtubules. CENP-E changes the activity of BUB1 once microtubules form attachments to the kinetochores on the chromosome. This then leads to the disabling of MAD2 making it incapable of binding to p55CDC (equivalent to cdc20 and Hct1/Cdh1 in budding yeast) (Gorbsky, 2001; Hwang *et al.*, 1998). The APC is thus activated and anaphase can therefore continue (Pennisi, 1998). See Fig 1.7 left side.

MAD2 linked to p55CDC can leave the uncaptured kinetochore even if only one kinetochore remains unattached to the spindle microtubule (Glotzer, 1996; Pennisi, 1998). The p55CDC protein coimmunoprecipitates with MAD2, Cdc27 and Cdc16 proteins, which form part of the APC. Anaphase onset is blocked by inactivation of APC by MAD2. See Fig 1.7 right side. Degradation of APC occurs via ubiquitination of the proteins marking them for rapid proteolysis. During anaphase, proteolysis commences and terminates only upon entering the subsequent S phase.

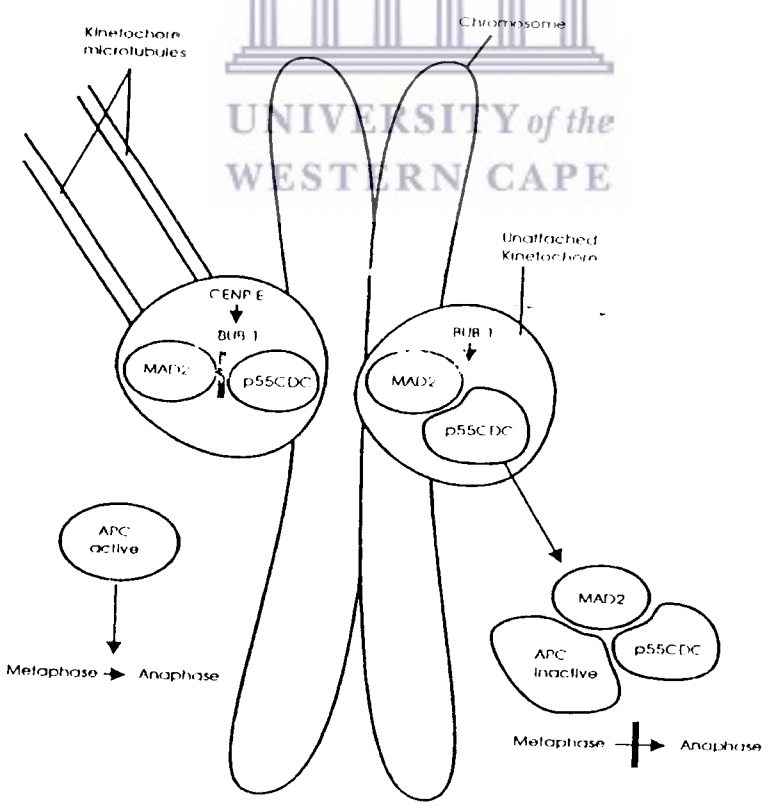


Fig. 1.7 How unattached kinetochores activate the spindle checkpoint in mammalian cells (Pennisi, 1998).

4.2.4.1.2 Tension differences

The spindle checkpoint is activated when both kinetochores of a single chromosome are captured by microtubules originating from the same spindle pole, even though there are no unattached kinetochores present. This could be a result of tension differences. A tension difference is created when both kinetochores of a chromosome are attached to the opposite poles compared to chromosomes attached to the same pole or one pole (Nicklas, 1997).

There is a biochemical difference between kinetochores that are under tension and those that are not (Nicklas *et al.*, 1995). Evidence for a tension-sensitive checkpoint was obtained from a phosphoprotein target recognized by the 3F3 antibody (Wells, 1996). The protein is expressed in the middle radiolucent zones of kinetochores of misaligned chromosomes, but as the movements of chromosomes occur towards the metaphase plate the protein is lost (Gorbsky and Ricketts, 1993; Gorbsky, 1997). Therefore, much more of this phosphoprotein is present on unattached kinetochores. Thus tension alters the conformation of a tension-sensitive protein leading to the dephosphorylation of the protein detected by 3F3/2 (Nicklas *et al.*, 1995). Mitosis is delayed upon injection of 3F3 antibodies, preventing anaphase onset because 3F3 antibodies prevent dephosphorylation of the protein thus locking the checkpoint on (Wells, 1996).

4.2.5 The end point: exit from mitosis

Mitosis comes to an end when $p34^{cdc2}$ is inactivated which is brought about by APC-mediated proteolytic degradation of its associated cyclin B (Wells, 1996). Cdc2 is subsequently inactivated by Thr 161 dephosphorylation. The two daughter cells are then in interphase.

Summary: If unattached kinetochores or tension differences are present, the spindle checkpoint arrests cells in metaphase (Bunz *et al.*, 1998). Excess numbers of spindle poles are not monitored by the spindle assembly checkpoint, which invariably leads to aneuploidy. Anueploidy is associated with Down's syndrome and the formation of higher grade, invasive tumors, therefore the spindle checkpoint must make sure that tetraploidy, which is often a result of anueploidy in the next mitosis does not occur (Andreassen *et al.*, 1996; Pennisi, 1998).

4.2.6 Checkpoints and tumorigenesis

Many studies support the view that the loss of the G_1/S checkpoint can lead to genomic instability, inappropriate survival of genetically damaged cells and the evolution of cells in malignancy. p53 is often mutated in many human cancers and this suggests that abnormalities in the G_1/S checkpoint are important in tumorigenesis (Hartwell and Kastan, 1994). The function of several cellular proteins is altered by the gene product of certain DNA cancer viruses [human papilloma virus (HPV) and adenovirus] and can affect cell cycle checkpoint function. HPV infection has already been linked to the development of certain human cancers, in particular cervical carcinoma (Hartwell and Kastan, 1994).

Many environmental carcinogens are DNA damaging agents. The G_1/S and G_2/M checkpoints may be important in protecting cells from exogenous sources of DNA damage but the damage can also be caused by intrinsic cellular processes, including gene rearrangement during development, cell senescence, and apoptosis (Hartwell and Kastan, 1994).

5. APOPTOSIS

In 1972, Kerr, Wyllie and Curie distinguished between two forms of cell death, necrosis and apoptosis (Wyllie *et al.*, 1990). Apoptosis, or programmed cell death (PCD) as it is also known (Altieri, 2001), is a central part of normal development as well as toxicities and disease (Corcoran *et al.*, 1994) and is morphologically distinct from necrosis (Ohta and Ishibashi, 1999). It is a genetically mediated form of cell death, which is involved in organogenesis, tissue homeostasis, and the editing of the immune system to remove autoreactive clones (Wyllie *et al.*, 1990) with unique biochemical and morphological characteristics (Altieri, 2001). Apoptosis is considered to be an active cell function, namely suicide, when a programmed sequence of events leads to cell death (Szende *et al.*, 1991). The importance of apoptosis is to mold the developing organism by eliminating outdated and unneeded structures, but is also central to the homeostasis of adult tissues by maintaining the balance between cell production and cell elimination (Altieri, 2001).

5.1 Morphological changes during apoptosis

Apoptosis is characterized by a series of stereotypical morphological changes (Cohen, 1997). In apoptosis the cells condense and shrink and cell to cell contacts are broken down. The chromatin becomes pyknotic and packed into small masses applied against the nuclear membrane (margination of chromatin or chromatin condensation). The fragmented chromatin takes on characteristic patterns as it marginates around the outside of the nucleus creating curved profiles such as crescents, halos, sickle, lancet, ship like and horse shoes (Majno and Joris, 1995; Meyn *et al.*, 1994). The nucleus may also break up (karyorhexis) and the protuberances of the cell surface separate into multiple membrane-bound bodies (apoptotic bodies), which contain nuclear remnants and intact organelles. These apoptotic bodies may be phagocytized by macrophages or neighboring cells (Bowen and Bowen, 1990; Cohen, 1997; Corcoran *et al.*, 1994; Earnshaw *et al.*, 1999; Fraser and Evan, 1996; Hale *et al.*, 1996; Harvey and Kumar, 1998; Majno and Joris, 1995; Nuñez *et al.*, 1998; Obeid and Hannum, 1995; Ohta and Ishibashi, 1999; Thornberry and Lazebnik, 1998; Wylie, 1997) (Fig1.8). Apoptosis is also characterized by mitochondrial damage and DNA fragmentation (Budihardjo *et al.*, 1999; Nuñez *et al.*, 1998) where the latter can be demonstrated by agarose gel electrophoresis, wherein a characteristic "ladder" develops (Cohen, 1997) due to specific cleavage between nucleosomes (Hale *et al.*, 1996; Majno and Joris, 1995).

Necrosis is signaled by irreversible changes in the nucleus and in the cytoplasm (Majno and Joris, 1995). It commences with an impairment of the cell's ability to maintain homeostasis, which leads to an influx of water and extracellular ions. The main features are organelle swelling, most notably the mitochondria, followed by swelling and rupturing (lysis) of the entire cell. Clumping of the nuclear chromatin in apparent but the density of the masses is less uniform than in apoptosis and their edges are less sharply defined. Ultimately the plasma membrane breaks down and the cytoplasmic contents are released into the extracellular fluid, which accelerates disintegration. Necrotic cell death occurring in vivo is often associated with extensive tissue damage resulting in an intense inflammatory response (Kerr and Harmon, 1991; Majno and Joris, 1995 and Wyllie, 1997).

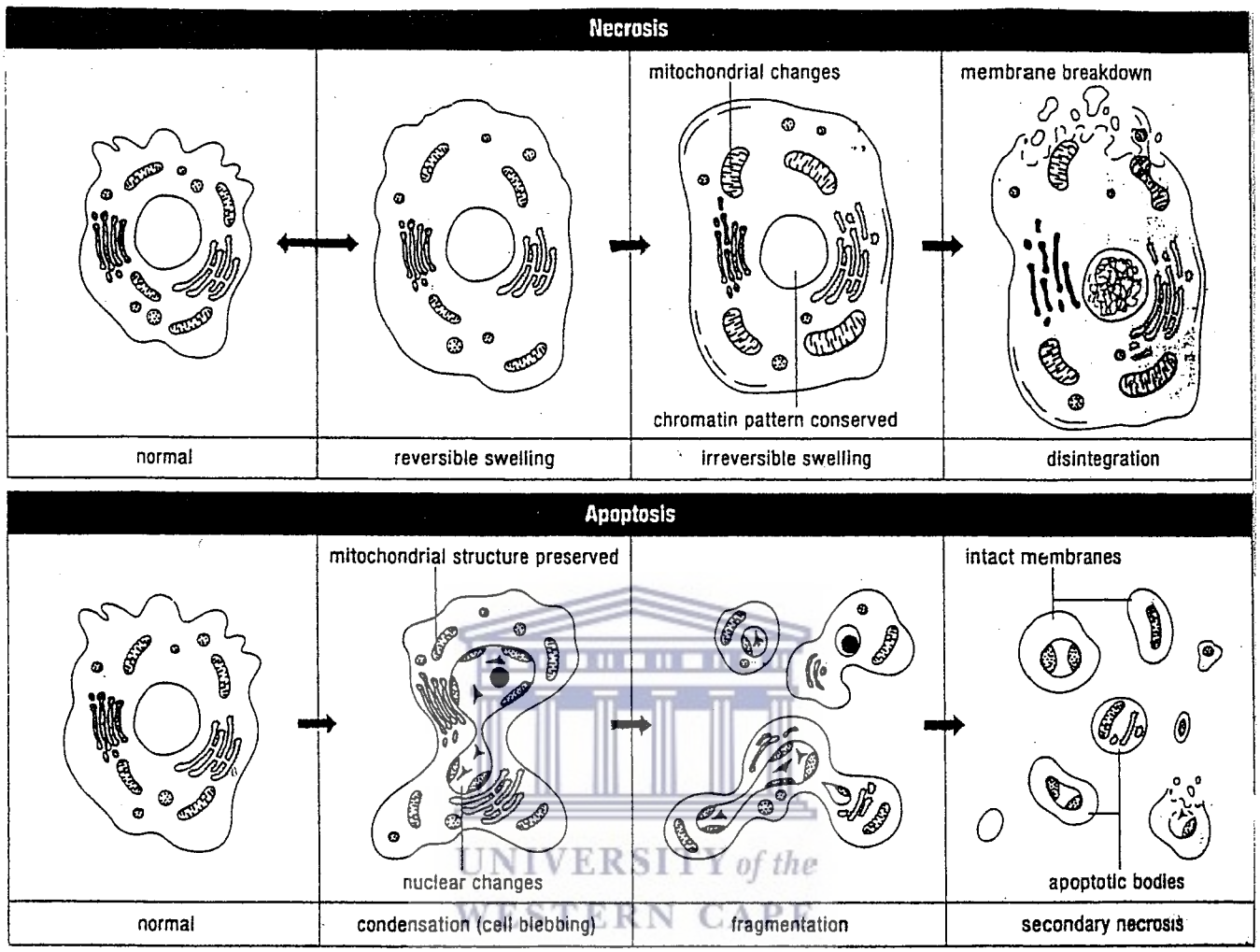


Fig. 1.8 The morphological features of necrosis and apoptosis (Boehringer Mannheim 's guide to cell proliferation and apoptosis methods, 1995).

5.2 Caspases and apoptosis

Apoptosis induction relies on the mitochondria, which release apoptogenic factors that activate caspases, a family of proteases that kill the cells via proteolysis of key substrates (Wolf and Green, 2002). The morphological appearances of apoptosis rely on the activation of these caspase-family cysteine proteases (Green and Kroemer, 1998; Harvey and Kumar, 1998; Nuñez *et al.*, 1998; Salvesen, 1999). They are the most important effector molecules that induce apoptosis and are synthesized as inactive precursors (proenzymes) that are activated either by autocatalytic cleavage or by other proteases (Nuñez *et al.*, 1998; Thornberry and

Lazebnik, 1998). The 'c' in caspase denotes the cysteine proteases and the 'aspase' referring to their ability to cleave after an aspartic acid residue thereby becoming activated (Cohen, 1997; Fraser *et al.*, 1996; Fraser and Evan, 1996). They all contain three domains: an NH2-terminal domain, a large subunit and a small subunit (Earnshaw *et al.*, 1999; Thornberry and Lazebnik, 1998;). Proteolytic processing between domains activates the caspases and this is followed by association of the large and small subunits to form a heterodimer. The association of two heterodimers then forms a tetradimer (Thornberry and Lazebnik, 1998). There are two ways by which caspases can be activated: firstly, a caspase cascade is initiated by upstream activated caspases cleaving downstream effector caspases (Nuñez *et al.*, 1998; Thornberry and Lazebnik, 1998). Caspases that are activated as a consequence of cell membrane signalling events are initiating or upstream caspases (e.g. caspase 2, 8, and 10) (Green and Kroemer, 1998). Downstream effector caspases include caspase 3, 6 and 7 (Earnshaw *et al.*, 1999; Green and Kroemer, 1998) are activated later than upstream caspases. Secondly, caspase 8 (FLICE, FADD like ICE) may be activated downstream of the Fas/Cd95 death receptor (Ashkenazi and Dixit, 1998).

The main function of caspases is in an 'executive' role to turn off protective pathways and turn on downstream activities, which in turn lead to cell death (Earnshaw *et al.*, 1999). Once caspases are activated during apoptosis they cleave a variety of intracellular polypeptides, which are key structural components of the cytoskeleton (actin, gelsolin, growth arrest specific gene Gas2, α-fodrin) (Harvey and Kumar, 1998) and nucleus (lamin A and B), numerous proteins involved in signaling pathways (proinflammatory cytokines pro-interleukin-1β), as well as components of the DNA repair machinery [poly (ADP-ribose) polymerase (PARP) and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs)] (Cohen, 1997; Earnshaw *et al.*, 1999).

Most of the apoptotic events such as DNA fragmentation, chromatin condensation, membrane blebbing and apoptotic bodies, are brought about by caspases (Thornberry and Lazebnik, 1998). The overall picture of how they do this is not fully understood. Thornberry and Lazebnik, 1998 suggest that a subset of effector caspases is responsible for the cellular changes that occur during apoptosis. They are directly accountable for the proteolytic

cleavages that lead to cell disassembly and they inactivate proteins that protect living cells from apoptosis (Thornberry and Lazebnik, 1998). The direct disassembly of cell structures is illustrated by the destruction of nuclear lamina. During apoptosis, lamins are cleaved at a single site by caspases, which causes the lamins to collapse and thereby contributing to chromatin condensation (Thornberry and Lazebnik, 1998).

As caspase 3 activation was investigated as a possible pathway in initiating apoptosis in WHCO3, it will briefly be discussed.

5.2.1 Caspase 3

Caspase 3 (CPP32, Yama, apopain) is one of the key executioners that is activated during apoptosis (Cohen, 1997). Active caspase 3 is a marker for cells undergoing apoptosis and consists of a heterodimer of 17 and 12 kDa subunits, which is derived from the 32 kDa proenzyme (Hale *et al.*, 1996) by cleavage at Asp-28-Ser-29 and Asp-175-Ser-176 (Cohen, 1997). It is either partially or totally responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme PARP as well as other caspases (Cohen, 1997). Caspase 3 activating pathways have been identified to be either dependent or independent of mitochondrial cytochrome c release and caspase 9 function (Porter and Jänicke, 1999). Caspase 3 is widely distributed with high expression in cell lines with lymphocytic origin. This suggests that it may be an important mediator of apoptosis in the immune system (Cohen, 1997). Knockout studies indicate that caspase 3 is necessary for the developmental death of neurons and essential for normal brain development (Porter and Jänicke, 1999). Caspase 3 is also required for some of the typical hallmarks of apoptosis and is also fundamental for apoptotic chromatin condensation and DNA fragmentation in many cell types. It is therefore essential for certain processes associated with the break down of the cell and the formation of apoptotic bodies. It may also function before or at the stage when commitment to loss of cell viability is made (Porter and Jänicke, 1999). It is thought that caspase 3 represent downstream effectors of the death pathway because they can act on nuclear substrates that are cleaved during apoptosis (eg. lamins, DNA repair and splicing RNA proteins) (Salvesen, 1999).

The most recently identified DNA fragmentation factor (DFF) induces DNA fragmentation following activation of caspase 3. Caspase 3 cleaves the DFF thereby generating the active factor capable of DNA fragmentation (Nuñez *et al.*, 1998; Harvey and Kumar, 1998). Following this caspase 3 mediated cleavage; no further requirement of caspase 3 or any other cytoplasmic factors are needed. Hereby, the activation of caspase 3 with the activation of DFF are directly linked which in turn is responsible for the mediating DNA fragmentation by an as yet unknown mechanism (Harvey and Kumar, 1998).

There are two caspase-activating cascades that regulate apoptosis and they are: (a) the initiation of the cell surface death receptor and, (b) the triggering of changes in mitochondrial integrity (Altieri, 2001; Nuñez *et al.*, 1998; Wolf and Green, 2002).

(a) Initiation of cell surface death receptor

One pathway that triggers the activation of caspase is initiated by the ligation of cell surface death receptors (Budihardjo *et al.*, 1999; Earnshaw *et al.*, 1999). Death activators such as tumor necrosis factor (TNF) bind to tumor necrosis factor receptor (TNFR1), Fas ligand (FasL) bind CD95/Fas/Apo-1, death receptor 3 (DR3) bind Apo3 ligand and DR4 and DR5, both bind Apo2TRAIL at the cell surface (Earnshaw *et al.*, 1999). They are integral membrane proteins with their receptor domains exposed at the surface of the cell. Binding of the complementary death activators transmits a signal to the cytoplasm that leads to the activation of caspase 8. Caspase 8 activates a cascade of caspases leading to cell death (Fig. 1.9). CD95/Fas/Apo-1 trimerizes on binding the FasL and initiates a chain of reactions, starting with its own cytosolic domain, which interacts with the adapter molecule FADD (Earnshaw *et al.*, 1999). The FADD triggers the caspase cascade (Wyllie, 1997). TNF binds to TNFR1 and may trigger binding proteins TNF receptor associated factor (TRAF1) and the adapter molecule TNF-R1 associated death domain protein (TRADD) (Earnshaw *et al.*, 1999; Wyllie, 1997).

(b) Changes in mitochondrial integrity

In a healthy cell, the outer membranes of its mitochondria express the protein bcl-2 on their surface. Perturbation of the outer mitochondrial membrane function leads to the leakage of

pro-apoptotic molecules [cytochrome c and apoptosis inducing factor (AIF)] from the intermembrane space into the cytosol. Here they participate in the activation of caspases and endonucleases (Green and Kroemer, 1998). Internal damage of the cell causes bcl-2 to release Apaf-1 (apoptotic protease activating factor-1) and to no longer keep cytochrome c from leaking out of the mitochondria (Budihardjo *et al.*, 1999). Cytochrome c interacts with Apaf-1 (Wolf and Green, 2002), which is released from bcl-2 (Green and Kroemer, 1998). The released cytochrome c and Apaf-1 bind to molecules of caspase 9. The resulting complex of cytochrome c, Apaf-1 and caspase 9 (and dATP, ATP) activates caspase 3 (Budihardjo *et al.*, 1999) and is called the apoptosome (Green and Kroemer, 1998; Green and Reed, 1998). The formation of this multimeric Apaf-1/cytochrome c complex may serve to increase the local concentration of caspases for intermolecular cleavage and to set the threshold of caspase activation relatively high so that occasional leakage of cytochrome c will not cause cells to commit to apoptosis (Budihardjo *et al.*, 1999). The apoptosome aggregate in the cytosol and leads to the digestion of structural proteins in the cytoplasm, the degradation of chromosomal DNA and death of the cell (Fig. 1.9).

5.3 Apoptosis and cancer

Apoptosis is also an element in the control of abnormal growth in tumors (Bowen and Bowen, 1990) and one of its physiological functions include terminating immune responses and eliminating virally infected or cancerous cells (Wolf and Green, 2002). Deficient apoptosis leads to cancer (Thornberry, 1998). Toxicity to cells and failure to kill cancer cells are the two main problems in the treatment of cancer. This is due to the indirect way in which chemotherapeutic drugs and irradiation damage cells in the hope to trigger apoptosis (Thornberry and Lazebnik, 1998). An abnormally increased cellular lifespan in cancer cells as a result of reduced apoptosis is ideal to support the insurgence of genetic mutations, as well as to safeguard transformed cells from death induced chemotherapy or radiotherapy, and to promote their survival at distant sites. Manipulation of apoptosis has therefore come forward as a new therapeutic strategy to preserve tissue integrity or to help eliminate cancerous cells (Altieri, 2001).

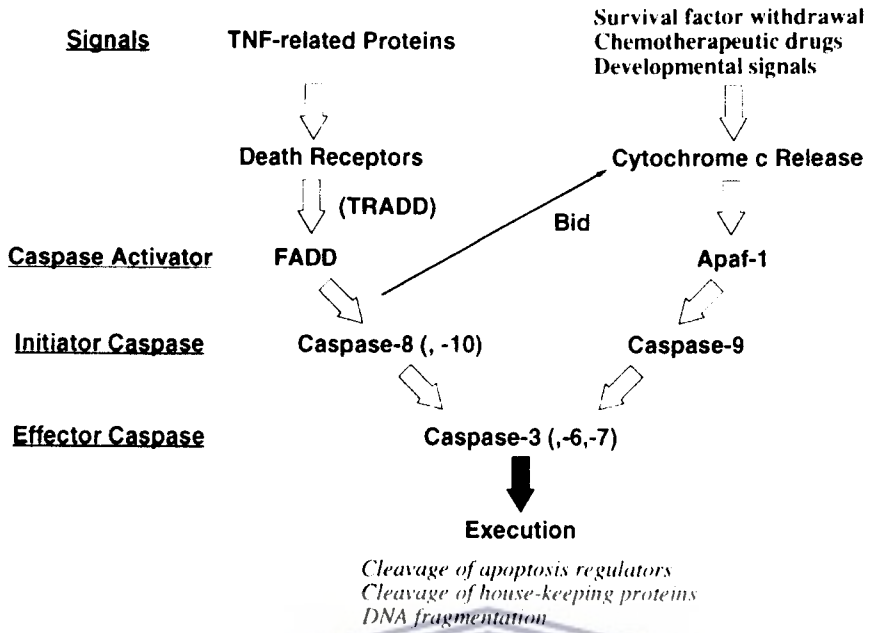


Fig. 1.9 Two main pathways of caspase activation. In the first pathway, activation of initiator caspase 8 or caspase 10 is triggered by ligation of death receptors including Fas, TNFR1 and DR3 through the adapter molecule FADD. In the second pathway, a variety of extracellular and intracellular death stimuli trigger the release of cytochrome c from the mitochondria. Cytosolic cytochrome c binds to Apaf-1 and Apaf-1 promotes activation of procaspase-9 in the presence of dATP or ATP. Active effector caspases mediate the cleavage of an overlapping set of protein substrates, resulting in morphological features of apoptosis and demise the cell. Caspase-8 cleaves Bid, a proapoptotic bcl-2 family member. Cleaved Bid binds to mitochondria and induces the release of cytochrome c. Thus, Bid links the caspase-8 and caspase-9 pathways (Nuñez *et al.*, 1998).

Apoptosis appears to be a mechanism, which removes cancer cells from the population that have sustained carcinogen-induced DNA damage. A block or inhibition of these cells by mutations in oncogenes or anti-oncogenes (bcl-2 or p53), allow the cells to be free to propagate their genetic abnormalities through further division (Meyn and McDonnell, 1994). After the formation of a tumor, cancer treatments such as radiotherapy and chemotherapy kill cells through the production of DNA damage. These same mutations in bcl-2 and p53 may have an influence in the effectiveness of these therapies because they have the ability to inhibit the apoptotic mode of cell death (Meyn and McDonnell, 1994).

EFAs and their metabolites that are capable of inducing cell death in a variety of tumor cells were found to trigger apoptosis in these cells. However, the exact mechanism by which these fatty acids are able to induce apoptosis is not clear.

6. Aims for this study

1. To detect structural changes affected by PGA_2 in WHCO3 (human oesophageal cancer cells) using morphological techniques in synchronized and unsynchronized cells and flow cytometry to quantitate chromatin condensation.

2. To study the effects of PGA_2 on the NOR (nucleolar organizer region) using silver staining.

3. To determine the effects of PGA_2 on cell cycle progression and apoptosis induction in transformed cells using flow cytometry (DNA-propidium iodide stain and caspase 3) to identify apoptotic cells as well as DNA fragmentation.

4. To study the effects of PGA_2 on the cytoskeleton (using α-tubulin) and nucleolar proteins of the cancer cells with the aid of indirect immunofluorescence and to identify apoptotic cells using Hoechst 33342.

CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell line

WHCO3 (human epithelial oesophageal carcinoma) cell line was obtained from Professor Thornley (Department of Zoology, University of the Witwatersrand, Johannesburg, South Africa) from a patient with squamous cell oesophageal cancer. These cells are poorly differentiated and non-keratinizing (Seegers, *et al.*, 2000).

2.1.2 Culture medium

Dulbecco's minimum essential medium containing nutrient mix F12 (DMEM FX12), supplemented with 5% fetal calf serum, 1% penicillin/streptomycin and gentamicin were continuously used as the culture medium. The medium, antibiotics, trypsin-EDTA and phosphate buffered saline (PBS) were all purchased from Gibco BRL (USA).

2.1.3 Sterile consumables

Sterile tissue culture flasks, petri dishes, cell scrapers, sterile 2ml, 10ml and 25ml pipettes (used to change the medium) as well as cryovials were purchased from Greiner. Sterile filters were obtained from Renner GMBH.

2.1.4 Chemicals

2.1.4.1 Amershampharmacia biotech supplied:

- ECL™ Western Blotting and Analysis system

2.1.4.2 Biorad supplied:

- Tris buffer
- 40% Acrylamide/Bis
- Temed
- Tris/glycine/SDS buffer for SDS PAGE

2.1.4.3 Fluka supplied

- ❖ Propidium Iodide (PI)

2.1.4.4 Pharmigen supplied:

- ❖ Polyclonal caspase 3 antibody (Western Blotting)
- ❖ FITC-(fluorescein isothiocyanate), Conjugated rabbit anti-active caspase 3 monoclonal antibody (Flow cytometry)

2.1.4.5 Roche, Germany supplied:

- ❖ Apoptotic DNA ladder kit

2.1.4.6 Sigma Chemical Company supplied:

- ❖ Monoclonal anti-α-tubulin, Clone DM 1A
- ❖ Monoclonal anti-FITC Biotin Conjugate Clone FL-D6
- ❖ Goat anti-mouse IgG (whole molecule) FITC Conjugate, affinity isolated antigen specific antibody
- ❖ Ammonium persulphate (APS)
- ❖ Hoechst 33342
- ❖ Maleic acid
- ❖ Tween 20

2.1.4.7 Merck (Germany) and BDH (UK) supplied:

- ❖ The rest of the analytical grade chemicals

2.1.5 Essential Fatty Acid

Prostaglandin A_2 [PGA_2 ($C_{20}H_{30}O_4$)] was purchased from Sigma Chemical Company USA (synthetic approx. 95%), 10mg/ml in methyl acetate. Methyl acetate was removed by evaporation in a sterile environment.

The fatty acid was dissolved in ethanol to give a concentration of 10mg/ml and stored at -20°C (Joubert *et al*, 1999). PGA_2 is light sensitive and was therefore sealed and stored in a dark vial at -20°C. When necessary, the stock solution was diluted to the appropriate concentrations at the time of their use.

2.1.5.1 The effects of ethanol on cell growth

Studies showed that ethanol ensured total solubility of GLA in the medium of cultured cells on the growth rate of various malignant and benign cell lines (De Kock, 1989 and Dippenaar, 1986). Up to a concentration of 0.3% ethanol could be used to bring in solution FAs in the aqueous culture medium. This concentration proved to have no significant effect on the growth rate of the cell lines in the presence or absence of ethanol (De Kock, 1989).

2.2 METHODS

2.2.1. Cell culture

WHCO3 cells were grown as monolayer cultures in DMEM FX12 supplemented with 5% heat inactivated fetal calf serum (FCS) and a 1% mixture of penicillin and streptomycin as well as gentamicin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Cells were re-supplemented 2-3 times a week and sub-cultured at weekly intervals. In all the experiments, the cells were exposed to doses of 2, 5, 20 and 50μg/ml of PGA_2 unless stated otherwise.

2.2.2 Method of PGA_2 addition

The required amount of PGA_2 was added to the medium prior to dispensing it into the flasks or petri dishes containing the attached cells. This was done because the experiments required a uniform level of FAs. This method rules out the effects of actual contact of the FA on cell viability when added directly (De Kock, 1989).

2.2.3 Morphological effects

2.2.3.1 Haematoxylin and Eosin staining

The cells were seeded onto heat sterilized cover slips in small petri dishes. Near confluent layers of WHCO3 cells were exposed to PGA_2 for a time period of 24 and 48 hrs. After the specified time periods, the experiment was terminated and it was found that the cells exposed to 50μg/ml PGA_2 affected the attachment of cells to the cover slips. It was then decided to reduce the time of exposure of 50μg/ml PGA_2 to a period of 3 hrs. The cover slips were removed from the petri dishes with a tweezers and fixed in Bouin's fixative for 60 minutes. The cells were left in 70% ethanol for 20 minutes, rinsed in water for approximately 5 minutes and stained for 15 minutes in Haematoxylin (Ehrlich). This was followed by another rinse in water for 1 minute and then stained for 2 minutes in Eosin. Cells were dehydrated in a graded series of ethanol twice each (70%, 90% and 100% ethanol) for 5 minutes respectively and cleared in xylene. The cover slips containing the cells were mounted with a mounting medium (DPX mountant) face down onto microscope slides (Kiernan, 1990) and viewed with a Zeiss microscope. Cytotoxic effects of doses of PGA_2 in WHCO3 cells including apoptotic body formation were studied. Magnifications in fig. 3.1-3.10 (40x and 100x) represent the magnification of the objective lenses.

2.2.3.2 Indirect Immunofluorescence

2.2.3.2.1 α-tubulin studies

During the M phase of the cell cycle, the spindle figure is required for segregation of chromosomes and is therefore essential for cell division. Anti-microtubule drugs or microtubule disrupting agents block the cell cycle at the M phase of proliferating cells by interfering with the function and integrity of the spindle microtubules during the M phase. This indicates the positive roles of microtubules in propelling the cell cycle (Gotoh *et al.*, 1991).

The cytoskeleton is important for cell shape, motility, migration, polarity, signal transduction, establishing linkages to adhesion molecules, and intercellular contacts to produce tissue architecture (Hurtley, 1998). The cytoplasm of higher eukaryotic cells consist of cytoskeletal proteins in highly organized arrays and it is these proteins that aid the cytoskeleton in its functions. Three different building blocks make up the cytoskeleton and they are

microfilaments, composed of actin; microfibrils composed of tubulin; and intermediate filaments composed of vimentin (Hurtley, 1998). As far as cancer research is concerned, the main function of microtubules is to form the mitotic spindle during cell division (Kreis and Vale, 1993). Tubulin is found in all cells and falls into two classes, α- and β-tubulin and a third class (γ-tubulin) has recently been identified (Kreis and Vale, 1993). α-Tubulin was used to investigate the morphology of the cytoskeleton of WHCO3 cells because it is commonly applied in our laboratory and the method is well known. It has a molecular weight of ~60 kDA and is used as a positive control for immunofluorescence.

The effects of PGA_2 on the cytoskeleton in the interphase and spindle formation in WHCO3 cells were determined with the aid of monoclonal antibodies against α-tubulin (monoclonal anti-α-tubulin), 3, 24 and 48 hrs after initial exposure using an indirect immunofluorescence staining technique (Ochs *et al.*, 1983; Seegers *et al.*, 1985). Specifically bound antibody is visualized by incubation with a biotinylated second step antibody (biotin conjugate monoclonal anti-FITC) against immunoglobulins, followed by incubation of a goat anti-mouse IgG antibody, which has been directly probed with FITC (Goat anti-mouse IgG FITC conjugate).

The effect of PGA_2 on the cytoskeleton of WHCO3 cells was studied by evaluating α-tubulin. Cells were seeded onto heat sterilized cover slips in small petri dishes and exposed to PGA_2 for 3, 24 and 48 hrs. The cells on the cover slips were fixed in 2% formaldehyde in PBS for 10 minutes at room temperature and washed three times for five minutes each time in PBS. Cells were permeabilized by placing the cover slips in 97% methanol at -20°C for 4 minutes. The cover slips were washed again in PBS as described above. 25µl of the primary antibody (monoclonal anti-α-tubulin) (1:500 in PBS) were added on a microscope slide and the cover slips were placed cells down onto the microscope slide and incubated in a humidified chamber at 37°C for 60 minutes. The cells were washed as described above and exposed to 25µl of biotin conjugated monoclonal anti-FITC antibody (1:400 in PBS) for 45 minutes, and again incubated in a humidified chamber at 37°C. The washing process was repeated and cells were incubated with the affinity isolated antigen specific antibody (FITC conjugated goat anti-mouse IgG) (1:32 in PBS) for 15 minutes at 37°C. Cells were extensively washed with PBS

and the cover slips were mounted in PBS/glycerol and an anti fading medium n-propylgalate. The slides were viewed for fluorescence with an Axiovert Zeiss microscope equipped with fluorescence attachments and excitation (488nm)-emission (515-540nm) filters for FITC. The inbuilt camera was an Axiocam and photos were analyzed using an Axiovision program. Magnification of photographs was 100x.

2.2.3.2.2. Viability staining

Hoechst 33342 were used to assess apoptosis in WHCO3 cells. Hoechst 33342 is a UV light excitable nucleic acid binding dye that is cell permeant. It uniformly stains the highly condensed chromatin of apoptotic cells and dimly stains the looser chromatin structure of viable cells. The apoptotic cells can take the form of crescents around the periphery of the nucleus or the entire nucleus can appear to be one or a group featureless, bright spherical beads. Cells were seeded onto heat-sterilized cover slips in small petri dishes and exposed to PGA_2 for 24 and 48 hrs. The growth medium was discarded and 2ml of 1µg/ml Hoechst 33342 (prepared in DMEM FX12) was added to the cells and incubated for 30 minutes at 37°C. After the incubation period the petridishes were placed under an Axiovert Zeiss microscope and viewed with a UV light and a blue filter (excitation 358nm, emission 461nm). Magnification of photographs was 40x.

2.2.3.2.3 Silver staining for NOR activity

This is a simple one step technique with the use of a protective colloidal developer in combination with aqueous silver nitrate. The NORs are stained black to observe nuclear activity, while the chromosome arms are stained yellow. The nucleolus is a highly specialized organelle, which functions to produce ribosomal RNA (rRNA). They are then assembled into preribosomal ribonucleoprotein particles and transported to the cytoplasm (Busch and Smetana, 1970). The rRNA gene is an important model for studying various gene control mechanisms. Ribosomal DNA (rDNA) is easily visualized by the specificity of silver staining for detecting the location of the ribosomal genes on metaphase chromosomes (Hubbel, *et al.*, 1980). Hybridization of rRNA has shown that silver localizes at the site of the NOR of metaphase chromosomes and detects only active genes (Howell, 1977). The amount of silver deposited in the cytochemical reaction is a reflection of the activity of the ribosomal genes and

therefore the identification of the NOR-specific silver staining has been of much interest (Howell, 1982).

WHCO3 cells were seeded onto heat-sterilized cover slips in petri dishes and exposed to PGA_2 for 3 hrs (50µg/ml) and 24 hrs (2, 5, 20 and 50µg/ml) PGA_2. After the indicated time period, cells were fixed in 2% formaldehyde in PBS. Cells were washed three times in PBS and permeabilized in 95% ethanol at -20°C for 5 minutes. Another washing process followed, three times in PBS and three times in water. Each wash was 5 minutes long. 2 drops of the colloidal developer and 4 drops aqueous silver nitrate ($AgNO_3$) were added to the surface of the cover slip containing the cells. The solution was mixed and covered with a slide. The slides were placed on the surface of a slide warmer, which was stabilized at 70°C. Within 30 seconds the silver staining mixture turned yellow and within 2 minutes it turned golden brown. The slide was then removed and rinsed three times with deionised water for 5 minutes. A dehydration process followed which included 25, 50, 75 and 100% alcohol and then cleared in xylene. The cover slips were mounted onto slides with DPX mounting medium and examined under a Zeiss microscope. Photographs were taken with a 100 ASA film.

The colloidal developer solution was prepared by dissolving 2g-powdered gelatin, USP into 100ml deionized water and 1ml pure formic acid. Constant stirring for 10 minutes was done until the gelatin was dissolved. This solution was stable for 2 weeks. An aqueous $AgNO_3$ solution was prepared by dissolving 4g $AgNO_3$ into 8ml-deionised water. This solution was then stable. Both solutions were stored in capped amber bottles.

2.2.4 Mitotic Indices

A time- and dose dependent study of the effects of PGA_2 in WHCO3 cells was done by determining the mitotic indices (MI). WHCO3 cells were seeded onto heat-sterilized cover slips in small petri dishes. Near confluent layers of cells were exposed to PGA_2 for periods of 3 hrs (50µg/ml), 24 and 48hrs (2, 5 and 20µg/ml). The cells were fixed in Bouin's fixative and stained with Haematoxylin and Eosin (H & E) using the procedure described in 2.2.3.1. MI were obtained by counting 1 000 cells on each cover slip. Total mitosis included all the normal phases of the dividing cells as well as the cells in abnormal metaphase (including

metaphase with lagging chromosomes and/or metaphases with highly condensed chromosomes). Apoptotic cells were also counted. These experiments were repeated 6 times.

2.2.5 Cell Synchronization

The duration of the S phase, G_2/M transition period and mitosis were determined in WHCO3. Cells were seeded onto heat-sterilized cover slips as described above and effectively blocked at the G_1/S boundary when confluent layers were exposed to 2ml 1.5mM hydroxyurea (HU) for 20 hrs (Lottering *et al.*, 1992). The block was removed by three times changes of DMEM without HU. Every hour for 20 hrs, duplicate cover slips were removed from the incubator after the removal of the G_1/S block. They were then fixed in Bouin's fixative and stained with H & E (Kiernan, 1990). Mitotic indices were determined by counting 1 000 H & E stained cells on each cover slip (Seegers *et al.*, 1989).

2.2.6 Protein Determination

For the protein electrophoresis and western blot experiments described later, the protein content of WHCO3 had to be determined. The media were removed and cells were rinsed with 1ml cold PBS and left on ice. Cells were harvested from the flasks with a cell scraper in the 1ml PBS, placed into eppendorfs and microcentrifuged for 1 minute at 2000 rpm. The supernatant was sucked off using a Pasteur pipette and cells were resuspended in 200µl cold PBS (cells could be stored at this point at –20°C until later analysis). A cell lysate was prepared by three freeze (-20°C) thaw (room temperature) cycles. An aliquot of ±50µl was then removed and the protein content of the lysate determined (in triplicate) using the method described by Lowry *et al.*, 1951. The proteins of the lysate were determined in a Beckman DB Spectrophotometer using a wavelength of 650nm. A similar study using unsynchronized cells was also prepared and the proteins determined as well.

2.2.7 Protein Electrophoresis

Experiments were carried out on synchronized cells to determine the effects of PGA_2 on specific events in a specific phase. Synchronized G_1 (media with block) and S phase cells were exposed to PGA_2 for 3 hrs. The S phase cells were allowed to move into the S phase for one hour before the PGA_2 was added. After the 3 hrs exposure time, both G_1 and S phase

experiments were terminated by placing the flasks with cells on ice. A cell lysate was prepared as described in Section 2.2.6, an aliquot removed and used for determination of proteins of the lysate. The remaining samples were diluted 1:3 with an electrophoresis sample buffer consisting of:

Deionised water

0.5M Tris-HCl, pH6.8

Glycerol

10% (w/v) SDS (sodium dodecyl sulfate)

0.5% bromophenol

β - mercaptoethanol

(Proteins are fully denatured by reduction with β - mercaptoethanol and saturated with SDS. β - mercaptoethanol was added just before the sample buffer was used). After the sample was added, the samples were heated for 4 minutes at 95°C. This reduced and denatured the proteins. 20µl aliquots of the sample were loaded per slot into the 10% SDS-polyacrylamide electrophoresis gel. A molecular weight protein standard was loaded simultaneously. The running buffer consisted of Tris base, glycine and SDS. Electrophoresis was carried out at 200V (constant voltage) with a current of 50mA for 2 hrs.

Separating gel was made as follows:

1.5M Tris buffer (adjusted with HCl to pH 8.8)

10% SDS

Deionised water

40% Acrylamide/bis

10% APS (ammonium persulfate)

Temed (N, N, N', N'-tetramethyl-ethylene diamine)

After the gel was poured, it was overlaid with 1ml water and allowed to set at room temperature for ±15 minutes.

The stacking gel was made up as the separating buffer but with a 0.5M Tris buffer and adjusted with HCl to pH 6.8.

When the gel was set, the water was poured off, the comb inserted and the stacking gel buffer carefully poured and allowed to set. The comb was removed and the samples loaded as described above.

2.2.8 Immunoblots (Western Blotting)

Western blotting is useful for the identification of specific proteins in complex mixtures of molecules and does not require labeling of the target protein. Detergents and reducing agents are used to solubilize the samples and separated by SDS polyacryamide gel electrophoresis, and then transferred to a solid support (nitrocellulose membrane), which may then be stained and probed with reagents that have specific sequences of amino acids. The antibodies react specifically with antigenic epitopes displayed by the target protein attached to the solid support. The electrophoretic separation of the target protein is usually performed under denaturing conditions; therefore problems concerning solubilization of the target proteins are eliminated. After transfers to the membrane, parts of the protein are renatured and native epitopes can react with their ligands or with polyclonal or monoclonal antibodies. The membrane is exposed to unlabeled antibodies specific for the target protein. A secondary immunological reagent detects the bound antibody.

20µl protein per lane was electrophoresed on a 10% SDS polyacrylamide gel as described in Section 2.2.7. An electroblotting apparatus was used to transfer the protein samples from the polyacrylamide gel onto a nitrocellulose membrane. A porous pad, filter paper and nitrocellulose membrane were soaked in transferring buffer before a sandwich was made. Using concentrated running buffer with methanol and deionised water made up the transferring buffer. A sandwich was assembled consisting of a porous pad, 2 filter papers, 1 nitrocellulose membrane (cut to the dimensions of the gel), another 2 filter papers and again the porous pad. This was done in a stacking order. Air bubbles were removed by rolling a glass tube over the filter papers. The gel was placed in the electroblotting apparatus and the transferring process was carried out at 52 mA for 1hrs30-2 hrs. The nitrocellulose membrane was removed after the specified time period and placed in a solution of Poncheau S to verify the transfer of proteins onto the nitrocellulose membrane.

A Stock solution of Poncheau S was made up by mixing:

2g Poncheau S

30g Trichloroacetic acid

30g Sulfosalicyclic acid to 100ml water

Poncheau S was diluted 1:9 with deionised water to make up a working solution. The stain is transient and is washed away during processing of the western blotting and is therefore completely compatible with all methods of immunological probing (Sambrook *et al.*, 1989).

The blotting was performed in the buffer system with methanol and therefore the membrane was washed 2 times with TBS (Tris buffered saline).

TBS pH7.5

50mM Tris base

150mM NaCl

This was made up in deionised water at pH 7.5 with 1mM HCl and diluted to 1 liter deionised water.

The membrane was incubated in 30 ml 1% blocking solution in maleic acid overnight in a refrigerator (4°C). The incubation was to block non-specific binding of the antibody.

Maleic acid:

100mM maleic acid, pH 7.5

150mM NaCl

10% Blocking solution:

10g blocking reagent (supplied by kit)

100ml maleic acid

The membrane was briefly rinsed in TBS-T (Tris buffered saline-Tween 20) using two changes of washing buffer then once for 15 minutes and twice for 5 minutes with fresh changes of the washing buffer at room temperature. Afterwards the membrane was incubated in 2ml primary antibody [(caspase 3) 2µg/ml] diluted in 1% blocking solution (w/v) for 1 hour. The membrane was washed as described above using large volumes of washing buffer

and incubated for another hour in diluted biotinylated secondary antibody (0.2µg/ml) and washed once for 15 minutes and then 4x5 minutes in fresh changes of washing buffer.

TBST: TBS-Tween 20:
0.1% Tween 20 (0.1ml Tween 20)
99.9ml TBS

Detection using the ECL™ method

The detection solutions (supplied by kit) were mixed in equal volumes sufficient to cover the membranes. The final volume was 0.125ml/cm^2 membrane. The excess washing buffer was removed from the membranes and placed on a piece of cling wrap, the protein side up. The detection reagent was added to the protein side of the membranes and incubated for 1 minute at room temperature without agitation. The excess detection reagent was drained and the membranes were wrapped in cling wrap. The air pockets were gently smoothed out and the membranes were placed in a film cassette. A sheath of hyperfilm was placed on the protein side of the membranes, the cassette closed and the membranes exposed for 15 seconds. The film was removed and placed in developer for 4 minutes and then fixed.

2.2.9 Flow cytometry

Flow cytometry is a method of measuring certain chemical and physical characteristics of cells and particles as they travel in suspension one by one past a sensing point. The cytometer passes cells through a tightly focussed beam of laser light. Interaction of the particle with the laser beam produces a light scatter in the forward direction (FSC-forward scatter, that correlates with cell size) and a lateral direction (SCC-side scatter, that correlates with granularity and/or cell density). A reduction of both SCC and FSC (probably due to a rapture of plasma membrane and leakage of the cell content) characterize necrotic death. An initial increase in SCC (probably due to a chromatin condensation) and a reduction in FSC (due to cell shrinkage) characterize apoptosis.

Flow cytometry was used to analyze the cell cycle and apoptosis induction in WHCO3 cells. A combination of mirrors and filters were used to separate the green (FITC) and the red PI.

Each analysis was based on 10 000 events. The same amount of WHCO3 cells was seeded into 25cm^2 flasks after trypan blue exclusion. Effects of the cell cycle and apoptosis induction were determined after the cells were exposed to PGA_2. Untreated cells were used as controls. After the specified exposure time (3 and 24 hrs for caspase 3 and 3, 24 and 48 hrs for PI) the medium was discarded into 15ml vials and cells were trypsinized. The trypsinized cells with the cells in the 15ml vials were then centrifuged at 2000rpm for 5 minutes. The supernatant was discarded and 3ml ice-cold methanol (-20°C) was added drop wise while vortexing. This is the permeabilization process to allow the primary antibody to move into the cell. The cells were stored in methanol at -20°C over night. Just before flow cytometry was performed, cells were centrifuged at 2000rpm for 5 minutes at 20°C, resuspended in 2ml 20% FCS in 3% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. Samples were again centrifuged as above and incubated in 1ml FITC-conjugated anti caspase 3 in 10% FCS in 3% BSA in PBS for 60 minutes at room temperature. This monoclonal antibody recognizes the active form of caspase 3 in human cells. Isotypic controls were exposed to 2% irrelevant mouse IgG2b-kappa-FITC for 60 minutes at room temperature. Subsequently, cell pellets were washed three times with PBS. WHCO3 cells that were stored in methanol over night were also stained with PI just before flow cytometry was performed. 1ml PI (18µg/ml) in PBS was added to the cells and filtered through a mesh lining and analyzed on a FACSCaliber flow cytometer. Caspase 3 and PI experiments were done separately.

2.2.10 DNA fragmentation

The effects of PGA_2 on cellular DNA fragmentation in WHCO3 cells were determined by means of an apoptotic DNA ladder kit (Roche Mannheim, Germany). This kit detects the typical DNA ladder, which is the hallmark of apoptotic cells by purifying nucleic acids from different sample materials.

Principle: A positive control consisting of lyophilized samples of apoptotic U937 cells (supplied by kit) was prepared in the same manner as the cell samples. After lysis of cultured cells in binding buffer, the lysate is applied to the filter tube with glass fiber fleece passaged through the glass fiber fleece by centrifugation. During this process nucleic acids bind to the

surface of the glass fibers in the presence of chaotropic salts. The residual impurities are removed by a wash step and subsequently DNA is eluted in the buffer (10mM Tris, pH 8.5).

WHCO3 cells were seeded into flasks and exposed to PGA_2 for 3 hrs (50µg/ml) and 24 hrs (20µg/ml). Cells were harvested from the flasks with a cell scraper. 1-2ml PBS was added and pipetted into eppendorfs and centrifuged for 1 minute at 2000rpm. The supernatant was discarded and $2x10^6$ cells in a sample volume of 200µl were used. 200µl binding buffer (supplied by kit) were added to a final volume of 400µl. This was mixed immediately. The samples were incubated at room temperature for 10 minutes. A volume of 100µl isopropanol was added and vortexed. During the 10 minutes incubation period, a filter tube and a collection tube (supplied by kit) were combined and put aside. The samples were pipetted into the upper reservoir of the collection tube and centrifuged for 1 minute at 8000rpm in a standard tabletop centrifuge. The flow through was discarded. 500µl washing buffer (20mM NaCl and 2mM Tris-HCl, pH 7.5) were added and centrifuged for 1 minute at 8000rpm. This was done three times and the final centrifugation was for 10 seconds at 13 000rpm to remove the residual buffer. The collection tube was discarded and the filter tube placed in a clean tube. Elution of the DNA was done by using 200µl of prewarmed (70°C) elution buffer (10mM Tris, pH 8.5). Elution buffer was added to the filter tube and centrifuged for 1 minute at 8000rpm. The samples/DNA were stored at -20°C for analysis by electrophoresis in 2.2.11.

2.2.11 DNA Electrophoresis

A cell lysate was prepared as described in Section 2.2.10, an aliquot removed and used to determine the DNA fragmentation of the lysate. A 1% agarose gel was made up to determine the DNA fragmentation. 150ml TBE was added to1.5g of agarose. The TBE consisted of Tris, boric acid and Na_2EDTA. The flask was heated in a microwave oven at maximum energy level for 2 minutes or until the agarose was completely dissolved. The solution was allowed to cool and 5µl ethidium bromide (EtBr) was added. EtBr (10g/ml) was covered with foil because of its light sensitivity and allowed to cool. The solution was poured into the prepared electrophoresis chamber and allowed to set for 30 minutes.

The samples were prepared by mixing 60µl of the purified DNA with 5µl of the loading buffer. The latter consisted of SDS, bromophenol blue, deionised water and glycerol. A DNA molecular weight marker was loaded into the first well and also mixed with loading buffer. The rest of the samples were loaded into the other wells. The voltage was set at 75V for an hour. The DNA samples were visualized by placing the gel into a UVP, white/UV transilluminator for analysis.

2.2.12 Statistical analysis

The quantification graphs of the morphology of the effects of PGA_2 on WHCO3 cells in figures 3.11-3.14 were analyzed for statistical differences. Using Medcalc (Schoonjans *et al.*, 1995) multiple comparison graphs with error bars for ±2 standard error of the mean (SEM) and 95% confidence interval (CI) and one way analysis of variance (ANOVA) were then used to visualize and quantify the influence of PGA_2 on each sample in an experimental group. The T-bars refer to the 95% CI. A p value of <0.05 was accepted as significant.

The histograms in figures 3.17-3.21 represent the flow cytometry results. The values are tabulated in tables 2 and 3 and are expressed as a percent of a 100% gated events of 10 000 cells counted. A Becton Dickinson Immunocytometer was used and the data were analyzed with a Cell Quest 3.3 program.

CHAPTER 3
RESULTS

3.1 MORPHOLOGICAL EFFECTS

3.1.1 HAEMATOXYLIN AND EOSIN STAINING

H & E staining was done to establish the effects of PGA_2 on the morphology of WHCO3 cells. These effects are illustrated in Fig 3.1-3.4 after 3, 24 and 48 hrs. Vehicle-exposed control cells are shown in Fig. 3.1A, 3.2A, 3.3A and 3.4A after 3, 24 and 48 hrs. Abnormal dividing cells are present in PGA_2 treated cells (Fig.3.1B, 3.2B-F, 3.3B, 3.4B-D). The morphological results show that PGA_2 caused abnormal metaphases (Fig. 3.2B-F, Fig. 3.4B and C) and an increase in apoptosis induction in the cells exposed to the higher concentrations of PGA_2 (Fig. 3.1B, 3.2D, 3.3B and 3.4D).

Fig. 3.1 H & E stained cells showing the effects of 50µg/ml PGA_2 on WHCO3 cell morphology after 3 hrs of exposure. (A) Untreated control showing normal interphase cells with prominent nucleoli (arrows); (B) PGA_2 caused WHCO3 cells to become apoptotic. Cytoplasmic blebbing (arrowhead) and an abnormal metaphase can be seen (arrow) (100x).

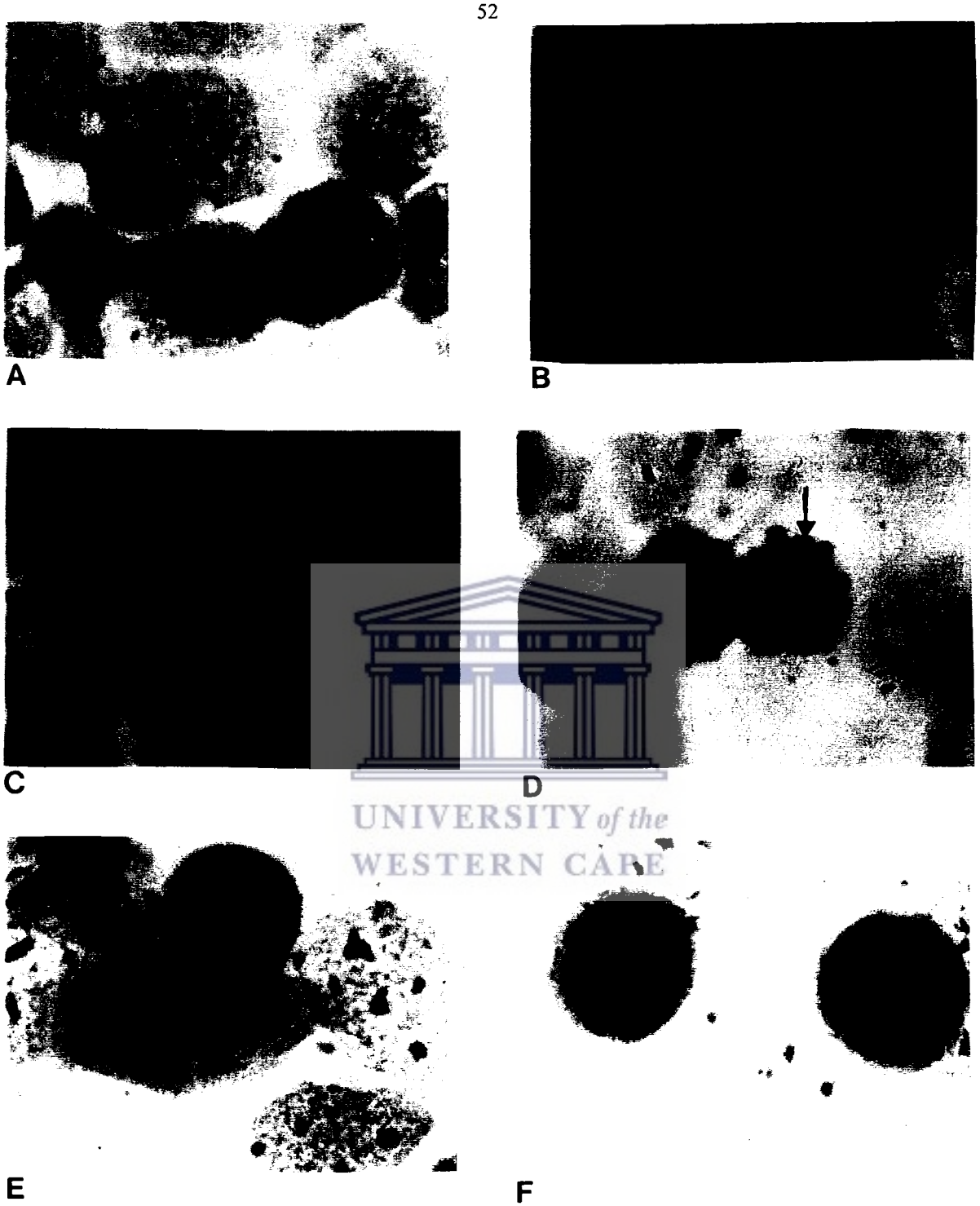


Fig. 3.2 H & E stained cells showing the effects of PGA_2 on WHCO3 cell morphology after 24 hrs of exposure. (A) Untreated control shows normal interphase cells with prominent nucleoli (arrows), a normal anaphase (large arrowhead) and a normal telophase (small arrowhead); (B) 2μg/ml PGA_2 treated cells show an abnormal metaphase (arrow); (C) 5μg/ml PGA_2 treated cells show an abnormal metaphase (arrow); (D), (E) & (F) cells exposed to 20μg/ml PGA_2; (D) shows condensation of chromatin (small arrowhead) as well as apoptotic cells with cytoplasmic blebbing (large arrow). A normal (large arrowhead) and abnormal metaphase are present (small arrow). Abnormal metaphases can be seen (arrows) in (E) & (F) (100x).

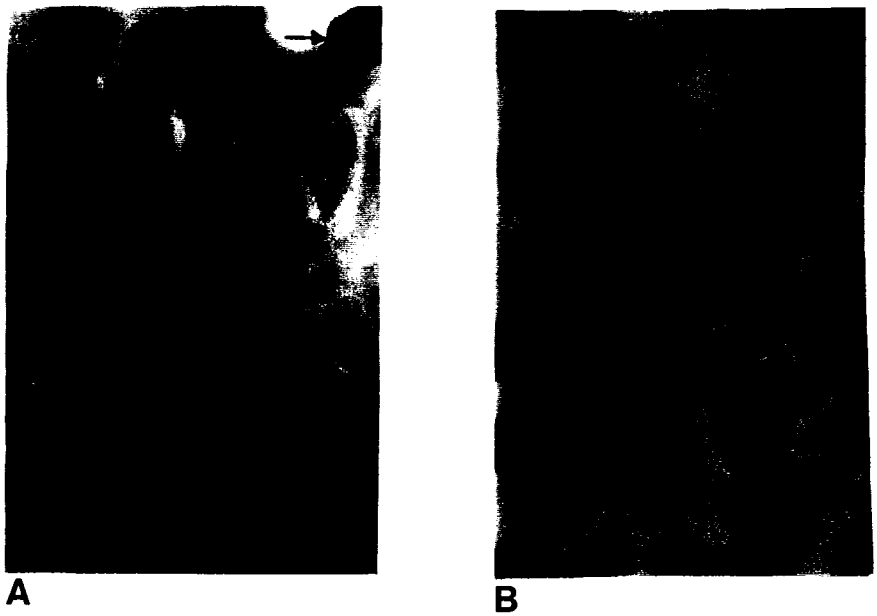


Fig. 3.3 Lower magnification of H & E stained WHCO3 cells exposed to 20µg/ml PGA_2 for 24 hrs. (A) Untreated control showing two normal metaphases (arrows) and a normal telophase (arrowhead); (B) Apoptotic cells showing cytoplasmic blebbing (arrows) and hypercondensation (arrowheads) of chromatin. A few interphase cells are also present (40x).

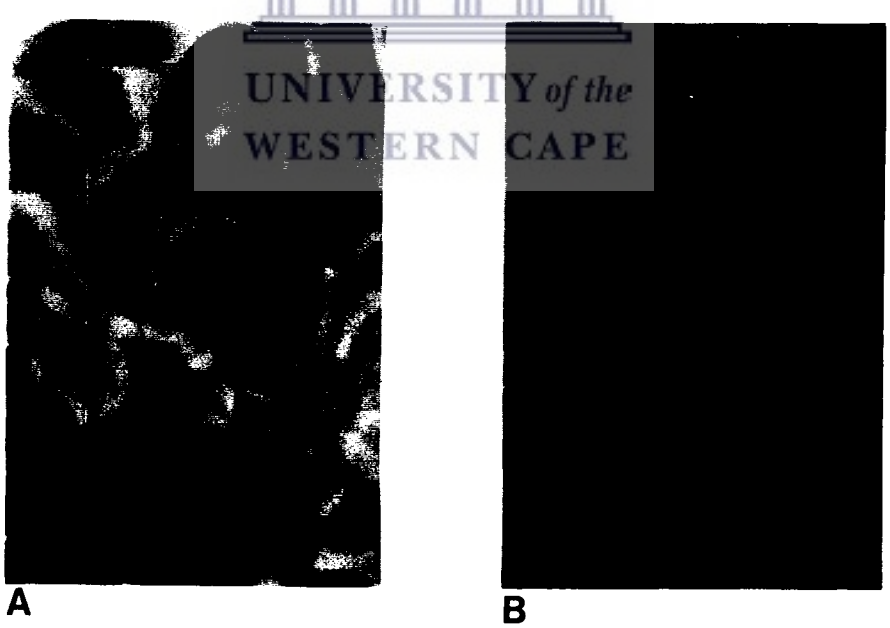


Fig. 3.4 H & E stained cells showing the effects of PGA_2 on WHCO3 cell morphology after 48 hrs. (A) Untreated control showing normal interphase cells with prominent nucleoli (arrowheads), and a normal anaphase (arrow); (B) 2µg/ml PGA_2 treated cells show normal interphase cells (arrows) as well as abnormal metaphases (arrowheads). Fig. 3.4 C and D on p 54 (40x).

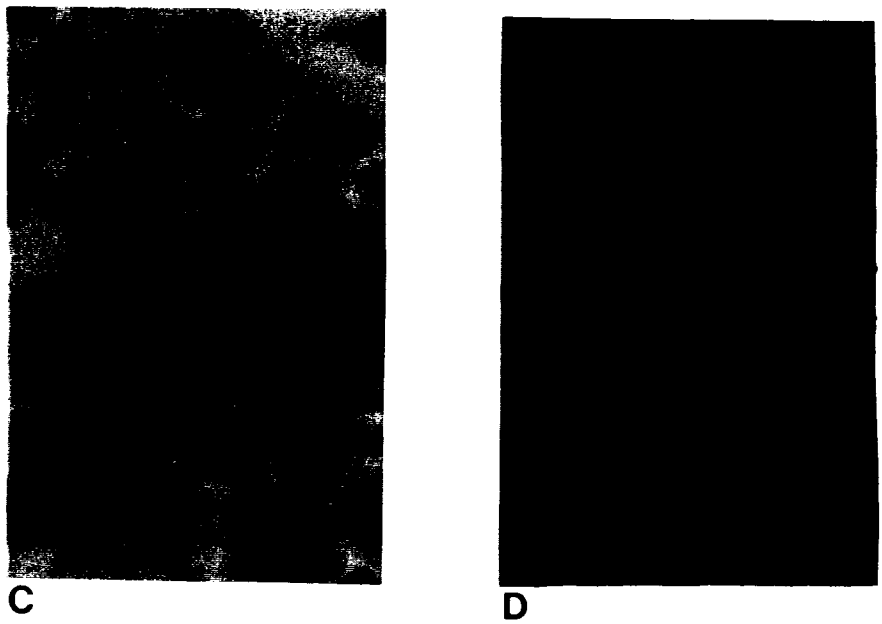
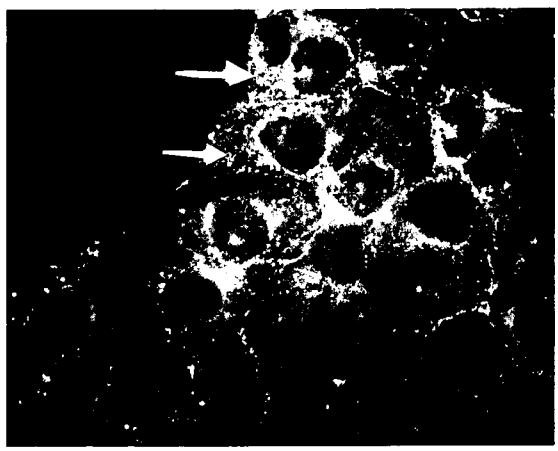


Fig. 3.4 C & D (C) 5μg/ml PGA_2 treated cells showing an abnormal metaphase (arrowhead); (D) cells exposed to 20μg/ml PGA_2 show condensation of chromatin (arrowheads) as well as apoptotic cells with cytoplasmic blebbing (arrows).

3.1.2 INDIRECT IMMUNOFLUORESCENCE

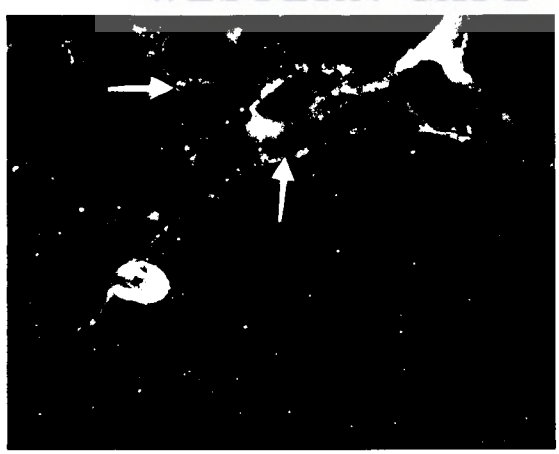
3.1.2.1 α-Tubulin studies

In view of the abnormal metaphase morphology seen in H & E stained cells, it was decided to evaluate the effects of PGA_2 on the microtubules (α-tubulin) of WHCO3 cells with indirect immunofluorescence (Fig. 3.5 & 3.6). Microtubules function as structural and mobile elements in mitosis, intracellular transport, flagella movement and the cytoskeleton. WHCO3 cells were exposed to 20 and 50μg/ml PGA_2 for 24 and 48 hrs only. Morphological studies of indirect immunofluorescent stained WHCO3 cells exposed to PGA_2 showed rearrangement of α-tubulin (Fig. 3.5C, 3.6C and D). However, some cells exposed to 20μg/ml PGA_2 for 48 hrs showed intact α-tubulin and normal spindle figures (Fig. 3.6B). The lower concentrations (2 and 5μg/ml PGA_2) showed no marked differences in α-tubulin arrangement and were therefore not included in the results.



A

B



C

Fig. 3.5 Indirect immunofluorescence of α-tubulin in WHCO3 cells exposed to PGA_2 for 24 hrs. (A) Control with arrows indicating normal cytoskeleton; (B) 20μg/ml PGA_2 did not have an outspoken effect on the arrangement of the α-tubulin after 24 hrs; (C) 50μg/ml PGA_2 treated cells displayed abnormal rearrangement of microtubules (arrows) (100x).

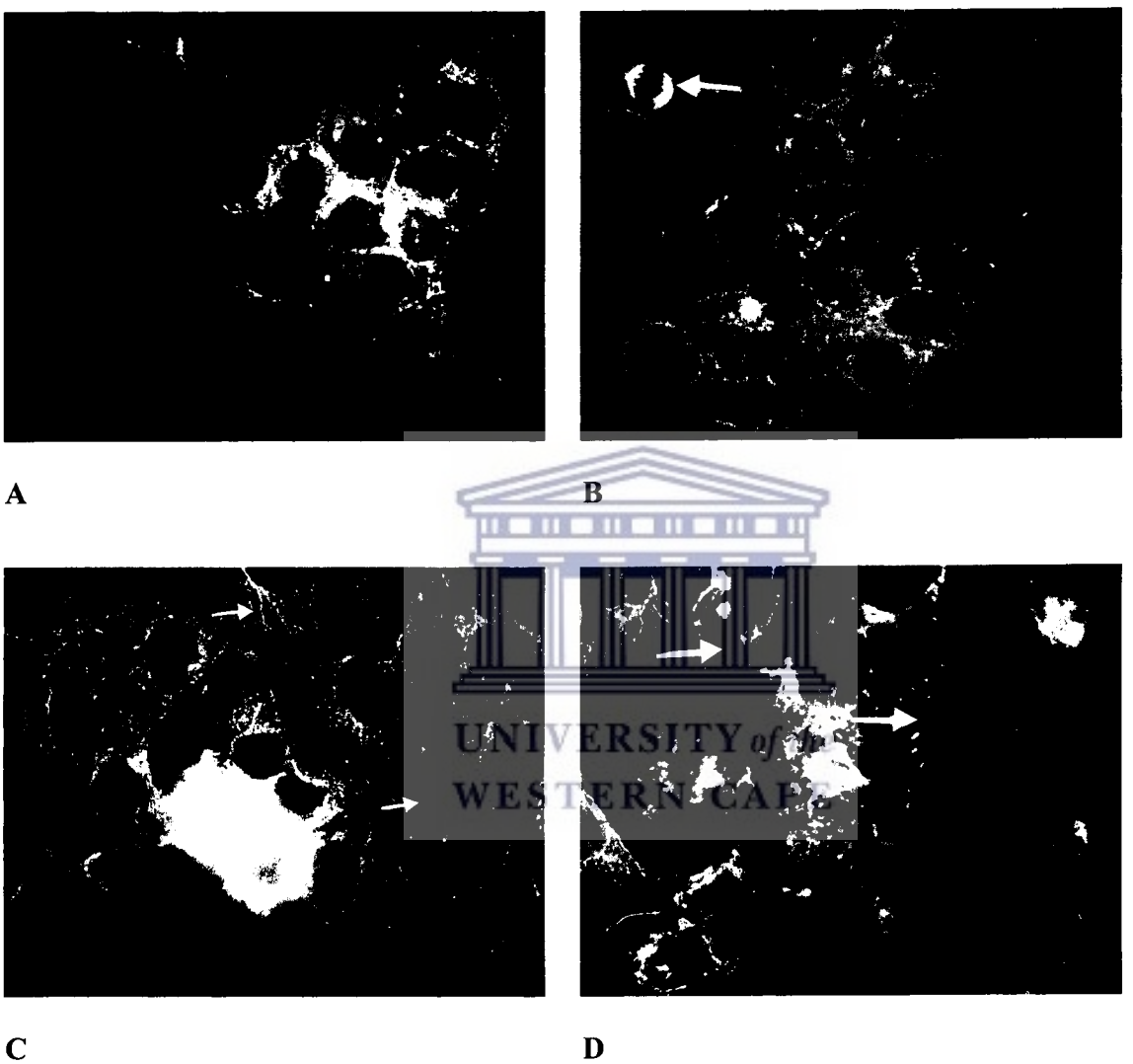


Fig. 3.6 Indirect immunofluorescence of α-tubulin in WHCO3 cells exposed to PGA_2 for 48 hrs. (A) Control cells displaying normal cytoskeleton; (B) and (C) cells exposed to 20μg/ml PGA_2 show a normal spindle formation (arrow) with disrupted microtubules (crisscross pattern) of α-tubulin (small arrows); (D) 50μg/ml PGA_2 show abnormal rearrangement of α-tubulin (arrows) (100x).

3.1.2.2 Viability staining using Hoechst 33342

Hoechst 33342 was used to asses apoptosis in WHCO3 cells. Apoptotic cells, due to a change in membrane permeability, show an increased uptake of the vital dye Hoechst 33342 compared to live cells. Increased cell death is evident in the higher concentrations of PGA_2 where they appear brightly stained (Fig. 3.7-3.8). The lower concentrations (2 and 5µg/ml) of PGA_2 showed no significant differences when compared to the control and are therefore not included in the results.

Fig. 3.7 Hoechst 33342 stained WHCO3 cells exposed to PGA_2 for 24 hrs. (A) Normal dividing cells appear in the control (arrows); (B) 20µg/ml PGA_2 treated cells exhibit apoptotic cells (arrows) as well as abnormal metaphases (arrowheads); (C) more apoptotic cells are evident when cells are exposed to 50µg/ml PGA_2 (arrows) (40x).

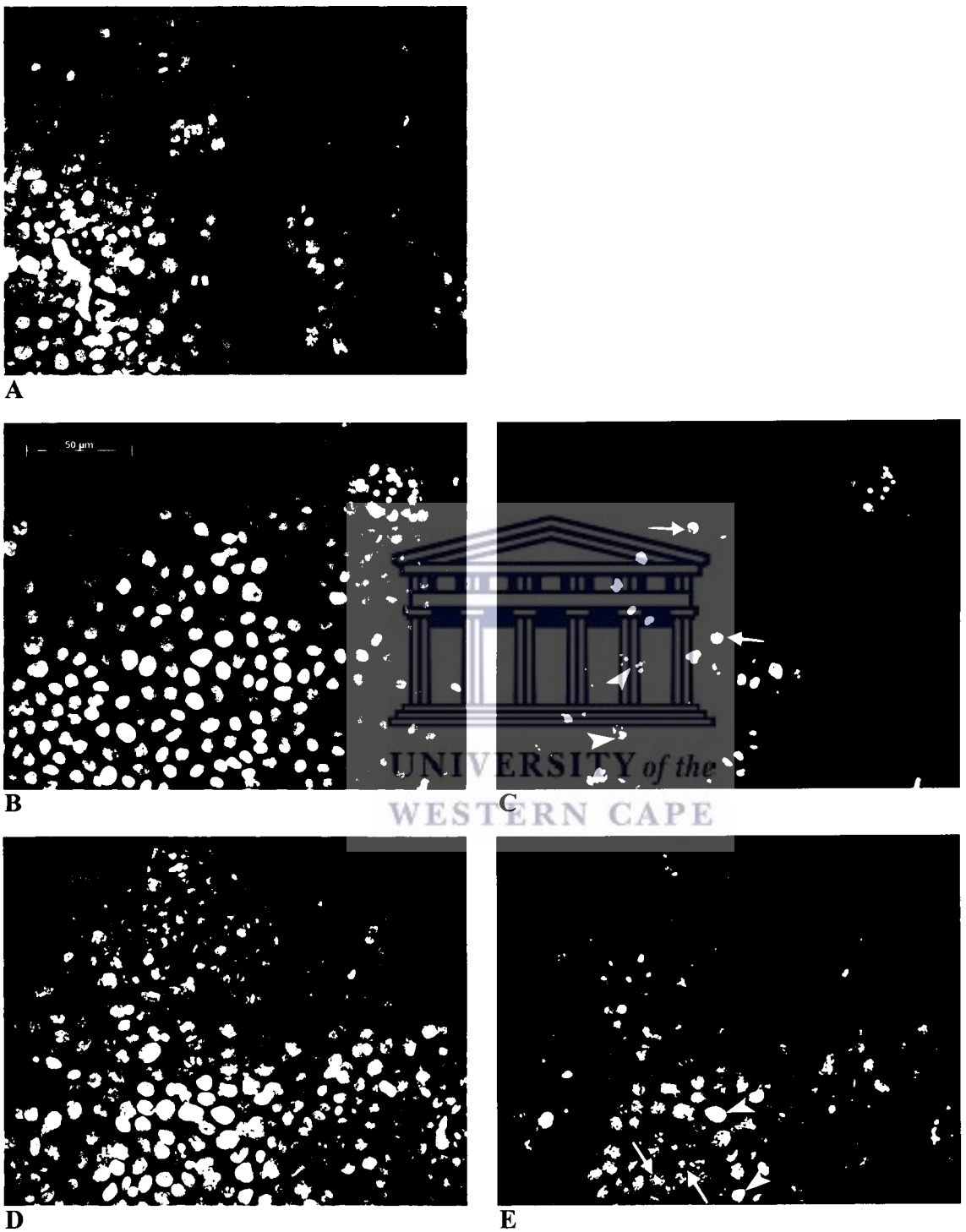


Fig. 3.8 Hoechst 33342 stained WHCO3 cells exposed to PGA_2 for 48 hrs. (A) Control showing normal dividing cells; (B) 20µg/ml display brightly stained apoptotic cells; (C) a converted image of B showing apoptotic cells (arrows) and cytoplasmic blebbing (arrowheads) only; (D) 50µg/ml treated cells show an increase in apoptotic cells; (E) is a converted image of D displaying fragments (arrows) of and apoptotic cells (arrowheads) (40x).

3.1.2.3 SILVER STAINING FOR NOR ACTIVITY

A specific stain for fibrillar phosphoproteins was used to determine whether the fragments in the nuclei and nucleoli contained fibrillar material. The major silver staining protein of the nucleolus is the phosphoprotein C_{23} that is either directly or indirectly associated with rDNA. Silver staining in the fragments would then confirm the presence of fibrillar phosphoproteins throughout the nucleoplasm and active transcription in the nucleolar organizing regions (NORs) of the nucleolus of control WHCO3 cells. Fig. 3.9 & 3.10 show the silver staining of WHCO3 cells before (controls) and after PGA_2 supplementation for 3 (50µg/ml PGA_2) and 24 hrs (2, 5, 20 and 50µg/ml PGA_2). In the controls (Fig. 3.9A and 3.10A) intense silver staining with intermingled fibrillar and granular components are observed confirming the presence of fibrillar phosphoproteins throughout the nucleoplasm, which is indicative of active transcription in the NOR regions of the nucleoli. The presence of fibrillar phosphoproteins is an indication of increased rRNA and subsequently increased protein synthesis (Lischwe *et al.*, 1981). Very little (Fig. 3.9B) fibrillar phosphoproteins could be observed in cells exposed to 50µg/ml PGA_2 after only 3 hrs. The nucleoli showed decreased silver staining mainly in intact nucleoli that also showed segregated fibrillar and granular components (Fig. 3.9B). This is an indication of a decrease in active transcription in the NOR regions of the nucleolus and cells which are metabolically more inactive than the untreated controls. The decreased silver staining could be an indication of decreased rRNA activity and ribosome production and subsequently less protein synthesis in the usually metabolically highly active malignant WHCO3 cells. At concentrations of 2 and 5µg/ml PGA_2, segregation of the nucleolar granular and fibrillar phosphoproteins can already be observed (Fig. 3.10B & C). A small degree of silver staining predominated in cells exposed to 20µg/ml PGA_2 and further segregation of nucleolar granular and fibrillar phosphoproteins were evident (Fig. 3.10D). As in fig. 3.9B, the cells exposed to 50µg/ml PGA_2 for 24 hrs (Fig. 3.10E) show the same segregation of nucleolar fibrillar and granular components.

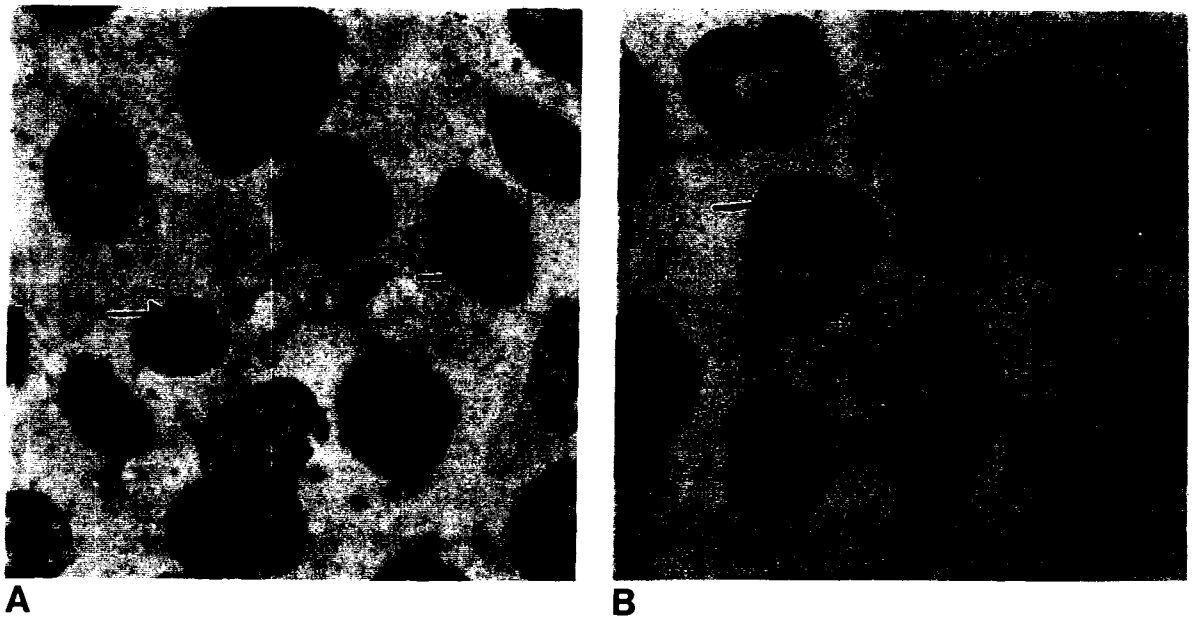


Fig. 3.9 Silver staining of WHCO3 cells exposed to 50µg/ml PGA_2 for 3 hrs. (A) Untreated control shows increased silver staining of nucleoli with intermingled fibrillar and granular components (arrows); (B) In PGA_2 treated cells decrease silver staining and very little or no fibrillar phosphoproteins could be observed (arrows) (100x).

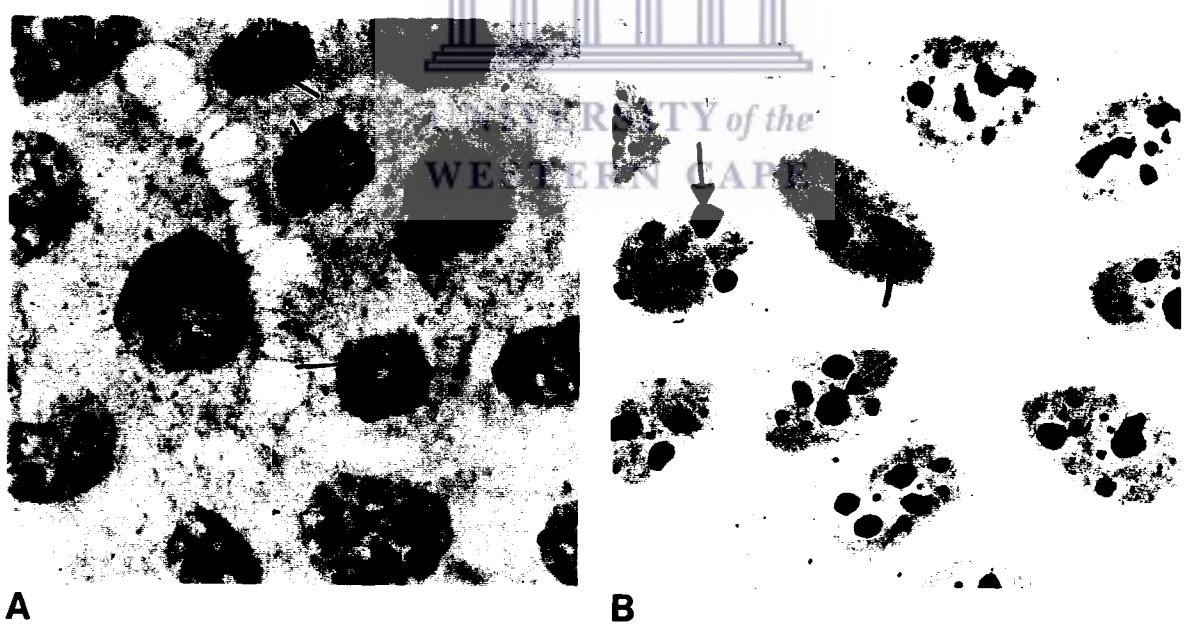


Fig. 3.10 Silver staining of WHCO3 cells exposed to PGA_2 for 24 hrs. (A) Untreated control shows increased silver staining of nucleoli with intermingled fibrillar and granular components (arrows); (B & C) 2 & 5µg/ml PGA_2 treated cells show the start of segregated nucleolar material and fibrillar phosphoproteins (arrows). Fig. 3.10 (C), (D) & (E) on p61 (100x).

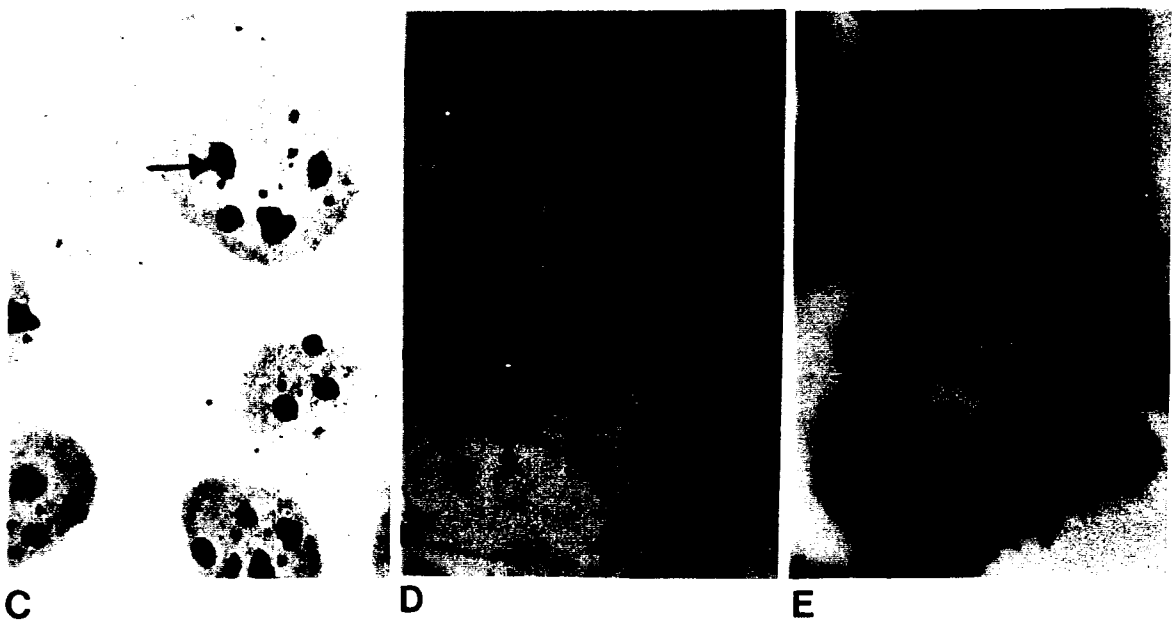


Fig. 3.10 (D) 20µg/ml PGA_2 treated cells show further segregation of granular and fibrillar components with decreased silver staining in the nucleoli (arrows); (E) cells exposed to 50µg/ml PGA_2 show no silver staining of nucleolar and fibrillar phosphoproteins (100x).

3.2 MITOTIC INDICES

The time and dose dependent effect of PGA_2 on $WHCO_3$ cell growth after 3, 24 and 48 hours, using H & E staining are illustrated in Figures 3.11-3.14. The study indicated that PGA_2 significantly inhibited the number of mitotic $WHCO_3$ cells in a time and dose dependent manner, thus showing that PGA_2 has an inhibitory effect on cell growth. Cells exposed to 20µg/ml PGA_2 for 24 hrs showed a significant increase of dividing cells in metaphase (abnormal metaphase included) (Fig 3.12B), while 5µg/ml PGA_2 caused a significant decrease of cells in metaphase. As mentioned earlier, the effect of PGA_2 is time and dose dependent and the most significant decrease on mitosis in the $WHCO_3$ cells were seen after the 48 hrs exposure time period (Fig. 3.13A). Apoptotic cells were observed in $WHCO_3$ cells exposed to 50µg/ml PGA_2 for 3 hrs (Fig 3.14A) and 20µg/ml PGA_2 for 24 and 48 hrs (Fig. 3.14B) when compared to controls. After 48 hrs, fewer apoptotic cells were observed in cells exposed to 20µg/ml PGA_2 than when exposed for 24 hrs (Fig. 3.14B). Although the apoptotic effects of 20µg/ml PGA_2 at 48 hrs in Fig 3.14B are decreased, growth (total mitosis) was not enhanced (Fig. 3.13A). Mitosis and total mitosis are indicative of mitotic indices.

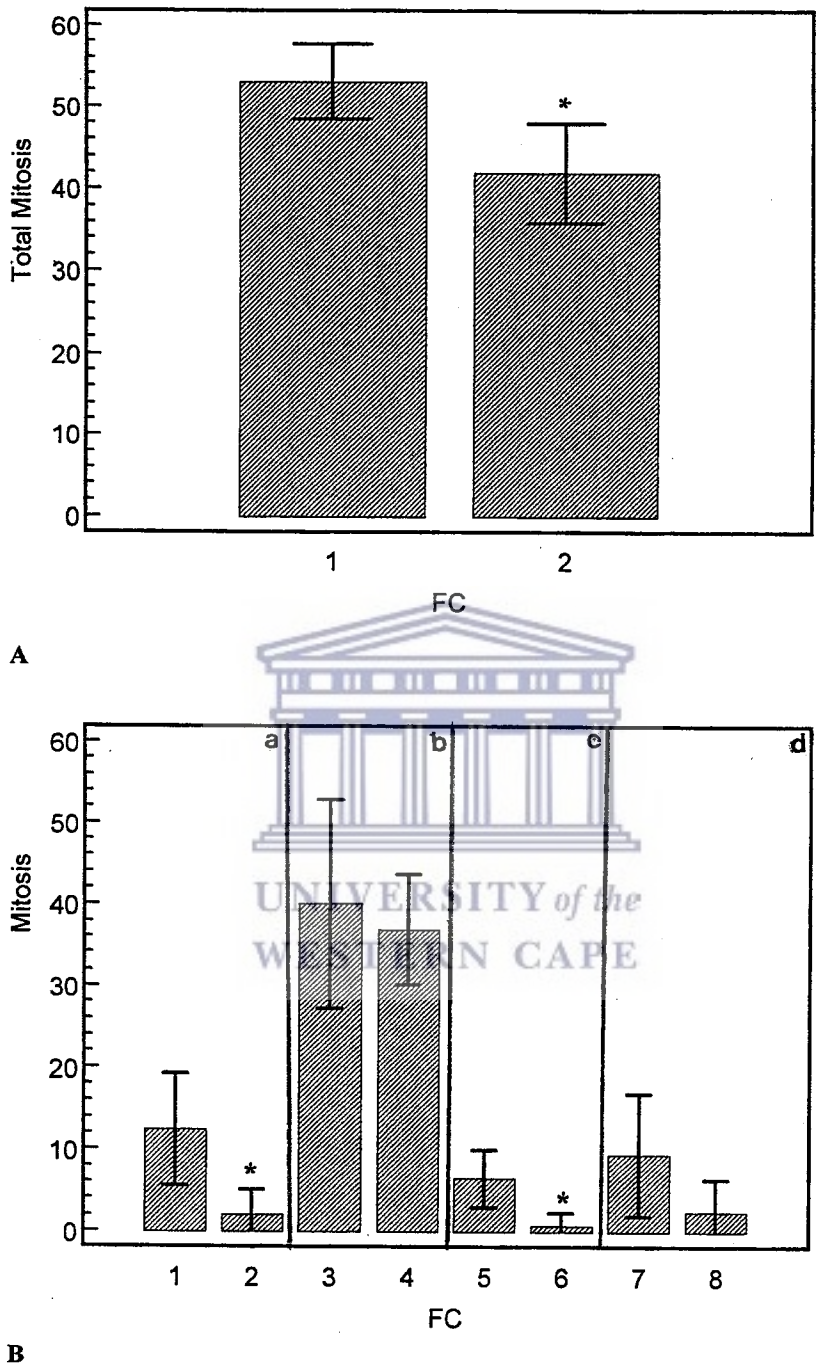


Fig 3.11 Quantification of the effects of PGA_2 (50µg/ml) on dividing WHCO3 cells exposed for 3 hrs are illustrated. (A) Total mitosis of control (FC=1) and cells exposed to 50µg/ml PGA_2 (FC=2). 50µg/ml PGA_2 caused a significant inhibition in the total mitotic cells after only 3 hrs; (B) A detailed breakdown of the effect of 50µg/ml PGA_2 on the different mitotic phases; (a) Prophase Control (FC=1), 50µg/ml (FC=2); (b) Metaphase Control (FC=3), 50µg/ml (FC=4); (c) Anaphase control (FC=5), 50µg/ml (FC=6); (d) Telophase control (FC=7), 50µg/ml (FC=8). Prophase and anaphase were significantly decreased after PGA_2 exposure with $p<0.05$ (*$p<0.05$). FC- Factor Codes.

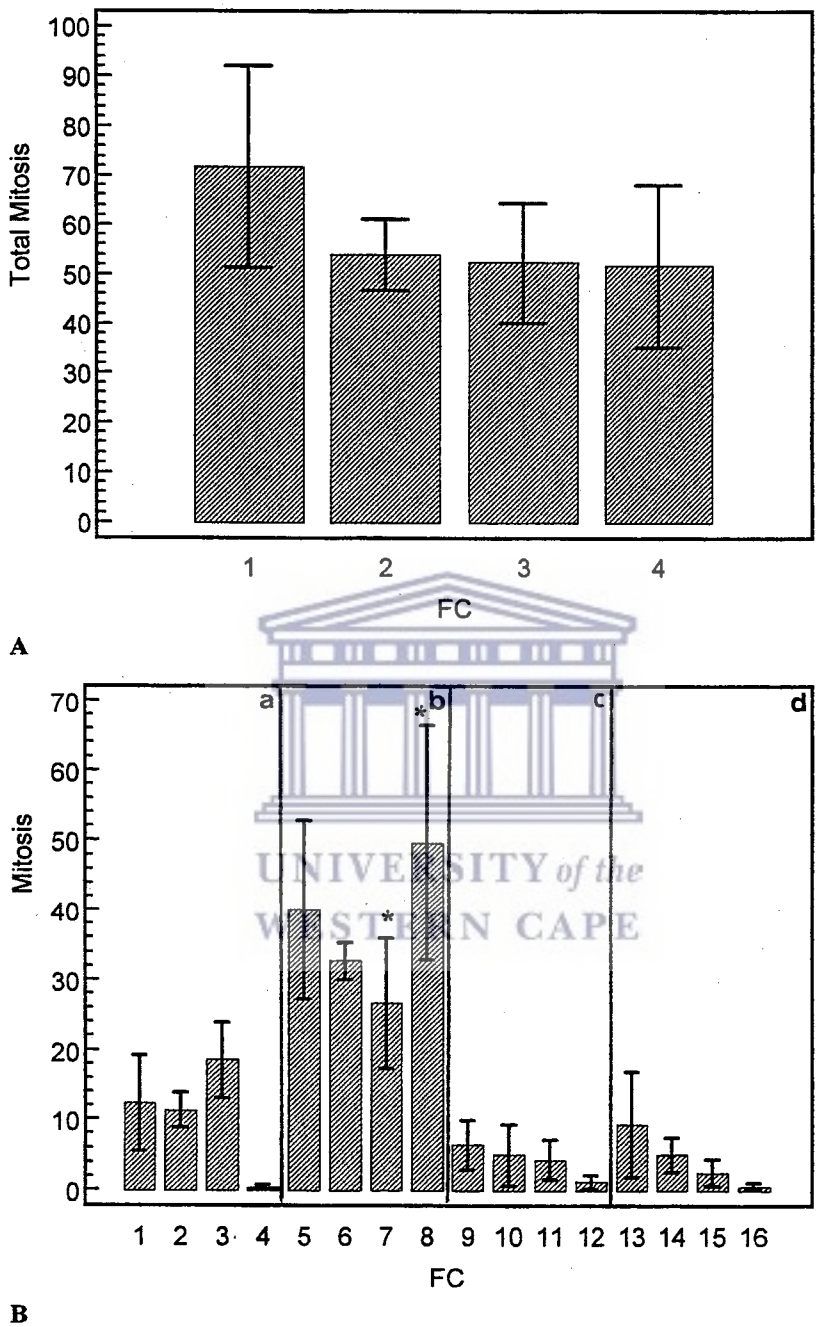


Fig **3.12** Quantification of the effects of PGA_2 on dividing WHCO3 cells exposed for 24 hrs are illustrated. (A) Total mitosis of control (FC=1) and cells exposed to 2μg/ml (FC=2), 5μg/ml (FC=3), and 20μg/ml (FC=4) PGA_2. No significant differences were observed; (B) A detailed breakdown of the effect of PGA_2 on the different mitotic phases; (a) Prophase Control (FC=1), 2μg/ml (FC=2), 5μg/ml (FC=3), 20μg/ml (FC=4); (b) Metaphase Control (FC=5), 2μg/ml (FC=6), 5μg/ml (FC=7), 20μg/ml (FC=8); (c) Anaphase Control (FC=9), 2μg/ml (FC=10), 5μg/ml (FC=11), 20μg/ml (FC=12); (d) Telophase Control (FC=13), 2μg/ml (FC=14), 5μg/ml (FC=15), 20μg/ml (FC=16). During metaphase a significant decrease was observed with 5μg/ml PGA_2 and a significant increase with 20μg/ml PGA_2 (* $p<0.05$).

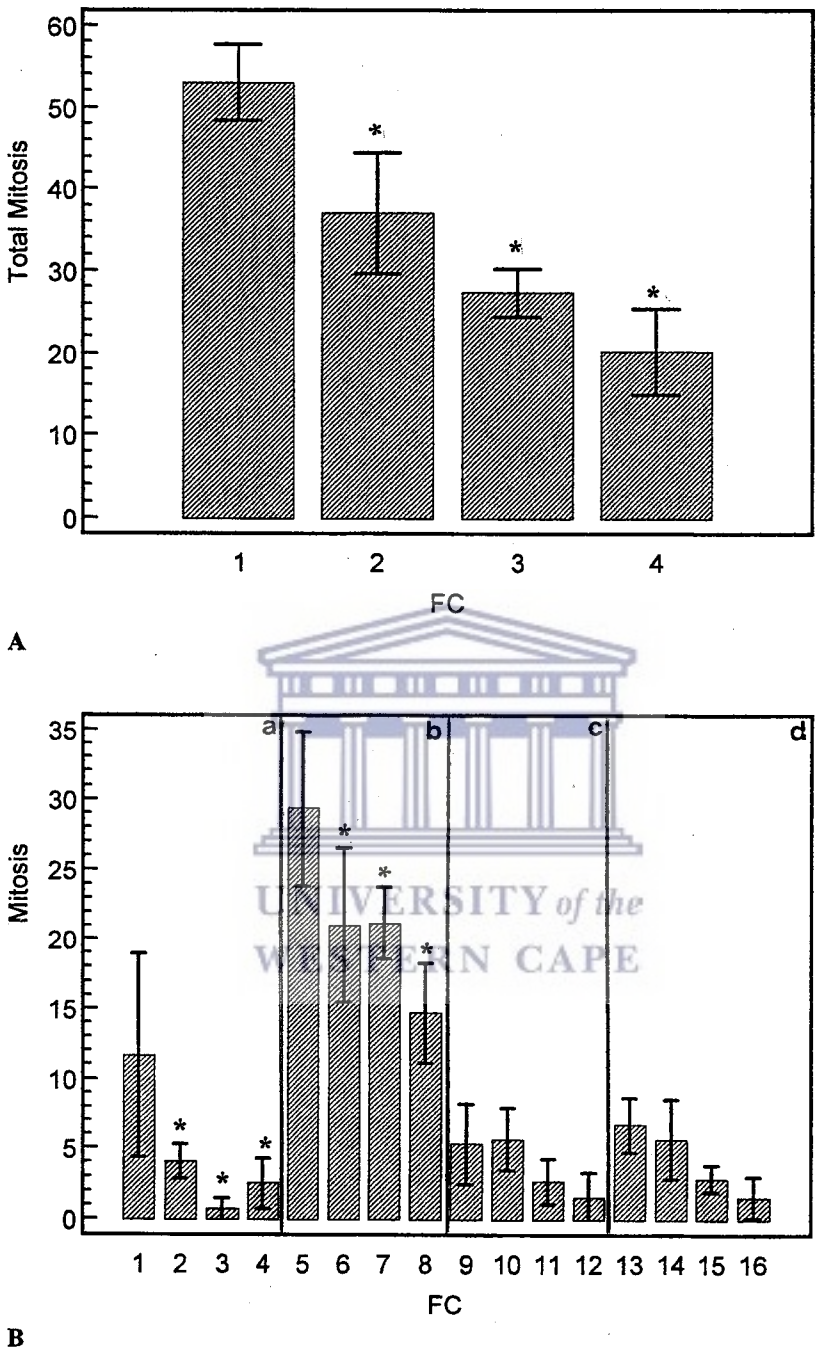
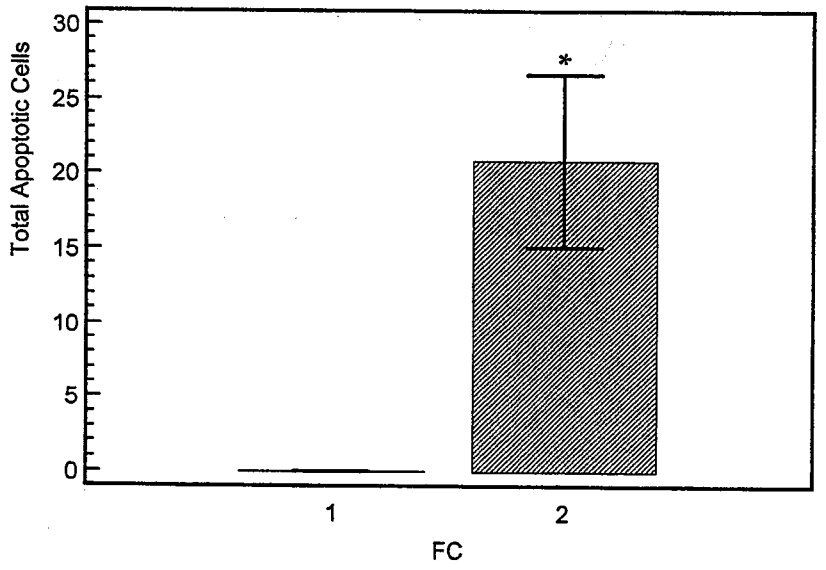
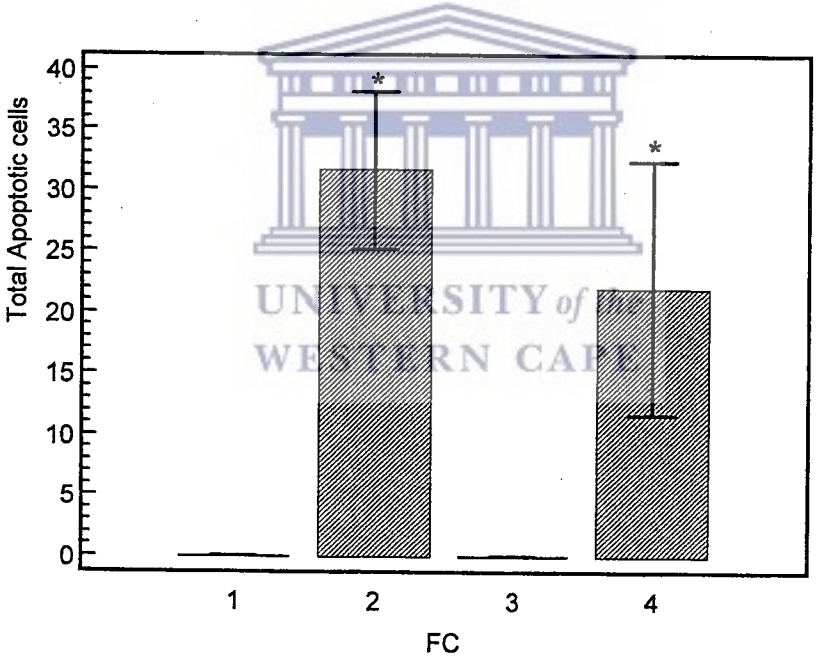


Fig **3.13** Quantification of the effects of PGA_2 on dividing WHCO3 cells exposed for 48 hrs are illustrated. (A) Total mitosis of control (FC=1) and cells exposed to 2μg/ml (FC=2), 5μg/ml (FC=3), and 20μg/ml (FC=4) PGA_2. All concentrations of PGA_2 caused a significant decrease in the total mitosis of WHCO3 cells; (B) A detailed breakdown of the effect of PGA_2 on the different mitotic phases; (a) Prophase Control (FC=1), 2μg/ml (FC=2), 5μg/ml (FC=3), 20μg/ml (FC=4); (b) Metaphase Control (FC=5), 2μg/ml (FC=6), 5μg/ml (FC=7), 20μg/ml (FC=8); (c) Anaphase Control (FC=9), 2μg/ml (FC=10), 5μg/ml (FC=11), 20μg/ml (FC=12); (d) Telophase Control (FC=13), 2μg/ml (FC=14), 5μg/ml (FC=15), 20μg/ml (FC=16). PGA_2 significantly decreased both prophases and metaphases after 48 hrs (*$p<0.05$).



A



B

Fig 3.14 Quantification of the number of apoptotic WHCO3 cells exposed to PGA_2 for 3, 24 and 48 hrs are illustrated. (A) 3 hrs: Control (FC=1), 50μg/ml (FC=2). A significant increase in apoptotic cells was observed. (B) 24hrs: Control (FC=1), 20μg/ml (FC=2), 48 hrs: Control (FC=3), 20μg/ml (FC=4). Both time intervals showed a significant increase in the number of apoptotic cells (*$p<0.05$).

3.3 CELL SYNCHRONIZATION

Mitotic indices (MI) were used to determine the duration of the S phase, G_2/M transition period and mitosis after the removal of the cell cycle block, hydroxyurea (Fig. 3.15). WHCO3 cells showed that for the first 6 hrs the MI were <2%, indicating that the cells were in the S phase. An increase in the MI was observed between 7 and 9 hrs after the release of the G_1/S block, representing a G_2/M transition period of approximately 3 hrs. A sharp increase in the MI 10 hrs after the release of the G_1/S block indicated the commencement of mitosis. A plateau was reached at 13 hrs.

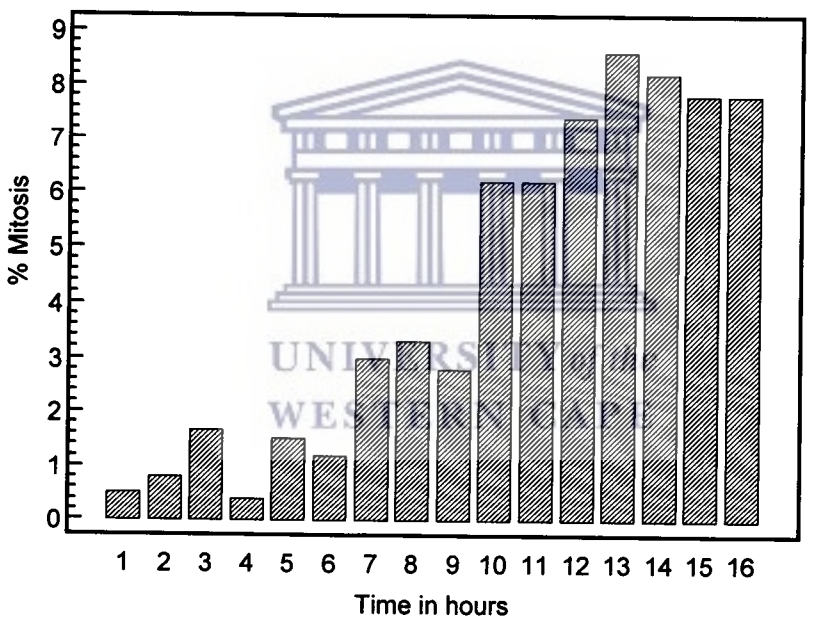


Fig. 3.15 Synchronized WHCO3 cells

3.4 IMMUNOBLOTTING (WESTERN BLOTTING)

Western blots were performed on synchronized as well as unsynchronized WHCO3 cells to see whether apoptosis in these cells is related to a downstream regulatory mechanism involving caspase 3 (Fig. 3.16). Caspase 3 is one of the key executioners that is activated during apoptosis (Cohen, 1997) and is a marker for cells undergoing apoptosis. Very little caspase 3 was expressed in cells exposed to 50µg/ml PGA_2 for 3 hrs in synchronized G_1 cells.

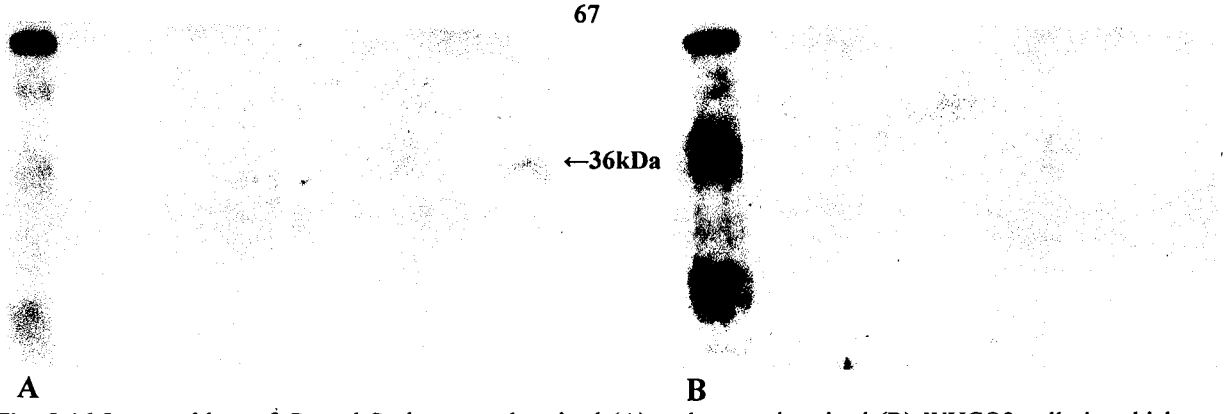


Fig. 3.16 Immunoblots of G_1 and S phase synchronized (A) and unsynchronized (B) WHCO3 cells in which equal amounts of proteins (20µg/slot) were electrophoresed. Synchronized WHCO3 cells were exposed to PGA_2 for 3 hrs only and unsynchronized for 24 hrs. In both immunoblots caspase 3 was not expressed in cells exposed to 2, 5 and 20µg/ml PGA_2. Caspase 3 was expressed in synchronized G_1 phase cells exposed to 50µg/ml PGA_2.

3.5 FLOW CYTOMETRY

To verify the results obtained in western blotting of the effects of PGA_2 on caspase 3 levels in WHCO3 cells, flow cytometry was carried out (Fig. 3.17 & 3.18). Cells were exposed to PGA_2 for 3 and 24 hrs. In each sample 10 000 cells were analyzed and expressed as a percentage of 100% gated events (Table 2). No marked differences in caspase 3 levels were seen in cells exposed to PGA_2 for 3 hrs (Fig. 3.17). After 24 hrs the higher concentrations (20 and 50µg/ml PGA_2) caused an increase in caspase 3 levels (Fig. 3.18 and Table 2).

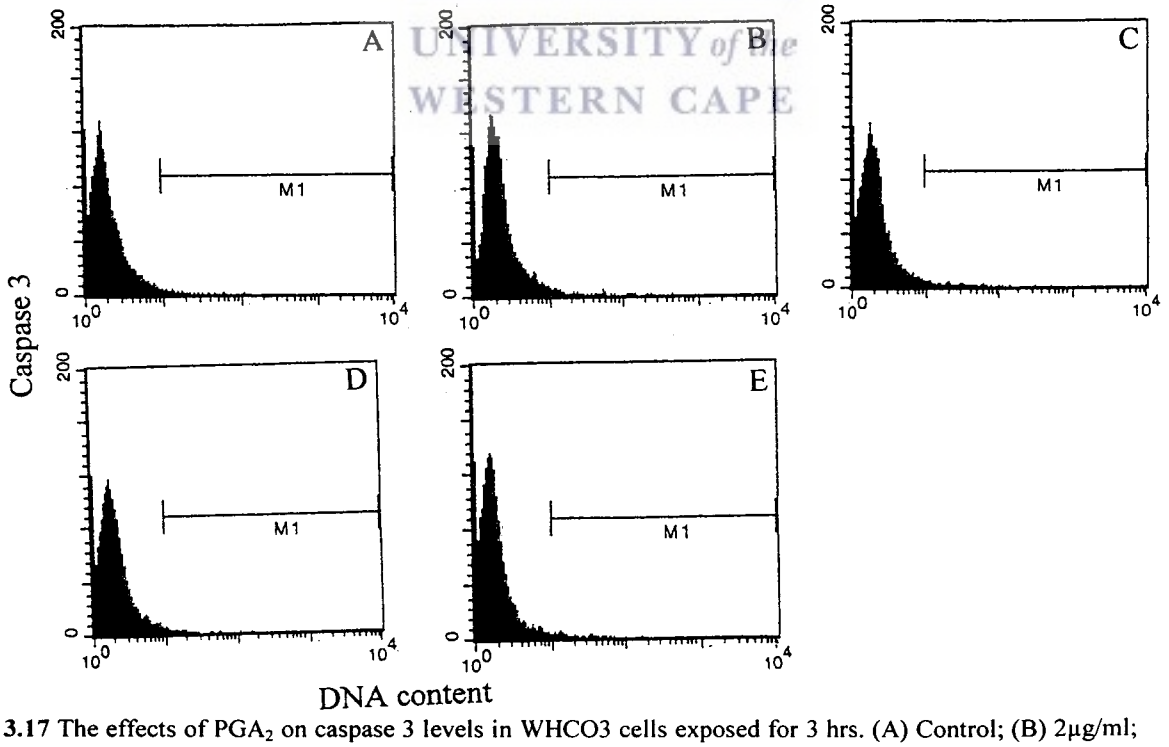


Fig. 3.17 The effects of PGA_2 on caspase 3 levels in WHCO3 cells exposed for 3 hrs. (A) Control; (B) 2µg/ml; (C) 5µg/ml; (D) 20µg/ml; (E) 50µg/ml. No significant differences were observed.

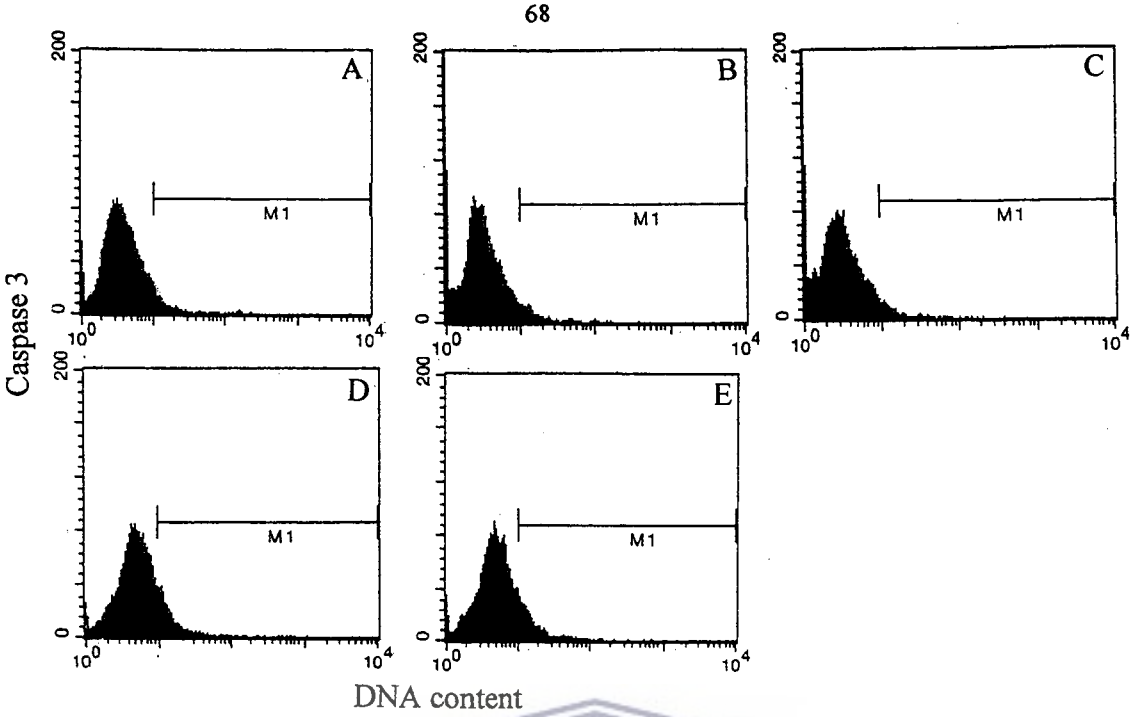


Fig. 3.18 The effects of PGA_2 on caspase 3 levels in WHCO3 cells exposed for 24 hrs. (A) Control; (B) 2µg/ml; (C) 5µg/ml; (D) 20µg/ml; (E) 50µg/ml. Cells exposed to the higher concentrations expressed an increase in caspase 3 levels (see Table 2).

Table 2: Flow cytometry results showing the effects of PGA_2 on caspase 3 levels in WHCO3 cells exposed for 3 and 24 hrs.

	PGA_2	M1 (% of 100% gated events)	Mean
3 hrs	Control	1.15	30.22
	2µg/ml	0.60	17.73
	5µg/ml	1.18	29.15
	20µg/ml	0.89	19.19
	50µg/ml	0.76	24.93
24 hrs	Control	4.42	18.51
	2µg/ml	4.10	18.47
	5µg/ml	3.57	18.99
	20µg/ml	12.66	19.15
	50µg/ml	13.19	17.75

At higher concentrations of PGA_2 (20 and 50µg/ml) for 24 hrs, caspase 3 was expressed which may be an indication of the apoptotic pathway used by WHCO3 cells during programmed cell death (Table 2).

Cell cycle analysis including apoptosis induction in WHCO3 cells were also studied with the aid of flow cytometry showing the DNA content of WHCO3 cells exposed to PGA_2 for 3, 24 and 48 hrs. The results of cells stained with PI are illustrated in Fig. 3.19-3.21. In each sample 10 000 cells were analyzed and expressed as a percentage of 100% gated events (Table 3). PGA_2 had little effect on the cycling WHCO3 cells exposed to 2, 5 and 20μg/ml for 3 hrs (Fig. 3.19B, C and D). These effects included small increases in the G_0/G_1 peak and slight decreases in the S phase. At 50μg/ml PGA_2, a very significant increase in G_2/M was observed (Fig. 3.19E), which could be an indication of an M phase arrest. At 24 hrs a marked increase in the number of cells at the sub G_0 peak was caused by the 20 and 50μg/ml PGA_2 which could be an indication of apoptotic cells (Fig. 3.20D and E). An increase of 18.28 and 28.93% respectively in the number of cells with DNA content at sub G_0 was seen compared to 0.48% in the control (Table 3). The marked increase in the number of apoptotic cells indicate that these doses of PGA_2 can be regarded as cytotoxic to WHCO3 cells. Apoptosis is frequently seen in untreated cells (Seegers, *et al.*, 1997) as observed in the controls especially at 3 hrs. The lower concentrations of PGA_2 (2 and 5μg/ml) showed an increase in the G_0/G_1 peaks (Fig. 3.20B and C) whereas a marked decrease was observed in cells exposed to 20 and 50μg/ml PGA_2 (Fig. 3.20D and E and Table 3). Increases in the S phases were seen in all treated WHCO3 for 24 hrs. During the morphological studies WHCO3 cells exposed to 20μg/ml PGA_2 for 24 hrs showed an increase of cells in metaphase (normal and abnormal) (Fig. 3.2D, E and F) and flow cytometry confirmed these results. This increase in cells in metaphase could be indicative of a metaphase block. Cells exposed to 20μg/ml PGA_2 for 24 hrs (Fig. 3.20D) showed a significant increase in cells in G_2/M phase. At 48 hrs (Fig. 3.21), slight differences in the sub G_0 cells were present at the lower concentrations (2 and 5μg/ml PGA_2) (Fig. 3.21B and C) whereas a marked increase of 15.88 and 51.63% (Table 3) of cells were observed at 20 and 50μg/ml PGA_2 (Fig. 3.21D and E) respectively compared to 0.80% in the control (Table 3). During G_0/G_1 phase a 3-fold decrease in cells exposed to 50μg/ml PGA_2 was observed (Fig. 3.21E and Table 3). No marked differences in the S and G_2M phases were seen (Table 3).

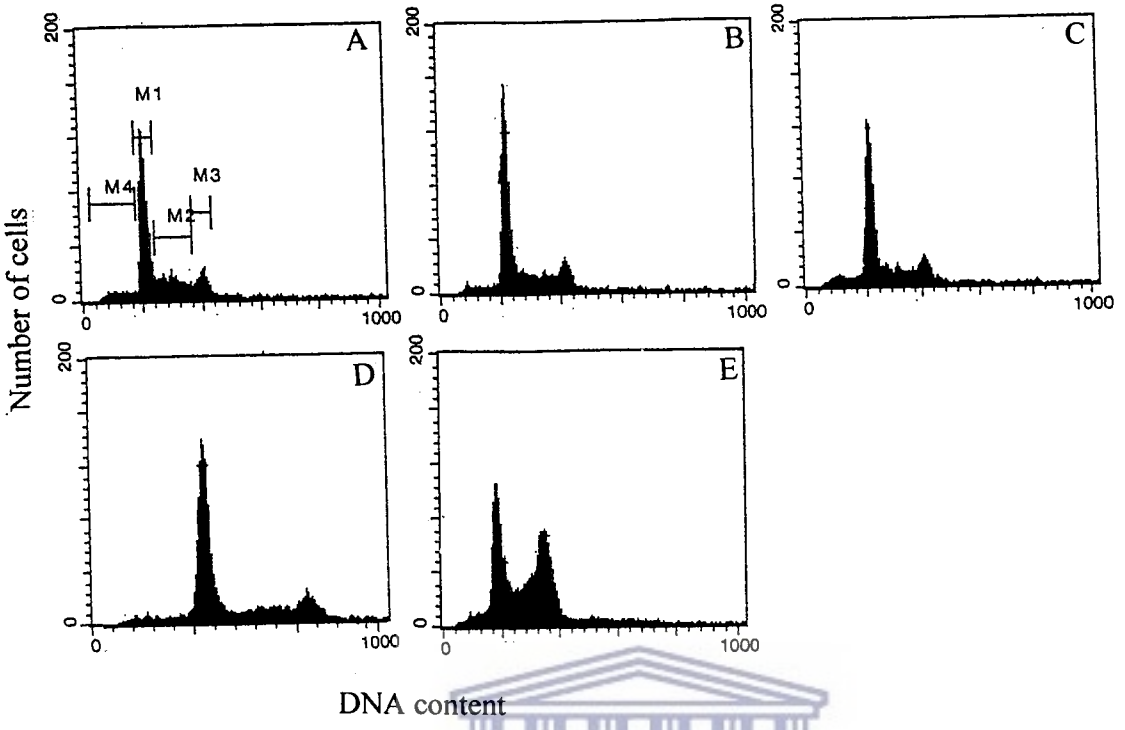


Fig. 3.19 DNA histograms showing the effects of PGA_2 on WHCO3 cells exposed for 3 hrs. (A) Control; (B) 2µg/ml; (C) 5µg/ml; (D) 20µg/ml; (E) 50µg/ml.

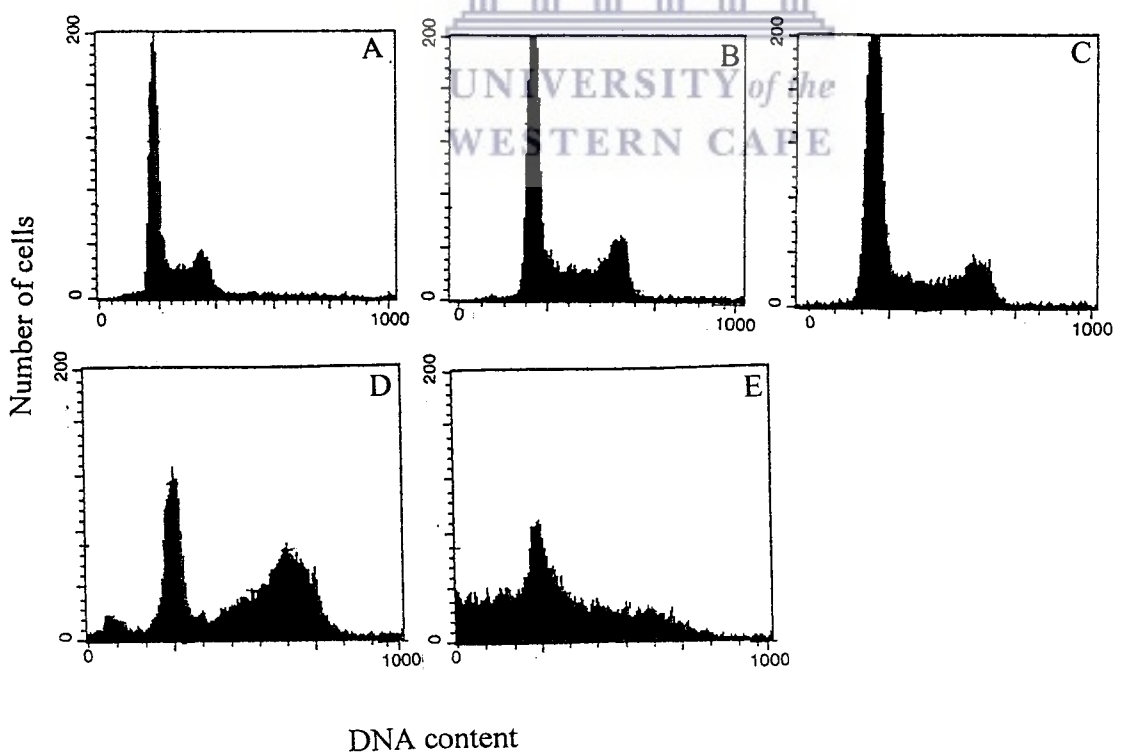


Fig. 3.20 DNA histograms showing the effects of PGA_2 on WHCO3 cells exposed for 24 hrs. (A) Control; (B) 2µg/ml; (C) 5µg/ml; (D) 20µg/ml; (E) 50µg/ml.

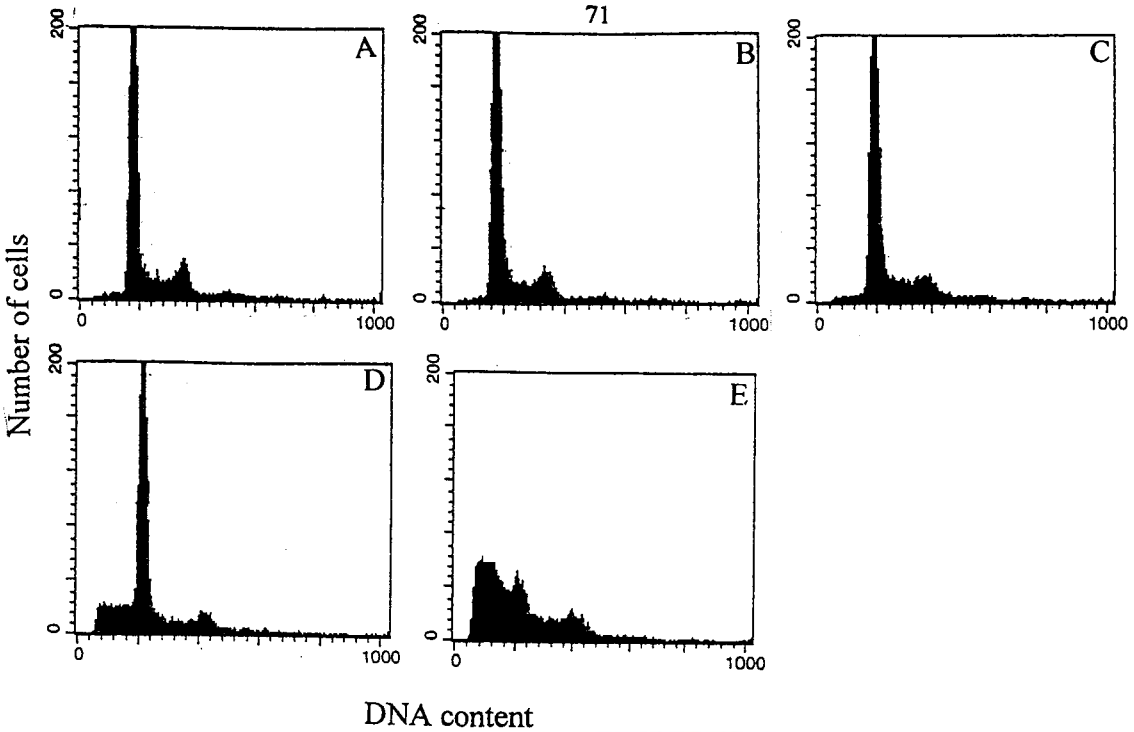


Fig. 3.21 DNA histograms showing the effects of PGA_2 on WHCO3 cells exposed for 48 hrs. (A) Control; (B) 2µg/ml; (C) 5µg/ml; (D) 20µg/ml; (E) 50µg/ml.

Table 3: Flow cytometry results showing the effects of PGA_2 (in µg/ml) on WHCO3 cells expressed as a % of 100% gated events for 3, 24 and 48 hrs.

	Cell cycle phases	Control	2µg/ml	5µg/ml	20µg/ml	50µg/ml
3 hrs	Sub G_0	6.18	4.26	6.04	5.38	4.09
	G_0/G_1	55.46	57.41	56.49	61.40	31.72
	S	21.03	18.85	17.82	15.24	20.15
	G_2/M	12.48	13.15	12.10	12.14	33.53
24 hrs	Sub G_0	0.48	0.77	0.82	18.28	28.93
	G_0/G_1	60.07	61.19	67.22	32.11	35.04
	S	15.70	15.76	16.80	20.16	21.67
	G_2/M	15.18	18.09	13.23	38.40	11.97
48 hrs	Sub G_0	0.80	0.48	1.12	15.88	51.63
	G_0/G_1	75.63	79.93	75.91	68.45	23.07
	S	10.17	7.11	9.36	6.54	10.53
	G_2/M	16.82	8.86	8.83	5.80	10.63

3.6 DNA FRAGMENTATION

The presence of apoptotic cells and abnormal chromosome condensation observed in H & E stained cells prompted further studies on PGA_2 induced DNA fragmentation using DNA electrophoresis.

Nuclear alterations are the greatest ultrastructural changes of apoptosis and are often associated with internucleosomal cleavage of DNA, recognized as a DNA ladder and long considered as a biochemical hallmark of apoptosis. WHCO3 cells treated with PGA_2 clearly revealed a typical ladder pattern of DNA fragments consisting of multiples of 180 bp (base pairs). In non-treated cells DNA fragments were not seen (Fig. 3.22). Cells were exposed to 20 and 50µg/ml PGA_2 only.

Fig 3.22 DNA fragmentation of WHCO3 cells by PGA_2 in which equal amounts of DNA (40µl per slot) were electrophoresed. Controls show no DNA fragmentation (lanes 1 and 3) while DNA fragmentation is observed at 20µg/ml for 24 hrs (lane 4) and 50µg/ml for 3 hrs (lane 2). A decrease in DNA fragmentation in the 20µg/ml (lane 4) is observed. 1=Control 3 hrs; 2=50µg/ml 3 hrs; 3=Control 24 hrs; 4=20µg/ml 24 hrs.

CHAPTER 4
DISCUSSION

The anti-proliferative effects of EFAs (AA and GLA) have been studied in a variety of human tumor cells *in vitro*. PGA_2 has been shown to exhibit anti-mitotic activity *in vivo* as well as *in vitro* (Joubert *et al.*, 1999) with characteristic morphological and biochemical features of apoptosis (Ahn *et al.*, 1998). PGs derived from PUFAs may inhibit proliferation of human and animal tumor cell lines and/or cause reverse transformation of transformed cells (Bégin *et al.*, 1985).

Investigation of PGA_2 treated WHCO3 cells for basic morphology showed that PGA_2 inhibited mitosis in a time and dose dependant manner (Fig. 3.11-3.13), caused chromatin condensation (Fig. 3.2D, 3.3B and 3.4D) as well as formation of apoptotic bodies (Fig. 3.1B, 3.2D, 3.3B and 3.4D). Chromatin condensation and apoptosis were more prevalent in WHCO3 cells exposed to 20µg/ml PGA_2 for 24 hrs and apoptosis induction more evident in the 50µg/ml PGA_2 treated cells. An increase in abnormal metaphases was observed in 20µg/ml PGA_2 treated cells for 24 hrs, which could activate the spindle assembly checkpoint. One of the functions of this checkpoint is to ensure that all chromosomes are attached to the spindle and aligned on the equatorial plane of the spindle to from a metaphase plate, thereby delaying anaphase until this has occurred. The abnormal metaphases obtained in this study show mostly circular metaphases (Fig. 3.2E) with sometimes lagging chromosomes, and therefore chromosomes are not aligned on the metaphase plate as should be. Cells are arrested in mitosis and it is possible that the maintained activity of the spindle checkpoint leads to the inhibition of anaphase onset and subsequent cell division in response to the metaphase arrest. When this checkpoint is activated it blocks anaphase (Gorbsky, 2001) and arrests cells in metaphase (Bunz *et al.*, 1998). This could occur when unattached kinetochores are present, if the tension on chromosomes is unbalanced (Bunz *et al.*, 1998; Glotzer, 1996) or if there is abnormal dynamic behavior of the microtubules attached to the kinetochore (Bunz *et al.*, 1998; Glotzer, 1996; Murray, 1994). Cells exposed to 20µg/ml PGA_2 show the accumulation of cells with condensed chromosomes (Fig. 3.2D), which is characteristic of a sustained

mitotic block (Cahill *et al.*, 1998). If during this checkpoint there is failure to repair the damage, cells die by means of apoptosis.

The mitotic arrest that was observed in 20µg/ml PGA_2 treated cells for 24 hrs was confirmed with flow cytometry (Fig. 3.20D) as well as the studies determining the MI (Fig. 3.12B). A mitotic arrest was observed in cells exposed to 50µg/ml PGA_2 for 3 hrs as well (Fig. 3.19E) and morphological studies showed circular metaphases (Fig. 3.1B). During flow cytometry, the G_2 and M phases are combined (G_2/M phase) (Fig. 3.19-3.21), thus the increase in the G_2/M phase could therefore also indicate the M phase arrest. This cell cycle arrest in M phase can be induced even if one of the many chromosomes fails to properly attach to the mitotic spindle and align at the metaphase plate.

The spindle assembly checkpoint also monitors for malfunctions such as DNA damage. Detection of defects leads to cell cycle arrest and if the damage is severe, apoptosis ensues (Gorbsky, 1997). The higher (20 and 50µg/ml) concentrations of PGA_2 possibly induced fatal DNA damage, which resulted in an M phase arrest that subsequently leads to apoptosis induction in these cells (Fig. 3.1B, 3.2D, 3.3B, 3.4D, 3.14, 3.20D and E and 3.21D and E). This arrest was upheld for 24 hrs. However, prolonged (48 hrs) exposure of WHCO3 cells to 20µg/ml PGA_2 showed a decrease in apoptosis (Fig. 3.14B and Table 3) and it appears that the cells were able to overcome the arrest (Fig. 3.21D). This could be the result of checkpoint adaptation in which cells have the ability, after some time, to overcome the checkpoint and progress into interphase, even without proper solution to the problem (Gorbsky, 1997). The decrease in apoptotic cells could be ascribed to the fact that PGA_2 activate a p53 dependent apoptotic pathway after prolonged exposure (Joubert *et al.*, 1999). It has been stated that p53 is a negative regulator of bcl-2 expression. The oncogene-like role of bcl-2 has the ability to prolong the survival of non-cycling cells and prevent cell death by apoptosis of cycling cells (Joubert *et al.*, 1999). Seegers *et al.*, 2000 also found enhanced p21 expression in WHCO3 cells, which protect tumor cells from the anti-proliferative effects of PGA_2. Also, $p34^{cdc2}$ kinase is essential for the movement of cells into mitosis and it is possible that the cells are kept in an M phase state by high $p34^{cdc2}$ kinase activity. So this could be possible explanations for the results found in WHCO3 exposed to 20µg/ml PGA_2 for 48hrs. The lower

concentrations (2 and 5μg/ml) of PGA_2 caused no significant differences during the 24 hrs exposure (Fig. 3.12A). It could be suggested that the lower concentrations of PGA_2 caused a reversible M phase arrest during which DNA damage can be repaired and cells continue in the cell cycle. When the cells were exposed for 48 hrs a significant decrease in cell viability was observed (Fig. 3.13A) proving that the effects of PGA_2 was time and dose dependent.

Indirect immunofluorescence of α-tubulin demonstrated abnormalities in the cytoskeleton (Fig. 3.5C, 3.6B-D). Cells exposed to 20μg/ml PGA_2 for 24 hrs appear to display fewer but intact microtubules (Fig. 3.5B). Disrupted microfilaments prevent the formation of apoptosis (Cotter *et al.*, 1992), therefore our observation indicates that apoptosis development, which is apparently dependent on intact microfilaments, was increased in WHCO3 exposed to 20μg/ml PGA_2 for 24 hrs as was found during the morphological studies (Fig. 3.3B) and the mitotic indices study of mitosis (Fig. 3.14B) as well as flow cytometry (Fig. 3.20D and Table 3). The results obtained from cells exposed to 20μg/ml PGA_2 for 48 hrs show that the cytoskeleton of cells was affected differently. Some cells showed normal spindle formations whilst others display abnormal microtubule arrangement (Fig. 3.6). The latter could possibly prevent the formation of apoptosis (Cotter *et al.*, 1992). It is therefore possible that the cells with the abnormal microtubule arrangement (Fig. 3.6C) were prevented from undergoing apoptosis and this could explain the decrease in apoptotic formation reflected in the morphological studies and the quantification of mitotic indices (Fig. 3.14B). The presence of some normal spindles could explain the absence of an M phase arrest when cells were exposed to 20μg/ml PGA_2 for 48 hrs. In 50μg/ml PGA_2 total rearrangement of microtubules were very much evident and this rearrangement could be a demonstration of apoptosis as indicated by the large amount of apoptotic cells (28.93 and 51.63% during 24 and 48 hrs respectively) during flow cytometry (Fig. 3.20E, 3.21E and Table 3). $p34^{cdc2}$ is associated with centrosomes and perhaps phosphorylation of centrosomal proteins by $p34^{cdc2}$ kinase could have a role in the rearrangement of the microtubular cytoskeleton at mitosis (Gorbsky, 2001; Nurse, 1990).

The effect of PGA_2 on the localization of nucleolar fibrillarin with the aid of silver staining is illustrated in fig. 3.9-3.10. At the lower concentrations (2 and 5μg/ml) of PGA_2 treated cells, nucleolar material and fibrillar phosphoproteins started to segregate (Fig. 3.10B and C). At

20µg/ml further segregation of granular and fibrillar components with decreased silver staining in the nucleoli were observed (Fig. 3.10D). Decreased (Fig. 3.9B) to non-existing (Fig. 3.10E) silver stained nucleoli were present in 50µg/ml PGA_2 exposed for 3 and 24 hrs respectively. The fact that nucleolar morphology was affected by PGA_2 causing segregated fibrillar and granular components is indicative that protein synthesis is inhibited in these cells and that the cells now have non-functional nucleoli (Hadjiolov, 1985).

In contrast to control WHCO3 cells, PGA_2 treated cells showed a decrease in silver staining mainly in intact nucleoli and this pointed to less active transcription in the NORs of the nucleoli and no or very little silver staining in the nucleoplasm indicating a decrease in the degree of rRNA production which indicates a decreased ribosome production and subsequently decreased protein synthesis in PGA_2 exposed cells. All of this indicates that the PGA_2 treated cells are metabolically more inactive than the untreated controls (Hadjiolov, 1985).

The quantification of the morphological features of WHCO3 cells exposed to PGA_2 is illustrated in Fig. 3.11-3.14. A significant dose responsive decrease in cell growth was observed. Previous studies done by Joubert *et al.*, 1999, showed that PGA_2 inhibited cell growth substantially in WHCO3 cells. In this study again it has been shown that PGA_2 exhibited anti-mitotic activity. Morphological changes in H & E and silver stained PGA_2 treated WHCO3 cultured cells indicated cells that were metabolically more inactive compared to the untreated controls. Decreased silver staining of the nucleoli pointing to less active transcription in the NORs, decreased rRNA and ribosome production which is in accord with decreased protein synthesis, abnormal metaphases and apoptosis induction were all observed in WHCO3 cells exposed to the higher concentrations (20 and 50µg/ml PGA_2). WHCO3 cells showed changes in the morphology of the nucleoli after PGA_2 supplementation and these changes coincide with the decrease in the mitotic indices observed (Fig. 3.11-3.14). Joubert *et al.*, 1999, proved that WHCO3 cells are very susceptible to the anti-mitotic effects of PGA_2 and that this susceptibility may be explained by the fact that they are less differentiated.

To identify which molecular processes are involved during apoptosis in WHCO3 cells, the effects on signal transduction pathways of specific proteins involved in control of cell cycle progression and apoptosis induction was envisaged. In the present study we demonstrated that WHCO3 cells incubated with cytotoxic amounts of PGA_2, showed many of the fundamentally important signs of apoptosis (such as chromatin condensation, DNA fragmentation and cytoplasmic blebbing), indicating that the cytotoxicity is mediated via an endogenous apoptotic pathway (Kim *et al.*, 1993).

The results demonstrate here that PGA_2 could induce apoptosis by the activation of caspase 3 (Fig. 3.16). Cells were synchronized with hydroxyurea and exposed to PGA_2 for 3 hrs. Caspase 3 was expressed only in 50µg/ml PGA_2 treated cells. Concentrations of 2, 5 and 20µg/ml PGA_2 treated cells exposed for 3 hrs did not induce apoptosis as demonstrated by the above experiments and therefore no caspase 3 was expressed. Fig. 3.16 shows that a single protein of ~36kDa reacted with the antibody only in the 50µg/ml PGA_2 treated cells in the synchronized G_1 phase. Unsynchronized cells show no expression of caspase 3. Flow cytometry was performed to verify these results (Fig. 3.17). However, the WHCO3 cells exposed to PGA_2 for 3 hrs (Fig. 3.17) show no expression of caspase 3 but after 24 hrs (Fig. 3.18) levels of caspase 3 increased during 20 and 50µg/ml PGA_2 (12.66 and 16.19% respectively). This corresponds with the results obtained as described above that apoptosis is induced at these concentrations and exposure time.

DNA fragmentation was also examined which is considered to be the hallmark of apoptosis (Ahn *et al.*, 1998) and was clearly induced by PGA_2 at the higher concentrations (20µg/ml for 24 hrs) and (50µg/ml for 3 hrs) as expected (Fig. 3.22) and therefore verifies apoptosis induction in WHCO3 cells by the higher concentrations. Caspase 3 is required for internucleosomal DNA degradation and chromatin condensation in cultured cells in which apoptosis occurred (Earnshaw *et al.*, 1999; Porter and Jänicke, 1999). The degradation of nuclear enzymes involved in DNA repair that results in their loss of function may assist the endonucleosomal cleavage of DNA that is characteristic of apoptotic cells (Harvey and Kumar, 1998; Wylie, 1997).

Other apoptotic events, which were found in this study, include chromatin condensation (Fig. 3.2), during which lamins are cleaved at a single site by caspase 3 causing lamina to collapse and contributing to chromatin condensation (Thornberry and Lazebnik, 1998), membrane blebbing, cell shrinkage and disassembly into membrane bound vesicles (apoptotic bodies) (Fig. 3.1, 3.2, 3.3 & 3.4) and caspase 3 is suggested to be responsible for these cellular changes that occur during apoptosis (Thornberry and Lazebnik, 1998). Caspases also part take in apoptosis in a manner reminiscent of a well-planned operation. They cut off contacts with surrounding cells, reorganize the cytoskeleton (Fig. 3.5 & 3.6), shut down DNA replication and repair, destroy DNA (Fig. 3.22) and disrupt the nuclear structure (Fig. 3.9-9.10) (Thornberry and Lazebnik, 1998). Multiple caspases may have a redundant function or act in concert to execute the apoptotic process in a cell-specific and stimulus dependent manner thereby contributing to the complex role of caspases and mammalian apoptosis (Nuñez *et al.*, 1998).

This study confirms that PGA_2 (an endogenous human metabolite) has potent anti-mitotic and cytotoxic effects in WHCO3 cells. The fact that PGA_2 inhibits cell growth in WHCO3 cells supports the hypothesis that the presence of a reactive α,β-unsaturated carbonyl group in the cyclopentane ring of this molecule is the cause for the anti-proliferative activity (Bregman *et al.*, 1986; Fukushima and Kato, 1984; Honn and Marnett, 1985; Narumiya and Fukushima, 1986; Santoro *et al.*, 1986; Sasaki and Fukushima, 1994).

CONCLUSION

The anti-mitotic effect of endogenous EFAs and their metabolites have been studied in a number of human tumor cells *in vitro* and PGA_2 has been shown to cause anti-proliferative activity (Joubert *et al.*, 1999), as well as to induce apoptosis in tumor cells (Das, 1999).

In this study it could be shown that PGA_2 inhibited cell growth significantly in WHCO3 cells. Different morphological effects were observed in cells after PGA_2 exposure and may be linked to apoptosis development. Cytoplasmic blebbing, chromatin condensation, DNA fragmentation and rearrangement of the cytoskeleton are observed in the higher concentrations (20 and 50µg/ml) of PGA_2 in a dose responsive manner. It appears that WHCO3 cells exposed to 20 µg/ml PGA_2 for 24 hrs exhibited the most pronounced effects. A significant M phase arrest was observed after the above treatment and could activate the spindle assembly checkpoint. The M phase arrest could be due to unattached chromosomes to microtubules, unbalanced tension on chromosomes or abnormal dynamic behaviour of microtubules attachment to kinetochores. The increased concentrations of PGA_2 also induced fatal DNA damage, which resulted in cells dying through the process of apoptosis. However, a decrease in apoptosis was observed after 48 hrs when WHCO3 cells were exposed to 20µg/ml PGA_2. It appears that the cells were able to overcome the arrest. It could be said that the cells adapted the ability to overcome the checkpoint and progress into interphase, even without fixing the problem (Gorbsky, 1997). The decrease in apoptotic cells could be due to PGA_2 activating a p53 dependent apoptotic pathway after prolonged exposure. It has been stated that p53 is a negative regulator of *bcl*-2 expression. The oncogene-like role of *bcl*-2 has the ability to prolong the survival of non-cycling cells and prevent cell death by apoptosis of cycling cells (Joubert *et al.*, 1999). It has been found that enhanced p21 expression in WHCO3 cells protect tumor cells from the anti-proliferative effects of PGA_2 (Seegers *et al.*, 2000). Lower concentrations of 2 and 5µg/ml PGA_2 did not adversely affect WHCO3 cells. Nucleolar morphology was affected in WHCO3 cells as well, causing segregated fibrillar and granular components. This shows that protein synthesis may be inhibited in the PGA_2 treated cells with non-functional nucleoli (Hadjiolov, 1985). Indirect immunofluorescence showed disruption of microfilaments at 48 hrs when cells were exposed to 20µg/ml PGA_2. This could also account

for the decrease in apoptosis found which corresponds to Cotter *et al.*, 1992 that disruption of the microfilaments prevents apoptosis. The rearrangement of the microtubules found at 50μg/ml PGA_2 might be a manifestation of apoptosis. Apoptosis induction is thought to be caspase 3 dependent as was found during western blotting and flow cytometry results. It is suggested that caspase 3 participate in apoptosis in a manner reminiscent of a well-planned and executed military manner. They cut off contacts with surrounding cells, reorganize the cytoskeleton, destroy the DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis and disintegrate the cell into apoptotic bodies (Thornberry and Lazebnik, 1998) as was surely found in this study.

Cancer therapy is dominated by the idea that treatment must aim at destruction of cancer cells. The goal is to exploit some differences between the cancer cells and the normal cells, which will allow the cancerous ones to be selectively destroyed. The above study demonstrates the ability of an EFA (PGA_2) to prevent the formation, inhibit the progression, and directly kill cancer WHCO3 cells. The anti-proliferative activity against tumor cells consists of the cyclopentenone PGs containing a α-β unsaturated ketone (Bregman *et al.*, 1986; Fukushima and Kato, 1984; Honn and Marnett, 1985; Narumiya and Fukushima, 1986; Santoro *et al.*, 1986; Sasaki and Fukushima, 1994). Also, the control mechanisms of mitosis may be altered in tumor cells, which may also explain the EFA-induced anti-mitotic and cytotoxic effects. Identifying the altered control mechanisms may therefore aid in understanding why EFAs inhibit mitosis and/or cause cytotoxic effects in malignant cells, but not normal cells (Begin *et al.*, 1985).

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