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THE ROLE OF PROSTAGLANDINS IN THE ANTI-APOPTOTIC ACTION OF TRANSFORMING GROWTH FACTOR ALPHA IN HEN GRANULOSA CELLS

by

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2. ABSTRACT

Most (> 99 %) ovarian follicles will succumb to atresia via a process known to involve apoptosis of granulosa cells. TGFα is mitogenic in hen granulosa cells and prostaglandins have been shown to mediate the actions of this growth factor in several cell types. Since most follicular atresia in the hen ovary occurs at the prehierarchal stage of follicular development (follicular diameter < 9 mm) when granulosa cells are less responsive to gonadotropins, the role of non-gonadotropin survival factors (e.g. TGFα) may be crucial in determining the eventual fate of prehierarchal follicles (atresia vs. ovulation).

Hematoxylin and eosin stained histological sections of healthy prehierarchal follicles (2-6 mm) appeared to be morphologically unspoiled, round, with intact layers of granulosa cells. In contrast, same size atretic follicles appeared to be morphologically spoiled, with a diminished yolk space containing detached granulosa cells. *In situ* 3'-end labelling of DNA in healthy follicles failed to display fragmented DNA in the granulosa or theca layers. However, granulosa cells of atretic follicles stained intensely for fragmented DNA, whereas theca layers showed little or no DNA fragmentation, consistent with the notion that ovarian follicular atresia is linked to apoptosis of granulosa, but not theca cells *in vivo*.

An *in vitro* cell culture system was used to investigate if TGFα suppresses granulosa cell apoptosis and whether prostaglandins (PGs) mediate the anti-apoptotic action of TGFα. Granulosa cells cultured in medium containing 10 % fetal bovine serum (FBS) showed no morphological or biochemical signs of apoptosis. In contrast, granulosa cells cultured in serum-free media underwent

spontaneous apoptosis as evident by DNA fragmentation and displayed morphologic features characteristic of apoptosis, including cellular shrinkage and rounding, membrane blebbing, fragmentation of cells into apoptotic bodies, chromatin condensation and nuclear fragmentation. TGFα inhibited the serum deprivation-induced apoptotic DNA fragmentation in a concentration-dependent manner, with a complete inhibition detectable at 20 ng TGFα/ml. Furthermore, cells cultured in the presence of TGFα appeared morphologically healthy.

Granulosa cell prostaglandin synthesis (PGE and PGF), determined by radio immuno assay, was increased by TGFα in a concentration-dependent manner. Maximum stimulation of PGE (2.5 fold) and PGF (4.5 fold) secretion was noted at 20 ng TGFα/ml. Inhibitors of cyclooxygenase (COX; NS398 and ibuprofen) and phospholipase A_2 [PLA_2; aristolochic acid (AC) and arachidonyl triflouro methyl ketone (TFMK)], markedly suppressed the TGFα-induced PG synthesis and induced significantly more apoptotic DNA fragmentation than control (no inhibitor), indicating that prostaglandins mediate the anti-apoptotic action of TGFα. The apoptotic effect of the COX inhibitor NS398, and PLA_2 inhibitor AC could be completely prevented by exogenous PGs ($PGF_{2\alpha}$, PGE_1, PGE_2) and arachidonic acid, respectively. Exogenous PGs, however, failed to prevent the PLA_2 inhibitor (AC)-induced apoptotic DNA fragmentation, implying that in addition to PGs, arachidonic acid, leukotrienes and/or arachidonic acid metabolites of the cytochrome P450 pathway (i.e. HETEs or EETs) may be important in mediating the anti-apoptotic action of TGFα.

In conclusion, these findings suggest that granulosa cell viability *in vitro* is dependent on the continual supply of survival/growth factors (e.g. serum, TGFα) and that removal of such factors spontaneously induces apoptosis. Prostaglandin

synthesis is a necessary, but not sufficient, event in the suppression of granulosa cell apoptosis by TGFα. Whether arachidonic acid, leukotrienes and/or arachidonic acid metabolites of the cytochrome P450 pathway are important in the anti-apoptotic action of TGFα in hen granulosa cells remains to be determined.

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4. LIST OF ABBREVIATIONS

$\alpha[^{32}P]$-dCTP	deoxy cytosine tri-phosphate
AC	aristolochic acid
AGE	agarose gel electrophoresis
bFGF	basic fibroblast growth factor
bp	base pair
cAMP	3',5' cyclic adenosine monophosphate
cGMP	3',5' cyclic guanosine monophosphate
Ci	curie
COX	cyclooxygenase
COX I	cyclooxygenase I
COX II	cyclooxygenase II
$cPLA_2$	cytosolic phospholipase A_2
cpm	counts per minute
DAB	diaminobenzidine tetrahydrochloride
dCTP	deoxycytidine 5'-triphosphate
DD	death domain
DFF	DNA fragmentation factor
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNase I	deoxyribonuclease I
dUTP	deoxyuridine 5'-triphosphate
ED_{50}	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
EETs	epoxyeicosatrienoic acids
EGF	Epidermal growth factor
FBS	fetal Bovine Serum
FSH	follicle stimulating hormone
GH	growth hormone
GLB	gel loading buffer
GnRH	gonadotropin releasing hormone
H&E	hematoxylin and eosin
hCG	human chorionic gonadotropin
HEPES	hydroxyethylpiperazine ethanesulfonic acid
HETEs	monohydroxyeicosatetraenoic acids
HRP	horseradish peroxidase
ID_{50}	half maximal inhibitory concentration
ICE	interleukin-1β-converting enzyme
IGFs	insulin-like growth factors

IGF-I	insulin-like growth factor-I
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGFBPs	insulin-like growth factor binding proteins
IL-1β	interleukin-1β
IL-6	Interleukin-6
ISEL	in situ 3'-end labelling
kb	kilo base
LH	leutininzing hormone
LMW	low molecular weight
LWF	large white follicle
MAPK	mitogen activated protein kinase
MEM	minimum essential medium
mRNA	messenger RNA
NBF	neutral buffered formalin
NDGA	nordihydroguaiaretic acid
NO	nitric oxide
NSB	non specific binding
ONO-RS-82	2-p-amylcinnamoyl amino-4-chlorobenzoic acid
PARP	poly(ADP)-ribose polymerase
PBS	phosphate buffered saline
PBSG	phosphate buffered saline-gelatin
PC	phosphatidylcholine
PG	prostaglandin
PGE	prostaglandin E
PGF	prostaglandin F
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGs	prostaglandins
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A_2
PPAR	peroxisome proliferator-activated receptor
PS	phosphatidylserine
RIA	radio immuno assay
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
SCC	side chain cleavage
SCF	stem cell growth factor
SEM	standard error of mean

SREBPs	sterol regulatory element binding proteins
TdT	terminal transferase
TFMK	arachidonyl trifloro methyl ketone
TGFα	transforming growth factor alpha
TNFα	tumour necrosis factor alpha
UI-snr	UI-small nuclear ribonucleoprotein
VIP	vasoactive intestinal peptide
vs	verses

5. LIST OF FIGURES

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8. INTRODUCTION

The primary function of the ovary is to deliver viable oocytes which can subsequently be fertilised to ultimately result in the birth of offspring. Oocytes are housed in follicles that have to develop from the primordial (smallest) to the preovulatory (largest) before ovulation. As ovarian follicles develop, the majority (> 99 %) become atretic and regress before reaching 9 mm in diameter (in hens) or before developing past the early antral stage (in mammals). However, for yet unknown reasons, once the ovarian follicle develops beyond the 9 mm size in hens (Johnson et al, 1986) or past the small antral stage in mammals (reviewed by Hirshfield, 1991), it is committed to ovulate and will generally not succumb to atresia.

Follicular atresia is a process known to involve apoptosis of granulosa cells (Hughes and Gorospe, 1991; Tilly et al, 1991a; Quirk et al, 1995) and controlled by a host of endocrine, autocrine, paracrine and neural signals. While the suppression of follicular atresia by gonadotropins (e.g. FSH) is well documented (Chun et al, 1996; Hirshfield and Midgley, 1978), less is known about the non-gonadotropin signals that regulate granulosa cell apoptosis and ultimately follicular atresia. In the hen ovary, since follicular atresia only occurs at the prehierarchal stage of follicular development (follicular size < 9 mm in diameter) when granulosa cells are much less responsive to gonadotropins compared to those from hierarchal (> 9 mm) follicles, the role of non-gonadotropin survival factors (e.g. TGFα) may be crucial in determining the eventual fate of prehierarchal follicles (atresia vs. ovulation).

TGFα is mitogenic in hen granulosa cells and prostaglandins have been shown to mediate the actions of this growth factor in several cell types (Li and Tsang, 1994; Li and Tsang, 1995; Harrison, 1994). Although, TGFα has been shown to prevent serum deprivation-induced apoptosis in rat granulosa cells (Tilly et al, 1992a), it is unknown whether the growth factor has a similar effect on hen granulosa cells. More importantly, it is unknown whether prostaglandins mediate the suppression of apoptosis by TGFα. Hence, the aims of this project were (i) to demonstrate differences in morphology and occurrence of apoptosis between healthy and atretic prehierarchal hen ovarian follicles *in situ*, and (ii) to investigate, *in vitro*, whether TGFα suppresses hen granulosa cell apoptosis and whether prostaglandins mediate the anti-apoptotic action of this growth factor.

9. LITERATURE REVIEW

9.1 Overview and Embryological Development of the Ovary

Unlike mammalian females that contain two (right and left) ovaries and accompanying oviducts, the egg-laying hen has only one left ovary and its oviduct. However, the right genital ridge is present in the early embryonic stages of the bird but regresses by day 10 of development since most of the primordial germ cells distribute preferentially to the left genital ridge (Franchi et al, 1962). The primordial germ cells in the embryonic ovary are called oogonia. Oogonia undergo a process of oogenesis to give rise to primary oocytes which are arrested at the diplotene stage of prophase I of meiosis in most vertebrate species (Franchi et al, 1962). Almost all primordial germ cells are converted to primary oocytes (arrested at the diplotene stage) by the time of birth or hatching of the bird. Within the first 4-5 days of life after hatching or birth, primary oocytes are covered by a layer of flat cells derived from the ovarian stroma, and the resulting structure is called a primordial follicle (Callebaut, 1976). The ovary of the immature (newly hatched) bird contains about 500,000 primordial follicles (Johnson, 1986). Marked by the onset of ovulation and egg laying, the hen reaches sexual maturity at the age of ~22 weeks, at which time there are about 4000 ova (in primordial follicles) remaining in the ovary. Hence, about 99.2 % of the ova die in the time between hatching and sexual maturity. Of these 4000 primordial follicles, about 800 oocytes are eventually ovulated during the reproductive life of the hen, as determined by the total number of eggs laid (Gilbert, 1971; Gilbert et al, 1983). However, before an oocyte can be ovulated it must progress from the immature stage of primordial follicle and pass through the intricate process of follicular

development which involves growth, proliferation and differentiation of the oocyte, granulosa and theca cells; it must along the way also escape the overwhelming possibility of atresia (death).

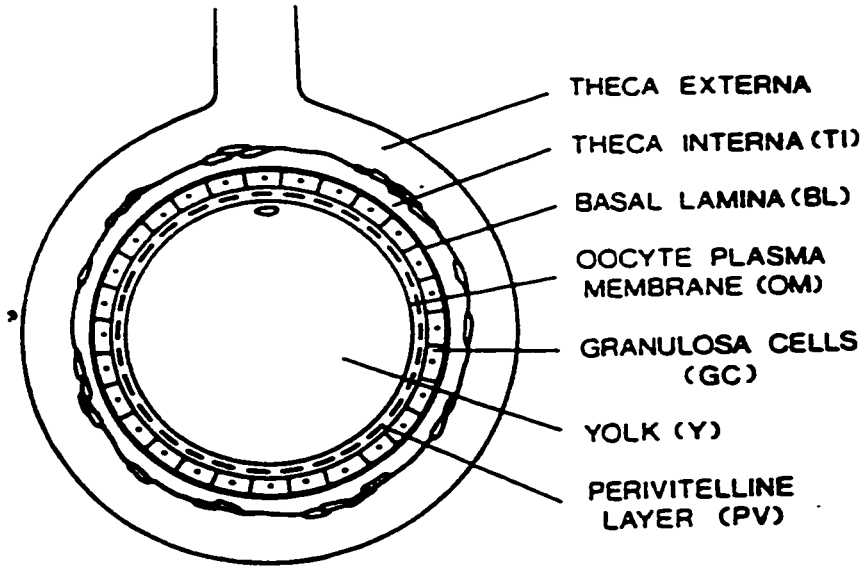
9.2 Anatomy, Structure and Follicular Hierarchy of the Avian Ovary

The ovary is attached to the cephalic end of the left kidney by the mesovarian ligament. The ovary receives its blood supply from the ovarian artery which arises from the left renolumbar artery which directly emanates from the aorta. The hen ovary is also well innervated by both adrenergic and cholinergic neurons, and the number of fibers innervating each follicle increases as the follicle progressively matures (Unsicker et al, 1983). Each follicle is attached to the ovarian tissue by a stalk and is vascularised with arterioles running through the stalk and branching throughout the theca, but not granulosa layers. Blood flow is greatest to the six largest preovulatory follicles in the hen (Johnson, 1986).

The follicle consists of concentric layers of tissue that surround the oocyte and yolk. As seen in Figure 1, immediately adjacent to and surrounding the yolk filled oocyte is the oocyte plasma membrane. Next in relation to the oocyte plasma membrane are the perivitellin plasma membrane, granulosa cells, basal lamina, theca (interna and externa) and epithelium.

Ovarian follicles range in size from the smallest (primordial; < 1 mm in diameter) to the largest (~ 40 mm in diameter) just prior to ovulation. Follicles that develop to a size greater than 9 mm in diameter are generally destined to ovulate and are called hierarchal follicles, owing to their embarkment on a path (hierarchy) leading to ovulation.

Figure 1. Cross-section of the hen ovarian follicle.



Johnson et al, 1986

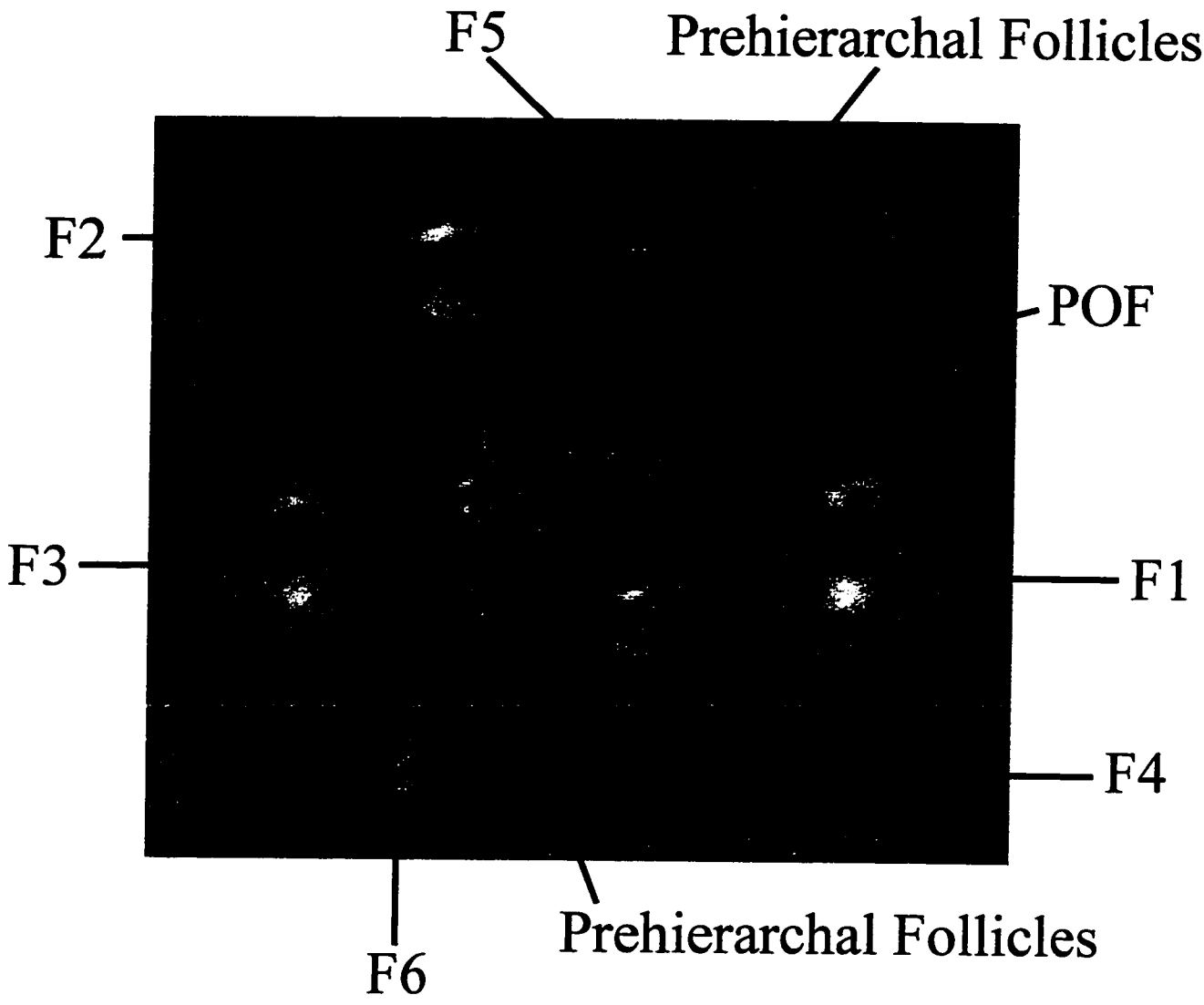
Daily egg laying hens have in their ovaries a clearly visible hierarchy of at least six preovulatory follicles. The largest follicle (~ 40 mm diameter) is called F1 and is the next to ovulate. The second biggest follicle is called F2; likewise the remaining follicles take on names (F3 to F6) based on decreasing size (Plate 1). After the F1 ovulates, the F2 (~ 35 mm) then becomes the largest follicle in the ovary and grows in 24 hours to be ovulated next as an F1. As F1 follicles ovulate, the follicles next in sequence develop and advance, and additional small follicles join the hierarchy at the F6 (~ 15 mm) level. In daily egg-laying hens the maintenance of such a follicular hierarchy is necessary to ensure the laying of an egg every 24 hours. Hierarchal follicles smaller than the F6 (i.e. between 9 mm and 15 mm) are numerous and the size difference is too small to warrant their classification. Prehierarchal follicles (< 9 mm) are prone to atresia (Johnson et al, 1996); only about 800 out of the 4000 (20 %) small follicles reach the 9 mm stage to be recruited into the hierarchy leading to ovulation (Gilbert, 1971; Gilbert et al, 1983).

9.3 Function of Ovary

The primary function of the ovary is to deliver viable oocytes to the oviduct where they can be fertilised to ultimately result in the creation of offspring. The follicle as a whole ensures that the oocyte is in an environment that is conducive for growth and development of the oocyte. The follicle protects the oocyte until it is successfully ovulated, if ovulation is its fate. The granulosa cells ensure that the oocyte is nourished properly.

Plate 1. A photograph of the hen ovary.

Upon sacrificing the hen, its entire ovary was excised, rinsed in PBS and photographed. F1 to F6 are the six largest preovulatory follicles in the ovary. Prehierarchal follicles are those whose diameter is less than 9 mm and their fate (ovulation vs. atresia) is yet undetermined. After the F1 ovulates, the remaining structure is called the post ovulatory follicle (POF).


F5
Prehierarchal Follicles
F2
POF
F3
F1
F4
F6
Prehierarchal Follicles

Both granulosa and theca cells serve to deliver steroids (e.g. estrogen and progesterone) that control ovulation by multiple pathways including, feed-back on to the pituitary to regulate gonadotropin secretion and induction of follicular prostaglandin synthesis which is necessary for ovulation (Koos and Clarke, 1982; Armstrong and Grinwich, 1972). In addition, steroids also control proliferation and/or differentiation of the granulosa, theca and oocyte via paracrine or autocrine mechanisms (Espey, 1986).

In hens, estrogen is needed to stimulate and maintain vitellogenisis, the processes by which the yolk granule in the oocyte is filled with proteins (lipovitellin, phosvitin, β-lipoproteins) and lipids (Redshaw and Follett, 1972)

9.3.1 Follicular Development, Selection and Ovulation

Growth of hen ovarian follicle can be divided into three stages (Johnson, 1986): (1) slow growth of primordial follicles to ~ 1 mm in diameter, lasting months to years; (2) the rapid growth of 1 mm follicles to ≤ 9 mm in diameter, lasting about months; (3) upon selection of follicle into the hierarchy for ovulation, the final rapid growth phase takes about 10-15 days before the follicle reaches its maximum preovulatory diameter of ~ 40 mm. Follicular growth/survival at various levels of development is stimulated by different factors as outlined in Table 1.

Although the mechanisms involved in selecting prehierarchal follicles into the hierarchy for ovulation are unknown, it involves switching the follicle from a non-steroidogenic to a steroidogenic one.

Table 1. A list of the different mammalian and avian ovarian follicle developmental stages and their respective survival factors

Mammalian Ovary	
Follicle Stage	*Growth/Survival Factor* *
Preantral	Estrogen > cGMP (IL-1β/NO)
Early antral	FSH > IGF-I, IL-1β > LH, EGF, bFGF, activin >>> GH
Preovulatory	FSH, LH/hCG, GH, IGF-I, EGF, bFGF, IL-1β

(taken from Kaipia and Hsueh, 1997)

Hen Ovary	
Follicle Stage	*Growth/Survival Factor* *
Prehierarchal (< 9 mm)	TGFα > VIP, IGF-I > EGF > FSH
Hierarchal (9-12 mm)	FSH > TGFα, IGF-I, IGF-II, >>> LH
Hierarchal (12-40 mm)	LH > FSH > TGFα, IGFs

* The growth/survival factors are listed in order of potency

While all follicles selected for ovulation are steroidogenic, it is not known whether the ability to produce steroids is a prerequisite for selection or is secondary to selection. Follicle stimulating hormone (FSH), insulin like growth factor-I (IGF-I), IGF-II and vasoactive intestinal peptide (VIP) have been shown to stimulate steroidogenesis in hen granulosa and theca cells (Johnson et al, 1994; Oganbesan and Peddie, 1995). Both IGF-I and VIP have an additive effect on FSH-stimulated steroidogenesis (Johnson et al, 1994; Oganbesan and Peddie, 1995). IGF-II mRNA, IGF receptor mRNA and FSH receptor levels peak in hen ovarian follicles of 6-8 mm, a stage in development when hen follicles are recruited into the hierarchy for ovulation (Armstrong and Hogg, 1996; Bahr and Johnson, 1984).

During periods of daily egg laying, ovulation in the hen occurs once every ~24 hours and is under the control of the LH-surge which precedes ovulation by ~5 hours (Johnson, 1986). In hierarchal hen ovarian follicles, granulosa cell progesterone synthesis is primarily under the stimulation of LH (Porter et al, 1989). A small amount of the progesterone is converted to testosterone and estrogen in the theca interna and theca externa cells, respectively (Huang and Nalbandov, 1979; Porter et al, 1989). Evidence suggests that ovulation in the hen is controlled by a positive feedback mechanism in which progesterone stimulates pituitary LH release which in turn induces granulosa cells progesterone synthesis (Johnson, 1986). A preovulatory progesterone-surge is necessary to produce the LH-surge which causes ovulation (Etches and Cunningham, 1976; Johnson and van Teinhoven, 1980)

9.3.2 Ovulation to Egg Laying

The oviduct is functionally divided into five parts: infundibulum, magnum, isthmus, shell gland (uterus) and vagina. The passage of the ovum/egg throughout the oviduct is aided by the peristaltic action of the longitudinal and circular muscles of the oviduct (Arjamaa and Talo, 1983). The ovulated oocyte is received and fertilised at the infundibulum (or funnel of oviduct). Sperms are stored in infundibular glands and retain their fertilising capacity for 7-14 days (Bakst, 1981). The ovum, whether fertilised or not, resides in the infundibulum for about 15 minutes and moves to the magnum (largest portion of the oviduct), in which albumen is deposited around the yolk filled ovum (Aitken, 1971). After about 2 hours, the ovum advances to the isthmus in which the inner and outer shell membranes are formed around the albumin, a process which takes about 1 hours to complete. It then progresses to the shell gland (uterus) in which the long (~ 15 hours) process of calcification of the egg is initiated. The egg remains in the uterus for about 21 hours after which the muscles of the uterus contract, possibly in response to a $PGF_{2\alpha}$ (Shimada and Asai, 1979) and expel the egg through the vagina. It takes roughly 24 hours from the time the ovum is ovulated to the time it is expelled as an egg. If the oocyte was fertilised, the eggs requires an incubation period of 20 days before the chick is hatched (Johnson, 1986).

9.4 Ovarian Atresia

9.4.1 Embryonic Germ Cell Loss and Ovarian Follicular Atresia

The Greek term 'atresia' means closure or deletion of a normal body orifice or passage. In the context of ovarian physiology, atresia refers to the process whereby germ cells/oocytes are lost from the female ovary by means other than ovulation. Deletion of primordial germ cells/oogonia and oocytes occurs during embryogenesis. By the time of birth or during the first few days after birth, all remaining oocytes are converted to primordial follicles and subsequent germ cell death occurs only by follicular atresia. In humans, deletion of oogonia and oocytes begins in utero at about 6 months of gestation (Baker, 1963), and in hens by the 17 th day of development of the chick embryo (Johnson, 1986). In most vertebrate species over two thirds of the potential germ cells are lost by attrition by the time of birth or hatching (Hughes, 1963). For example, the human fetal ovary has ~ 7 x 10^6 germ cells by 20 weeks of gestation while only about 2 x 10^6 germ cells (primary oocytes) remain at birth (Baker, 1963; Forabosco et al, 1991). Within the first few (1-4) days of life after hatching or birth, the primary oocytes are covered by a layer of cells derived from the ovarian stroma, forming the primordial follicle (Callebaut, 1976). Those oocytes that remain naked (i.e. fail to become enclosed by follicular cells) usually degenerate (Ohno, 1964). About 50 % of the oocytes degenerate due to failure to form primordial follicles, leaving about 1 x 10^6 primordial follicles in the neonatal human ovary. The ovary of the newly hatched bird contains about 500,000 primordial follicles with oocytes arrested at prophase I (Johnson, 1986). The hen reaches sexual maturity at the age of ~ 22 weeks with about 4000 follicles remaining in the ovary (Gilbert, 1971; Gilbert et al, 1983). Of these 4000, only about 800 are ovulated during the reproductive life of the hen, as

indicated by the total number of eggs laid (Gilbert, 1971; Gilbert et al, 1983). Hence, 80 % of the follicles remaining at the onset of sexual maturity in hen undergo atresia, which is comparable to the percentage of follicles degenerating during the 'reproductive' life in lower vertebrates such as mouse and rat (Jones, 1978).

In light of the vast majority (> 99%) of follicles that undergo degeneration, ovulation can be viewed as a rescue process requiring active intervention to prevent an otherwise inevitable demise. Why germ cell/follicular atresia occurs is a mystery. It is not immediately clear why nature would first establish a large germ cell endowment during embryogenesis only to subject it to life-long decline. Furthermore it is equally unclear why a cohort of hundreds of follicles is nurtured and grown in every ovulatory cycle during reproductive life only for most follicles to be wasted.

I attempt to forward two speculations as to why follicular atresia occurs: In mammals, it is possible that the cohort of recruited follicles serve an endocrine/paracrine function. The granulosa cells of recruited follicles release estrogen to ensure that the dominant follicle receives ample estrogen for growth (paracrine), and estrogen can also feedback to the hypothalamus and pituitary to control gonadotropin release (endocrine) and thus control the timing of ovulation. The non-selected follicles, however, undergo demise at various times along the ovulation cycle. Alternately, follicular atresia may be a way of eliminating oocytes that are less likely to survive post-ovulation and/or less likely to result in successful zygote formation. The oocyte may communicate with the adjacent granulosa cells and regulate the follicle's capacity to respond to survival factors. As a result, oocytes with the greatest capacity to succeed post-ovulation may

bestow their follicles the highest ability to respond to survival factors, resist atresia and continue to grow. In the same light, follicular atresia may also be a way of eliminating oocytes that have tumerogenic potential (Kaipia and Hsueh, 1997).

9.4.2 Morphological Aspects of Follicular Atresia

Based on morphological criteria, atresia of mammalian (rat) antral follicles can basically be divided into three stages:

Stage I is characterised by a small percentage (< 10 %) of granulosa cells with pyknotic nuclei, as demonstrated initially by chromatin condensation and subsequently by disruption of the nuclear membrane and breaking of the nucleus into dense, membrane bound fragments. These first few dying cells are usually close to the antrum (follicular fluid). Although the total [^{3}H]thymidine incorporation by granulosa cells is reduced compared to a healthy same-size follicle, many granulosa cells are still in mitosis (Byskov, 1974).

A stage II atretic follicle contains more (~ 30 %) granulosa cells with pyknotic nuclei, little or no granulosa cell [^{3}H]thymidine incorporation, few cells in mitosis, and cellular debris in the follicular fluid. The basement membrane loses its integrity, allowing leukocytes to infiltrate the granulosa layers. In the oocyte, meiotic-like changes are evident.

Stage III is characterised by a reduction in the number of granulosa cells (Bomsel-Helmreich et al, 1979; McNatty et al, 1979), detachment of cells from the basement membrane (Hirshfield and Midgley, 1978; Hay et al, 1976),

fragmentation of the basement membrane (Bavagandos et al, 1983) and collapse of the follicle. The oocyte also undergoes germinal vesicle breakdown (Erickson et al, 1985; Himelstein-Braw et al, 1976). In contrast, the theca cells increase in size and become incorporated as interstitial cells in the stroma of the ovary (Himelstein-Braw et al, 1976; Erickson et al, 1985; Hilliard et al, 1967), where they are believed to function as steroidogenic cells (Erickson et al, 1985).

Although such morphological classification of hen ovarian follicles undergoing atresia has not been reported, evidence from studies in this thesis and from *in vitro* studies (Johnson et al, 1996) shows that the morphological characteristics of hen follicular atresia are similar to those of mammalian.

9.4.3 Biochemical Aspects of Follicular Atresia

In addition to the morphological changes described in the previous section, marked biochemical and endocrinological alterations occur during follicular atresia. These include reduced DNA synthesis in granulosa cells (Greenwald, 1989; Hirshfield, 1989), suppressed expression of connexin 43 (a gap-junction protein; Wiesen and Midgley, 1994), decreased gonadotropin receptors (Uilenbroek et al, 1980; Carson et al, 1979), and in mammalian ovaries suppressed P450-aromatase mRNA expression and $C_{17,\ 20}$-lyase activity (Tilly et al, 1992b), resulting in decreased estrogen synthesis and increased progesterone release (Maxson et al, 1985; Carson et al, 1981). In contrast, increased expression of several genes is observed during follicular atresia. These include insulin-like growth factor binding proteins (IGFBPs; Nakatani et al, 1991), sulfated glycoprotein-2 (Kaynard et al, 1992), cathepsin-D (a lysosomal aspartyl endopeptidase; Dhanasekaran and Moudgal,

1989), and angiotensin II receptor (Daud et al, 1988; Horiuchi et al, 1997). It is believed that the increases in IGFBPs sequester endogenous IGF-I and deprive the follicle of the survival effects of the growth factor. Cathepsin-D may mediate protein degradation events associated with atresia, while the increase in angiotensin II receptor may facilitate the putative atretogenic actions of angiotensin II. The exact role of these proteins in initiating or maintaining follicular atresia is unclear.

9.5 Apoptosis

9.5.1 Physiological Significance of Apoptosis

Apoptosis (or programmed cell death) is a normal, physiological process of cell death (Wyllie et al, 1980; Kerr et al, 1972). It is a genetically controlled process by which unwanted cells are neatly eliminated. It has been postulated that all differentiated cells in multicellular organisms are capable of undergoing apoptosis through a highly conserved suicidal program (Raff, 1992; Raff et al, 1993; Thompson, 1995). The apoptotic death programme may be activated by partial or complete deprivation of survival factors, or by an apoptosis-inducing ligand binding to its receptor, or by irreversible damage (e.g. by radiation) to cellular macromolecules (Kaipia et al, 1996; Jacobson et al, 1997; Hsueh et al, 1994).

Apoptosis at any time serves one or more of four purposes: 1) sculpting body structures; 2) deleting unneeded cellular structures; 3) controlling cell numbers; and 4) eliminating abnormal, harmful or non-functional cells. Apoptotic cell death is abundant throughout pre- and post-natal life, as exemplified below.

In the developing embryo, widespread apoptosis actively sculpts the body shape during organogenesis (Clarke, 1990). Digits (fingers and toes) emerge as a result of apoptotic elimination of cells between developing digits (Milligan et al, 1995; Jacobson et al, 1996). Similarly, apoptosis is involved in hollowing out solid structures to create the gut, from the mouth to anus (Coucouvanis and Martin, 1995).

In the course of animal development, various structures are formed which are later removed by apoptosis, including those that are needed at one stage of development but not later, or needed in one sex but not in the other. Subplate neurons are required transiently during development of the mammalian cerebral cortex but are subsequently removed by apoptosis (Jocobson et al, 1997). The Mullerian duct forms the uterus and oviducts in female mammals, but is not needed in the male where it regresses by apoptosis under the influence of mullarian inhibition factor (Jocobson et al, 1997). On the other hand, the Wolffian duct forms the vas deferens, epididymis, and seminal vesicle in the male, but is not needed in females where it regresses by apoptosis due to the lack of testosterone, a survival factor for cells in the Wolffian duct (Jocobson et al, 1997).

In many organs, cells are first over produced and then deleted by apoptosis. In the vertebrate nervous system, for example, both neurons and oligodendrocytes are generated in excess during development. Half or more of the neurons and oligodendrocytes are eliminated by apoptosis, apparently to match the number of neurons to the number of target cells requiring innervation and the number of oligodendrocytes to the number of axons requiring myelination (Barde, 1989; Oppenheim, 1991; Barres et al, 1992).

Apoptosis also serves to remove abnormal, non-functional, or potentially dangerous cells. In the vertebrate immune system for example, developing T and B lymphocytes that fail to produce useful antigen-specific receptors or that produce self-reactive receptors that render themselves potentially dangerous are removed by apoptosis (Duke et al, 1996). If sufficient DNA is damaged by radiation for example, mammalian cells can activate their self-destruct programme by various mechanisms, one of which is by activating the p-53 tumour-suppresser protein (Clarke et al, 1993; Lowe et al, 1993). This response not only serves as an anti-cancer mechanism but also prevents the birth of defective offspring (Norimura et al, 1996). An additional example is presented by intestinal cells which are generated at the base of the villus but over several days migrate to the tip of the villus where they die by apoptosis and are sloughed off (Duke et al, 1996).

9.5.2 Morphological Features of Apoptosis

Apoptosis can occur in scattered, individual cells in a tissue. Early in the apoptotic process the cell looses its volume (cytoplasmic condensation) and pulls away from its neighbouring cells. Blebs form at the cell membrane, while the chromatin in the nucleus condenses and migrates to the nuclear envelop. The other organelles (e.g. mitochondria, golgi, etc.) retain their structure. In the final stages of apoptosis, the nucleus disintegrates into spherical dense fragments, and the cell begins to fragment as the membrane blebs lead to budding off of membrane-bound vesicles (apoptotic bodies; Kerr et al, 1972; Kerr et al, 1994). Apoptotic bodies, containing cytoplasmic and nuclear material of the dying cell, are quickly removed by neighbouring cells via phagocytosis (Savill, 1995). In this manner, the

apoptotic cell is neatly removed; no cellular contents spill into the extracellular space, and no immune response is mounted (Figure 2).

In contrast, necrotic cell death affects a continuous tract of cells in a tissue. Necrosis is a consequence of physical injury or traumatisation, such as an ischemic insult. This leads to loss of cell structure and swelling as the cell looses its ability to maintain the ionic potential across its cell membrane, and ultimately cell rupture. The cytoplasmic contents are released into the extracellular space resulting in an immune response and inflammation (Hsueh et al, 1994).

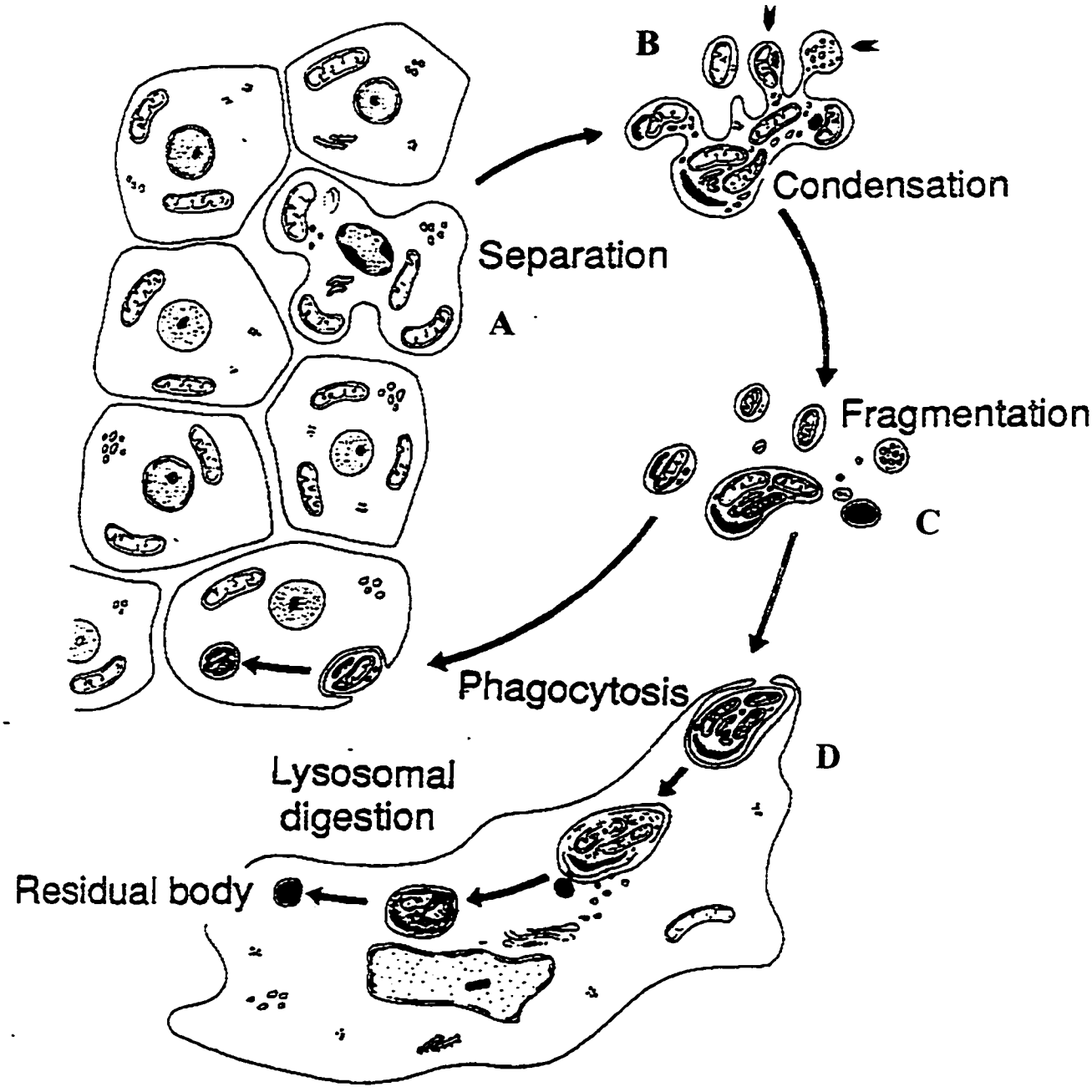
9.5.3 Biochemical Features of Apoptosis

Apoptosis is an active, energy-utilising, gene-directed process. Synthesis of new mRNA and protein appears to be required for apoptotic cell death in most but not all cells (Martin et al, 1988; Schwartz and Osborne, 1993). A prominent feature of apoptosis is the activation of DNase I, a Ca^{2+}/Mg^{2+}-dependent endonuclease, that degrades genomic DNA in multiples of 180 base pairs (Peitsch et al, 1993a; Peitsch et al, 1993b; Boone et al, 1995; Boone and Tsang, 1997). This is due to the fact that about 180 bp of DNA is packed around each nucleosome (i.e. 150 bp of DNA wrapped around the nucleosome plus 30 bp DNA in the linker region between nucleosomes), and the endonuclease can only cleave the free, unbound DNA in the linker region between nucleosomes. This characteristic DNA profile can be seen as "DNA ladders" when DNA from apoptotic cells is resolved on agarose gels. In contrast, necrotic cell death is passive, does not use ATP nor involve new protein or RNA synthesis. The breakdown of DNA in necrotic cells is not by an endonuclease, but is random and generalised, resulting in a 'smear' when the DNA is resolved on agarose gels (Hsueh et al, 1994).

Figure 2. The morphological features of apoptosis.

A: First the apoptotic cell undergoes cytoplasmic condensation and pulls away from its neighbouring cells. B: Blebs (arrow head) are then formed at the cell membrane, as the cell prepares to fragment. The nucleus also disintegrates in the cells and chromatin is found in discrete dense fragments. C: The cell begins to fragment into membrane-bound vesicles called apoptotic bodies. D: The apoptotic bodies are quickly removed by neighbouring cells via phagocytosis, and digested in lysosomes.

Morphological Features of Apoptosis



modified from Kerr et al, 1972

Type II transglutaminase is a Ca^{2+}-dependent cytosolic protein which is selectively upregulated in cells undergoing apoptosis (Piacentini et al, 1994). During apoptosis the large elevation of cytoplasmic calcium is sufficient to activate the transglutaminase which catalyses irreversible cross links between the glutamine residue of one protein and the lysine residue of another (Piacentini et al, 1994). Cross linking of intracellular proteins stabilises the cytoplasm of the dying cells, hence preventing leakage of cytoplasmic contents into the extracellular space, an event which could lead to an immune response. Immunological characterisation of the cross-linked matrix in apoptotic cells indicated the presence of actin, fibronectin, vinculin, annexin II, and other uncharacterised proteins (Knight et al, 1993). As well, a rearrangement of the actin microfilament network occurs in the cytoplasm of apoptotic cells to allow for cellular condensation and fragmentation into apoptotic bodies (Hale et al, 1996; Martin and Cotter 1990; Cotter et al, 1992).

In healthy living cells, the anionic membrane lipid phosphatidylserine (PS) is located in the inner plasma membrane, inaccessible from the extracellular space, while sphingomyelin, phosphatidylcholine (PC), and neutral phospholipids are found on the outer plasma membrane of the bilayer (Fadok et al, 1992). During apoptosis, PS is exposed on the outer membrane surface. For this reason, apoptotic but not normal cells acquire the ability to recognise annexin V, a protein with high affinity for PS (Homburg et al, 1995). It has been proposed that the appearance of PS in the outer membrane is important for phagocytosis of apoptotic cells/bodies by neighbouring cells or macrophages (Fadok et al, 1992).

Although the mechanisms involved in inducing the nuclear, cytoplasmic and membrane events are largely unclear and not well understood, it appears that they can proceed in parallel (Jacobson et al, 1994). Furthermore, during apoptosis the

cytoplasmic and nuclear events can occur independently. For example, DNA fragmentation is not required for the cytoplasmic changes to occur (Jacobson et al, 1994).

9.5.4 Molecular Aspects of Apoptosis

There is growing evidence suggesting that the process of apoptosis is very highly conserved through evolution, and that the diverse signals inducing or suppressing apoptosis eventually converge on a limited number of evolutionary conserved genes. Bcl-2 and interleukin-1β-converting enzyme (ICE) are the two most studied gene families involved with apoptosis. ICE members are cysteine proteases that cleave peptides/proteins at sites after aspartic acid. Therefore, ICE members are now called 'caspases' (cysteine aspases), and 10 caspases have been identified to date (Nagata, 1997). All caspases exist in cells in inactive forms (zymogens) which must be proteolytically activated to be functional. Caspases operate in a cascade where upstream caspases activate downstream caspases by proteolytic cleavage, in addition to cleaving their own substrate to destroy or activate it. Over-expression of any one of the 10 caspases results in apoptosis (Alnemri et al, 1996). Caspases active in the apoptosis process degrade proteins required for cell viability and function including poly(ADP)-ribose polymerase (PARP; an enzyme involved in DNA repair), DNA-dependent protein kinase (DNA-PK), sterol regulatory element binding proteins (SREBPs), small nuclear ribonucleoproteins (important in spliceosome assembly and mRNA processing; e.g U1-snr), and rho-GDI (a protein that maintains cellular actin cytoskeleton) (Martin and Green, 1995; Nagata, 1997). Caspases also degrade proteins required for cell structure and support such as lamin, actin, and structural proteins of the nuclear

scaffold (Martin and Green, 1995). Recently, a missing link between caspase activation and apoptotic DNA fragmentation was reported: caspase-3 cleaves and activates DNA fragmentation factor (DFF), a cytosolic protein involved in the induction of apoptotic DNA fragmentation (Liu et al, 1997). Table 2 contains a list of the 10 known caspases and their substrates reported to date. Substrates for caspases 4, 5 and 10 have not been identified. It is safe to predict that in reality the number of substrates for caspases are far greater then those known to date.

There is evidence suggesting that caspase-8 (also known as FLICE or machα1) is the first to be activated in the cascade which then activates downstream events (Boldin, 1996). One way in which caspase-8 is activated is by association with the intracellular domain of an activated tumour necrosis factor alpha (TNFα) receptor or fas receptor (fas antigen) (Muzio et al, 1996; Boldin et al, 1996). Both TNFα and fas ligand are well known apoptosis inducing signals in many cell types, including granulosa cells (Quirk et al, 1995; Witty et al, 1996).

Whereas the ICE family of proteases work to induce or facilitate the apoptotic process, the bcl-2 proto-oncogene family members can both suppress and promote apoptosis. There are at least nine mammalian members in the bcl-2 gene family that can form homodimers and heterodimers with one another. *Bcl-2*, *bcl-xlong*, *bcl-w* and *mcl-1* inhibit apoptosis, whereas *bcl-xshort*, *bax*, *bad*, *bik* and *bak* promote apoptosis (reviewed by Hale et al, 1996). Consequently, each monomer can either enhance (e.g. *bcl-2/bcl-xlong*) or suppress (e.g. *bcl-2/bcl-xshort*) the function of the other in dimers of the bcl-2 family. In this way, the ratio of inhibitors to activators may determine the cell's propensity to undergo apoptosis.

Table 2. Members of the caspase family and their known substrates

Protease	*Substrate*
Caspase-1 (ICE)	pro-interleukin 1β, pro-caspase 3 and 4
Caspase-2	PARP
Caspase-3 (cpp-32; apopain)	Actin, PARP, DNA-PK, SREBP, rho-GDI, U1, DFF
Caspase-4	?
Caspase-5	?
Caspase-6	lamin A
Caspase-7	PARP, pro-caspase 6
Caspase-8	Pro-caspase 8 (by autocatalysis)
Caspase-9	PARP
Caspase-10	?

A '?' implies the substrate for that caspase is yet unknown.

Over-expression of *bcl-2* or *bcl-xlong* blocks apoptosis induced by various triggers such as serum- or survival factor-deprivation, irradiation, c-myc, fas ligand, TNFα and anti-cancer drugs (Itoh et al, 1993; Boise et al, 1995; Nagata, 1997).

The apoptosis suppressers *bcl-2* and *bcl-xlong* are integral membrane proteins, localised mostly in the outer mitochondrial membrane but with smaller amounts present in the endoplasmic reticular and nuclear membranes. They exist as dimers facing the cytosol (Nguyen et al, 1993; Krajewski et al, 1993). CED-4 is a *Caenorhabditis elegans* protein necessary for apoptosis (Yuan and Horvitz, 1992). Homodimers of apoptosis suppressers (e.g. *bcl-2* or *bcl-xlong*) can bind to an adapter protein believed to be the mammalian homologue of CED-4 (Wu et al, 1997; Chinnaiyan et al, 1997). Mammalian CED-4 also binds to pro-caspase-8, the inactive form of caspase-8. This tripartite association (bcl-xlong/CED-4/pro-caspase-8) keeps the caspase-8 inactive, thus preventing the onset of apoptosis. This explains how over-expression of *bcl-2* or other apoptosis suppresser members prevents apoptosis. However, heterodimerisation of suppresser bcl-2 members with apoptosis promoters (e.g. *bcl-xshort*, *bax*, *bad*, *bik*, *bak*) causes CED-4/procaspase-8 to dissociate from the bcl-2 complex (Chinnaiyan et al, 1997). This dissociation results in CED-4-induced autoproteolysis of pro-caspase-8 to produce to active caspase-8, leading to the activation of other downstream caspases and apoptotic death of the cell. Activation of cysteine proteases in this pathway depends directly on the nature of dimerisation of bcl-2 family members. The availability of various apoptosis promoting and suppressing members of bcl-2 are regulated by receptor-mediated signals or intracellular macromolecular damage. This demonstrates a point of convergence for the bcl-2 (apoptosis suppressing) and caspase protease (apoptosis promoting) pathways.

Evidence suggests that certain cell survival factors suppress apoptosis by preventing apoptosis-promoting members from forming heterodimers with suppresser members of bcl-2. In the presence of survival factors, *bad* (a proapoptotic member of bcl-2) is phosphorylated on serine residues (Zha et al, 1996). Phosphorylated *bad* looses its affinity for *bcl-2*; instead, phosphorylated *bad* binds to 14-3-3, a multifunctional protein that binds various signalling molecules (Zha et al, 1996). The kinase pathways leading to the phosphorylation of bcl-2 members are not fully known. Activation of RAF-1, a serine/threonine kinase, has been shown to phosphorylate *bad* and prevent apoptosis (Wang HG et al, 1996). Recently, activated protein kinase B (PKB) has been shown to prevent c-myc-induced apoptosis in Rat-1 cells (Kauffmann-Zeh et al, 1997).

9.5.5 Apoptosis in the Ovary

There is compelling evidence suggesting that apoptosis is involved in atresia of germ cells, oocytes and follicles in the ovary (Hirshfield, 1991; Kaipia and Hsueh, 1997). Evaluation of cellular and nuclear morphology revealed that degeneration of fetal mouse oogonia and oocytes occurs by apoptosis (Coucouvanis et al, 1993). While primordial germ cell proliferation is stimulated by stem cell growth factor (SCF) and basic fibroblast growth factor (bFGF), deprivation of the growth factors rapidly results in apoptosis (Dolci et al, 1993; Pesce et al, 1993; Resnick et al, 1992).

In addition to oogonia and oocytes, degeneration of ovarian follicles involves apoptotic granulosa cell death. In contrast, theca cells do not undergo apoptosis, but become incorporated into the ovarian stroma (Erickson et al, 1985). The first

evidence of the involvement of apoptosis in follicular atresia came from the detection of apoptotic DNA fragmentation in granulosa cells from atretic, but not healthy, chicken and porcine follicles (Tilly et al, 1991a). Following this report, several studies demonstrated morphological and biochemical evidence of apoptosis in atretic rat (Hughes and Gorospe, 1991; Palumbo and Yeh, 1994), bovine (Jolly et al, 1994), and human (Quirk et al, 1995) ovarian follicles.

9.5.6 Hormonal Control of Follicular Apoptosis

9.5.6.1 Survival factors

Follicle growth, selection for ovulation, maturation and eventual ovulation require the concerted effort of gonadotropins and intraovarian autocrine/paracrine factors. Many studies on the hormonal regulation of follicular atresia utilise an *in vitro* culture of isolated granulosa cells or whole ovarian follicles. While culture of granulosa cells and ovarian follicles in serum free medium induces apoptosis (Tilly et al, 1992a; Flaws et al, 1995a; Johnson et al, 1996), gonadotropins (FSH, LH, hCG), VIP, 8-bromo-cAMP, TGFα/EGF, bFGF, IGF-I and growth hormone (GH) have been shown to inhibit granulosa cell apoptosis and follicular atresia (Chun et al, 1994; Chun et al, 1996; Eisenhauer et al, 1995; Tilly et al, 1992a; Flaws et al, 1995a; Johnson et al, 1996). In addition to inhibiting apoptosis, IGF-I synergises with gonadotropins to promote granulosa cell differentiation. TGFα is antagonistic to the actions of gonadotropin but stimulates granulosa cell mitosis (Adashi et al, 1985; Davoren et al, 1986; Onagbesan and Peddie, 1995). While FSH, LH and VIP increase intracellular cAMP and subsequently activate PKA, TGFα/EGF, IGF-1 and bFGF signal via receptor tyrosine phosphorylation.

The cytokine interleukin-1β (IL-1β) also inhibits apoptosis of rat preovulatory follicles (Chun et al, 1995). In rat granulosa cells, as well as other cell types, IL-1β stimulates intracellular nitric oxide (NO) production which in turn activates a guanyl cyclase to increase cGMP, the second messenger for NO (Schmidt and Walter, 1994). cGMP analogues and sodium nitroprusside, a NO donor, are also effective at suppressing rat ovarian follicle apoptosis (Chun et al, 1995).

Estrogen is indispensable for growth and maturation of follicles in mammalian, but not avian ovaries. Estrogen promotes granulosa cell division (Williams, 1940). Atretic ovine follicles exhibit a decreased estrogen/androgen ratio, suggesting the importance of local estrogen for maintenance of healthy follicles (Carson et al, 1981). Estrogen markedly decreases the occurrence of follicular apoptosis in immature rats *in vivo* (Hsueh et al, 1994). Progesterone has also been shown to decrease the apoptosis of cultured granulosa cells (Peluso and Pappalardo, 1994).

In mammalian ovaries, although follicular apoptosis and atresia can occur at any stage, the majority of follicles are lost at the preantral to early antral transition (Kaipia and Hsueh, 1997). Follicles that develop past the early antral stage are resistant to apoptosis. Likewise, hen follicles that grow to a size greater than or equal to 9 mm are resistant to atresia and committed to ovulate (Johnson et al, 1996). Pathways mediating survival signals during follicle development are redundant, and the potencies of different survival factors vary with the developmental stage of the follicle. The different mammalian and avian ovarian follicle developmental stages and their respective trophic/survival factors are listed in Table 1.

Although responsive to gonadotropins, preantral follicles can grow to the antral stage without gonadotropin support. Gonadotropins are ineffective in rescuing preantral follicles from atresia (Hirshfield, 1991). In contrast, FSH at high concentrations can act as an anti-apoptotic factor in cultured hen granulosa cells from prehierarchal follicles (4-8 mm in diameter; Johnson et al, 1996).

Several members of the bcl-2 and ICE gene families have been identified in the ovary at the mRNA and protein level (Tilly, 1996). Whereas caspase-2 mRNA levels remain at low levels throughout development of healthy ovarian follicles in hens, atretic follicles have 3 fold higher caspase-2 mRNA levels than healthy ones (Johnson et al, 1995). Serum-deprivation increases *bax* (apoptosis-inducer) mRNA expression in cultured rat ovarian follicles while *bcl-2* and *bcl-xlong* (apoptosis-suppressers) mRNA levels remain unchanged (Tilly et al, 1995). Furthermore, treatment of immature rats with gonadotropins significantly reduces granulosa cell *bax*, caspase-2 and caspase-3 mRNA expression while bcl-2 and bcl-xlong mRNA levels remain unchanged (Tilly et al, 1995; Flaws et al, 1995b). It appears that regulation of follicular apoptosis is mostly by caspases and apoptosis-promoting members of bcl-2, while the apoptosis-suppressing members of bcl-2 remain at constitutive levels. Although further studies are needed to fully understand the regulation of *bcl-2* and ICE, whether other survival factors (e.g. TGFα/EGF, bFGF, IGF-I) also regulate these genes in the ovary remains to be determined.

9.5.6.2 Atretogenic Factors

A number of intraovarian factors induce follicular atresia via granulosa cell apoptosis. Tumour necrosis factor-α (TNFα), a cytokine expressed in the oocyte and granulosa cells, signals through type I TNFα receptor (TNFα-RI) (Tartaglia et al, 1993) and induces apoptosis in cultured rat follicles (Kaipia et al, 1996) and hen granulosa cells (Witty et al, 1996). Ceramide, a lipid second messenger of TNFα, can also mimic the apoptotic effect of TNFα (Witty et al, 1996; Kaipia et al, 1996). TNFα-RI has a death domain (DD) on its intracellular side, and upon receptor activation the DD recruits and activates caspase-8 via various adapter proteins, thereby directly activating the proteolytic cascade leading to apoptotic cell death (Hsu et al, 1996a; Chinnaiyan et al, 1995; Muzio et al, 1996; Boldin et al, 1996).

Fas, another death domain containing receptor, belongs to the TNFα receptor family and has been identified in rat and mouse granulosa cells (Hakuno et al, 1996; Kim and Tsang, unpublished data). Fas ligand binds and activates fas receptor *in vivo*. Activation of fas receptor induces apoptosis in cultured human granulosa/luteal cells (Quirk et al, 1995). In atretic rat follicles, oocytes have been shown to express fas ligand while granulosa cells expressed fas (Hakuno et al, 1996). Fas-induced apoptosis could also be mediated by activation of caspases via the DD of fas (Stanger et al, 1995; Hsu et al, 1996b; Duan and Dixit, 1997).

Interleukin-6 (IL-6), another cytokine produced by granulosa cells, also induces apoptotic DNA fragmentation in cultured rat granulosa cells (Gorospe and Spangelo, 1993). In addition, gonadotropin releasing hormone (GnRH) inhibits follicle differentiation and induces apoptosis via direct interaction with GnRH

receptors on granulosa cell membrane (Billig et al, 1994). Whereas estrogen is a survival factor in mammalian granulosa cells, androgens promote ovarian follicular apoptosis (Billig et al, 1993). Treatment of estrogen-primed rats with testosterone increases granulosa cells apoptosis (Billig et al, 1993; Zeleznik et al, 1979).

9.6 Transforming Growth Factor alpha

TGFα, a 50 amino acid peptide, is a close relative of epidermal growth factor (EGF) and is believed to act via binding to EGF receptors (Marquant et al, 1984) on the same cell (autocrine stimulation) or neighbouring cells (paracrine stimulation) to initiate its signal transduction cascade. The first event subsequent to TGFα-receptor binding is receptor subunit dimerisation and tyrosine auto-phosphorylation (Kumar et al, 1995). Activation of the Ras-mediated kinase cascade is linked to the activated receptor by Grb2 (growth-factor receptor bound protein 2) and Sos (sons of sevenless). Grb2 interacts directly with the tyrosine kinase domain of the activated receptor at one end and Sos at the other. Sos then interacts directly with Ras and activates Ras by promoting the replacement of GDP by GTP. Activated Ras-GTP initiates the mitogen activated protein (MAP) kinase which regulates nuclear transcription factor activity as well as cell proliferation (Kumar et al, 1995; Marais and Marshall, 1996). Recently, TGFα-induced activation of MAP kinase has been shown to upregulate *fos* and *myc* message and protein levels in intestinal epithelial cells (Oliver et al, 1995). In contrast, Gotoh et al (1997) showed that TGFα upregulated *myc* in NIH 3T3 cells via pathways independent of MAP kinase.

Other signalling molecules can also activate signalling cascades distil to receptor stimulation in a cell-specific manner. For instance, phospholipase C_γ (PLC_γ) can also interact with phosphotyrosines on the activated receptor, leading to phosphorylation and activation of the PLC_γ. Activated PLC_γ increases phophatidylinositol turnover, with subsequent activation of PKC and mobilisation of intracellular calcium (Cochet et al, 1991). Recently, other cellular proteins, called signal transducers and activators of transcription (STAT), that transduce TGFα/EGF signals have been identified. STATs are phosphorylated by direct interaction with the activated receptor. Alternatively, the phosphorylation of STATs following receptor activation is mediated by JAK1, a member of the janus kinases (Darnell et al, 1994; Shuai et al, 1993). Phosphorylated STATs then form either homodimers or heterodimers which translocate into the nucleus and function as transcription regulators (Ruff-Jamison et al, 1993).

The avian granulosa cell EGF/TGFα receptor binds human TGFα with 300 fold greater affinity than human EGF (Onagbesan et al, 1996). The presence of TGFα receptors and its ligands (EGF and TGFα) has been reported in the granulosa, theca interna and theca externa layers of the hen ovarian follicle at all stages of follicular development (Onagbesan et al, 1994). This suggests an autocrine and paracrine mode of action for TGFα. The highest concentration of both TGFα receptor and ligand is in the smallest follicles, with levels decreasing as follicular size increases (Onagbesan et al, 1994). This is consistent with the observation that the TGFα-induced thymidine incorporation in granulosa cells decreases with increasing follicular maturation (Onagbesan et al, 1994), suggesting that TGFα plays a vital role at the early (prehierarchal) stages of hen follicular development.

Generally, TGFα stimulates proliferation/mitosis in hen granulosa cells and suppresses their differentiation into steroid producing cells (Li and Tsang, 1995). TGFα has been shown to inhibit VIP- and FSH-induced increases in P450 side chain cleavage (scc) mRNA, P450 17α-hydroxylase mRNA and progesterone levels in granulosa cells from 6-8 mm hen follicles (Johnson et al, 1994; Li and Johnson, 1993a). In another study, TGFα suppressed FSH- and 8-bromo-cAMP-stimulated androstenedione synthesis in granulosa cells from 3-12 mm hen follicles (Li and Johnson, 1993b). Similarly, in differentiated granulosa cells from F3 to F1 hen follicles, TGFα inhibits basal and IGF-I- and LH-stimulated progesterone production (Onagbesan and Peddie, 1995). Likewise in theca cells from 9-12 mm hen follicles, EGF inhibits LH- and IGF-I-stimulated aromatase activity (Onagbesan et al, 1994b).

9.7 Ovarian Eicosanoid Biosynthesis

Considerable efforts have been made in recent years in defining the nature and the regulation of eicosanoids in various physiologic functions of the ovary (Olofsson and Leung, 1996). The biosynthesis of eicosanoids involves the activation and/or induction of two key enzymes: Phospholipase A_2 (PLA_2) and cyclooxygenase (COX; Smith et al, 1996; Figure 6). Phospholipase A_2 catalyses the hydrolysis of the sn-2-ester bond of phospholipids. Membrane phospholipids such as phosphatidylcholine (PC) are metabolised by cytosolic phospholipase A_2 ($cPLA_2$) to arachidonic acid and lysophosphatidic acid (LPC). PLA_2 can be divided into two broad categories: the low MW secreted form ($sPLA_2$; 14 kDa) and the high MW cytosolic form ($cPLA_2$; 100 kDa). Cytosolic PLA_2 exists in Ca^{2+}-dependent (nM range of Ca^{2+}) and -independent subtypes (Clark et al, 1995).

The Ca^{2+}-dependent $cPLA_2$ is activated by phosphorylation and specifically hydrolyses membrane phospholipids containing only arachidonic acid at their sn-2 position (Clark et al, 1995). In contrast $sPLA_2$ is also Ca^{2+}-dependent but requires a calcium concentration in the micromolar range for activity (Murakami et al, 1995). It does not require to be phosphorylated to be active and is not specific for arachidonic acid containing phospholipids (Murakami et al, 1995).

The cyclooxygenase (COX) enzyme catalyses the first and rate limiting step in the conversion of arachidonic acid to PGs, prostacylins and thromboxanes (Smith et al, 1996). The 'cyclooxygenase' activity of COX inserts two oxygen molecules in arachidonic acid to convert it to PGG_2 and then the 'peroxidase' of COX reduces PGG_2 to PGH_2 (Smith et al, 1996). PGH_2 is then converted to various prostanoids (e.g. PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 or thromboxane A_2) via the activity of separate enzymes. For instance prostaglandin endoperoxide reductase converts PGH_2 to $PGF_{2\alpha}$, whereas, prostaglandin endoperoxide E-isomerase converts PGH_2 to PGE_2. Two isoforms of cyclooxygenase (COX 1 and COX II), encoded by separate genes, have been identified to date (Kraemer et al, 1992; Kujubu and Herschman, 1992). The COX I and II proteins (~ 72 kDa) are located in the lumen of the endoplasmic reticulum and nuclear envelope, have almost identical kinetic properties (V_{max} and K_m) with respect to arachidonic acid metabolism, and appear to act on different pools of arachidonic acid (Lanuville et al, 1994; Morita et al, 1995). COX 1 is a constitutive enzyme present in many but not all cells (O'Neill and Ford-Hutchinson, 1993). On the other hand, COX II is inducible by mitogens and cytokines, and has been implicated in the inflammatory response (Moncada and Vane, 1979), ovarian follicular rupture (Armstrong and Grimwhich, 1972), luteolysis (Auletta et al, 1990; Olofsson and Leung, 1994) and parturition (Williams et al, 1979). Sirois (1992) et al have shown that FSH, LH and hCG can

upregulate both COX II mRNA (4.4 kb) and protein (72 kDa) in rat preovulatory ovarian follicles and granulosa cells. In contrast, COX I mRNA (2.8 kb) was not detected in both hen (Li et al, 1996) and rat (Sirois et al, 1992) granulosa cells.

Although, prostaglandins (PGs) have been shown to mediate the action of TGFα in numerous physiologic systems (Harrison, 1994), including the mitogenic response of differentiated hen granulosa cells (Li and Tsang, 1995) and of rat hepatocytes (Skouteris and McMenamin, 1992), it is not known whether PGs are important in the anti-apoptotic action of TGFα in the control of hen granulosa cell apoptosis. Hence, an objective of the present studies was to examine the role and regulation of these eicosanoids in effecting granulosa cell survival in response to TGFα during ovarian follicular development and atresia.

10. HYPOTHESIS

TGFα is an important survival factor for hen granulosa cells. The suppression of granulosa cell apoptosis by TGFα is mediated by increased prostaglandin synthesis and action.

11. OVERALL OBJECTIVES

1. To demonstrate that hen ovarian follicular atresia is associated with granulosa cell apoptosis in situ.

2. To elucidate the relationship between TGFα and hen granulosa cell apoptosis *in vitro*.

3. To investigate the involvement of prostaglandins in mediating the suppression of granulosa cell apoptosis by TGFα.

12. SPECIFIC AIMS

A. Role of Granulosa Cell Apoptosis in Hen Ovarian Follicular Atresia

1. To demonstrate differences in morphology between healthy and atretic prehierarchal hen ovarian follicles *in situ.*

2. To demonstrate differences in display of apoptosis between healthy and atretic prehierarchal hen ovarian follicles *in situ.*

B. TGFα as a Cell Survival Factor for Hen Granulosa Cells

1. To determine if serum deprivation induces apoptotic cell death in granulosa cells from hen prehierarchal follicles *in vitro.*

2. To determine if TGFα inhibits serum deprivation-induced apoptosis in granulosa cells *in vitro.*

C. Involvement of Prostaglandins in the Anti-apoptotic action of TGFα

1. To determine whether TGFα increases prostaglandin synthesis in cultured granulosa cells.

2. To determine if inhibitors of COX and PLA_2 suppress TGFα-induced PG production and induce apoptosis in granulosa cells.

3. To determine if exogenous prostaglandins or arachidonic acid can prevent the apoptotic effect of the COX or PLA_2 inhibitors, respectively.

4. To determine if exogenous prostaglandins alone can mimic the anti-apoptotic actions of TGFα by suppressing serum deprivation-induced apoptosis in granulosa cells *in vitro*.

13. MATERIALS

QIAamp Blood Kit, QIAquick Nucleotide Removal Kit and ribonuclease A (RNase A) were purchased from Qiagen Inc. (Chatsworth, CA, USA). Arachidonyl trifluoromethyl ketone (TFMK), N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS398) and arachidonic acid were purchased from Cayman Chemical Company (Ann Arbor, MI, USA)

Culture media (Minimum Essential Medium and M199), culture media reagents (L-glutamine, non essential amino acids, penicillin, streptomycin and fungizone) and fetal bovine serum (FBS) were purchased from Gibco/Bethesda Research Laboratories (Burlington, Ontario, Canada). Hydroxyethylpiperazine ethanesulfonic acid (HEPES), sodium dodecyl sulphate, bromophenol blue and trizma base were obtained from BDH (Toronto, Ontario, Canada).

Klenow polymerase was purchased from New England Biolabs (Beverly, MA, USA). α-[^{32}P]-dCTP (250μCi/vial) and obtained from Amersham Life Science (Oakville, Ontario, Canada).

Recombinant human transforming growth factor alpha (TGFα) was purchased from Collaborative Research (Bedford, MA, USA). 2-p-amylcinnamoyl amino-4-chlorobenzoic acid (ONO-RS-82), agarose, aristolochic acid, calf thymus DNA, Collagenase-1A, ethylenediaminetetraacetic acid (EDTA), 3,3'-diaminobenzidine tetrachloride (DAB), ethidium bromide, salmon sperm DNA, trypsin-inhibitor (type I), Hoescht 33258 dye, and sodium citrate were obtained from Sigma Chemical Co. (St Louis, MO, USA). Biotinylated 16-dUTP, Protinase-K and

terminal transferase were purchased from Boehringer Mannhein (Montreal, Quebec, Canada).

[5,6,8,9,11,12,14,15-^{3}H(N)]-$PGF_{2\alpha}$ and [3,6,8,11,12,14,15-^{3}H(N)]-PGE_2 (100-200 Ci/mmol), and scintillation fluid (ScintiVerse) were obtained from Dupont/NEN Research Products (Mississauga, Ontario, Canada).

Charcoal, dextran, Gelatin, Permount and Eosin Y were purchased from Fisher Scientific (Nepean, ON, Canada). Prostaglandins (PGE_1, PGE_2 and $PGF_{2\alpha}$) were purchased from Biomol Research Laboratories, Inc (Plymouth Meeting, PA, USA). PGE_2 and $PGF_{2\alpha}$ antisera were gifts from Dr. N.R. Mason (Lilly Research Laboratories, Indianapolis, IN) and Dr. D.T. Armstrong (University of Western Ontario, London, Ontario, Canada), respectively.

14. METHODS

14.1 Experimental Animals

White Leghorn hens in their first year of egg laying were obtained from a local farm (Berry's Egg Farm, Kempville, ON) and were housed individually in cages in an air conditioned (22 °C), windowless room with free access to food and water. The daily light cycle of the holding room was 10 hours of darkness followed by 14 hours of light. Hens were monitored on video camera for egg laying pattern and only hens which laid an egg during the first three hours of the light cycle qualified for experimentation. Hens were sacrificed by cervical dislocation approximately 10 - 13 hours after egg laying, at a time as far from the previous LH surge as possible, so as to avoid additional complications resulting from the endocrine influence of the gonadotropin on the granulosa cells.

Unless mentioned otherwise, all biochemical and cellular procedures were carried out at room temperature.

14.2 Histology

Whole hen ovarian follicles (2-6 mm; healthy and atretic) were fixed at 4°C in 10 % neutral buffered formalin (NBF, pH 7.4) for 24 h, dehydrated through a graded series of ethanol [50 % (30 min), 80 % (30 min), 100 % (30 min) v/v] and xylene (30 min), and embedded in paraffin. The paraffin blocks were cut into 4-6 μm thick sections which were mounted onto positively charged glass slides. The

sections were deparafinized (60 °C, 10 min), slowly rehydrated at RT by immersing in xylene (5 min) followed by a graded series of ethanol [96 % (3 min), 90 % (1 min), 80 % (1 min)], and finally in deionized water (1 min). Histological sections were processed for either Hematoxylin & Eosin Staining (H & E) staining or *in situ* 3'-end labelling of DNA.

14.2.1 Hematoxylin & Eosin Staining

Hematoxylin stains the nucleus while Eosin stains the cytoplasm. Follicular sections attached on slides were stained with hematoxylin for 30 seconds, then briefly soaked in ammonium hydroxide (0.1%), followed by a rinse in water (5 min). They were then immersed in eosin stain for 30 seconds and dehydrated by immersing in a graded series of ethanol [50 % (2 min), 80 % (1 min), 100 % (1 min) v/v], followed by xylene (5 min). Cover slips were mounted on the slides with Canada balsam as a mounting medium.

14.2.2 *In situ* 3'-end labelling of DNA

To localise apoptotic cells *in situ,* follicular sections were examined for DNA fragmentation using the nonradioactive protocol of Gavrieli et al (1992). This method uses the terminal transferase (TdT) enzyme to attach biotinylated-dUTP to free 3' hydroxyl ends of DNA. Streptavidin (a biotin binding molecule) conjugated to horseradish peroxidase (HRP) is then allowed to bind to the biotinylated-dUTP. The enzyme HRP acts on its substrate [diaminobenzidine

tetrahydrochloride (DAB)] to yield a brown coloured product, which is visualised under a light microscope.

Hydrated follicular sections mounted on glass slides are treated for 30 min at 37 °C with proteinase-K (10 μg/ml in 20 mM tris and 2 mM $CaCl_2$, pH 7.4) and then washed for 15 min in tris buffer (100 mM tris and 150 mM NaCl, pH 7.5). The slides were then dipped in methanol containing 0.3 % H_2O_2 (RT, 20 min) to inhibit endogenous peroxidase activity and subsequently washed by dipping in deionized water (15 min). The sections were soaked in the TdT reaction buffer [tris (25 mM, pH 6.6), sodium cacodylate (200 mM), cobalt chloride (5 mM), bovine serum albumin (250 μg/ml)] for 15 min and then incubated in a humidified chamber (37 °C) with 50 μl of a mix containing TdT enzyme (10 Units) and 1 nmol biotinylated 16-dUTP for 60 min. The reaction was stopped by soaking sections in 2X SSC (300 mM sodium chloride, 30 mM sodium citrate), followed by washing in PBS at RT for 15 min. Next, sections were incubated (RT, 30 min) with horseradish peroxidase-conjugated streptavidin (Dako, Denmark) as per manufacturer's instructions to allow streptavidin to bind to the biotin on dUTP.

The slides were washed by dipping in PBS (15 min), and then the peroxidase colouring reaction was allowed to occur by immersing slides in DAB containing buffer [tris (0.05 M, pH 7.6), DAB (0.3 mg/ml), sodium azide (0.65 mg/ml), imidazole (10 mM), and H_2O_2 (0.003%)] for 10 min. Sections were then immersed in buffer containing methyl green (5 %) and veronal acetate (0.1 M, pH 4.0) for 2 min, at RT to counterstain the nuclei green. The negative control slides received H_2O instead of the TdT enzyme. Cover slips were mounted on the slides with Canada balsam as a mounting medium. Sections were viewed under light

microscopy using an Axioskop MC 100 microscope (Carl Zeiss Inc, Germany) and photographed at magnification 50 X, 100 X and 400 X.

14.3 Isolation of Granulosa Cells

Hen ovaries were excised and placed in cold Medium 199 supplemented with HEPES (25 mM). Only morphologically healthy follicles (2-6 mm in diameter), as determined by gross morphology (round, not flaccid, white coloured and yolk filled), were used for studies described in this thesis. The follicle was punctured and then gently squeezed using a pair of forceps to express sheets of granulosa cells into a petri dish of culture media. Granulosa cell layers from approximately 30 follicles were pooled in a 15 ml conical tube, and treated with 2 to 4 ml of Medium 199 containing collagenase (270 units/ml) and trypsin inhibitor (0.01%) at 37 °C for 5-10 minutes to facilitate cell dissociation. The cell layers were aspirated up and down with a glass pasture pipette for 30 seconds once every four minutes during the collagenase digestion. When no "clumps" of more than four cells (as monitored under a light microscope) were evident, 10 ml of Medium 199 was added with gentle mixing. Cell were collected by centrifugation (10 min, 400 x g) and washed in 10 to 12 ml of Medium 199, and finally resuspended in 4 to 6 ml of Minimum Essential Medium (MEM).

To determine granulosa cell viability, 20 µl of the cell suspension was mixed with 20 µl trypan blue dye and 60 µl of MEM, and the number of viable cells in the cell suspension was determined with a hemacytometer. Viable cells exclude trypan blue staining whereas dead cells take up the dye and appear blue under the microscope. Granulosa cell viability in the preparations was over 90 %.

14.4 Culture of Granulosa cells

Granulosa cells (1 x 10^6/dish) were first plated in 35 mm (diameter) plastic culture dishes for 6 hours in 1 ml of MEM containing 10% fetal bovine serum (FBS) at 39 °C under an atmosphere of 5% CO_2 : 95% air. This plating period allowed cells to attach to the culture surface before medium change and addition of test agents. The cells were then cultured for 24 hours in 1.5 ml of either fresh MEM with FBS (10%), or serum-free MEM containing TGFα, COX or PLA_2 inhibitors, and prostaglandins or arachidonic acid. At the end of the culture period, media was collected and centrifuged (5 min, 10,000 x g) to pellet down any floating cells. The cell-free media was supplemented with 2.0 ml absolute ethanol and stored at -20 °C until assayed for prostaglandins, whereas the cells recovered following centrifugation as well as those attached to the culture dish were pooled and stored at -80 °C, pending DNA extraction.

14.5 Prostaglandin Measurements by RIA

PGE and PGF in the culture media were measured by radio-immuno assay according to the method of Jaffe et al (1973). Appropriate volumes (25-200 µl) of the ethanolic extracts (in duplicates) and standard PGE_2 or $PGF_{2\alpha}$ (in triplicates) were dispensed into 12 x 75 mm glass tubes and evaporated to dryness in a 37 °C water bath under a stream of nitrogen. The tubes then received 100 µl of the appropriate radiolabelled PG (^{3}H-PGE_2 or ^{3}H-$PGF_{2\alpha}$; 14000 cpm/tube) in phosphate buffered saline-gelatin (PBSG; 0.1% gelatin in PBS) followed by 100 µl of PBSG. The tubes were vortexed briefly, and 100 µl of the appropriate antisera

in PBSG (at a concentration giving 25-30 % binding of the radiolabelled PG in the absence of unlabelled PG) was added. The final assay volume was 300 µl/tube.

The assay also included tubes in triplicates for "total counts" (the total cpm of labelled PG added to each assay tube); "B_0" (^{3}H-PG bound to the antibody in the absence of unlabelled PG); and "NSB" (non-specific binding; an assay blank subtracted from all values). All tubes were gently vortexed and allowed to equilibrate overnight at 4 °C. The next morning, tubes were incubated on ice for 30 minutes, and then 700 µl of an ice cold charcoal/dextran mixture (0.25% charcoal and 0.025% dextran in PBS) was added (except for the "total counts" tubes which received 700 µl of PBS). Charcoal/dextran serves to adsorb the ^{3}H-PG and PGs that are not bound to the antibody. Both ^{3}H-PG and unlabelled PG compete equally for specific binding sites on the antibody (limiting reagent) and, as the unlabelled PG/^{3}H-PG ratio increases the amount of ^{3}H-PG bound to the antibody decreases. The tubes were centrifuged (15 min, 2000 rpm, 4 °C) to pellet down the charcoal, and the supernatants which contained the antibody-bound ^{3}H-PG or unlabelled PG were decanted into scintillation vials. Four millilitres of scintillation cocktail (ScintiVerse, Fisher Scientific) was added and the vials were capped. The radioactivity in each vial was determined using a Liquid Scintillation Analyzer (Canberra Packard 2200CA). A standard curve [% of total ^{3}H-PG bound to antibody versus amounts of unlabelled PG standard added] was generated, and the amount of unlabelled PG in samples was determined. Since the antisera against PGE_2 and $PGF_{2\alpha}$ had significant cross-reactivity against PGE_1 (16.8 %) and $PGF_{1\alpha}$ (125%), respectively (Lewis et al, 1978), the data is reported as PGE and PGF equivalents.

14.6 Biochemical Assessment of Apoptosis (DNA Ladders)

14.6.1 DNA Extraction and Quantification

DNA was isolated using the QIAamp Blood Kit (Qiagen, Inc) as per manufacturer's instructions. Briefly, cells were lysed with an acidic, chaotropic salt solution and subject to RNase and proteinase K treatment to digest cellular RNA and protein. Samples were loaded on to spin columns containing silica membranes which specifically bound DNA (80 bp to 50 kb in size). Salt was removed from the columns by washing twice with an ethanolic solution, and finally DNA was eluted with tris buffer (10 mM, pH 9). Eluted DNA was stored at -80 °C pending 3'-end labelling with α-[^{32}P]-dCTP (deoxycytidine 5'-α-[^{32}P]triphosphate). To quantify the DNA in the samples, 50 µl of the above DNA elute was added to 450 µl tris (10 mM, pH 9). The absorbance of this final solution was measured in a quartz cuvette at 260 nm, using a Milton Roy Spectronic 1201 spectrometer. Fifty ng DNA /µl gives an absorbance (OD) unit of one.

14.6.2 DNA 3'-end Labelling

To quantify the extent of DNA fragmentation in cultured cells, the isolated DNA was radiolabelled at the 3'-end with α-[^{32}P]-dCTP, using the Klenow reaction as described by Rosl (1992) and resolved on agarose gel electrophoresis (AGE). In this reaction, the Klenow polymerase ligated the α-[^{32}P]-dCTP to the free 3' hydroxyl group (on the 5'→3' strand) when the nucleotide base on the complementary DNA strand (3'→5') was guanine. Briefly, equal amounts of

DNA (~ 400 ng) were mixed with Klenow Polymerase (2.5 units/sample) and an excess of α-[^{32}P]-dCTP (4 μCi/sample) and the labelling reaction was allowed to proceed at RT for 30 minutes. The labelled DNA was purified with the use of the Nucleotide Removal Kit (Qiagen Inc.) as per manufacturer's instructions. Briefly, this involved adsorption of the DNA onto a silica membrane in a spin column under low pH and high chaotropic salt conditions, followed by washing of the column with an ethanolic solution to remove salt. DNA (labelled) was eluted with tris buffer (10 mM, pH 8.5) and stored at -20 °C pending AGE.

14.6.3 Agarose Gel Electrophoresis and Autoradiography

Labelled DNA of different sizes was resolved by AGE according to the method of Sambrook et al (1989). Briefly, radiolabelled DNA was mixed with gel loading buffer (GLB; 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water), loaded 25 μl/well in agarose gel [2 % in tris-acetate EDTA buffer [(TAE; tris (4.84 g/L), glacial acetic acid (1.14 ml /L), EDTA (0.001M)] and resolved at 60-85 volts for about 150 minutes. GLB provided a dense medium for reliable sample loading and contained a dye (bromophenol blue migrated at the same rate as 300 bp double stranded DNA) to monitor the extent of electrophoresis. The gel was dried under negative pressure (no heat) in a gel dryer (DrygelsSr, Model SE 1160) for 3 hours and exposed to X-ray film for 0.5-24 hours (depending on the specific activity of the α-[^{32}P]-dCTP used). The X-ray film was then developed in a Kodak M35A X-OMAT processors. Alternatively, the gel was exposed to a phosphor screen for 0.5-24 hours which was subsequently scanned on a Molecular Dynamics Phosphorimager (Bio Rad, Mississauga, ON, Canada) to yield a computer generated image, similar to the X-ray film. The

relative intensity of labelled low molecular weight (fragmented) DNA in each lane (sample) was determined from the image using Molecular Analyst Software (Bio Rad, Mississauga, ON, Canada).

14.7 Morphological Assessment of Apoptosis

14.7.1 Phase Contrast Microscopy

At the end of the culture period, cells were viewed under phase contrast microscopy, using an Axiovert 35 inverted microscope (Carl Zeiss Inc, Germany) and photographed in bright field at low (100 X) and high (200 X) magnifications.

14.7.2 Hoechst Staining of Cultured Cells

At the end of the culture period, the medium (which contains floating cells) was transferred to a 1.5 ml eppendorf tube. The cells were detached from the culture surface by incubating in trypsin (0.025 g/ml in PBS; 37 °C; 2 min) and combined with the floating cells in the medium fraction, and fixed with 37 % formaldehyde (10 % v/v; RT, 10 min). Next, cells were incubated with Hoechst 33258 dye (2 μg/ml in PBS; RT, 10 min). Hoechst 33258 dye, which intercalates between the strands of double stranded DNA and emits visible light when excited by UV, was used to assess nuclear morphology of granulosa cells. An aliquot of the cell suspension was placed on a glass slide, viewed under UV light under a Zeiss IM 35 microscope (Carl Zeiss Inc, Germany) and photographed at magnification of 250 X. Cells with fragmented nuclei or highly condensed nuclei (which stain

brightly) were considered apoptotic. Between 75-220 cells were counted per treatment in each experiment.

14.8 Statistical Analysis

All data presented is from experiments that were repeated at least 3-4 times. Studies were statistically analysed by one-way analysis of variance. Significant differences between groups were determined by the Newman Kuels test. Statistical difference was inferred at $P < 0.05$.

15. RESULTS

15.1 Morphologically Healthy vs. Atretic Follicles

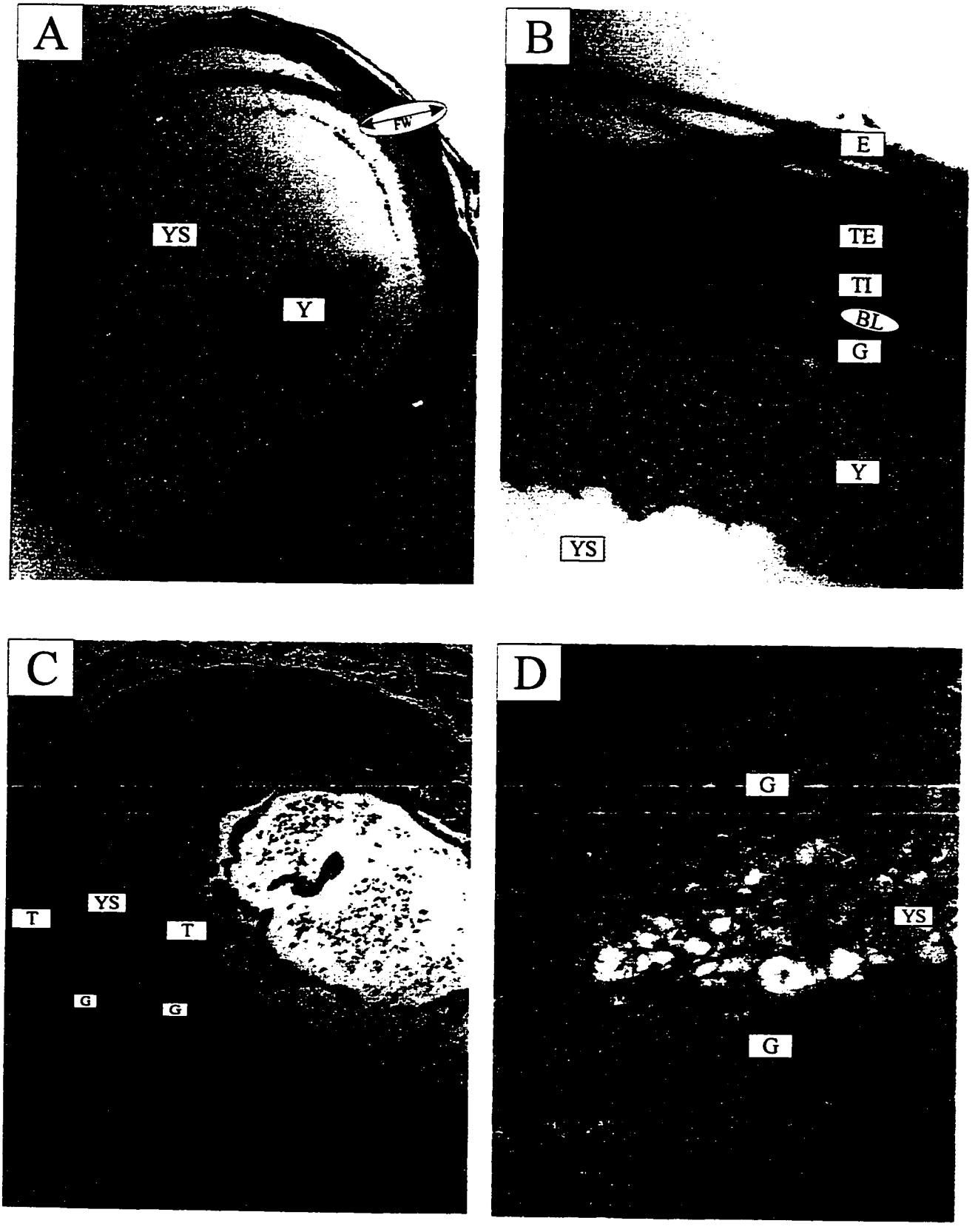
In the hen ovary, the fate of the follicle (ovulation verses atresia) is determined at the prehierarchal level of development, i.e. before the follicle grows to about 9 mm in diameter. Hen follicles that grow to a size of ≥ 9 mm (now called hierarchal follicles) generally continue to grow and are committed to ovulate. The healthy follicles appear firm, round, clear and are full of yolk. On the other hand, atretic follicles appear shrunk, flaccid, opaque and are yolk depleted. Healthy follicles of diameter 2-6 mm were conservatively considered prehierarchal with a yet undetermined fate and were thus used in this study.

To further examine differences in morphology at the cellular level between healthy and atretic hen prehierarchal ovarian follicles, histological sections of follicles (2-6 mm) were prepared. Ovarian follicles identified as healthy by gross appearance were morphologically unspoiled and circular under H & E staining (Plate 2A). The healthy follicle had intact layers of granulosa cells surrounded by a basal lamina which was in turn surrounded by multiple theca cell layers (Plates 2A and 2B). In contrast, ovarian follicles identified as atretic appeared under H & E staining to be morphologically spoiled, collapsed and not circular (Plate 2C). Furthermore, the intrafollicular yolk space was seriously diminished and many detached granulosa cells were present (Plate 2D; arrows).

There is much evidence suggesting that ovarian follicular atresia is linked to programmed cell death or apoptosis of follicular cells (Tilly et al, 1991a; reviewed by Hsueh et al, 1994).

Plate 2. Hematoxylin and eosin stained healthy and atretic hen prehierarchal ovarian follicle.

Morphologically healthy (panel A) and atretic (panel C) 2-6 mm ovarian follicles were extracted, fixed, paraffin embedded and sectioned. The sections were then stained with H & E and photographed at magnification of 50 X (panels A and C) and 400 X (panels B and D). In panel A (healthy follicle): YS, intrafollicular yolk space; Y, yolk. Panel B: magnification of the follicular wall (FW) area of the follicle in panel A; E, epithelium; TE, theca externa; TI, theca interna; BL, basal lamina; G, granulosa; and Y, yolk remnants. The large intrafollicular yolk space (YS) would be filled with yolk in the intact follicle. In panel C (atretic follicle): G, granulosa; T, theca; YS, intrafollicular yolk space. Panel D: magnification of the atretic follicle in panel C; the intrafollicular yolk space (YS) is reduced and contains numerous detached granulosa cells (black arrows).


A
FW
YS
Y
B
E
TE
TI
BL
G
Y
YS
C
YS
T
T
G
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D
G
YS
G

To examine differences in occurrence of apoptotic cell death between healthy and atretic follicles, serial histological sections were processed for *in situ* 3'-end labelling of fragmented DNA. Both granulosa and theca layers failed to display DNA degradation in healthy follicles (Plates 3A and 3B). At a higher magnification it was evident that only cells of the surface epithelium exhibited heavy staining for DNA degradation (Plate 3B). On the other hand, granulosa cells of the atretic follicles stained heavily for fragmented DNA, as evident by the dark staining in Plate 3C (compare to H & E staining in Plate 2C). Many of the detached granulosa cells in the intrafollicular yolk space also stained positively (Plate 3D). Compared to granulosa cells, theca cells in the atretic follicle displayed little or no DNA fragmentation (Plate 3D).

15.2 Serum Deprivation Induced Apoptosis

To investigate the regulation of granulosa cell apoptosis, it was important to study granulosa cells alone in a simple *in vitro* system. It has been reported in many cell types that serum-deprivation *in vitro* induces apoptosis (Raff, 1992). To determine whether granulosa cell apoptosis could be induced by serum withdrawal *in vitro*, cells were plated for 6 hours in the presence of 10 % FBS, then further cultured for 24 hours in media with or without serum. DNA from granulosa cells cultured in serum-free media displayed more extensive apoptotic fragmentation (3.8-fold) compared to that from cells cultured in media containing 10 % FBS ($p < 0.01$; Figure 3). Since a 24 hour culture of granulosa cells in serum-free medium was capable of inducing significant apoptosis, serum-deprivation was used in subsequent cultures as a model to investigate the regulation of granulosa cell apoptosis by TGFα *in vitro*.

Plate 3. In situ DNA 3'-end labelled healthy and atretic hen prehierarchal ovarian follicle.

Morphologically healthy (panel A) and atretic (panel C) 2-6 mm ovarian follicles were extracted, fixed, paraffin embedded and sectioned. The sections were then treated with DAB-dUTP and TdT enzyme (to label the 3' free hydroxyls on fragmented DNA) and photographed at magnification of 50 X (panels A and C), 100 X (panel D) and 400 X (panel B). Panel B is a magnification of the follicular wall (FW) area of the healthy follicle in panel A. As seen in panel B, only the epithelium (E) stained positive (dark staining) for fragmented DNA, while the theca externa (TE), theca interna (TI), basal lamina (BL), granulosa (G), and yolk remnants (Y) show no staining. Panel D: magnification of the atretic follicle in panel C; the theca (T) stained negative while the granulosa (G) layer stained positive for fragmented DNA, and most of the detached granulosa cells in the intrafollicular yolk space (YS) were also positive for fragmented DNA.

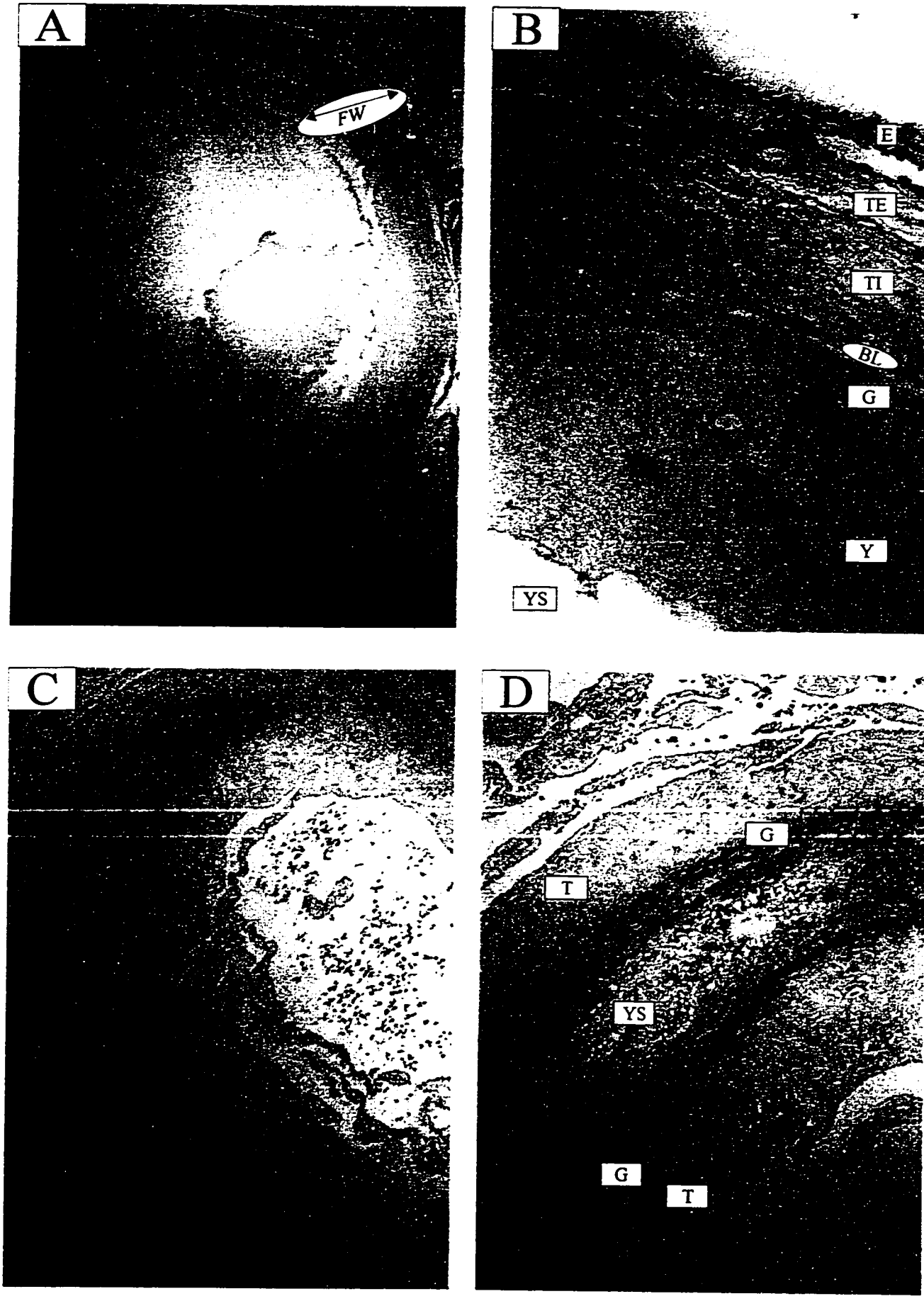
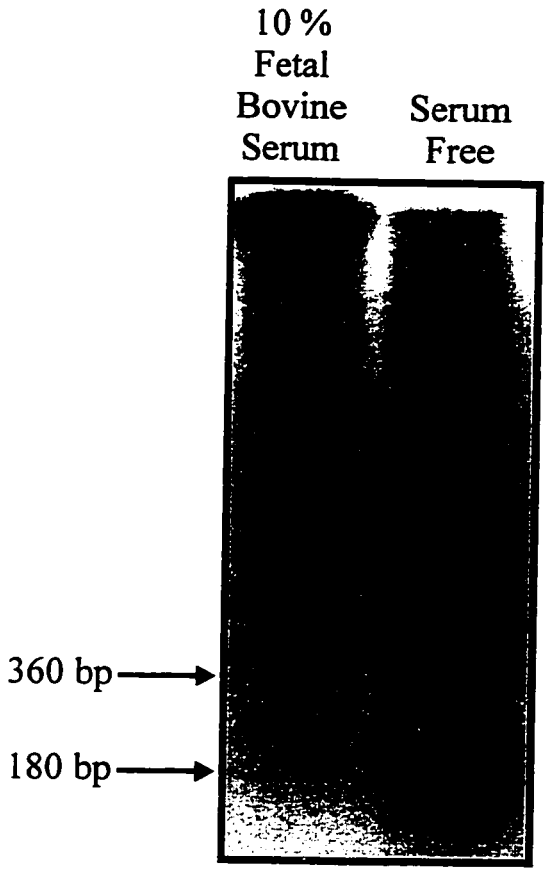
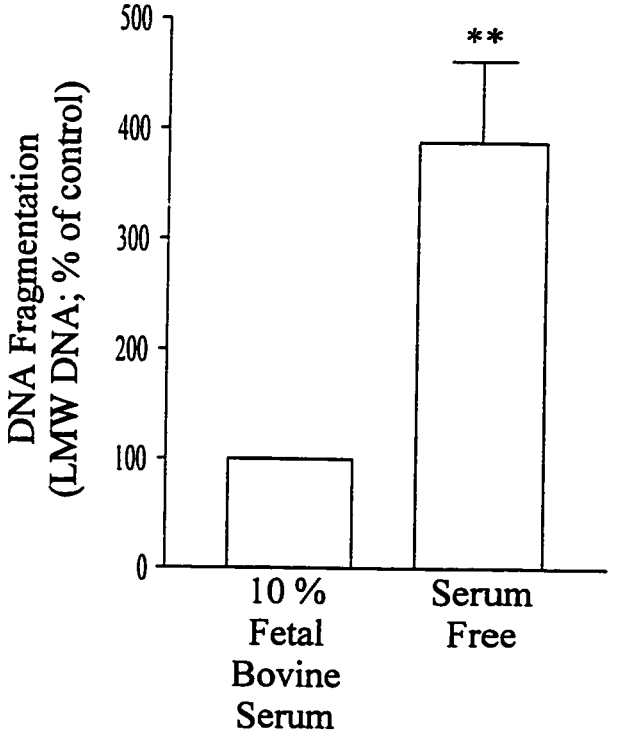

A
FW
B
E
TE
TI
BL
G
Y
YS
C
D
G
T
YS
G
T

Figure 3. Serum deprivation caused granulosa cell apoptosis *in vitro*.
Granulosa cells were plated for 6 hours in media containing 10 % FBS, then cultured for 24 hours in serum-free media or media containing 10 % FBS. DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA ($\leq$ 15 kb). Control (FBS) is set at 100 %. Data is expressed as mean % of control $\pm$ SEM; N = 5 experiments. ** $P < 0.01$ vs. control.

A.


10 % Fetal Bovine Serum
Serum Free
360 bp
180 bp

B.


DNA Fragmentation (LMW DNA; % of control)
500
400
300
200
100
0
**
10 % Fetal Bovine Serum
Serum Free

15.3 TGFα Suppressed Serum Deprivation-induced Apoptosis

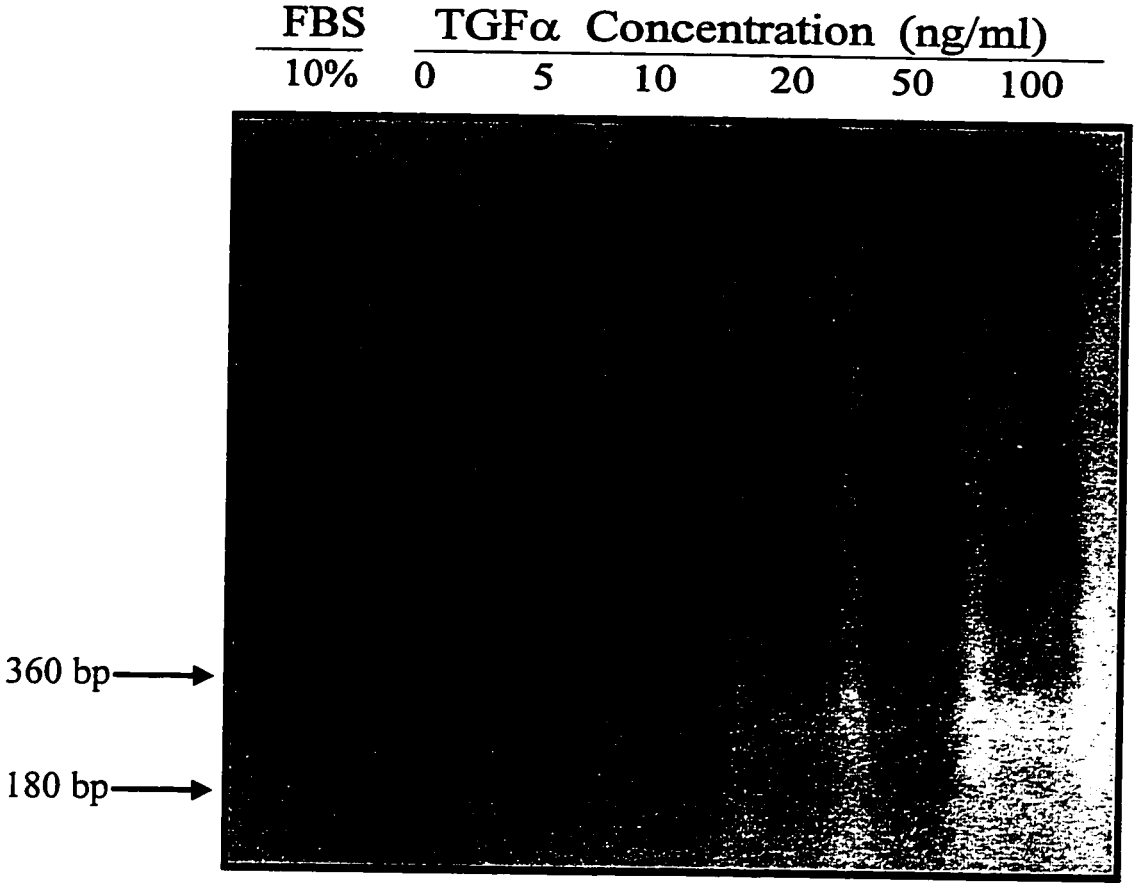
To determine if TGFα suppresses serum deprivation-induced apoptosis, granulosa cells were cultured in serum-free medium containing TGFα. TGFα (5-100 ng/ml) inhibited serum deprivation-induced apoptotic DNA fragmentation in a concentration-dependent manner (Figure 4). Maximum inhibition was observed at the TGFα concentration of 20 ng/ml which completely suppressed the serum-deprivation-induced DNA fragmentation ($p < 0.001$). The ID_{50} for TGFα in the suppression of DNA fragmentation induced by the serum withdrawal was 5 ng/ml.

15.4 TGFα Stimulated Prostaglandin Production

TGFα has been shown to exert its effects by increasing prostaglandin (PG) levels in many cell types (Li and Tsang, 1995; Hanke et al, 1996). If PGs mediate the actions of TGFα in granulosa cells, PG synthesis would be expected to be stimulated by the presence of TGFα. Granulosa cells secreted appreciable levels of PGE (12.9 ± 1.9 pmoles/10^6 cells) and 3 times more PGF (39.6 ± 3.3 pmoles/10^6 cells) during a 24 hour culture in serum-free medium containing no TGFα. Addition of TGFα (5-100 ng/ml) to the cell cultures significantly ($p < 0.01$) increased both PGE and PGF secretion in a concentration-dependent manner (Figure 5). Maximum stimulation was seen at a concentration of 20 ng/ml TGFα which enhanced PGE and PGF secretion to 2.5- and 4.5-fold above the control (zero TGFα), respectively. The ED_{50} for TGFα for both PGE and PGF secretion was 5 ng/ml, which is similar to its ID_{50} value for inhibition of serum deprivation-induced DNA fragmentation as seen in Figure 4.

Figure 4. TGFα suppressed serum deprivation-induced apoptosis in granulosa cells *in vitro.*

Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing the indicated concentrations of TGFα (black histograms) or in media containing only 10 % FBS (clear histogram). DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Corresponding histograms are positioned directly below their DNA ladders. The control (zero TGFα) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 4 experiments. ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control.


FBS
10%
TGFα Concentration (ng/ml)
0
5
10
20
50
100
360 bp
180 bp

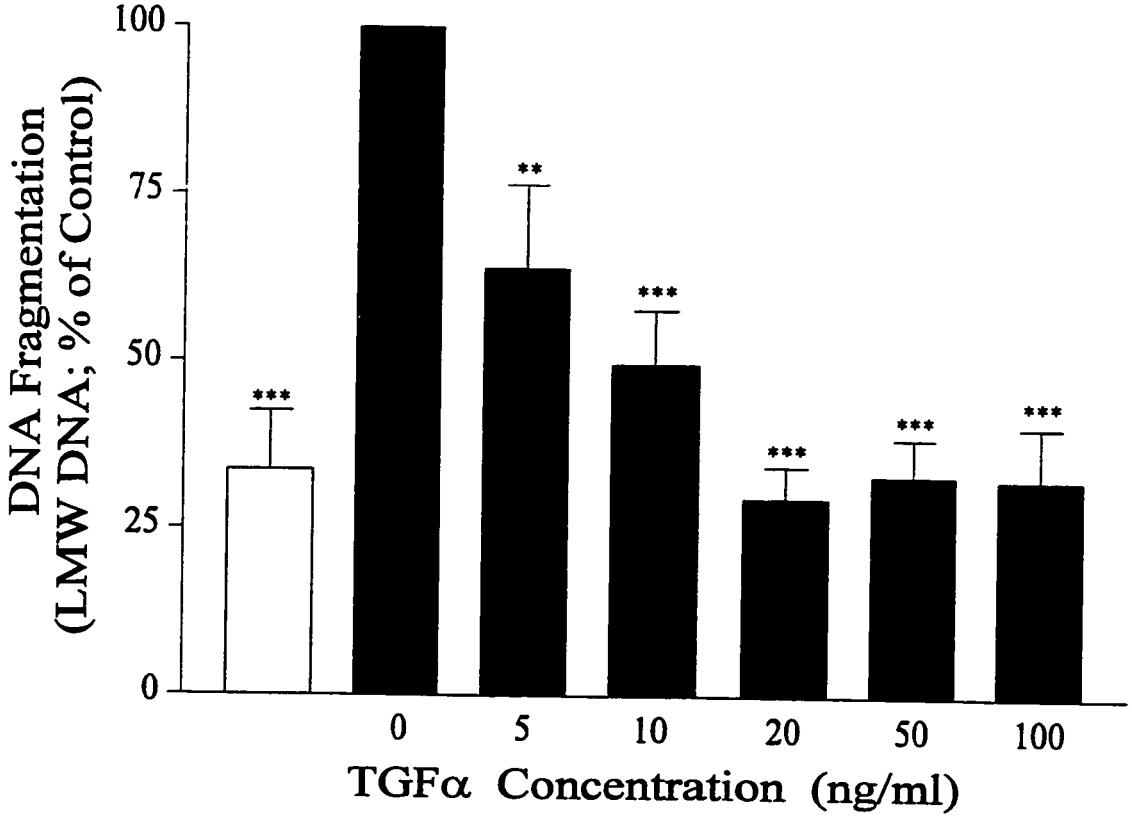
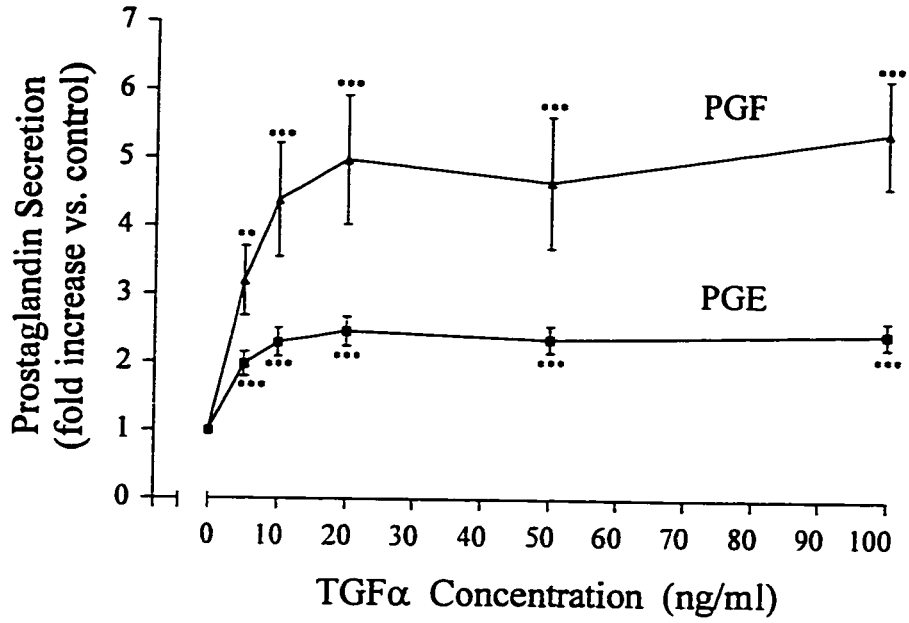

DNA Fragmentation
(LMW DNA; % of Control)
100
75
50
25
0
***
**
***
***
***
***
0
5
10
20
50
100
TGFα Concentration (ng/ml)

Figure 5. Stimulation of granulosa cell prostaglandin secretion by TGFα *in vitro*. Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing the indicated concentrations of TGFα. The amount of prostaglandins secreted in the culture media was determined by RIA. PGE and PGF secretion in the control groups (zero TGFα; set at 100 %) was 12.9 ± 1.9 pmoles/10^6 cells and 39.6 ± 3.3 pmoles/10^6 cells, respectively. Data is expressed as mean fold increase over control ± SEM; N = 9 (PGE) and 6 (PGF) experiments. ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control.


Prostaglandin Secretion
(fold increase vs. control)
7
6
5
4
3
2
1
0
PGF
PGE
0
10
20
30
40
50
60
70
80
90
100
TGFα Concentration (ng/ml)

15.5 COX Inhibitors Attenuated the TGFα-induced Suppression of Apoptosis

To determine whether the TGFα-induced prostaglandin synthesis mediate the anti-apoptotic action of TGFα, COX inhibitors were used to block prostaglandin synthesis (Figure 6). NS398 is a selective COX II inhibitor, whereas ibuprofen and indomethacin inhibit both COX I and COX II (Futaki et al, 1994; Lanuville et al, 1994; Kurumbail et al, 1996). Granulosa cells were cultured in serum-free media containing TGFα (20 ng/ml) and either NS398 (5-20 μM), ibuprofen (50-200 μM) or indomethacin (12.5-50 μM). Concentrations of COX inhibitors were decided bases on previously published studies (Li and Tsang, 1995; Futaki et al, 1994). NS398 and ibuprofen, but not indomethacin, effectively attenuated the TGFα-induced suppression of granulosa cell apoptosis. The lowest concentration of NS398 used (5 μM) significantly increased (180 %; $p < 0.05$) apoptotic DNA fragmentation compared to cells not exposed to NS398 (Figure 7). Higher concentrations of NS398 (10 μM and 20 μM) failed to significantly afford a further increase in DNA fragmentation. Similarly, cells cultured in the presence of ibuprofen (50 μM) displayed significantly greater (190%; $p < 0.01$) apoptotic DNA fragmentation compared to cells not exposed to ibuprofen (Figure 8). A concentration-dependent response to ibuprofen was not evident ($p > 0.05$). In contrast, cells cultured in the presence of indomethacin displayed only a small increase (140-160%) in apoptotic DNA fragmentation compared to cells cultured in the absence of the inhibitor (Figure 9). However, due to large inter-experimental variations, these responses were statistically not significant ($p > 0.05$).

Figure 6. Prostaglandin biosynthesis pathway and its inhibitors.
Membrane phospholipids such as phosphatidylcholine (PC) are metabolised by cytosolic phospholipase A_2 ($cPLA_2$) to arachidonic acid and lysophosphatidic acid (LPC). Cyclooxygenase (COX) then converts arachidonic acid to prostaglandins (PGs). Alternatively, lipoxygenase can metabolise arachidonic acid to leukotrienes. Aristolochic acid (AC), arachidonyl tri-floromethyl ketone (TFMK), and ONO-RS-82 inhibit the PLA_2 enzyme. NS398, ibuprofen and indomethacin inhibit the COX enzyme.

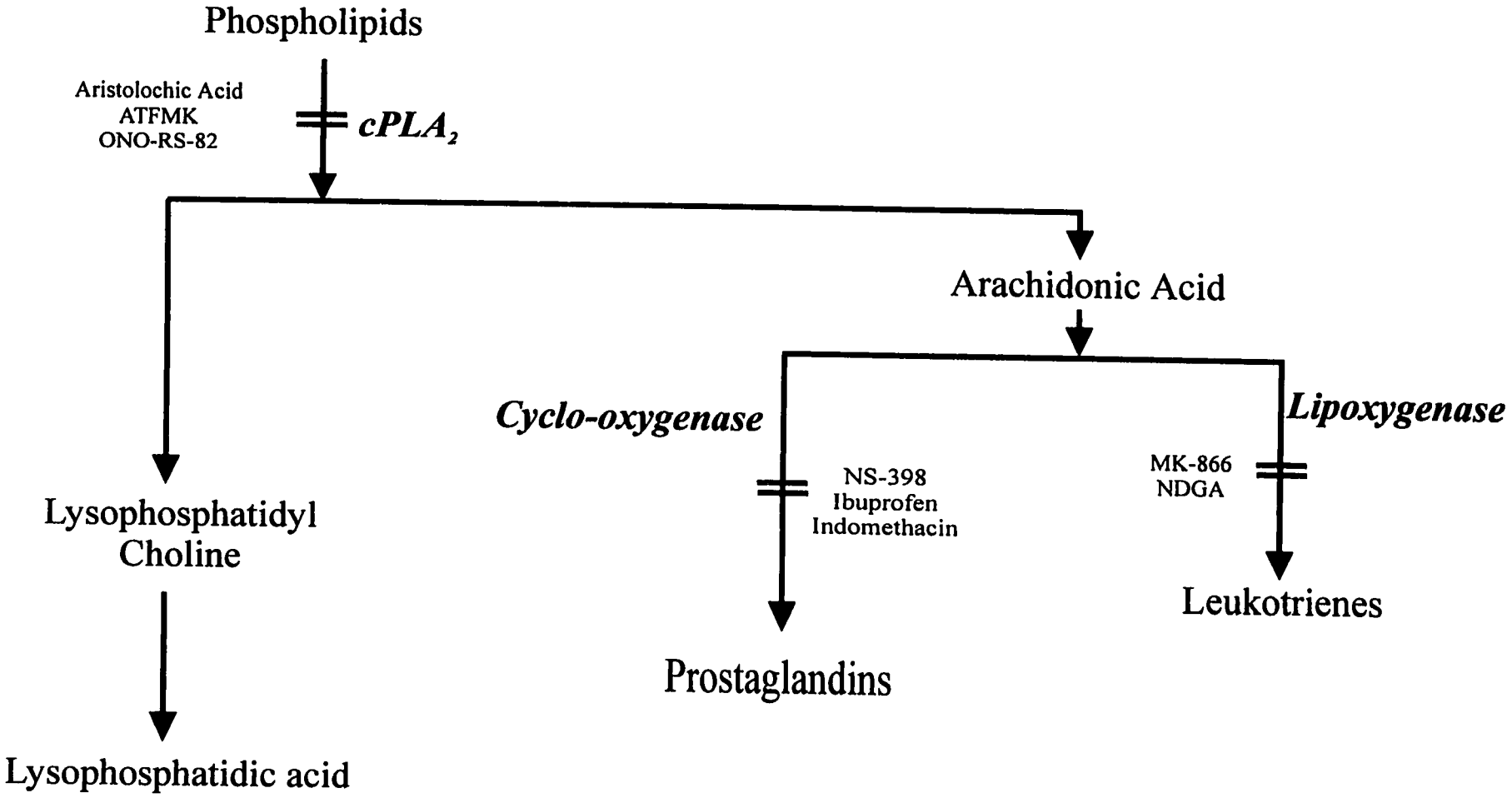
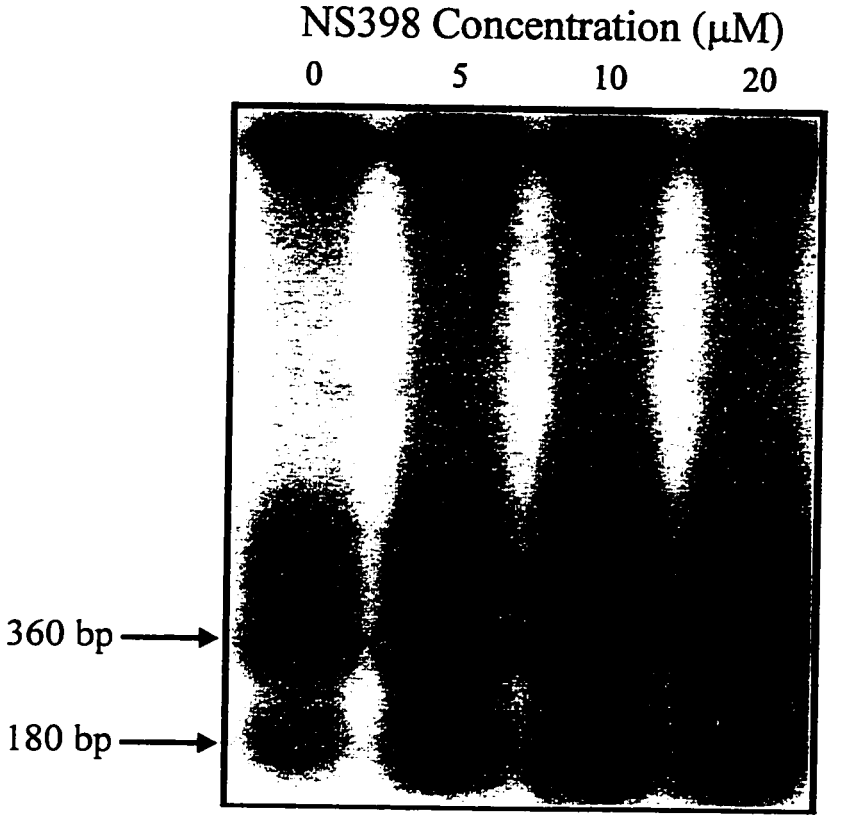

Phospholipids
Aristolochic Acid
ATFMK
ONO-RS-82
cPLA2
Arachidonic Acid
Cyclo-oxygenase
Lipoxygenase
NS-398
Ibuprofen
Indomethacin
MK-866
NDGA
Lysophosphatidyl
Choline
Leukotrienes
Prostaglandins
Lysophosphatidic acid

Figure 7. The COX II inhibitor NS398 attenuated the anti-apoptotic action of TGFα in granulosa cells *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the indicated concentrations of NS398. DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Control (20 ng/ml TGFα; no NS398) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 7 experiments. * $P < 0.5$ vs. control; ** $P < 0.01$ vs. control.

A.


NS398 Concentration (μM)
0
5
10
20
360 bp
180 bp

B.

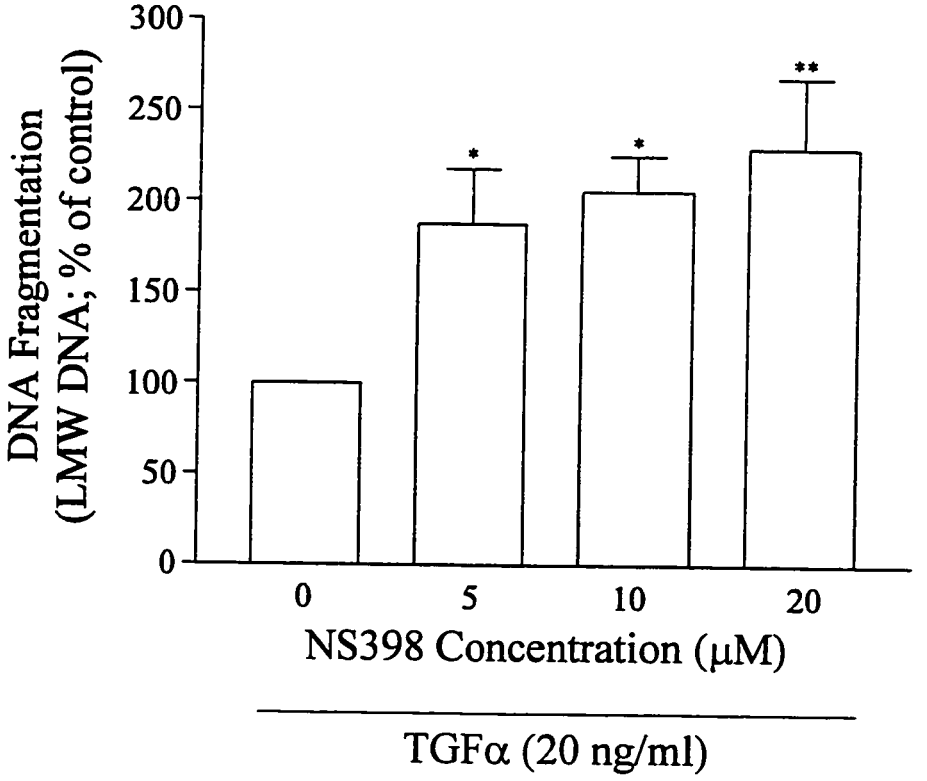
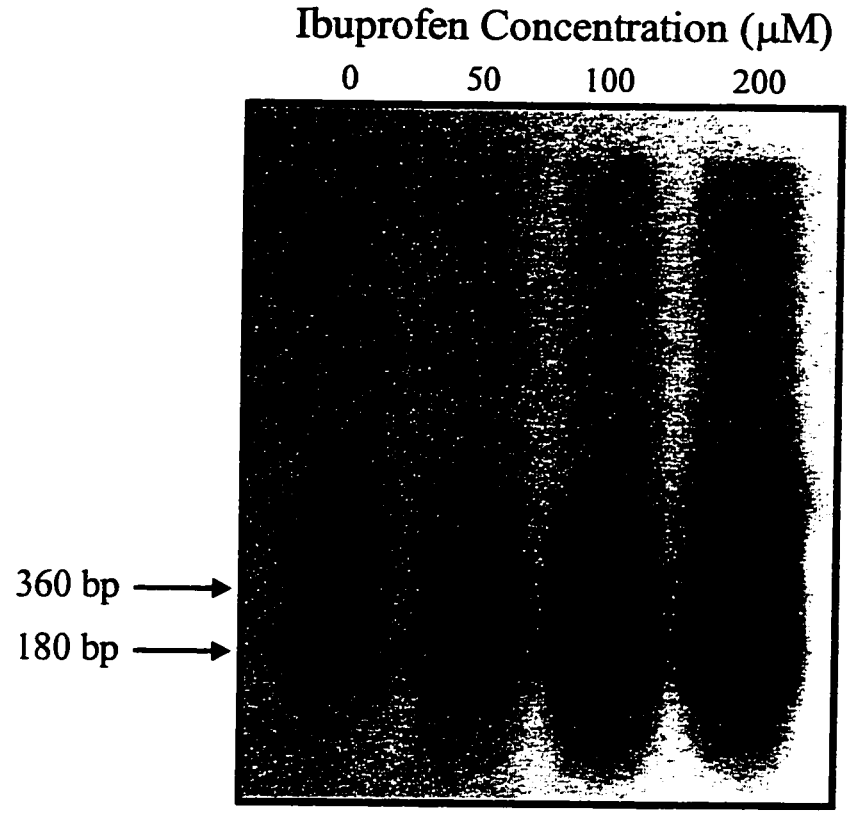

DNA Fragmentation
(LMW DNA; % of control)
300
250
200
150
100
50
0
*
*
**
0
5
10
20
NS398 Concentration (μM)
TGFα (20 ng/ml)

Figure 8. The COX inhibitor ibuprofen attenuated the anti-apoptotic action of TGFα in granulosa cells *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the indicated concentrations of ibuprofen. DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Control (20 ng/ml TGFα; no ibuprofen) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 7 experiments. * $P < 0.5$ vs. control; ** $P < 0.01$ vs. control.

A.


Ibuprofen Concentration (μM)
0
50
100
200
360 bp
180 bp

B.

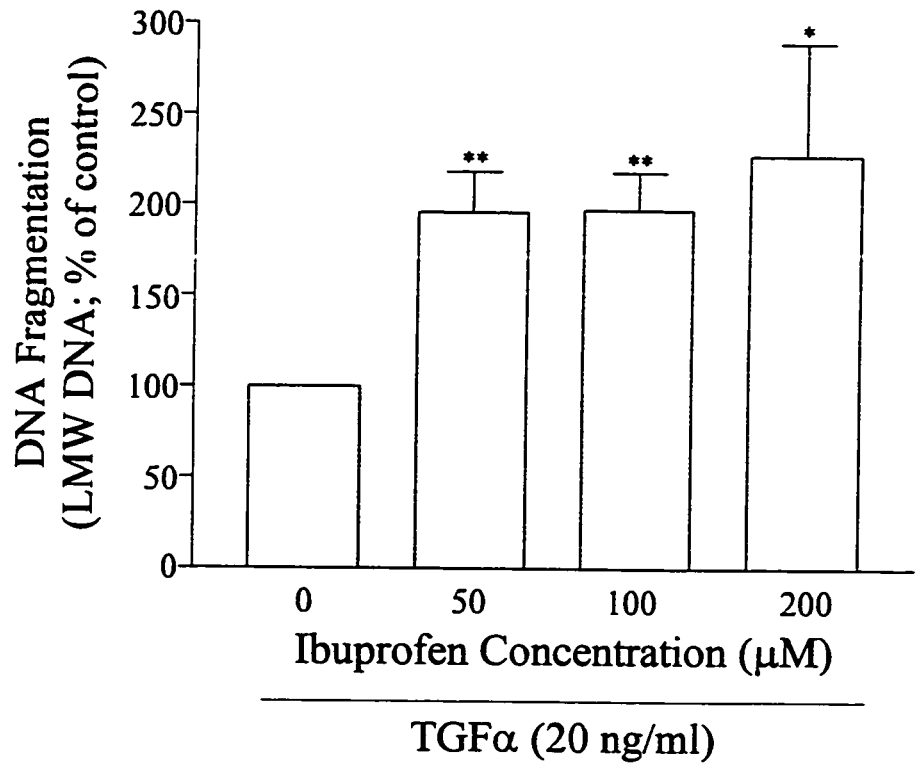
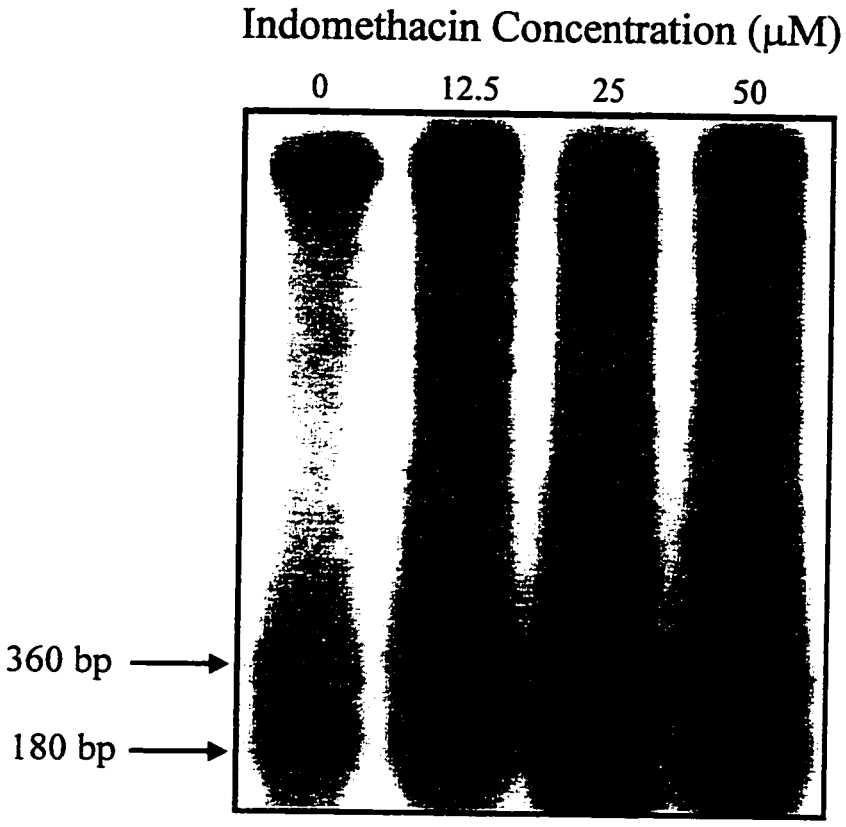
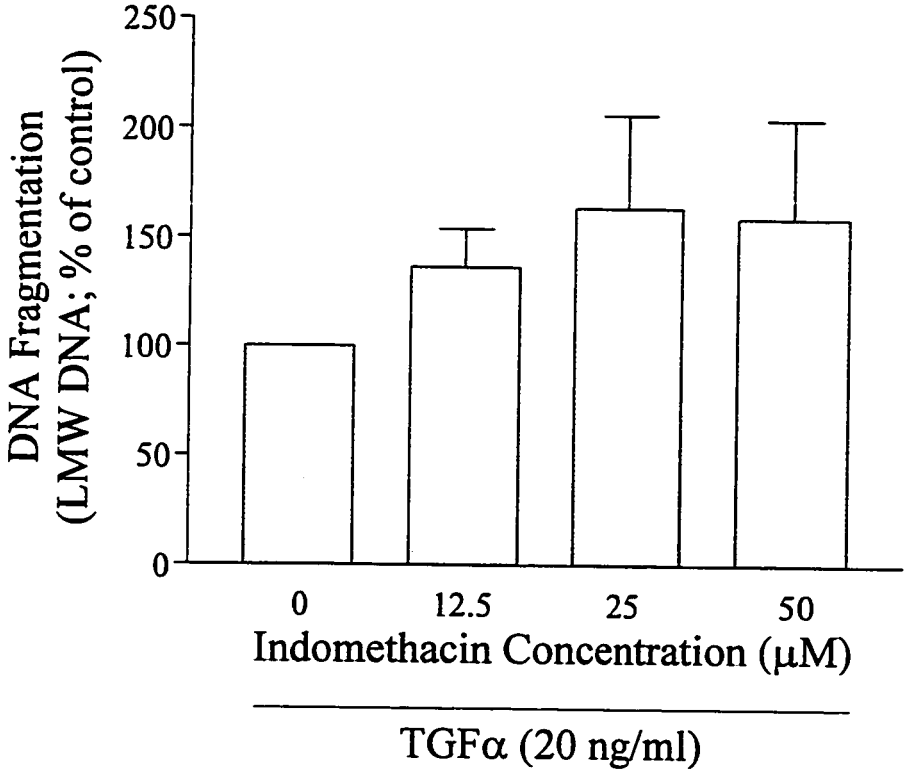

DNA Fragmentation
(LMW DNA; % of control)
300
250
200
150
100
50
0
**
**
*
0
50
100
200
Ibuprofen Concentration (μM)
TGFα (20 ng/ml)

Figure 9. Attenuation of the TGFα-induced suppression of apoptosis by the COX inhibitor indomethacin in granulosa cells *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the indicated concentrations of indomethacin. DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Control (20 ng/ml TGFα; no indomethacin) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 4 experiments. There are no significant differences between treatment and control groups ($p > 0.05$).

A.


Indomethacin Concentration (μM)
0
12.5
25
50
360 bp
180 bp

B.


DNA Fragmentation
(LMW DNA; % of control)
250
200
150
100
50
0
0
12.5
25
50
Indomethacin Concentration (μM)
TGFα (20 ng/ml)

15.6 COX Inhibitors Attenuated the TGFα-induced Prostaglandin Production

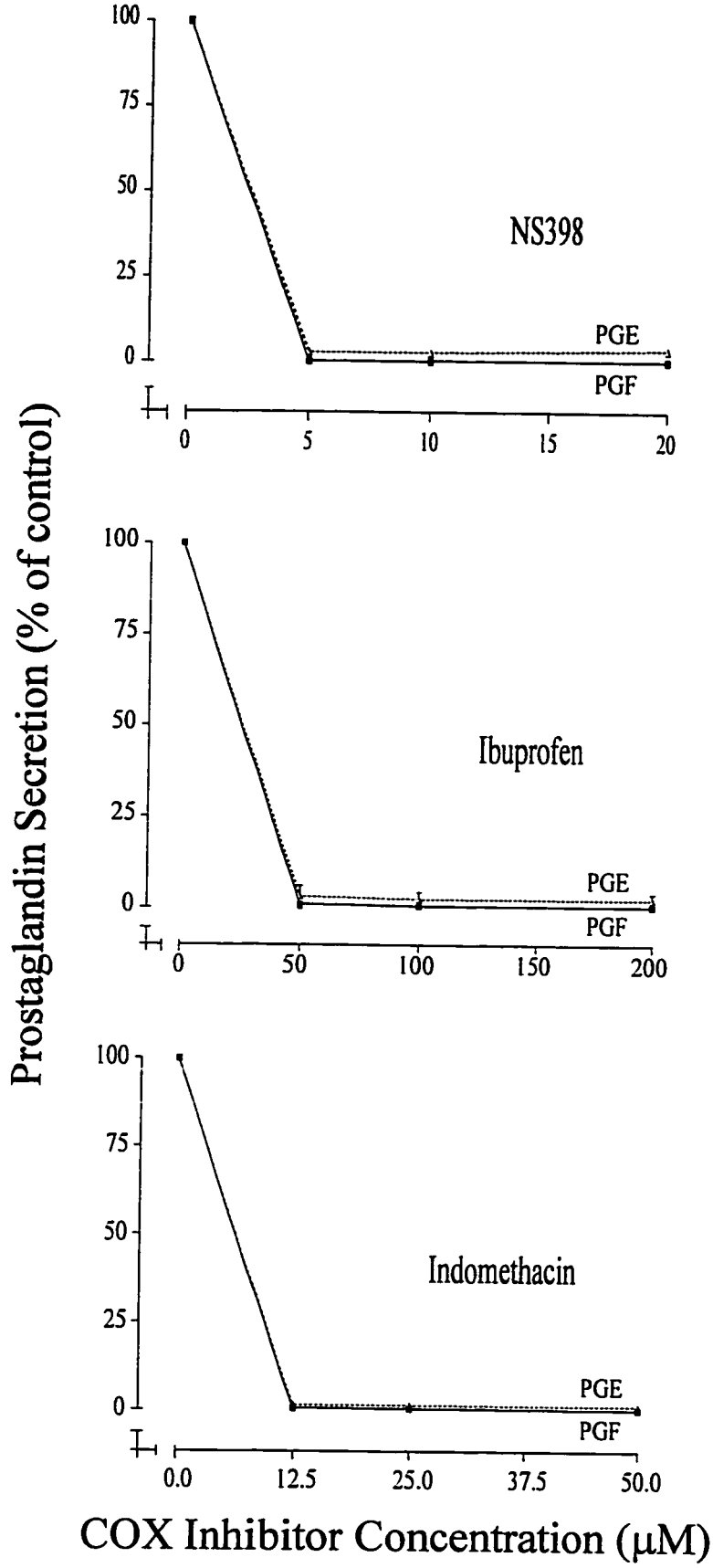
To confirm that the COX inhibitors were achieving their intended purpose (i.e. suppressing the TGFα-induced PG synthesis), PGE and PGF were measured in the medium from 24 hour granulosa cell cultures containing TGFα (20 ng/ml) and NS398 (5-20 μM), ibuprofen (50-200 μM) or indomethacin (12.5-50 μM). Granulosa cells cultured in serum-free medium for 24 hours secreted basal levels of PGE (12.9 ± 1.9 pmoles/10^6 cells) and PGF (39.6 ± 3.3 pmoles/10^6 cells; Figure 5). In the absence of COX inhibitors, the TGFα-induced secretion of PGF (185.1 ± 22.5 pmoles/10^6 cells) was 6 folds greater than that of PGE (30.0 ± 3.8 pmoles/10^6 cells; Figure 10). Addition of each COX inhibitor to cell cultures, even at its lowest concentration, caused a complete (~ 100%) inhibition of PG secretion into the media compared to the TGFα-treated control cultures (no inhibitor; $p < 0.001$). It appears that lowest concentrations of all three COX inhibitors not only fully attenuated the TGFα-induced PG synthesis (Figure 10), but also suppressed PG secretion to levels below those observed in cells cultured in serum-free medium containing no growth factor. Higher concentrations of the COX inhibitors elicited no further inhibition of PGE or PGF secretion.

15.7 Exogenous Prostaglandins Prevented the Apoptotic Effect of NS398

To determine whether the COX inhibitor-induced DNA fragmentation was a consequence of non-specific effects of the inhibitors (i.e. actions unrelated to the observed inhibition of PG synthesis), the ability of exogenous PGs to prevent the effect of NS398 was studied.

Figure 10. COX inhibitors suppressed TGFα-induced granulosa cell prostaglandin secretion *in vitro.*

Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the indicated concentrations of NS398 (top panel), ibuprofen (middle panel), or indomethacin (bottom panel). The amount of prostaglandins secreted in the culture media was determined by RIA. PGE and PGF secretion in the control group (no inhibitor, 20 ng/ml TGFα; set at 100 %) was 30.0 ± 3.8 pmoles/10^6 cells and 185.1 ± 22.5 pmoles/10^6 cells, respectively. Data is expressed as mean % of control ± SEM; N = 5-8 experiments. $P < 0.001$ for each data point vs. respective control.


Prostaglandin Secretion (% of control)
100
75
50
25
0
NS398
PGE
PGF
0
5
10
15
20
Ibuprofen
0
50
100
150
200
Indomethacin
0.0
12.5
25.0
37.5
50.0
COX Inhibitor Concentration (μM)

TGFα (20 ng/ml)

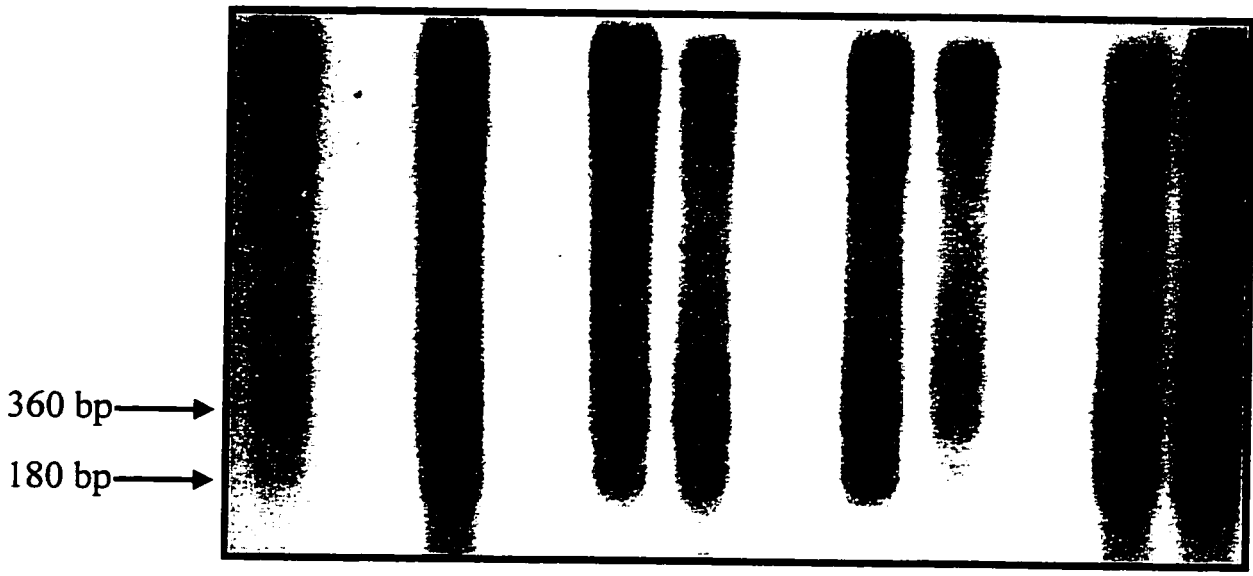
Addition of NS398 (10 μM) to granulosa cell cultures markedly induced apoptosis when compared to the TGFα (20 ng/ml)-treated control (no NS398; Figures 7 and 11). However, as shown in Figure 11, the presence of PGE_1, PGE_2 or $PGF_{2\alpha}$ (1 or 20 μM) effectively prevented the effect of this inhibitor ($p < 0.05$). Twenty micromolar concentration of $PGF_{2\alpha}$ afforded the strongest suppression (65%) of the NS398 effect ($p < 0.001$). On the other hand, PGE_2 (1 or 20 μM) appeared to be least effective; it suppressed the NS398 effect by only 38% ($p < 0.05$). Interestingly, PGE_1 and $PGF_{2\alpha}$ at 20 μM suppressed the NS398-induced apoptotic DNA fragmentation to levels below those observed in cells cultured with only TGFα (20 ng/ml). Complete attenuation of the COX inhibitor-induced DNA ladders with exogenous PGs has thus confirmed that the effects of NS398 were related to its inhibition of PG synthesis, and that the anti-apoptotic action of TGFα may be mediated by PGs.

15.8 PLA_2 Inhibitors Attenuated the TGFα-induced Suppression of Apoptosis

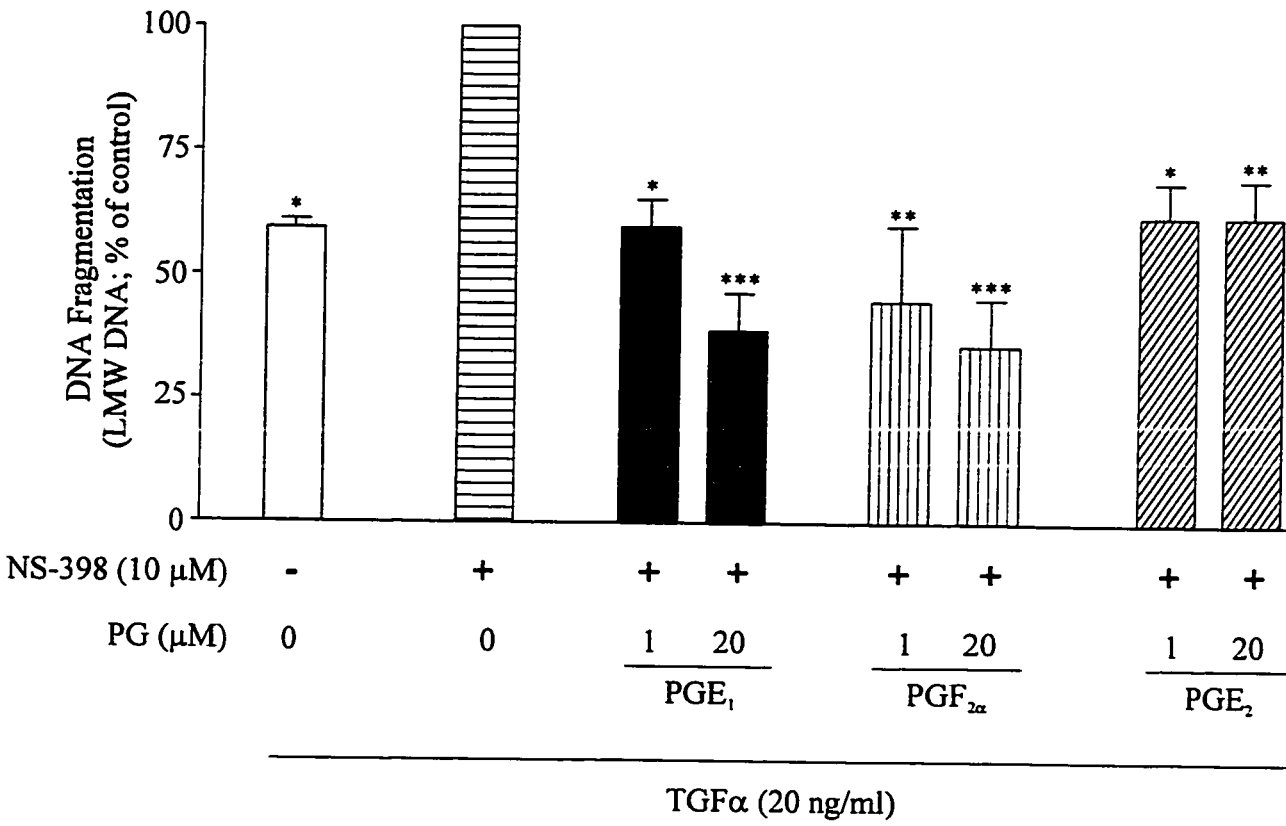
To further examine the possible involvement of PGs in mediating the suppression of apoptosis by TGFα, granulosa cells were cultured in serum-free media containing TGFα (20 ng/ml) and a PLA_2 inhibitor [aristolochic acid (AC; 20-40 μM), arachidonyl triflouro methyl ketone (TFMK; 5-20 μM), or 2-p-amylcinnamoyl amino-4-chlorobenzoic acid (ONO-RS-82; 5-20 μM)] to block arachidonic acid synthesis (Figure 6). Concentrations of AC and ONO-RS-82 were decided bases on previously published studies (Li and Tsang, 1995). All three PLA_2 inhibitors significantly induced, in a concentration-dependent manner and with varying degrees of effectiveness, greater apoptotic DNA fragmentation in

Figure 11. Prevention of the NS398-induced granulosa cell apoptosis by exogenous prostaglandins *in vitro.*
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the COX inhibitor NS398 (10 μM) or NS398 plus exogenous prostaglandins (1 or 20 μM). DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Control (no PG, 10 μM NS398) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 3 experiments. * $P < 0.5$ vs. control; ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control.

A.


360 bp
180 bp

B.


DNA Fragmentation
(LMW DNA; % of control)
100
75
50
25
0
*
*
***
**
***
*
**
NS-398 (10 μM) - + + + + + + +
PG (μM) 0 0 1 20 1 20 1 20
PGE1
PGF2α
PGE2
TGFα (20 ng/ml)

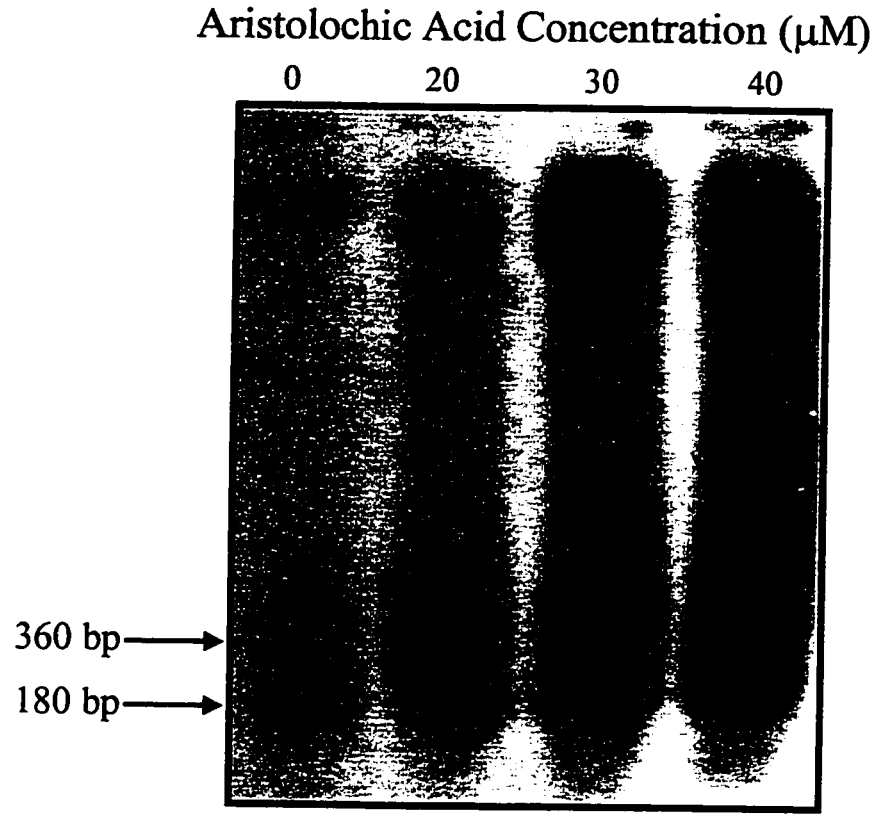
granulosa cells compared to TGFα-treated control cultures containing no inhibitor (Figures 12, 13, and 14). Although AC only elicited a slight (150%) and statistically non-significant effect ($p > 0.05$) at 20 μM, greater and more significant responses [220% ($p < 0.05$) and 270% ($p < 0.01$)] were noted at higher concentrations (30 and 40 μM, respectively; Figure 12). In contrast, TFMK at concentrations as low as 5 and 10 μM elicited significant DNA fragmentation [150% ($p < 0.05$) and 200% ($p < 0.01$), respectively], while a higher concentration (20 μM) of this inhibitor failed to afford a statistically significant response ($p > 0.05$) due to higher inter-experimental variations (Figure 13). In addition, although more extensive DNA fragmentation was observed with high concentrations of ONO-RS-82 (10 and 20 μM), interpretation of this data was complicated by the presence of random DNA degradation indicative of necrotic cell death (Figure 14). Correction for the contribution from necrotic DNA degradation by subtraction of the DNA background signal (smear) around the 180 and 360 bp bands by densitometric analysis, however confirmed that the intensity of the apoptotic DNA ladder was indeed proportional to the concentrations of ONO-RS-82 in the granulosa cell cultures (control, 100%; 5 μM, 182 ± 33 %; 10 μM, 237 ± 39 %; 20 μM, 395 ± 44 %).

15.9 PLA_2 Inhibitors Attenuated the TGFα-induced Prostaglandin Production

Previous studies by Li and Tsang (1995) have demonstrated that inhibitors of PLA_2 suppress PGE and PGF production in differentiated hen granulosa cells (form preovulatory hen follicles) in response to TGFα.

Figure 12. The PLA_2 inhibitor aristolochic acid attenuated the anti-apoptotic action of TGFα in granulosa cells *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the indicated concentrations of aristolochic acid. DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Control (no aristolochic acid, 20 ng/ml TGFα) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 5 experiments. * $P < 0.5$ vs. control; ** $P < 0.01$ vs. control.

A.


Aristolochic Acid Concentration (μM)
0
20
30
40
360 bp
180 bp

B.

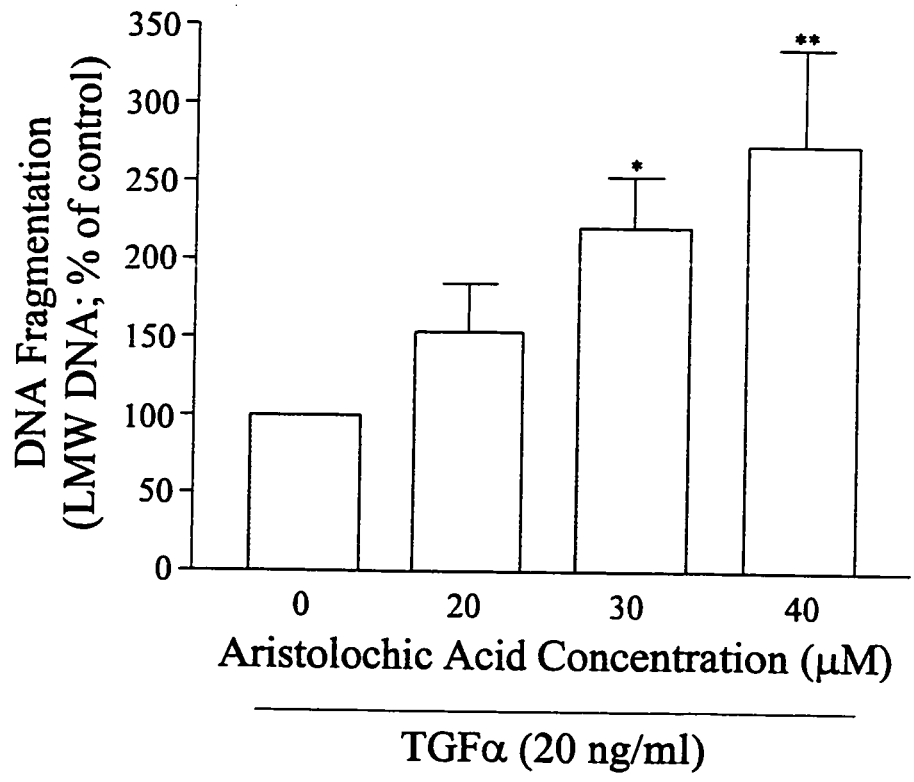
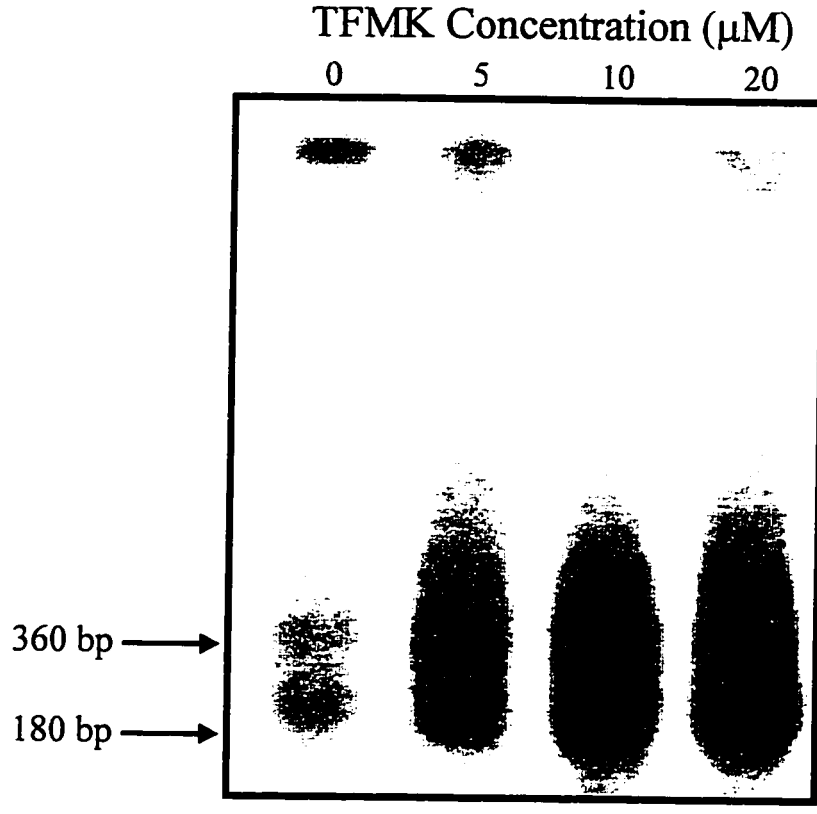

DNA Fragmentation
(LMW DNA; % of control)
350
300
250
200
150
100
50
0
*
**
0
20
30
40
Aristolochic Acid Concentration (μM)
TGFα (20 ng/ml)

Figure 13. The PLA_2 inhibitor TFMK attenuated the anti-apoptotic action of TGFα in granulosa cells *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the indicated concentrations of TFMK. DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Control (no TFMK, 20 ng/ml TGFα) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 3 experiments. * $P < 0.5$ vs. control; ** $P < 0.01$ vs. control.

A.


TFMK Concentration (μM)
0
5
10
20
360 bp
180 bp

B.

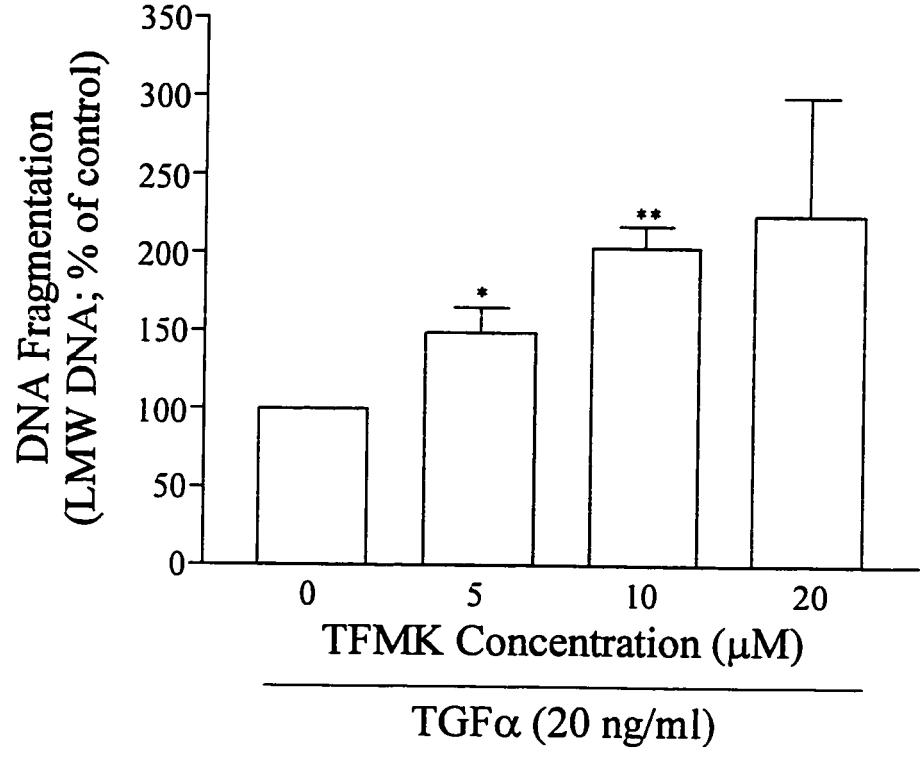
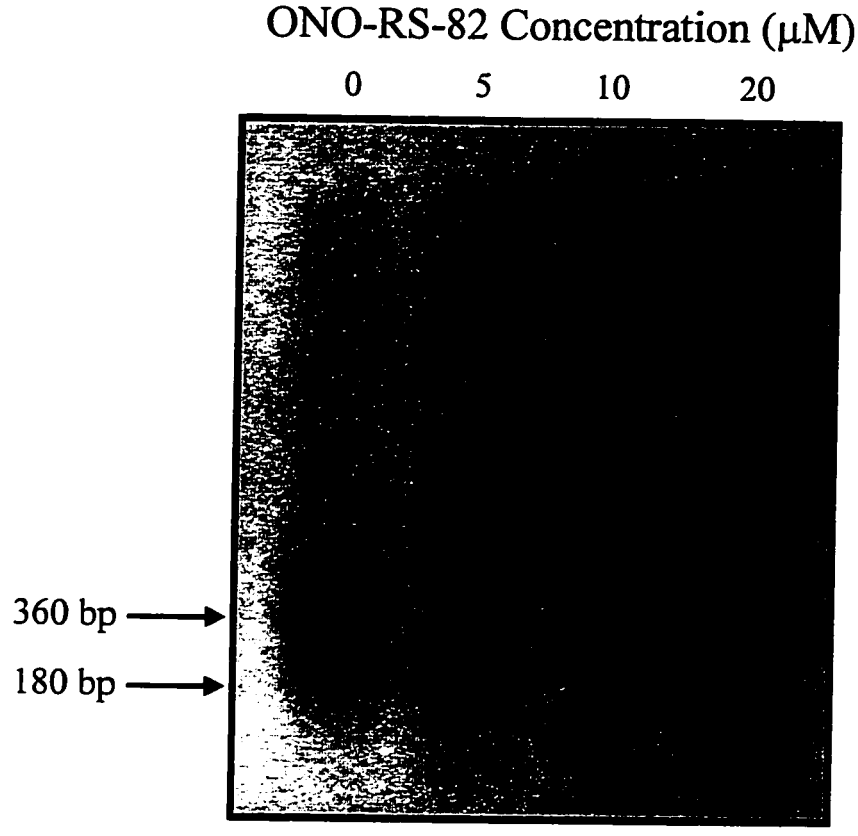
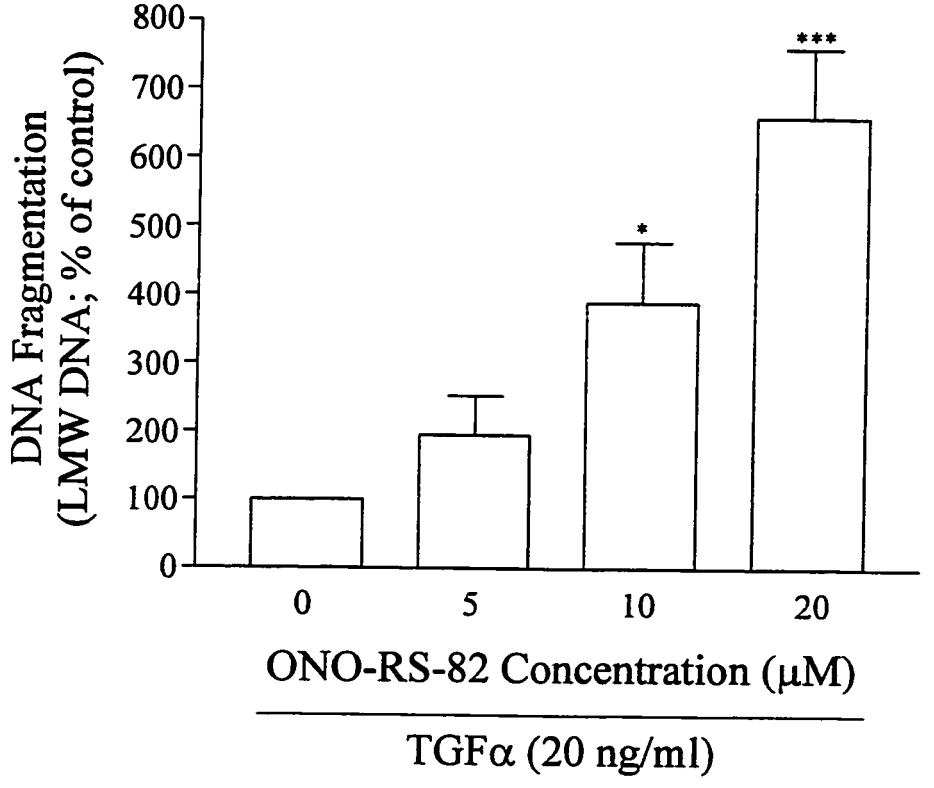

DNA Fragmentation
(LMW DNA; % of control)
350
300
250
200
150
100
50
0
*
**
0
5
10
20
TFMK Concentration (μM)
TGFα (20 ng/ml)

Figure 14. Attenuation of the TGFα-induced suppression of granulosa cell apoptosis by the PLA_2 inhibitor ONO-RS-82 *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the indicated concentrations of ONO-RS-82. DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Control (no ONO-RS-82, 20 ng/ml TGFα) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 4. * $P < 0.5$ vs. control; *** $P < 0.001$ vs. control.

A.


ONO-RS-82 Concentration (μM)
0
5
10
20
360 bp
180 bp

B.

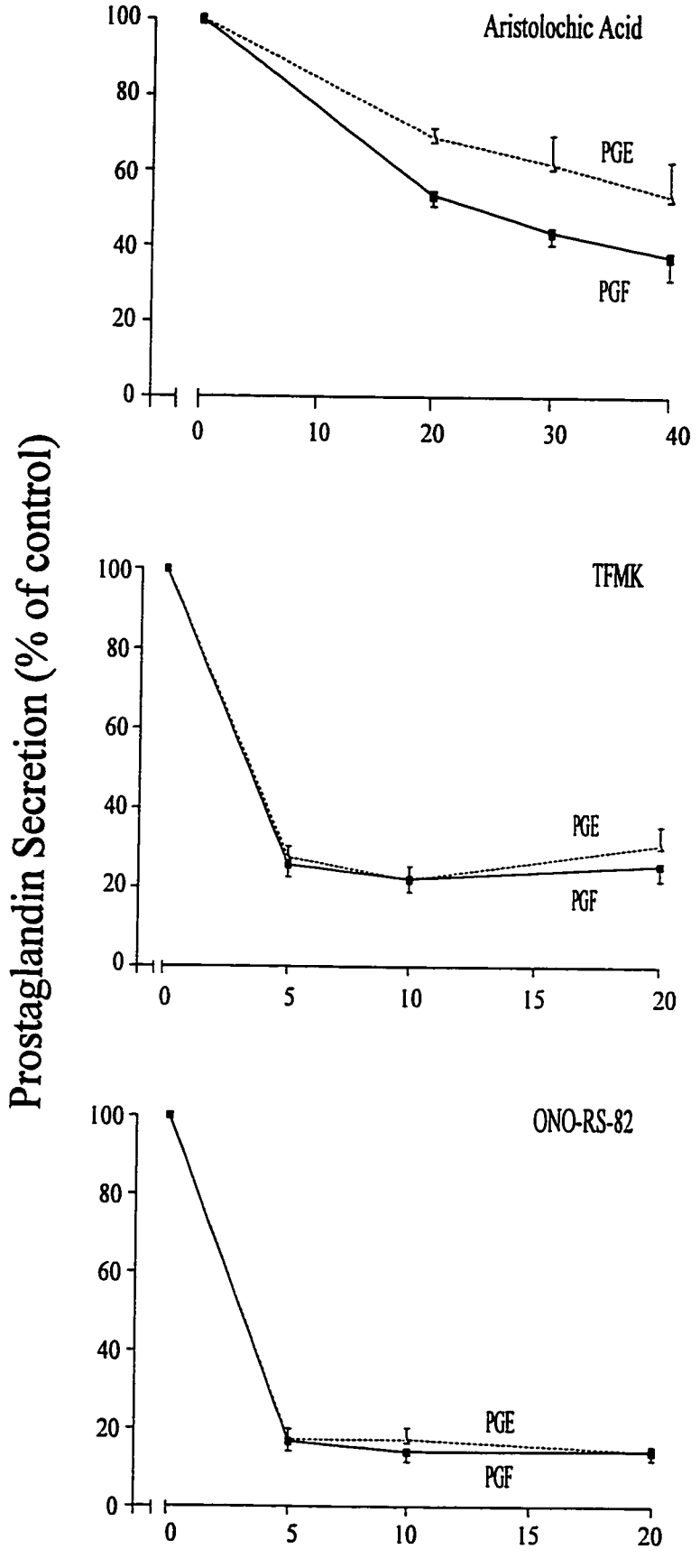

DNA Fragmentation
(LMW DNA; % of control)
800
700
600
500
400
300
200
100
0
*
***
0
5
10
20
ONO-RS-82 Concentration (μM)
TGFα (20 ng/ml)

To confirm that the PLA_2 inhibitors indeed suppressed the TGFα-induced PG synthesis in granulosa cells from prehierarchal follicles, PGE and PGF in the medium from 24 hour granulosa cell cultures (from large white follicles) containing TGFα (20 ng/ml) and either AC (20-40 μM), TFMK (5-20 μM) or ONO-RS-82 (5-20 μM) were measured. In the absence of the PLA_2 inhibitors, the TGFα-induced secretion of PGE and PGF was 30.0 ± 3.8 pmoles/10^6 cells and 185.1 ± 22.5 pmoles/10^6 cells, respectively (Figure 15). Addition of the PLA_2 inhibitors to the cell cultures resulted in a significant ($p < 0.001$) suppression of the TGFα-induced PG secretion, although with varying degrees of effectiveness. Aristolochic acid suppressed PGE and PGF secretion in a concentration dependent manner, reaching 55% and 40 % of their respective controls at 40 μM of the inhibitor. In contrast, both TFMK and ONO-RS-82 showed no preference to inhibition of PGF over PGE; secretion of both PGs was reduced by the same degree. Moreover, even at concentrations as low as 5 μM they elicited maximal inhibition of PGE and PGF secretion (75% and 82%, respectively). Unlike the COX inhibitors which almost completely (~ 100%) inhibited all PG synthesis, the PLA_2 inhibitors failed to afford such complete suppression of PG secretion.

15.10 Exogenous Arachidonic Acid Prevented the Apoptotic Action of Aristolochic Acid

To further assess if the inhibitor-induced apoptotic DNA fragmentation observed in section 15.8 was specific to their action on PLA_2, the ability of exogenous arachidonic acid in preventing the apoptotic effect of AC was examined.

Figure 15. Suppression of the TGFα-induced granulosa cell prostaglandin secretion by PLA_2 inhibitors *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the indicated concentrations of aristolochic acid (top panel), TFMK (middle panel), or ONO-RS-82 (bottom panel). The amount of prostaglandins secreted in the culture media was determined by RIA. PGE and PGF secretion in the control group (no inhibitor, 20 ng/ml TGFα; set at 100 %) was 30.0 ± 3.8 pmoles/10^6 cells and 185.1 ± 22.5 pmoles/10^6 cells, respectively. Data is expressed as mean % of control ± SEM; N = 5-8 experiments. $P < 0.001$ for each data point vs. respective control.


Aristolochic Acid
PGE
PGF
TFMK
PGE
PGF
ONO-RS-82
PGE
PGF
Prostaglandin Secretion (% of control)
100
80
60
40
20
0
0
10
20
30
40
0
5
10
15
20
PLA2 Inhibitor Concentration (μM)
TGFα (20 ng/ml)

As observed previously (Figure 12), addition of AC (30 μM) to the cell cultures markedly increased (~220%; $p < 0.05$) DNA fragmentation compared to the TGFα (20 ng/ml)-treated control (no AC; Figures 12 and 16). However, as shown in Figure 16, the presence of arachidonic acid (0.5 μM) completely ($p < 0.01$) prevented the AC (30 μM)-induced response. In contrast, exogenous PGE_1, PGE_2 or $PGF_{2\alpha}$, at concentration which effectively attenuated the COX II inhibitor-induced apoptosis (1 or 20 μM), failed to prevent the AC-induced DNA fragmentation (Figure 17). These findings suggest that, in addition to PGs (as demonstrated in studies with COX inhibitors and exogenous PGs), arachidonic acid and/or its other metabolites (i.e. leukotrienes, HETEs or EETs) may be important in the anti-apoptotic action of TGFα in hen granulosa cells.

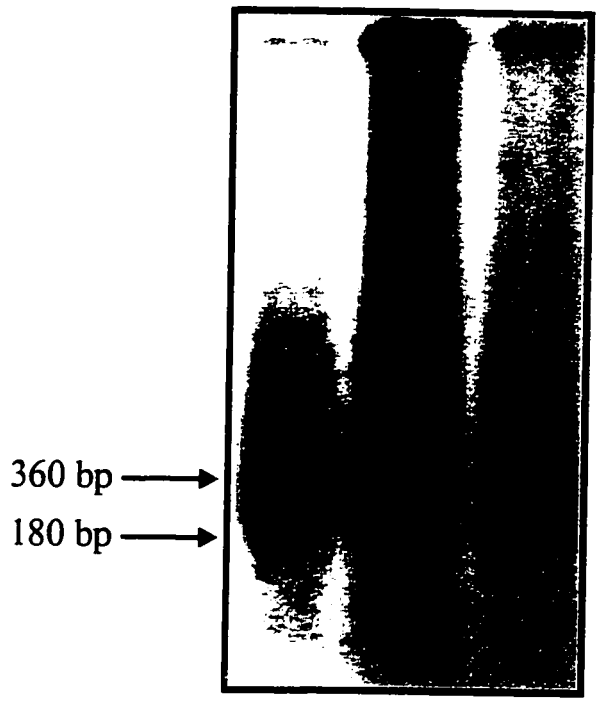
15.11 Morphological Assessment of Apoptosis in Cell Cultures

15.11.1 General Cellular Morphology

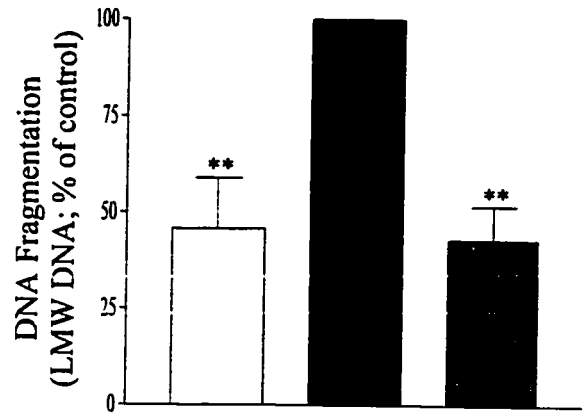
In addition to the biochemical assessment of DNA fragmentation in previous sections, the possible involvement of PGs in the anti-apoptotic action of TGFα in hen granulosa cells was examined morphologically. Granulosa cells appeared, after culture in medium containing serum (10 % FBS) for 24 hours, morphologically healthy (plates 4A). The cells were firmly attached to the culture dish, had intact cell membranes (not blebbed) and extensive cell-cell contacts through cellular projections (Plates 4A).

Figure 16. Prevention of the aristolochic acid-induced apoptosis in granulosa cells by exogenous arachidonic acid *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the PLA_2 inhibitor aristolochic acid (30 μM) or aristolochic acid plus arachidonic acid (0.5 μM). DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Control (no arachidonic acid, 30 μM aristolochic acid) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 4 experiments. ** $P < 0.01$ vs. control.

A.



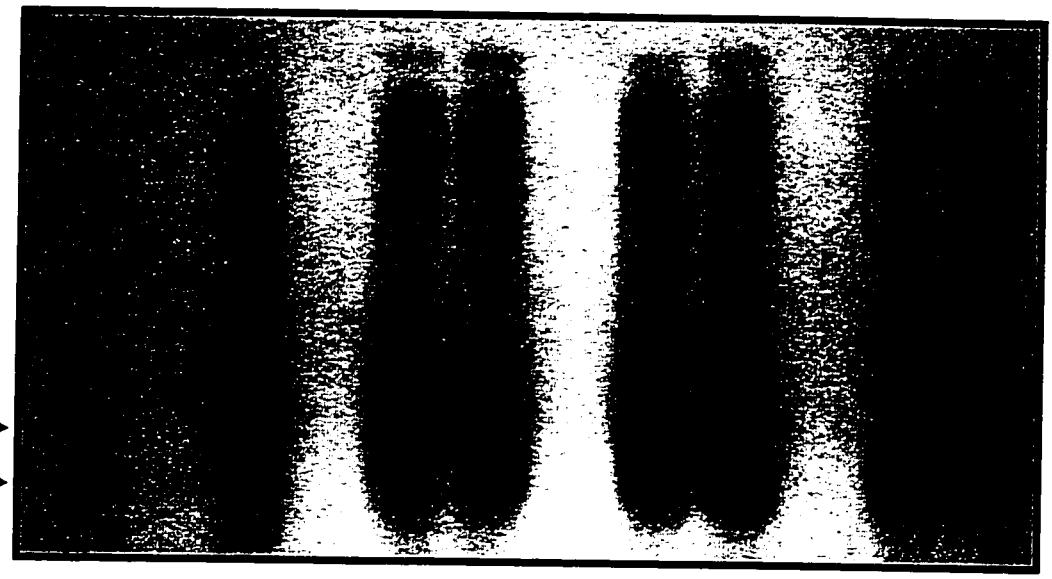
B.



TGFα (20 ng/ml)	+	+	+
Aristolochic Acid (30 μM)	-	+	+
Arachidonic Acid (0.5 μM)	-	-	+

Figure 17. Failure of exogenous prostaglandins to prevent aristolochic acid-induced granulosa cell apoptosis *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing TGFα (20 ng/ml) and the PLA_2 inhibitor aristolochic acid alone (30 μM) or aristolochic acid plus exogenous prostaglandins (1 or 20 μM). DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Data is expressed as mean % of control ± SEM; N = 4 experiments. * $P < 0.05$ vs. control.

A.


360 bp
180 bp

B.

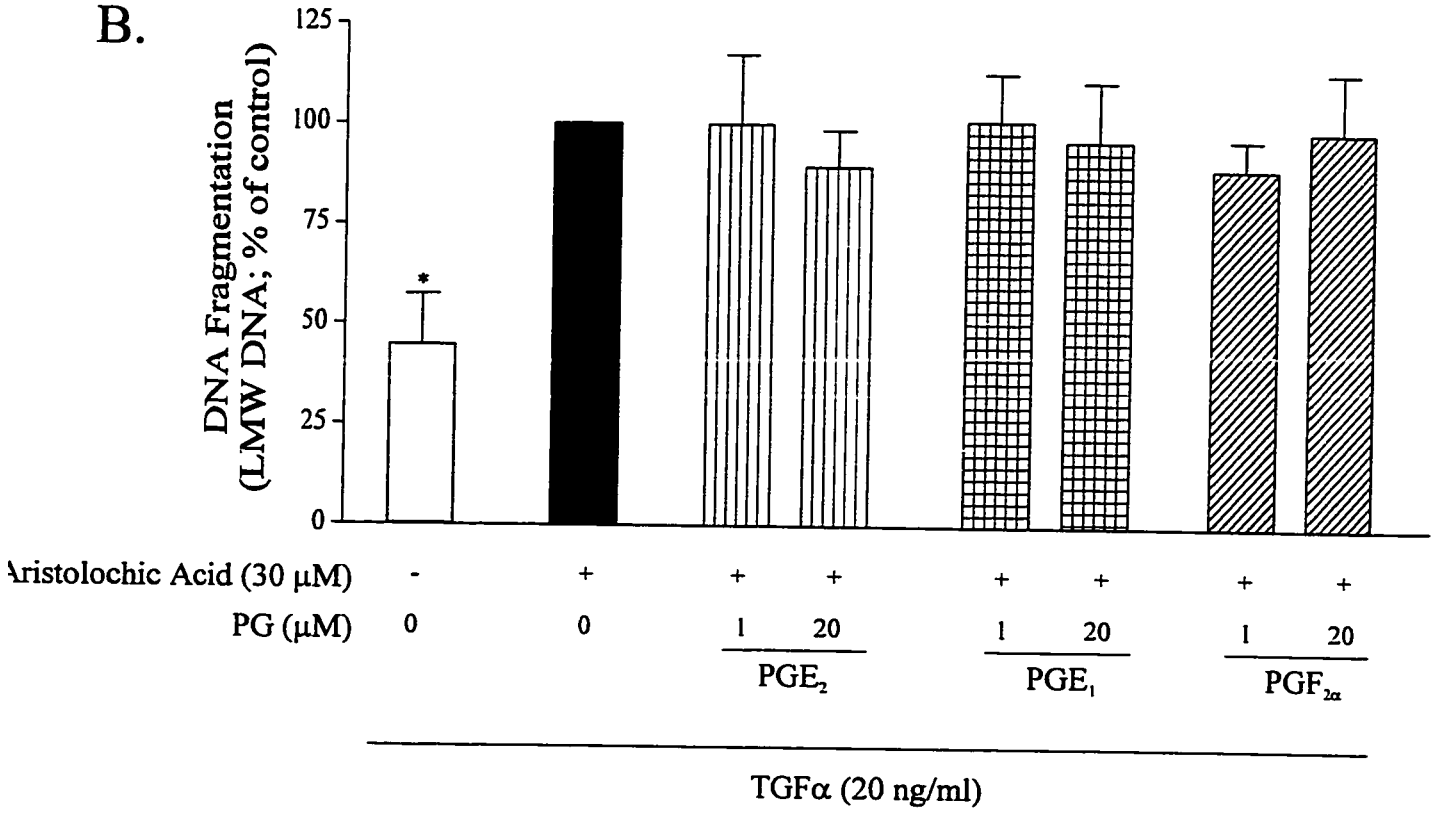
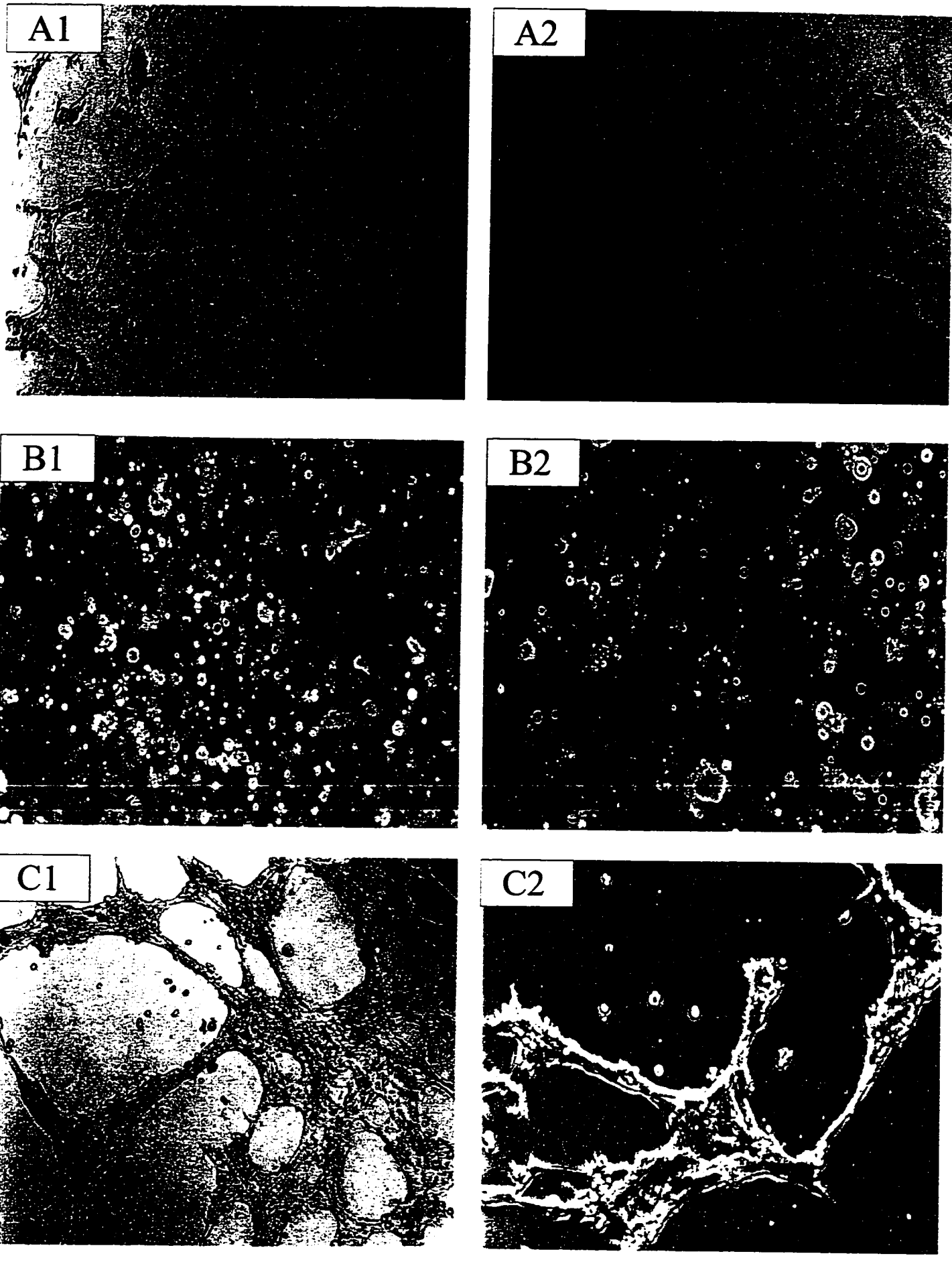

DNA Fragmentation
(LMW DNA; % of control)
125
100
75
50
25
0
*
Aristolochic Acid (30 μM) - + + + + + + +
PG (μM) 0 0 1 20 1 20 1 20
PGE2
PGE1
PGF2α
TGFα (20 ng/ml)

Plate 4. Phase contrast photomicrographs of granulosa cells cultured in medium with and without serum or TGFα.

Granulosa cells were allowed to plate on plastic culture dishes for 6 hours in medium containing 10 % FBS, and further cultured for 24 hours in medium containing 10 % FBS (panels A1 and A2) or in serum-free medium (panels B1 and B2) or in serum-free medium containing 20 ng TGFα/ml (panels C1 and C2), then photographed at magnification 100 X (panels A1, B1 and C1) and 200 X (panels A2, B2 and C2). Cells in panels A and C were adherent to the culture surface, and healthy as evident by morphological features such as cell-cell contact via projections (arrow), and intact cell membranes. Cells in panels B displayed morphological features of apoptosis, including membrane blebbing (arrow head), cellular condensation, cellular fragmentation as evident by the numerous floating apoptotic bodies (short arrow) and loss of cell-cell contact. Additionally many cells were also floating in the medium.


A1
A2
B1
B2
C1
C2

On the other hand, culture of granulosa cells in serum-free medium for 24 hours (Plates 4B) resulted in the appearance of features characteristic of apoptosis, including cellular shrinkage and rounding, membrane blebbing, loss of cell-cell contact, fragmentation of cells into membrane bound vesicles, and cellular detachment from culture surface. Addition of TGFα (20 ng/ml) prevented the morphological features of apoptosis evident during serum deprivation, and cells appeared morphologically healthy (Plates 4C). However, cellular morphology indicative of apoptosis was apparent when granulosa cells were cultured in the presence of TGFα (20 ng/ml) and an inhibitor of COX (NS398, 10 μM; Plates 5A) or PLA_2 (AC, 30 μM; Plates 6A). The apoptotic effect of the COX or PLA_2 inhibitor could be prevented by exogenous $PGF_{2\alpha}$ and arachidonic acid, respectively. Hence, morphological characteristics of healthy cells were apparent in granulosa cells cultured in the presence of TGFα (20 ng/ml), NS-398 (10 μM) and $PGF_{2\alpha}$ (20 μM, Plates 5B) or TGFα (20 ng/ml), AC (30 μM) and arachidonic acid (0.5 μM, Plates 6B).

15.11.2 Nuclear Morphology

The DNA fragmentation data only provides an index for the general levels of apoptosis in a given cell population and does not reveal the percentage of cells undergoing apoptosis in that population. To estimate the % of cultured granulosa cells undergoing apoptotic cell death, nuclear morphology was assessed at the end of the culture, using the nuclear stain Hoechst 33258. Nuclei that were fragmented or highly condensed were considered apoptotic, whereas healthy nuclei were those that were intact (i.e. not fragmented or highly condensed).

Plate 5. Phase contrast photomicrographs of granulosa cells cultured in serum-free medium containing TGFα and the COX inhibitor NS398 or NS398 plus $PGF_{2\alpha}$. Granulosa cells were allowed to plate on plastic culture dishes for 6 hours in medium containing 10 % FBS, and further cultured for 24 hours in serum-free medium containing 20 ng TGF/ml and either 10 μM NS398 (panels A1 and A2) or 10 μM NS398 plus 20 μM $PGF_{2\alpha}$ (panels B1 and B2), then photographed at magnification 100 X (panels A1 and B1) and 200 X (panels A2 and B2). Cells in panels A displayed morphological features of apoptosis, including membrane blebbing (arrow head), cellular condensation, cellular fragmentation as evident by the numerous floating apoptotic bodies (short arrow) and loss of cell-cell contact. Additionally many cells were also floating in the medium. Cells in panel B were adherent to the culture surface, and healthy as evident by morphological features such as cell-cell contact via cellular projections (arrow), and intact cell membranes.

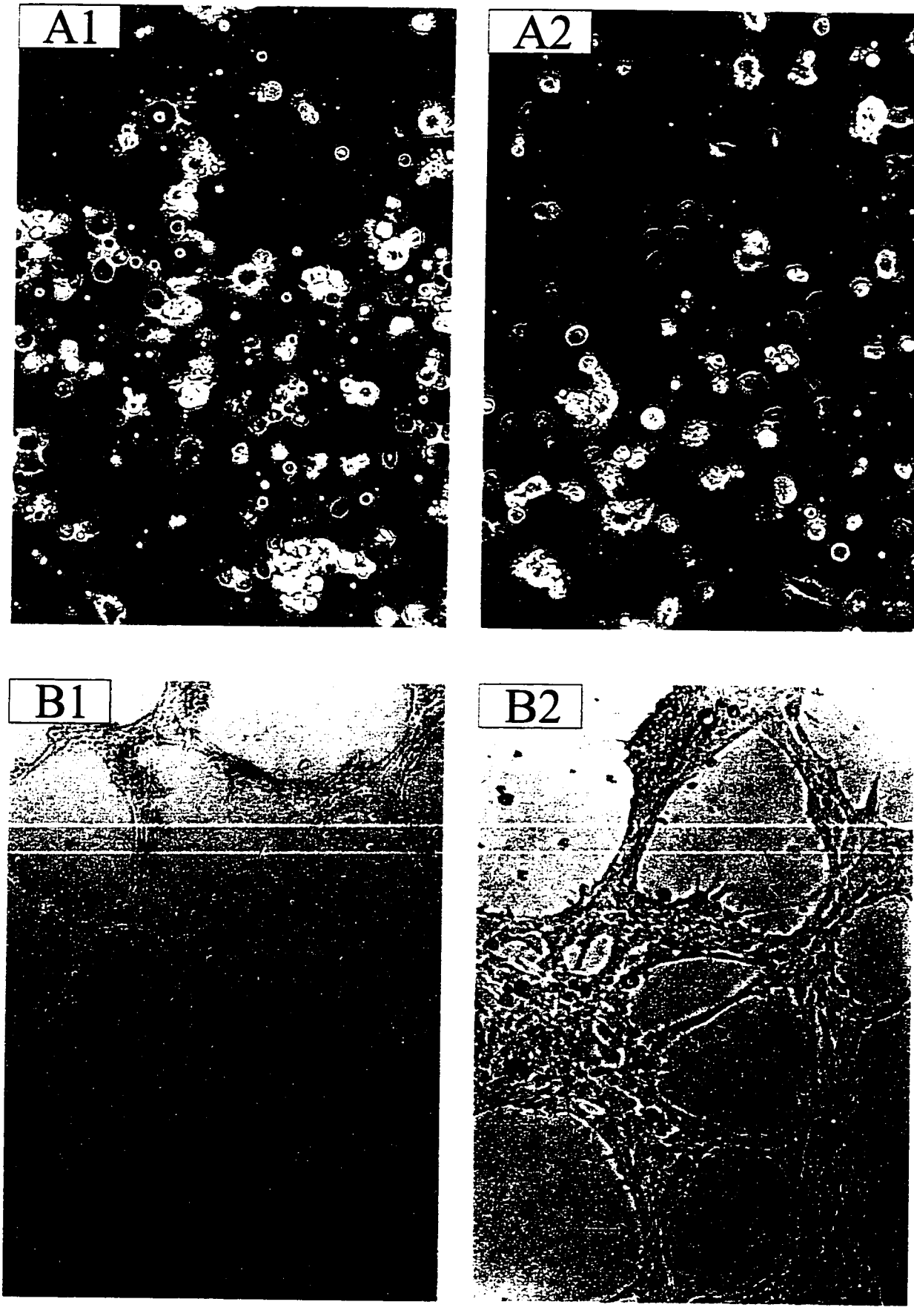
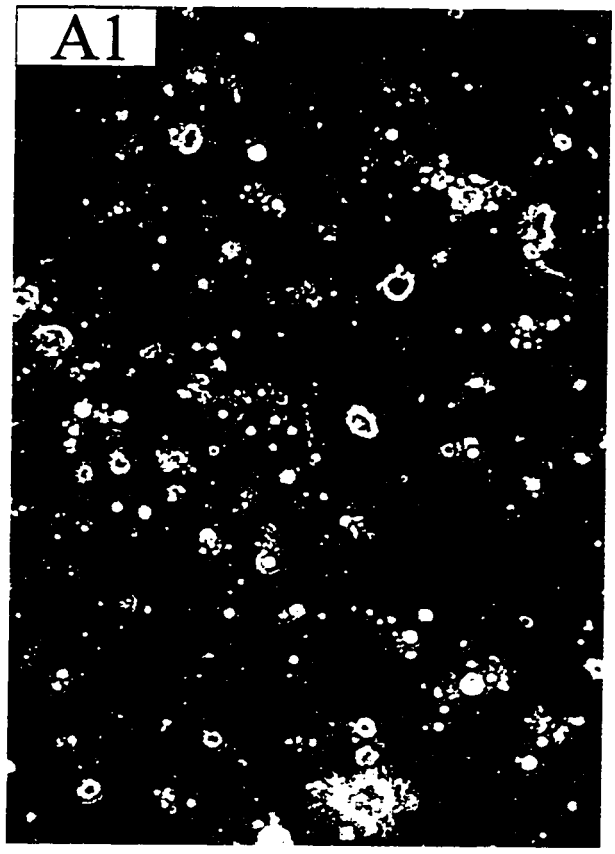
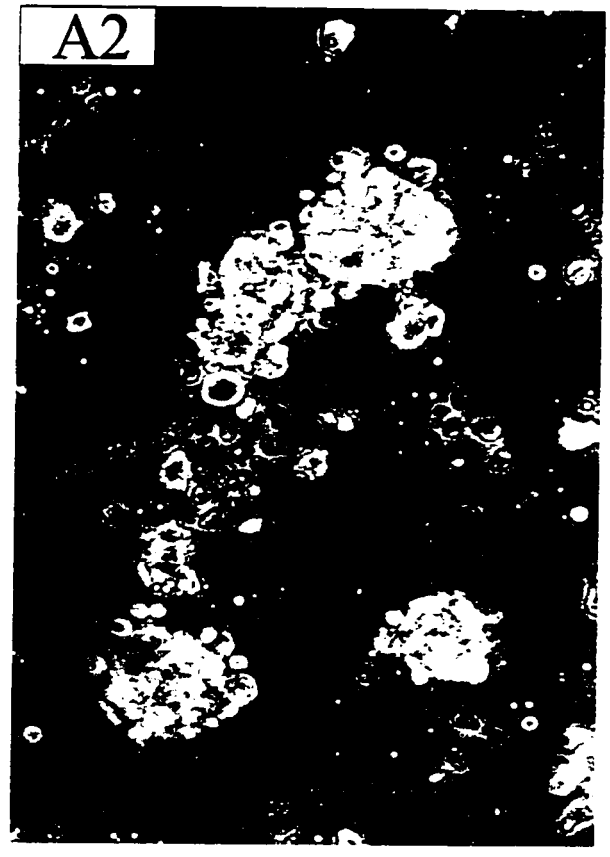

A1
A2
B1
B2

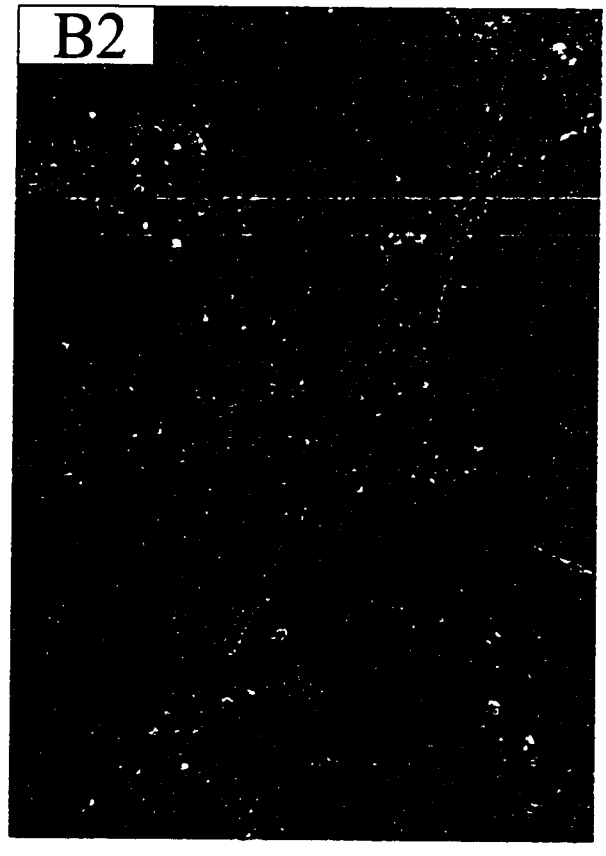
Plate 6. Phase contrast photomicrographs of granulosa cells cultured in serum-free medium containing TGFα and the PLA_2 inhibitor aristolochic acid (AC) or AC plus arachidonic acid.

Granulosa cells were allowed to plate on plastic culture dishes for 6 hours in medium containing 10 % FBS, and further cultured for 24 hours in serum-free medium containing 20 ng TGF/ml and either 30 μM AC (panels A1 and A2) or 30 μM AC plus 0.5 μM arachidonic acid (panels B1 and B2), then photographed at magnification 100 X (panels A1 and B1) and 200 X (panels A2 and B2). Cells in panel A displayed morphological features of apoptosis, including membrane blebbing (arrow head), cellular condensation, cellular fragmentation as evident by the numerous floating apoptotic bodies (short arrow) and loss of cell-cell contact. Additionally many cells were also floating in the medium. Cells in panel B were adherent to the culture surface, and healthy as evident by morphological features such as cell-cell contact via cellular projections (arrow), and intact cell membranes.


A1


A2


B1


B2

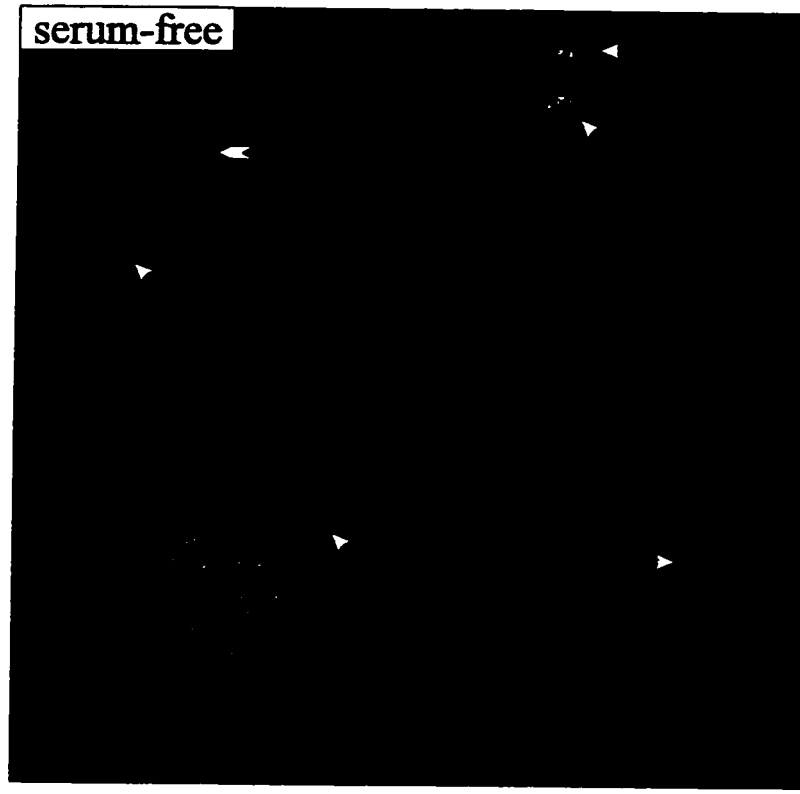
Consistent with the DNA fragmentation data reported in this thesis (Figure 4), serum-deprivation induced maximal percentage of granulosa cell apoptosis (18 %) which was markedly suppressed by TGFα (Plate 7 and Figure 18). Addition of NS398 (10 μM) induced significantly more (4-fold) granulosa cell apoptosis than TGFα control cultures, and the NS398 (10 μM)-induced apoptosis was prevented by exogenous $PGF_{2\alpha}$ (20 μM) which reduced the percentage of apoptotic cells to TGFα control levels (Figure 18). NS398 [(1 μM; at one fifth the concentration which induced maximal granulosa cell DNA fragmentation (Figure 7) and completely inhibited the TGFα (20 ng/ml)-induced prostaglandin synthesis (Figure 10)] significantly, but not maximally, induced granulosa cell apoptosis in the presence of TGFα. Cells cultured in the presence of TGFα and 1 μM NS398 displayed significantly more (2-fold) apoptosis than TGFα control cells, but significantly less apoptosis (2-fold) than cells cultured in the presence of TGFα and 10 μM NS398 (Figure 18). This concentration-dependent relationship is significant in light of the absence of concentration-dependent effects on DNA fragmentation and PG production with higher concentrations of NS398 (5, 10 and 20 μM; Figures 7 and 10).

15.12 Prostaglandins Alone Failed to Mimic the Anti-apoptotic Action of TGFα

Having established that TGFα stimulates PG production in granulosa cells (Figure 5), and that inhibition of the TGFα-induced PG production results in granulosa cell apoptosis (Figures 7-9 and 18; Plates 5A, 6A and 7), it was of interest to determine whether PGs or arachidonic acid alone could mimic the anti-apoptotic action of TGFα.

Plate 7. Detection of granulosa cell apoptosis by assessment of nuclear morphology.

Granulosa cells were plated on plastic culture dishes for 6 hours in medium containing 10 % FBS, then further cultured for 24 hours in serum-free medium (top panel) or serum-free medium containing 20 ng TGFα/ml (bottom panel). Cells were then stained with Hoechst 33258 dye and photographed at magnification 250 X. Fragmented or highly condensed and brightly stained nuclei (arrows) were considered apoptotic. Apoptotic bodies (arrow heads; small, brightly stained cell fragments containing nuclear material) were also detected. In contrast, healthy nuclei appeared morphologically intact (i.e. not fragmented or condensed).


serum-free

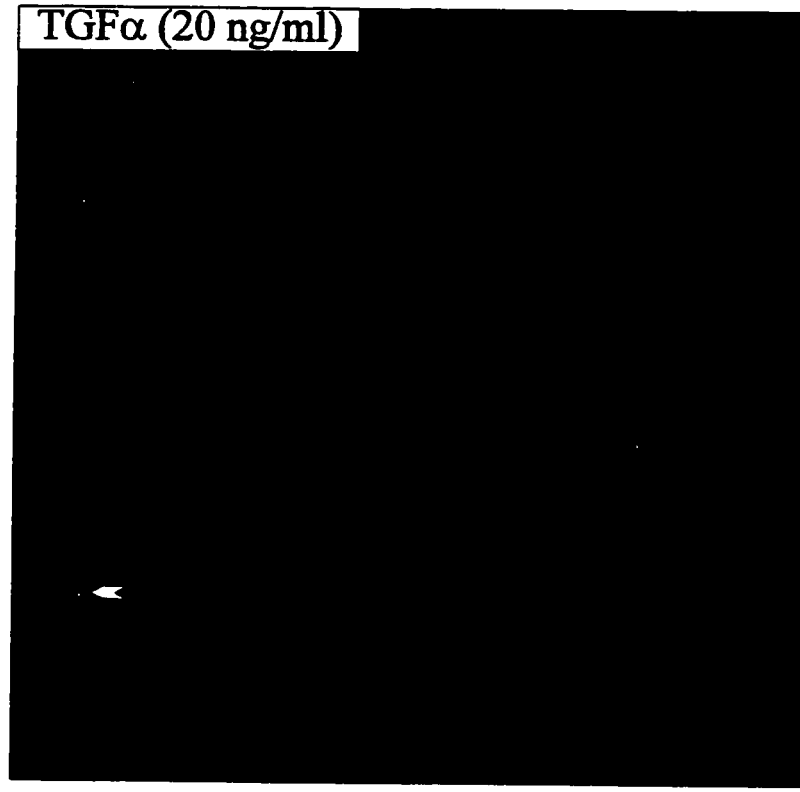
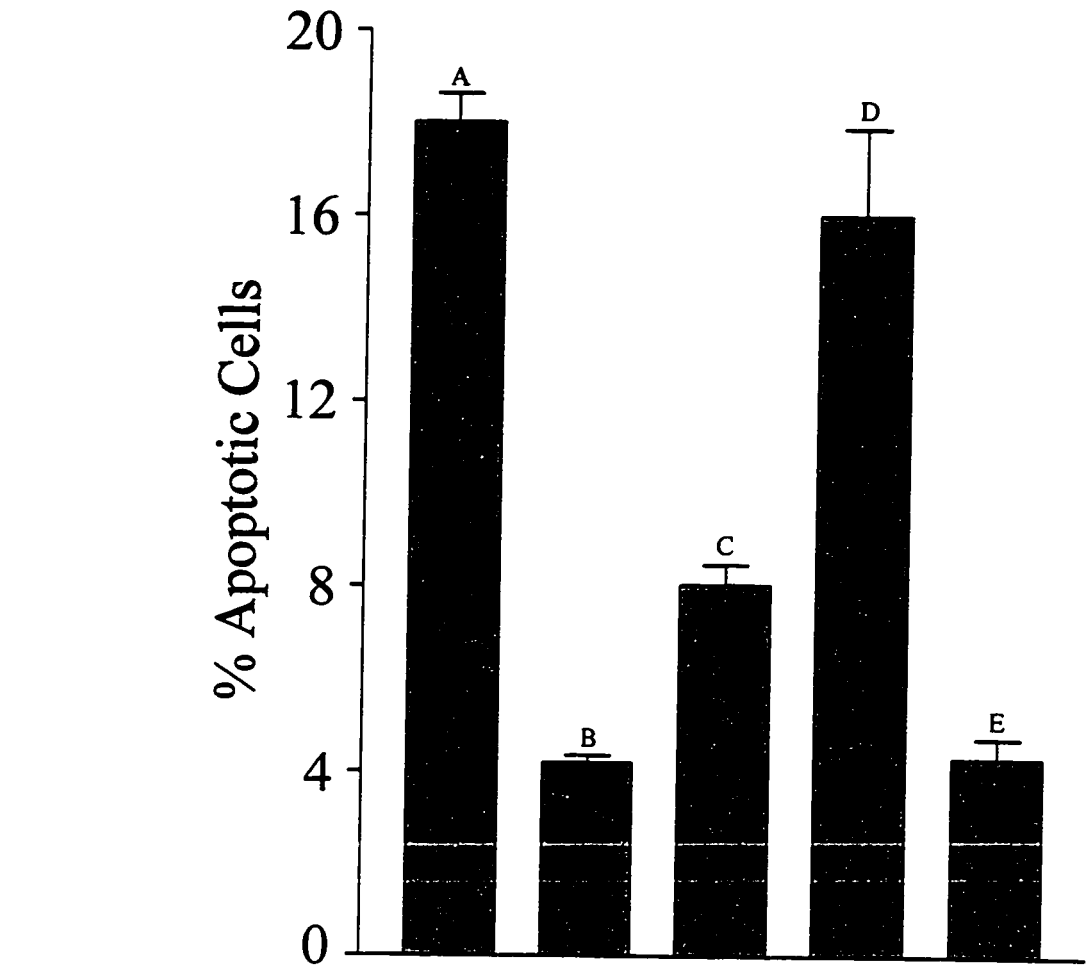

TGFα (20 ng/ml)

Figure 18. Influence of TGFα, NS398 and $PGF_{2\alpha}$ on apoptosis as determined by nuclear morphology.

Granulosa cells were plated on plastic culture dishes for 6 hours in medium containing 10 % FBS, then further cultured for 24 hours in serum-free medium (A) or serum-free medium containing 20 ng TGFα/ml (B) and either 1μM NS398 (C) or 10 μM NS398 (D) or 10 μM NS398 plus 20 μM $PGF_{2\alpha}$ (E). Cells were then stained with Hoechst 33258 dye and the % of apoptotic cells was determined by assessing nuclear morphology. Cells with fragmented or highly condensed nuclei were considered apoptotic. Data is expressed as mean ± SEM; N = 3-5 experiments. $p < 0.001$, A vs B; $p < 0.05$, C vs B; $p < 0.001$, C vs D; $p < 0.001$, B vs D; $p < 0.001$, E vs D.

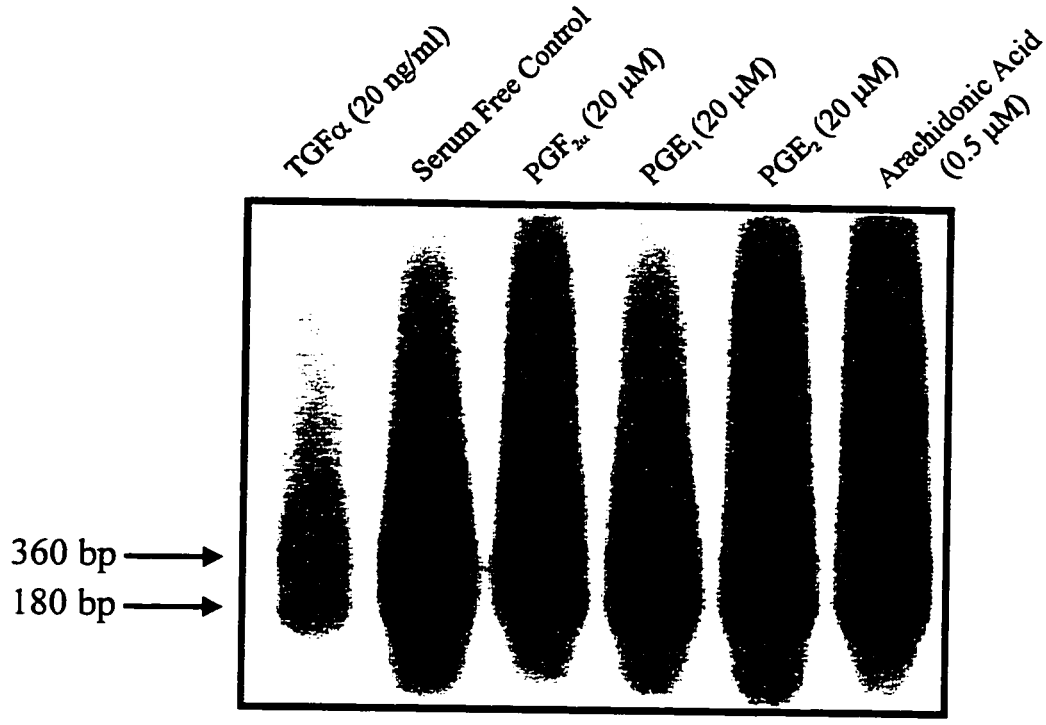


TGFα (ng/ml)	0	20	20	20	20
NS398 (μM)	0	0	1	10	10
$PGF_{2\alpha}$ (μM)	0	0	0	0	20

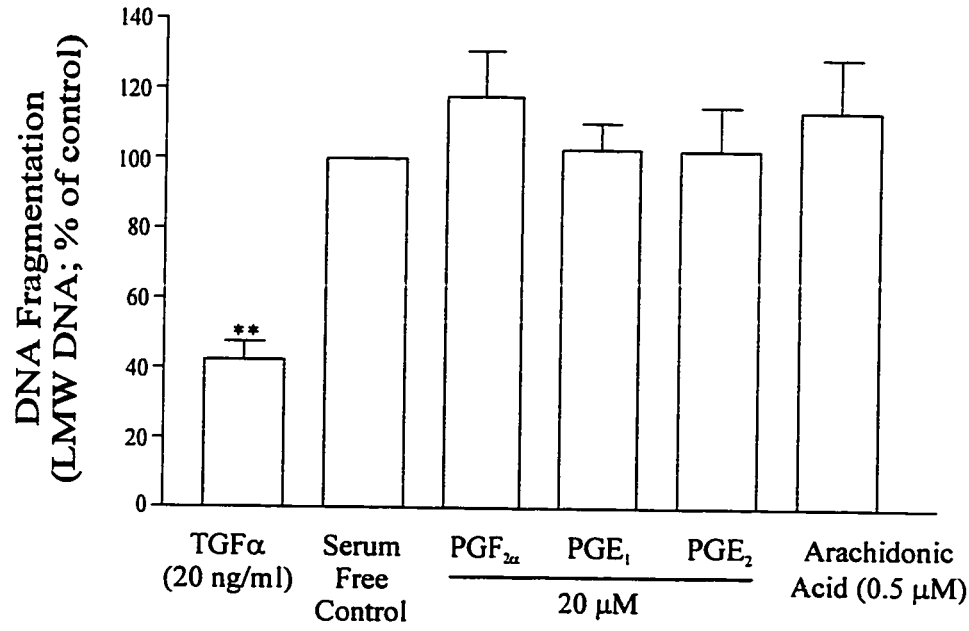
Granulosa cells were cultured in serum-free medium with or without $PGF_{2\alpha}$ (20 μM), PGE_1 (20 μM), PGE_2 (20 μM), arachidonic acid (0.5 μM) or TGFα (20 ng/ml). All three PGs and arachidonic acid alone failed to prevent the serum deprivation-induced apoptosis (Figure 19). Compared to serum deprivation-induced DNA fragmentation (100%), the presence of $PGF_{2\alpha}$ (118 %), PGE_1 (100 %), PGE_2 (100 %), or arachidonic acid (115 %) resulted in no significantly different extent of apoptotic DNA fragmentation. These findings demonstrate that PGs or arachidonic acid alone do not account for the anti-apoptotic action of TGFα. Hence, other molecules, in addition to PGs, must be necessary to effect the suppression of apoptosis by TGFα.

Figure 19. Failure of prostaglandins and arachidonic acid alone to mimic the anti-apoptotic effect of TGFα *in vitro*.
Following a 6 hour plating period in 10% FBS, granulosa cells were cultured for 24 hours in serum-free media containing PG [PGE_1, PGE_2, or $PGF_{2\alpha}$ (20 μM)] or TGFα (20 ng/ml). DNA was isolated, 3'-end labelled with [α^{32}P]-dCTP and resolved by AGE. Panel A: representative autoradiogram showing apoptotic DNA ladders. Panel B: densitometric quantification of the LMW DNA (≤ 15 kb). Control (TGFα only) is set at 100 %. Data is expressed as mean % of control ± SEM; N = 4 experiments. ** $P < 0.01$ vs. control.

A.


TGFα (20 ng/ml)
Serum Free Control
PGF2α (20 μM)
PGE1 (20 μM)
PGE2 (20 μM)
Arachidonic Acid (0.5 μM)
360 bp
180 bp

B.


DNA Fragmentation (LMW DNA; % of control)
140
120
100
80
60
40
20
0
**
TGFα (20 ng/ml)
Serum Free Control
PGF2α
PGE1
PGE2
20 μM
Arachidonic Acid (0.5 μM)

16. DISCUSSION

16.1 Follicular Atresia *In vivo*

The primary function of the ovary is to deliver viable oocytes which can subsequently be fertilised and ultimately result in the birth of offspring. Follicles containing the oocytes must develop from the primordial to the preovulatory stage before ovulation can occur. Following recruitment of a cohort of primordial follicles into the developmental pool, a majority of them become atretic and regress. Precisely why and under what conditions follicular atresia occurs in the ovary is still unknown. Nevertheless, recent evidence indicates that follicular atresia is an intricate, genetically programmed process controlled by a host of autocrine, paracrine, endocrine and neural signals, and involves interplay between oocyte, theca cells, granulosa cells and immune cells residing within the ovary.

Follicular atresia is found to occur in ovaries of all vertebrates studied to date, including mammalian (reviewed by Hirshfield, 1991), avian (Gilbert, 1979) and fish (Janz and Vanderkraak, 1997) species. In the hen ovary, follicles grow from a size less than 1 mm (primordial) to about 40 mm (preovulatory) just prior to ovulation. However, not all the developing follicles eventually ovulate. Over 99 % of the developing follicles become atretic before they reach 9 mm in diameter (Gilbert et al, 1983). Follicles that continue to develop past 9 mm in size usually do not succumb to atresia and are called 'hierarchal' follicles by virtue of the fact that they have embarked on a developmental hierarchy (path) that leads to ovulation. Hence the fate of the follicles (ovulation or atresia) is determined at the prehierarchal (< 9 mm) level of development.

Much evidence suggests that follicular atresia is a process that involves apoptosis (or programmed cell death) of selected follicular cells, ultimately leading to the demise of the follicle (Tilly et al, 1991a; Hughes and Gorospe, 1991; Hurwitz and Adashi, 1992; reviewed by Hsueh et al, 1994; Palumbo and Yeh, 1995). Apoptosis of granulosa cells and to a lesser degree of theca cells from atretic hen ovarian follicles (4-6 mm) has been demonstrated biochemically by apoptotic DNA fragmentation (the DNA ladder of 180 bp multiples) by Tilly et al. (1991a). Since this report, several other reports in other species have claimed that follicular apoptosis is predominantly restricted to the granulosa compartment and that the theca cells undergo little or no apoptosis (Hurwitz and Adashi, 1992; Palumbo and Yeh, 1994; Manabe et al, 1996; Johnson et al, 1996). Likewise, our results show that fragmented DNA (determined by in situ 3'-end labelling) in hen atretic follicles is predominantly localised in the granulosa cell layers, with little or no staining in the theca compartment. It is possible that the DNA ladders observed by Tilly et al. (1991a) in theca cell preparations from atretic hen follicles might have resulted from apoptotic granulosa cell contamination. This is not surprising since the basal lamina separating the granulosa and theca compartments disintegrates during atresia (Bavagandoss et al, 1983; Boone et al, 1997), allowing for easy detachment of apoptotic granulosa cells during isolation of theca layers. Furthermore, we demonstrate a complete absence of fragmented DNA in morphologically healthy prehierarchal ovarian follicles (2-6 mm), an observation consistent with earlier results of Tilly et al. (1991a) that demonstrated a complete absence of apoptotic DNA ladders in DNA isolated from both the theca and granulosa cells of healthy hen ovarian follicles (4-6 mm).

The fact that apoptotic cell death in the ovarian follicle *in vivo* is confined to granulosa cells, with the neighbouring theca cells being resistant to apoptosis may

be related to their different embryological origin: granulosa cells are epithelial-derived whereas theca cells have a mesenchymal origin. In addition, the theca compartment is vascularised while the granulosa layers receive no direct blood supply. Granulosa cells, but not theca, are dependent on diffusion of nutrients and other serum-factors (e.g. gonadotropins) across the basal lamina. As a result, theca cells are constantly exposed to survival/growth factors in serum which may further render the theca cells resistant to apoptosis. During atresia most of the theca cells are reincorporated into the ovarian interstitium (O'Shea JD et al, 1978; Erickson et al, 1985; Hurwitz and Adashi, 1992) while the granulosa cells fragment into apoptotic bodies that are rapidly phagocytosed by resident immune cells (such as phagocytes, macrophages, etc.). Due to this rapid 'clean-up' process it may be difficult to demonstrate abundant morphological features characteristic of apoptosis in *in vivo* studies.

Apoptosis of surface epithelium cells in preovulatory sheep follicles has previously been reported (Ackerman and Murdoch, 1993). Interestingly, we observed that the surface epithelium of some healthy hen follicles displayed fragmented DNA. Although the reasons for this are unknown, it is possible that continuous remodelling of the epithelial layer is needed to accommodate for follicular growth in healthy prehierarchal follicles that are destined to ovulate and apoptosis is involved in this epithelial remodelling process. Alternatively, the surface epithelium may be the first cell type to undergo apoptosis in healthy prehierarchal hen follicles that are doomed to atretic degeneration.

In summary, granulosa and theca cells in healthy prehierarchal hen ovarian follicles (2-6 mm) are completely free of apoptosis, whereas same size atretic follicles display extensive granulosa cell apoptosis which might have resulted in

the demise of the entire follicle. There is no evidence of theca cell apoptosis in atretic hen follicles.

16.2 TGFα as a Survival Factor

The fate of the granulosa cells during follicular development is regulated by a host of endocrine, autocrine, paracrine and neural factors. While the suppression of granulosa cell apoptosis by gonadotropins is well documented, less is known about the non-gonadotropin signals that regulate the fate of granulosa cells and ultimately the fate of the follicle (atresia vs. ovulation). About 4000 follicles are present at the onset of sexual maturity in the hen ovary, and about 80 % of these become atretic at the prehierarchal (< 9 mm) stage of development during the reproductive life of the hen (Gilbert et al, 1983). The physiological difference between prehierarchal (< 9 mm) and hierarchal (> 9 mm) follicles is that the granulosa cells of prehierarchal follicles are highly proliferative and undifferentiated, as demonstrated by their inability to secrete substantial amounts of steroids in response to FSH or LH. In contrast, granulosa cells from hierarchal follicles are not as proliferatively active as their prehierarchal counterparts but are more differentiated, as evident by their ability to secrete extensive amounts of progesterone in response to FSH (at the 9-12 mm stage) and subsequently (at the > 12 mm stage) in response to LH (Tilly et al, 1991b). Since follicular atresia only occurs in prehierarchal follicles when the granulosa cells are much less responsive to gonadotropins, non-gonadotropin survival factors (e.g. TGFα) may play an important role in determining the fate of the follicle (atresia vs. growth) at this stage of development. In hen ovarian follicles, both theca and granulosa cells express TGFα throughout follicular development (Onagbesan et al, 1994). As

well, both theca and granulosa cells express TGFα receptors at all stages, suggesting an autocrine/paracrine mode of action for TGFα. However, the highest density of both TGFα receptor and ligand in immature (prehierarchal) follicles is consistent with the higher proliferative activity of the granulosa cells from these follicles (Onagbesan et al, 1994; Lafrance et al, 1993). It is possible TGFα receptor and ligand expression declines before the onset of atresia. Although such a study has not been reported in granulosa cells, down-regulation of TGFα mRNA expression occurs in HuH-7 cells undergoing retinoid-induced apoptosis (Nakamura et al, 1996).

Culture of hen granulosa cells from prehierarchal follicles (2-6 mm) in serum-free medium induced spontaneous apoptosis in these cells, as demonstrated by the presence of distinct DNA ladders when DNA was resolved by agarose gel electrophoresis, and by the presence of morphological features characteristic of apoptosis, including membrane blebbing, cellular rounding and condensation, cellular fragmentation into membrane bound apoptotic bodies, chromatin condensation and nuclear fragmentation. These results are in agreement with other reports of serum-deprivation induced apoptosis in hen granulosa cells from 4-8 mm follicles (Flaws et al, 1995), gonadotropin-treated rat granulosa cells (Tilly et al, 1992), and several non-ovarian cell types (Raff, 1992; Thompson, 1995). Apoptosis induced by serum- or survival factor-deprivation *in vitro*, as observed in hen granulosa cells form prehierarchal follicles, suggests that these cells must be under the constant 'protective' influence of anti-apoptotic factors *in vivo*. When these cell survival factors are 'removed' *in vitro*, the tonic suppression of apoptosis no longer exists and, as a result, the genetic death program is turned on. Recently, evidence for this spontaneous and intrinsic drive for prehierarchal hen granulosa cells to activate their death program came from a report of a time-dependent

increase in the level of the nuclear transcription factor *myc* in cells incubated in serum-free medium (Johnson et al, 1997). In the absence of growth factors the *myc* protein is known to induce apoptosis by activating the tumour-suppresser gene p53 in several cell types, including granulosa cells (Keren-Tal et al, 1995; Askew et al, 1991; Galaktionov et al, 1996). In addition to spontaneous expression of apoptosis-inducing molecules (e.g. *myc*, p53), deprivation of survival factors may also lead to loss of intracellular kinase activity, resulting in the dephosphorylation (or loss of phosphorylation) of pro-apoptotic bcl-2 family members (Zha et al, 1997). Unphosphorylated *bad* (a pro-apoptotic dimerising partner of *bcl-2*) has been shown to associate with *bcl-2* and promote apoptosis (Zha et al, 1997). In contrast, phosphorylated *bad* has no affinity for *bcl-2* and hence keeps the apoptotic pathway inactivated (Zha et al, 1997).

Although apoptosis is the primary mechanism of granulosa cell death *in vitro* as demonstrated in my studies by nuclear and cytoplasmic morphology and DNA fragmentation pattern characteristic of apoptosis, it is not possible to rule out the possibility of cell death due to necrosis. In fact, the DNA smear observed between the 'rungs' of the apoptotic ladder might have been the result of necrotic cell death.

The observed differences in intensity between loading regions on the DNA gels might have been due to differences in fractions of fragmented high molecular weight DNA (> 15 kb), as seen in the centre lane (one containing the most fragmented DNA) of Figure 16. Alternatively, despite precautions taken to ensure that the DNA was indeed in solution, it may be possible that unequal amounts of DNA could have been loaded onto the gel, hence resulting in the observed differences in the intensity around the loading region.

In the present studies, we have demonstrated that addition of TGFα to the cell cultures effectively prevented the serum deprivation-induced granulosa cell apoptosis, while continual maintainance of these cells in serum-containing medium demonstrated minimal apoptotic response. Recently, Johnson et al. (1996) also demonstrated that TGFα suppressed serum deprivation-induced DNA fragmentation in granulosa cells from hen ovarian follicles (4-8 mm). Our findings are consistent with the anti-apoptotic role of TGFα reported in rat granulosa cells (Tilly et al, 1992a), teleost fish ovarian follicles (Janz and Vanderkraak, 1997), mouse mammary cells (Laufey et al, 1996), human keratinocytes (Reinartz et al, 1996) and a human hepatoma cell line (Nakamura et al, 1996). Although TGFα was capable of inhibiting the serum deprivation-induced apoptosis in this study, about 4 % of the cultured granulosa cells were apoptotic even in the presence of the maximally effective TGFα concentration (20 ng/ml), and as a result some residual apoptotic DNA fragmentation persisted. This is probably due to the inability of TGFα *in vitro* to emulate the *in vivo* scenario where multiple apoptosis-inhibitory (survival) factors may simultaneously be impinging on the granulosa cells.

It is important to note that the dependency of granulosa cells *in vitro* on survival factors is related to the developmental stage of the follicle from which the granulosa cells are isolated. Granulosa cells from mature hen follicles (> 9 mm; hierarchal) do not appear to be dependent on exogenous survival factors *in vitro*. Several studies have reported that granulosa cells from hierarchal follicles cultured in serum-free medium for as long as 21 hours exhibited no decrease in cell viability (Li and Tsang, 1994; Lafrance et al, 1993). Johnson et al. (1996) reported that differentiated hen granulosa cells from hierarchal follicles (> 9 mm) expressed

markedly more (5 folds) bcl-xlong mRNA compared to undifferentiated granulosa cells from prehierarchal (3-8 mm) follicles. The *bcl-xlong* protein is a member of the bcl-2 family and is a potent inhibitor of apoptosis (reviewed by Hale et al, 1996). This may explain the apparent resistance of differentiated granulosa cells to spontaneous apoptotic cell death when cultured under survival factor-free conditions. On the other hand, low levels of *bcl-xlong* in undifferentiated granulosa cells failed to prevent their spontaneous apoptotic death *in vitro*. It is possible that differentiated granulosa cells express, in addition to *bcl-xlong*, other gene products that also render these cells resistant to apoptosis and/or suppress expression of proteins that promote apoptosis.

In summary, this data confirms that undifferentiated granulosa cells from hen (prehierarchal) follicles need constant trophic support, without which a program for death is spontaneously activated. TGFα prevents serum deprivation-induced apoptosis of granulosa cells, a phenomena consistent with its role as a survival factor in other systems.

16.3 The Role of Prostaglandins in the Anti-apoptotic Action of TGFα

Prostaglandins (PGs) have been shown to mediate the action of TGFα in numerous physiologic systems (Harrison, 1994). TGFα-induced increases in PG secretion is believed to be important for DNA synthesis in differentiated hen granulosa cells (Li and Tsang, 1995) and in rat hepatocytes (Skouteris and McMenamin, 1992). However, to my knowledge, the present studies represent the first that link the TGFα-induced increases in PGs to apoptosis in any cell type. TGFα (20 ng/ml) significantly increased granulosa cell PGE and PGF secretion to 2.5- and 4.5-fold

above non-treated controls, respectively. TGFα has been recently shown to activate $cPLA_2$ (Li et al, 1997) as well as up-regulate the expression of COX II mRNA and protein (Li et al, 1996) in hen granulosa cells. Both $cPLA_2$ and COX II are involved in the biosynthesis of prostaglandins (Figure 6).

Since PGs are not stored or retained in the cell but are immediately secreted upon synthesis, the amounts detected in the culture media could be considered an accurate reflection of those synthesised by the granulosa cells. PGF was synthesised in greater amounts than PGE in both control and TGFα-treated granulosa cells (3- and 6-fold more PGF than PGE, respectively). The reason for these differences is not as yet apparent but it is possible that the larger amount of PGF detected could in part be due to either greater activity of PG endoperoxide reductase relative to that PG endoperoxide E-isomerase or enhanced conversion of PGE to PGF by PG 9 keto reductase (Franchi et al, 1985). The physiological significance of this finding is presently unclear and awaits knowledge on PG receptor profiles on hen granulosa cells. The concentration of TGFα (5 ng/ml) that elicited half maximal stimulation of PGE and PGF synthesis was similar to that needed to suppress serum deprivation-induced DNA fragmentation by 50 %. This observation is consistent with the concept that PGs mediate the TGFα-induced suppression of granulosa cell apoptosis *in vitro*.

Studies with COX and PLA_2 inhibitors were conducted to determine if the TGFα-induced increases in prostaglandins are associated with the anti-apoptotic action of TGFα. NS398, ibuprofen and indomethacin inhibit COX activity by binding to the active site of the enzyme and suppress the conversion of arachidonic acid to PGs (Kurumbail et al, 1996). NS398 is a selective COX II inhibitor while ibuprofen and indomethacin inhibit both COX I and II (Futaki et al, 1994;

Lanuville et al, 1994; Kurumbail et al, 1996). In the present studies, NS398 and ibuprofen completely suppressed the TGFα-induced PG synthesis and induced more extensive (2-4 fold) granulosa cell apoptosis compared to TGFα-treated control cultures, as revealed by DNA fragmentation and morphological features characteristic of apoptosis. Although initial studies with different concentrations of NS398 (5, 10, 20 μM) failed to demonstrate a concentration-response relationship in PG production and DNA fragmentation (Figures 7 and 10), subsequent investigations with a lower concentration range of the inhibitor (0, 1 and 10 μM) elicited a concentration-dependent induction of granulosa cell apoptosis, as determined by nuclear morphology [proportion of apoptotic cells: 4 % (control), 8 % (1 μM NS398), 16 % (10 μM NS398)]. Moreover, the NS398-induced apoptosis was completely prevented by exogenous PGs ($PGF_{2\alpha}$, PGE_1, PGE_2). These findings suggest that the effect of the inhibitor was related to the suppression of PG synthesis and that the inhibitor was not toxic to the cells. In addition, this data provides further evidence for the involvement of PGs in the suppression of granulosa cell apoptosis by TGFα.

Although both indomethacin and ibuprofen completely suppressed the TGFα (20 ng/ml)-induced PG synthesis, the former inhibitor failed to significantly induce DNA fragmentation compared to control. This might be due to non-specific effects of indomethacin which were unrelated to its COX suppression. A known non-specific effect of indomethacin in hen granulosa cells is stimulation of (^3H) thymidine uptake (Li and Tsang, 1995). Hence, it is possible that indomethacin might have either directly or indirectly suppressed the apoptotic machinery in the cell.

It is interesting to note that the selective COX II inhibitor NS398 suppressed the TGFα-induced PG synthesis to levels below those observed in the absence of the growth factor, as did the other two COX inhibitors. This suggests that COX II may be the primary if not only COX isoenzyme responsible for the synthesis of PGs in hen granulosa cells. This interpretation is consistent with a recent study which reported undetectable COX I message in hen granulosa cells (Li et al. 1996).

Aristolochic acid is a non-competative inhibitor of PLA_2 which binds to a site on the enzyme other than the active site and changes the secondary structure of PLA_2 (Vishwanath et al, 1987). TFMK is a competative inhibitor of PLA_2 (Riendeau et al, 1994; Street et al, 1993). All three PLA_2 inhibitors significantly, though incompletely, reduced the TGFα-induced PG synthesis. Even at high concentrations of the PLA_2 inhibitors, a low but significant level of PG production was still detectable. While the lowest concentration (5 μM) of TFMK and ONO-RS-82 caused a maximal suppression of PG synthesis, presumably a consequence of maximal inhibition of $cPLA_2$ activity, the maintenance of tonic levels of PG production was evident. The reason for the latter response is unclear; it is possible that arachidonic acid could be synthesised via pathways independent of PLA_2. Since COX, but not PLA_2, is active in the presence of PLA_2 inhibitors, any available arachidonic acid could be metabolised to PGs. Gamma linolenic acid can be elongated (by addition of carbons to the chain) and then desaturated to yield arachidonic acid (Carnielli et al, 1996). Alternatively, arachidonic acid-containing membrane phospholipids can be metabolised by phospholipase C (PLC) and subsequently by di-acylglycerol lipase to release arachidonic acid (Migas and Steverson, 1996). Arachidonic acid may also be released during de-esterification of arachidonic acid containing cholesterol-esters (Delton et al, 1995). Although

these non-PLA_2 pathways are believed to be minor routes to arachidonic acid synthesis in animal cells, it is unknown whether hen granulosa cells exhibit these pathways and to what extent can they account for the observed tonic release of PGs in the presence of PLA_2 inhibitors. In contrast, the pattern of PG synthesis inhibition when cells were cultured in the presence of the PLA_2 inhibitor aristolochic acid (Figure 15) indicates that the PLA_2 enzyme may not be completely inhibited even at the highest concentration of the inhibitor. Hence, larger amounts of PGE and PGF were detected in media from these cells compared to those cultured with TFMK or ONO-RS-82.

In the present studies, granulosa cells cultured in the presence of TGFα plus PLA_2 inhibitors (AC or TFMK) displayed significantly more extensive (1.5-2.5 fold) apoptotic DNA fragmentation compared to TGFα-treated control cells (no inhibitor). In addition, these cells also exhibited morphological features characteristic of apoptotic cell death whereas TGFα-treated control cells (no inhibitor) were healthy. This AC-induced apoptosis could be completely prevented by the addition of arachidonic acid (0.5 μM) to the cultures, thereby confirming that the observed AC-induced apoptosis is due to the suppression of arachidonic acid synthesis and not due to non-specific effects of the inhibitor. In contrast, exogenous PGE_1, PGE_2 or $PGF_{2\alpha}$, at concentrations which effectively attenuated the COX II inhibitor-induced apoptosis (1 or 20 μM), failed to prevent the AC-induced DNA fragmentation. Since arachidonic acid, but not PGs, could prevent the apoptotic action of AC, it is suggested that PGs are necessary (as demonstrated in studies with COX inhibitors) but not sufficient in mediating the anti-apoptotic action of TGFα. It is possible that, in conjunction with PGs, arachidonic acid and/or its metabolites (i.e. leukotrienes, HETEs or EETs) may be important for TGFα action in hen granulosa cells. Indeed, the production of both

PGs and leukotrienes following EGF stimulation appeared important in the growth factor-induced changes in cytoskeletal dynamics in A431 cells (Peppelenbosch et al, 1993). In addition to cyclooxygenase and lipoxygenase which metabolise arachidonic acid to prostaglandins and leukotrienes, respectively, the cytochrome P450 system converts arachidonic acid to monohydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs). The EETs can further undergo hydrolysis by epoxide hydrolase to form diol derivatives of EETs (Oliw et al, 1981). Whether arachidonic acid metabolites of the cytochrome P450 pathway are also involved in mediating the physiological actions of TGFα in granulosa cells is unknown.

Although inhibition of TGFα-induced PG production resulted in granulosa cell apoptosis, $PGF_{2\alpha}$, PGE_1, or PGE_2 alone (in the absence of TGFα or serum) failed to mimic the anti-apoptotic action of TGFα (i.e. PGs alone failed to prevent the serum-deprivation induced apoptotic DNA fragmentation in granulosa cells). This finding is consistent with the concept suggested by the PLA_2 inhibitor studies that PGs are necessary but not sufficient in mediating the suppression of apoptosis by TGFα. To produce the anti-apoptotic action of TGFα, PGs must work in conjunction with other, yet undefined, factors down-stream of TGFα receptor activation. In hen granulosa cells, TGFα/EGF has been shown to increase tyrosine phosphorylation (Tilly et al, 1992a), activate mitogen-activated protein (MAP) kinase (Keel et al, 1995), PKC and Na^+/H^+ antiporter (Li et al, 1991) as well as to induce *myc* protein expression (Johnson et al, 1997). Whether any of these TGFα-induced signals co-operate with PGs to effect the TGFα-mediated suppression of apoptosis is unknown.

Since Flaws et al. (1995) demonstrated that 8-bromo-cAMP and vasoactive intestinal peptide (VIP; which is known to act via cAMP/PKA) prevent serum deprivation-induced apoptosis in hen granulosa cells from prehierarchal (4-6 mm) follicles, it is unlikely PGF or PGE stimulate any receptors that result primarily in the elevation of intracellular cAMP (i.e. EP_2 and EP_4 receptors). Western blot studies in our laboratory have demonstrated the presence of EP_3 receptors (which bind $PGE_2 = PGE_1 > PGF_{2\alpha}$) in hen granulosa cell (Li and Tsang, unpublished). EP_3 receptor activation decreases cAMP levels by inhibiting adenylate cyclase. Although human and bovine granulosa cells are known to express FP ($PGF_{2\alpha} > PGF_{1\alpha}$) and EP_3 receptors (Ristimaki et al, 1997; Tsai et al 1996), it is unknown whether hen granulosa cells express FP or EP_1 (PGE_2) receptors. Studies on the expression of FP receptors during follicular development will provide further insight into the role of PGs, especially $PGF_{2\alpha}$, in the anti-apoptotic action of TGFα in hen granulosa cells.

Secreted PGs most probably act via G-protein coupled receptors (i.e. EP and FP receptor subtypes) on granulosa cell membranes. However, peroxisome proliferator-activated receptor (PPAR) is a nuclear receptor that is activated by various fatty acids, including leukotriene B_4 and prostaglandins of the A, D and J series (Yu et al, 1995; Devchand et al, 1996). PPAR is a transcription regulator and has been shown to maintain lipid homeostasis in the liver, promote adipocyte differentiation, and control the duration of the inflammatory response in the liver (Devchand et al, 1996; Shalev and Meier, 1996; reviewed by Wolf, 1996). It is unknown whether granulosa cells express PPAR.

Myc is a central regulator of cell proliferation and apoptosis. *Myc* is a nuclear transcription factor that relays external signals to the apoptotic or cell-cycle

machinery of the cell by regulating the transcription of several oncogenes, tumour suppresser genes and cell cycle genes (reviewed by Desbarats et al, 1996). *Myc* induces apoptosis in the absence of growth/survival factors, whereas in their presence *myc* induces proliferation (Desbarats et al, 1996; Hale et al, 1996). Recently Johnson et al (1997) demonstrated increased *myc* protein expression in a time-dependent manner in cultures of hen granulosa cells from 6-8 mm follicles in serum-free medium, suggesting *myc*-dependent apoptosis of granulosa cells. Furthermore, over-expression of *myc* in granulosa cells cultured in serum-free medium induced significantly more apoptosis than control cell cultured in serum-free medium (Johnson et al, 1997). However, addition of TGFα increased granulosa cell *myc* protein to levels above those induced by serum-deprivation, implying *myc*-mediated suppression of apoptosis by TGFα (Johnson et al, 1997). Johnson et al (1997) clearly demonstrate how *myc* can either promote or suppress apoptosis in hen granulosa cells depending on the absence or presence of TGFα, respectively. For *myc* to promote cell growth and suppress apoptosis, it has to heterodimerise with *max* (reviewed by Desbarats et al, 1996; Galaktionov et al, 1996). In the absence of growth factors, expression of both *myc* and *max* suppresses apoptosis and promotes growth (Kohlhuber et al, 1995; Hurlin et al, 1997; Peters et al, 1997), whereas over-expression of only *myc* strongly induces apoptosis (Johnson et al, 1997; Hunter, 1997; Hale et al, 1996).

In my studies, no difference was observed between the extent of DNA fragmentation in granulosa cells cultured in serum-free medium containing only PGs (no TGFα) and control cells cultured in serum-free medium. It is possible that PG receptor activation does not lead to an increase in *myc* levels. Hence, the TGFα-induced increase in *myc* is probably due to intracellular signals other than PGs. Since PGs mediate the suppression of apoptosis by TGFα, it is speculated

that the TGFα-induced increases in PGs result in increased *max* expression, hence allowing *max/myc* heterodimers to suppress apoptosis. In the presence of TGFα, inhibition of PG synthesis may lead to suppression of *max* synthesis resulting in *myc*-induced apoptosis. Whether these indeed are the physiologic basis for the present observations remains to be determined.

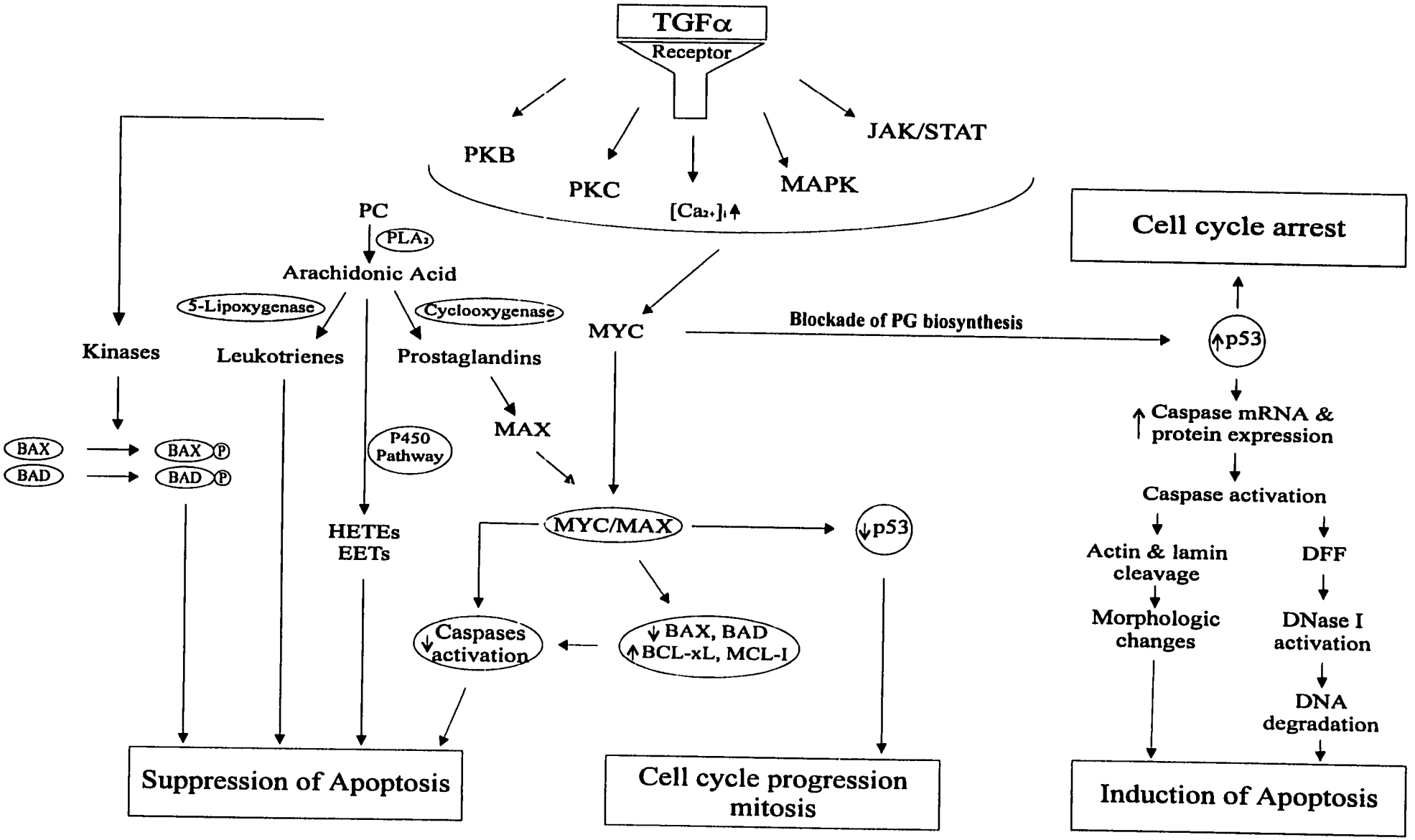
Figure 20 is a hypothetical model attempting to illustrate the steps between TGFα receptor activation and the suppression of apoptosis in granulosa cells. This model serves to focus future studies in elucidating the actions of TGFα and PGs in granulosa cells.

Irrespective of the molecular pathways by which PGs mediate the suppression of apoptosis by TGFα, ultimately all models of apoptosis must converge on to a handful of evolutionary conserved gene families, such as bcl-2 and ICE, that regulate apoptotic cell death (reviewed by Clarke and Clarke, 1996; Nagata, 1997; Hale et al, 1996). *Bad*, *bax*, *bak*, *bcl-xshort* are apoptosis promoting members of the bcl-2 family, whereas *bcl-xlong*, *mcl-1* and *bcl-2* are those which suppress apoptosis. ICE-related proteins (now called caspases) are proteases that induce apoptosis when activated. Caspases degrade proteins necessary for cellular viability and structure (such as PARP, U1-small nuclear ribonucleoprotein, DNA-dependent protein kinase, actin, lamin, rho-GDI etc.). Along with protein degradation, caspase-3 also cleaves and activates DNA fragmentation factor (DFF) which is necessary for the cleavage of genomic DNA into nucleosomal fragments of 180 base pair multiples by a Ca^{2+}/Mg^{2+}-dependent endonuclease (Liu et al, 1997; Zeleznik et al, 1989; Boone et al, 1995).

Figure 20. A hypothetical model illustrating the role and regulation of eicosanoid biosynthesis in the suppression of granulosa cell apoptosis following TGFα receptor activation.

TGFα receptor activation results in PG synthesis as well as the activation of kinases such as MAPK (Keel et al, 1995), PKC (Li et al, 1991). It is possible that signals other than PGs result in the TGFα-induced increases in *myc* protein. It is speculated that PGs induce the expression of *max* protein, which binds its heterodimerising partner, *myc*. The *max/myc* heterodimer is essential for the promotion of mitosis and suppression of apoptosis (Kohlhuber et al, 1995; Hurlin et al, 1997; Peters et al, 1997). In addition, PGs or other TGFα-induced signals can suppress apoptosis by activating kinases that phosphorylate and inactivate apoptosis-promoting members of bcl-2 (Zha et al, 1996; Wang HG et al, 1996; Kauffmann-Zeh et al, 1997). Suppression of the TGFα-induced PG synthesis results in apoptosis. This could be due to the inhibition of *max* synthesis, resulting in the TGFα-induced upregulation of *myc*. *Myc*, in the absence of *max*, is a potent inducer of apoptosis (Johnson et al, 1997; Hunter, 1997; Hale et al, 1996). *Myc*-induced apoptosis is known to be mediated by the tumour suppresser gene p53 (Askew et al, 1991; Levine, 1997; Keran-Tal et al, 1995). p53 arrests the cell-cycle by upregulating expression of proteins (such as waf-1, p21 and cip-1) that inactivate cyclins and cyclin-dependent kinases, hence inhibits proliferation (Levine, 1997). p53 upregulates *bax* and down-regulates transcription of the bcl-2 gene (Miyashita and Reed, 1995; Miyashita et al, 1994), leading to the activation of caspases and progression of apoptosis (Nagata, 1997). Caspase 3 can cleave and activate DNA fragmentation factor (DFF) which probably induces DNase-I to degrade genomic DNA to multiples of 180 bp fragments (Liu et al, 1997; Boone et al, 1995; Boone and Tsang, 1997). p53 also upregulates mRNA and protein levels of insulin-like growth factor binding proteins (IGFBPs) which can sequester insulin-like growth factors, hence suppressing their anti-apoptotic and mitogenic activity (Levine, 1997). Inhibition of PG synthesis may result in a loss of PG receptor-mediated kinase activity, which may in turn cause a lack of phosphorylation of *bax* or *bad*. Unphosphorylated *bax* or *bad* can bind to and antagonising the function of apoptosis-suppressers such as *bcl-xlong* and *mcl-1*, hence promote apoptosis by activating caspases (Chinnaiyan et al, 1997; Zha et al, 1996; Wang HG et al, 1996; Kauffmann-Zeh et al, 1997). ↑, upregulation or activation; ↓, down-regulation or suppression of activity.

A Hypothetical Model for the Role and Regulation of Eicosanoid Biosynthesis in the Suppression of Granulosa Cell Apoptosis by TGFα



Apoptosis inducing signals (e.g. serum-deprivation, fas ligand, TNFα, radiation, etc.) ultimately lead to the activation/upregulation of caspases and apoptosis-promoting relatives of bcl-2, and simultaneous suppression of the anti-apoptotic members of the bcl-2 family. On the other hand, signals which suppress apoptosis (e.g. TGFα and/or other growth factors) ultimately upregulate/activate the apoptotic-suppressing members of the bcl-2 family and down-regulate or maintain in an inactive state the caspases and apoptosis-promoting members of the bcl-2 family. Investigations on the expression of *max*, p53, *bcl-xlong*, *mcl-1*, *bax*, *bad*, caspase 3 and 8 in granulosa cells under serum-deprived conditions and on their responses to TGFα and inhibitors of PG synthesis (e.g. NS398) will provide insight into the molecular and cellular mechanism of action of this growth factor as a cell survival factor.

In summary, I have confirmed that granulosa cell viability *in vitro* is dependent on the continual supply of survival/growth factors (e.g. serum) and removal of such factors spontaneously induces apoptosis. In addition, I have shown that TGFα is a survival factor for undifferentiated hen granulosa cells, as demonstrated by the capacity of the growth factor to suppress serum deprivation-induced apoptosis. Prostaglandin synthesis is a necessary, but not sufficient, event in the suppression of granulosa cell apoptosis by TGFα. Whether arachidonic acid or leukotrienes or arachidonic acid metabolites of the cytochrome P450 pathway (i.e. HETEs or EETs) are important in the anti-apoptotic action of TGFα remains to be determined. To address these aspects, it may be necessary to study the effect of inhibitors of the lipoxygenase [e.g. nordihydroguaiaretic acid (NDGA) or MK-866] and cytochrome P450 [SKF-525A] pathways on the anti-apoptotic action of TGFα and to determine if the effects of the PLA_2 inhibitors can be suppressed by exogenous leukotrienes. It is possible prehierarchal hen ovarian follicles need a

continual supply of survival factors (such as TGFα) to maintain their viability and protect them from atresia. The availability of survival factors or the capacity of granulosa cells to respond to them may be important determinants of the fate of hen prehierarchal ovarian follicles (atresia vs. ovulation).

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18. BIBLIOGRAPHY

Name: Raman Manchanda

Education

M.Sc.: Physiology, University of Ottawa (1995-1997)

B.Sc. Honours *(Summa Cum Laude)*: Biochemistry, University of Ottawa (1991-1995)

Awards and Scholarships

International/National Awards

1. The Cornelia Post Channing Young Investigator Award at the XIth Ovarian Workshop (July 26, 1996, London, ON); this award is presented once every two years to a student or postdoctoral fellow.

2. Natural Sciences and Engineering Research Council of Canada (NSERC) Post Graduate Scholarship A (1995-1997)

3. NSERC summer research awards (1993 & 1995)

4. Canada Scholarship (1991-1994)

Provincial Awards

5. Ontario Graduate Scholarship (1995, declined)

Local awards

6. University of Ottawa Excellence Scholarship (1995-1997)

7. University of Ottawa Merit A+ Scholarships (1992 & 1994)

8. University of Ottawa Admission Scholarship (1991)

9. University of Ottawa, Faculty of Science Dean's Honour List (1992-1995)

10. Best Research Paper Award at the 16th Annual Ottawa Reproductive Biology Workshop (June 14, 1997)

Research Experience

1. **Graduate research project:** "The role of prostaglandins in the anti-apoptotic action of transforming growth factor α in hen granulosa cells" conducted under the supervision of Dr. Benjamin Tsang at the Ottawa Civic Hospital, Loeb Research Institute (Sept. 95 - July 97).

2. **Summer research project:** I studied the role of vitamin E as an anti-oxidant in rat mitochondria using high performance liquid chromatography (HPLC) in Dr. Alvin Chan's lab, University of Ottawa. I also examined the interaction of Vitamin E with phospholipids in liposomes using infrared spectroscopy (May - August, 1995).

3. **Honours research project**: "The Inhibited Oxidation of β-Carotene" conducted under the supervision of Dr. Graham Burton at the National Research Council of Canada (Sept. 94 - April 95).

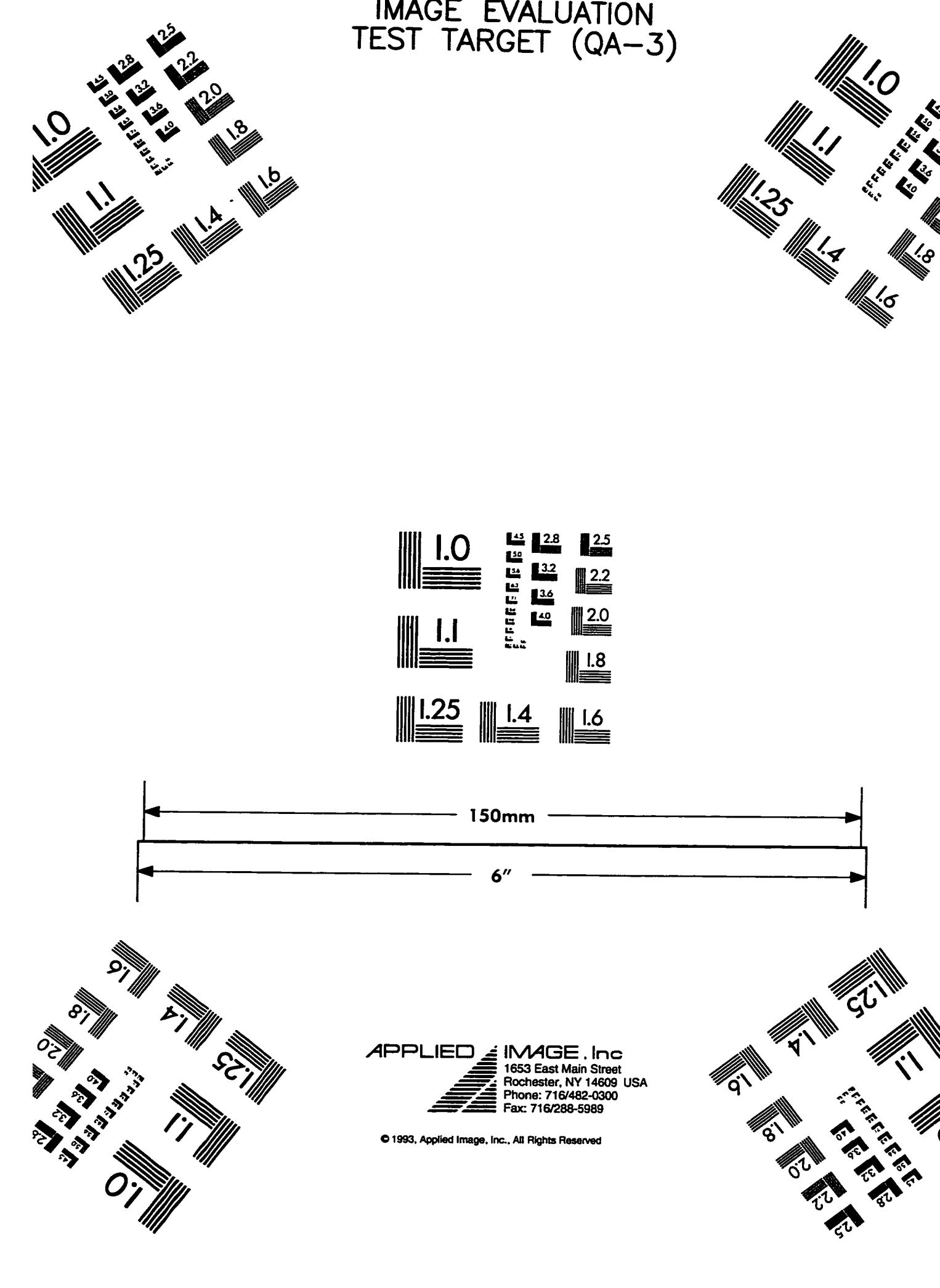
4. **Summer research project:** I worked on a breast cancer project in Dr. Martin Tenniswood's lab, University of Ottawa. I monitored surgery on mice and measured tumour growth and regression (May - August, 1993).

Research Presentations

1. **Manchanda R**, Kim JM, Tsang BK. *Involvement of TGFα and Prostaglandins in the Regulation of Apoptosis in Hen Granulosa Cells.* 15th Annual Ottawa Reproductive Biology Workshop, June 1996, Ottawa, Ontario, Canada.

2. **Manchanda R**, Kim JM, Tsang BK. *Transforming Growth Factorα Inhibits Apoptosis in Hen Granulosa Cells.* XIth Ovarian Workshop: Ovarian Cell Growth, Apoptosis and Cancer, July, 1996, London, Ontario, Canada.

3. **Manchanda R**, Kim JM, Tsang BK. *Prostaglandins Mediate the Suppression of Apoptosis by TGFα in Granulosa Cells from Hen Ovarian Follicles.* 16th Annual Ottawa Reproductive Biology Workshop, June 1997, Ottawa, Ontario, Canada.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