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The Role and Regulation of Deoxyribonuclease I in Rat Ovarian Apoptosis

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A thesis submitted to the School of Graduate Studies and Research, University of Ottawa, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Apoptosis is a physiological form of cell death and is the cellular basis of ovarian follicular atresia and luteal regression. Apoptosis has distinct morphological and biochemical features including the degradation of DNA by the action of a Ca^{2+}/Mg^{2+}-dependent endonuclease. This characteristic DNA degradation (DNA "ladders") is evident in atretic follicles and regressing luteal tissue of diverse species. Hormones, growth factors and cytokines that induce or suppress follicular atresia or luteal regression also induce or suppress the characteristic apoptotic DNA degradation. In order to elucidate the cellular and molecular mechanisms of ovarian apoptosis, the Ca^{2+}/Mg^{2+}-dependent endonuclease responsible for DNA degradation must be identified. The objectives of the present work were to identify the endonuclease responsible for ovarian apoptotic DNA degradation and its possible regulation during follicular atresia and luteal regression. Immature female rats were sequentially treated with diethylstilbestrol (DES; DES group; preantral follicles), DES+eCG (eCG group; antral follicles) or DES+eCG+hCG (hCG group; luteal tissue). There was a developmental pattern of ovarian endonuclease activity such that nuclei from the eCG- and hCG-, but not the DES-group, degraded their DNA in an apoptotic fashion when exposed to Ca^{2+} and Mg^{2+} *in vitro*. Nuclear protein extracts contained three nuclease activities: a Mg^{2+}-dependent 27 kDa protein which was active on single-stranded DNA and did not display a developmental pattern of activity, and a doublet of 32/34 kDa

which was Ca^{2+}/Mg^{2+}-dependent, active on double- or single-stranded DNA and present in the extracts of eCG and hCG, but not DES, groups. The 32/34 kDa activity was biochemically, immunologically and functionally indistinguishable from deoxyribonuclease I (DNase I). The mRNA encoding DNase I was also present, in a developmental pattern, in the rat ovary. Immunohistochemistry localized DNase I in nuclei of ovarian cells susceptible to apoptosis including luteal cells, granulosa cells from antral but not preantral follicles, and oocytes from preantral but not antral follicles. The possibility that caspase cleavage of actin and/or poly(ADP ribose) polymerase (PARP) regulates the activity of DNase I during ovarian apoptosis was investigated using an anti-eCG antibody *in vivo* to induce atresia and $PGF_{2\alpha}$ to induce luteal regression. Apoptosis during $PGF_{2\alpha}$-induced luteal regression, but not anti-eCG antibody-induced atresia, was associated with actin and PARP cleavage. Caspase-3 was immunolocalized in luteal cells and in theca, but not granulosa cells, of healthy follicles, and was also detected in granulosa cells of atretic follicles from anti-eCG antibody treated rats. These results demonstrate that the Ca^{2+}/Mg^{2+}-dependent endonuclease responsible for DNA "ladders" in ovarian cells is DNase I. Further, the expression and cellular distribution of DNase I during follicular development correlates with the susceptibility of ovarian cells to undergo apoptosis, which suggests that DNase I is responsible for the DNA "ladders" associated with follicular atresia and luteal regression. Although DNase I activity does not appear to be regulated by the caspase-mediated cleavage of actin or PARP, caspase-3 may be involved in the

regulation of DNase I activity during ovarian apoptosis. Studies of the regulation of DNase I expression and activation will provide further insight into the cellular and molecular mechanisms of ovarian follicular atresia and luteal regression.

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LIST OF ABBREVIATIONS

A_{450}	absorbance of light at the wavelength 450 nanometres
ADP	adenosine diphoshate
AES	3-amino-propyltriethoxysilane
Asp	aspartic acid
ATP	adenosine triphosphate
AUR	aurintricarboxylic acid
bFGF	basic fibroblast growth factor
BH-2	bcl-2 homology domain - 2
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanidine monophosphate
CL	corpus luteum
CRE	cAMP response element
CrmA	cow pox virus cytokine response modifier
DAB	3,3-diaminobenzidene tetrahydrochloride
dCTP	deoxy-cytidine triphosphate
ddATP	dideoxy-ATP

DED	death effector domain
DES	diethylstilbestrol
DFF	DNA fragmentation factor
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNase II	deoxyribonuclease II
DNase I	deoxyribonuclease I
dtt	dithiothreitol
dUTP	deoxyuridine triphosphate
eCG	equine chorionic gonadotropin
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbant assay
ER	endoplasmic reticulum
FA	fatty acid
FADD	fas-associated death domain
FasL	fas ligand
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLICE	FADD-like ICE
FSH	follicle stimulating hormone

GH	growth hormone
GnRH	gonadotropin releasing hormone
GRE	glucocorticoid response element
hCG	human chorionic gonadotropin
HRP	horse radish peroxidase
i.p.	intraperitoneal
IAA	iodoacetic acid
ICE	interleukin-1β-converting enzyme
IFNγ	interferon gamma
IGF-1	insulin-like growth factor-1
IGFBP	insulin like growth factor binding protein
IgG	immunoglobulin G
IL-6	interleukin-6
IL1β	interleukin-1β
IRP	ICE-related protease
IU	international unit
IVF	in vitro fertilization
kDa	kilodalton
LH	luteinizing hormone
LDL	low density lipoprotein
LDL-R	low density lipoprotein receptor

lpr	lymphoproliferative disorder
LWF	large white follicle
mA	milliamperes
MAP	myristolated acidic protein
NGF	nerve growth factor
NRS	normal rabbit serum
OPD	*o*-phenylenediamine hydrochloride
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PIP_2	phosphatidylinositol bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
s.c.	subcutaneous
SAM	sodium aurothiomaleate

SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error measured
SLE	systemic lupus erythmatosis
SREBP	sterol regulatory element binding protein
TAE	Tris-acetate-electrophoresis
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween
TE	Tris-EDTA
TGFα	transforming growth factor alpha
TNFα	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TRADD	TNFR associated death domain protein
TRE	thyroid hormone response element
TUNEL	terminal transferase dUTP nick end labelling
U	units
U1-70 sNRP	U1-70 small nuclear riboprotein
UV	ultraviolet
V	volt
VIP	vasoactive intestinal peptide

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1.0 INTRODUCTION

The essential function of the ovary is to produce oocytes for fertilization leading to the successful reproduction of the species. The production of oocytes is facilitated by the coordinated development and subsequent ovulation of follicles and the production of ovarian steroids to prepare the reproductive tract for pregnancy. The process of follicular development involves the recruitment, development, ovulation and luteinization of the follicle. Many follicles are recruited and begin to develop during an ovarian cycle, but only one (humans) or a few (rats) follicles completely mature and ovulate. The remaining follicles degenerate during development by a process known as atresia. Follicles that ovulate subsequently form the corpus luteum which persists for a period of time, depending on the occurrence of pregnancy, but eventually degenerates by a process called luteal regression. In recent years, it has become apparent that the cellular basis of follicular atresia and luteal regression is apoptosis (Tilly 1996; Hsueh et al 1994). Granulosa cells of the follicle and luteal cells of the corpus luteum undergo apoptosis during atresia and regression, respectively. Menopause occurs when all the oocytes and their associated follicles are removed from the ovary. Only a small number of these are removed by ovulation during the reproductive lifespan whereas most follicles are removed by atresia. The timing of the ovarian cycle depends on the regression of the corpus luteum of the previous cycle. Apoptosis in the ovary, therefore, is the most common fate of follicular granulosa cells and is a critical event

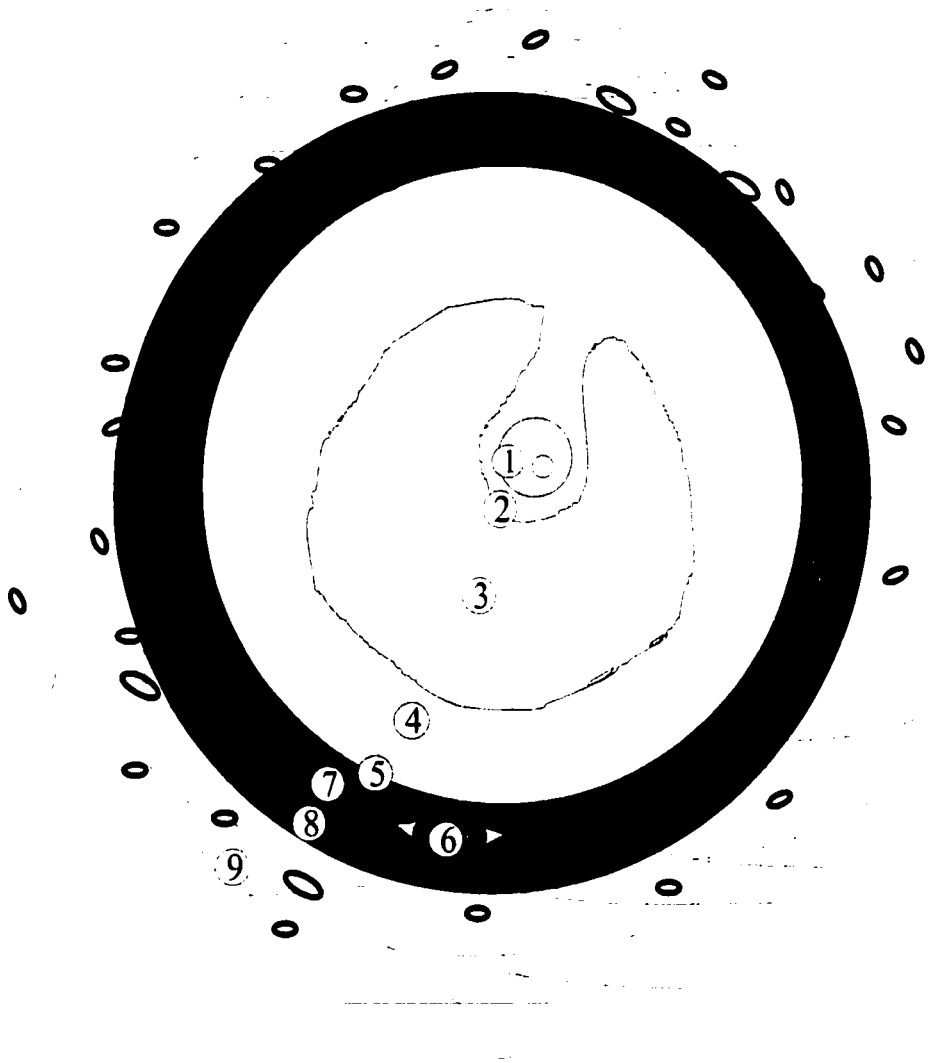
in luteal cells for the timing of each ovarian cycle. Despite being the predominant and critical event in the fate of ovarian cells, the cellular and molecular mechanisms that control apoptosis in the ovary are not completely understood.

1.1 THE OVARIAN FOLLICLE

1.1.1 Ovarian follicular anatomy

The follicle is the basic functional unit of the ovary. A mature Graafian follicle is an elegant structure composed of an oocyte, granulosa cells, follicular fluid, theca cells, a blood and nerve supply, connective tissue, and smooth muscle (fig. 1). The oocyte projects into the fluid-filled antrum of the follicle on a stalk of cumulus granulosa cells. The inner wall of the follicle is delineated by a basement membrane (lamina propria) which is covered by a stratified epithelium of membrana granulosa cells several layers thick. The cells lining the basement membrane are columnar in shape, while those closer to the antrum are polyhedral. Theca cells form layers outside the basement membrane and are divided into the theca interna and externa. Theca cells produce the type IV collagen, fibronectin and laminin that make up the basement membrane (Lipner 1988). As the follicle grows, the basement membrane must be continuously remodelled and synthesized to accommodate the increasing size of the structure. It is estimated that the basement membrane must increase over 400-fold in

Figure 1: Anatomy of the ovarian follicle. The oocyte (1) projects into the antrum on a stalk of granulosa cells and is surrounded by cumulus granulosa cells (2). The fluid-filled antrum (3) contains proteins, growth factors and steroids locally produced or filtered from the circulation. Granulosa cells (4) line the antral cavity in layers upon the basement membrane (5). The granulosa cells and antrum, within the basement membrane, are avascular and acquire nutrients from blood vessels (6) within the theca interna (7), which lies outside the basement membrane. Both the theca interna (7) and theca externa (8) contain blood vessels, nerves and connective tissues. Theca cells are derived from the cells of the interstitial tissue (9) of the ovary.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size from the time of initial growth to the mature preovulatory stage (Hirshfield 1991). The theca interna has a vascular supply that forms an anastomotic network terminating adjacent to the basement membrane, so that the granulosa layer does not have a direct blood supply and instead depends on the passage of nutrients through the basement membrane. Theca cells are the major source of androgens in the follicle and therefore play an important role in the regulation of steroid production (Gore-Langton and Armstrong 1988). The outermost layers of the follicle are composed of fibroblast and smooth muscle fusiform cells making up the theca externa. Among these cells are bundles of fibres composed of type I and type III collagen. Adrenergic and cholinergic innervation is present in both the theca externa and interna of most species (Lipner 1988). Both the granulosa and theca cell layers are functional syncytia due to the presence of gap junctions between cells that have been shown, for example, to spread FSH induced signals throughout the population of granulosa cells (Albertini et al 1975; Lawrence et al 1978).

1.1.2 Ovarian folliculogenesis

Formation of ovarian follicles begins during fetal development. Germ cells migrate to the genital ridge and colonize an area of mesoderm in the dorsal body wall where they interact with mesenchyme that will form the somatic components of the ovary (Hirshfield 1991). Both the germ cells and somatic cells proliferate and the somatic

cells become organized into cord-like structures which grow into epithelioid and interstitial compartments, separated by a basement membrane. Germ cells stop dividing and enter meiosis, becoming arrested at the diplotene stage of the first meiotic division. These cells remain arrested at this stage until the follicle is recruited, a period of quiescence that may last as long as forty years. Somatic cells continue to proliferate and envelop the germ cells to form primordial follicles. Each primordial follicle consists of an oocyte surrounded by a few flattened granulosa cells within a basement membrane. These follicles do not have theca layers or an independent blood supply. It is only during more advanced stages of follicular growth that theca cells begin to organize, forming multiple layers of flattened cells around the outside of the basement membrane, and the capillary network begins to form (Hirshfield 1991).

1.1.3 Ovarian follicular growth and development

Follicular development is commonly divided into discrete stages. Primordial follicles become primary follicles at the onset of growth, when the enlarged oocyte is surrounded by a single layer of cuboidal granulosa cells. Mitotic divisions create multiple layers of granulosa cells prior to the appearance of an antrum, which results in the formation of the secondary follicle. Theca cells surround the basement membrane of secondary follicles in some, but not all, species. The appearance of an antrum marks the conversion of the secondary follicle into a tertiary follicle. During

the last phase of growth, fluid-filled spaces begin to appear between granulosa cells and this fluid eventually coalesces into a single fluid-filled large antral cavity. Follicular fluid is derived from filtration of blood through the basement membrane, which acts as a molecular sieve excluding approximately 50% of 250,000 kDa molecules and all proteins greater than 850,000 kDa (Shalgi et al 1973). Antral fluid contains high concentrations of proteoglycans, principally chondroitin sulfate and heparan sulfate, which are diluted as the follicle enlarges and acquires more fluid, creating an antral fluid similar in protein content to serum (Lipner 1988). Mature follicles contain high concentrations of estrogen (1-2 μg/ml) in the follicular fluid due to the synthesis of this steroid by granulosa cells and the presence of steroid binding proteins in the antral fluid (Lipner 1988; Hirshfield 1991). The accumulation of follicular fluid marks a period of rapid growth as the follicle increases from 200-300 μm to 700 μm in size (rat) within 2 days (Hirshfield 1991). Near the end of this time, granulosa cells acquire receptors for LH and become competent, along with the theca cells, to respond to the ovulatory surge of LH. Following ovulation, the basement membrane breaks down completely, blood vessels invade the ruptured follicle and the granulosa and theca cells luteinize to form the corpus luteum (Lipner 1988). Luteinization involves extensive hypertrophy of the granulosa and theca cells, which develop increased cytoplasmic lipid droplets and produce large amounts of progesterone to prepare and sustain the uterine lining during pregnancy.

1.1.4 Gonadotropins and early follicle development

The role of gonadotropins in early follicle development is controversial (Greenwald and Terranova 1988; Hirshfield 1991). Hypophysectomy to remove the pituitary, the source of circulating gonadotropin, does not completely prevent the onset and early development of follicles, which suggests that gonadotropins are not critical for this process (Hirshfield 1991). Other evidence, however, suggests that gonadotropins are involved in the process of recruitment and early follicle development. Administration of eCG increases the number of primary and secondary follicles and variations in follicular growth initiation can be correlated to the estrous cycle of the rat (Greenwald and Terranova 1988). Hypophysectomy or suppression of gonadotropin by GnRH antagonists results in reduced numbers of small growing follicles (Hirshfield 1991). The number of primordial follicles is reduced as animals age and recruit them into development, but hypophysectomized or gonadotropin-suppressed animals retain significantly more primordial follicles as they age (Greenwald and Terranova 1988). Gonadotropin therefore appears to influence preantral follicular development but its role remains unclear. There may be important intraovarian effects of steroids or growth factors or of growth regulatory signals produced by the oocyte. These may act in response to gonadotropin or autonomously to initiate early follicle growth (Greenwald and Terranova 1988; Hirshfield 1991). Experimentally, the administration of estradiol to hypophysectomized rats is sufficient to produce large numbers of preantral follicles

containing multiple layers of granulosa cells (Billig et al 1993). The granulosa cells in preantral follicles are undifferentiated and mitotically active. Antrum formation begins when the follicle grows beyond 300 μm. The highest rates of mitosis are observed prior to antrum formation, in follicles between 200-300 μm in size (Greenwald and Terranova 1988). Mitosis continues after the formation of the antrum, but the rate steadily declines as the follicle matures.

1.1.5 Gonadotropins and follicular maturation

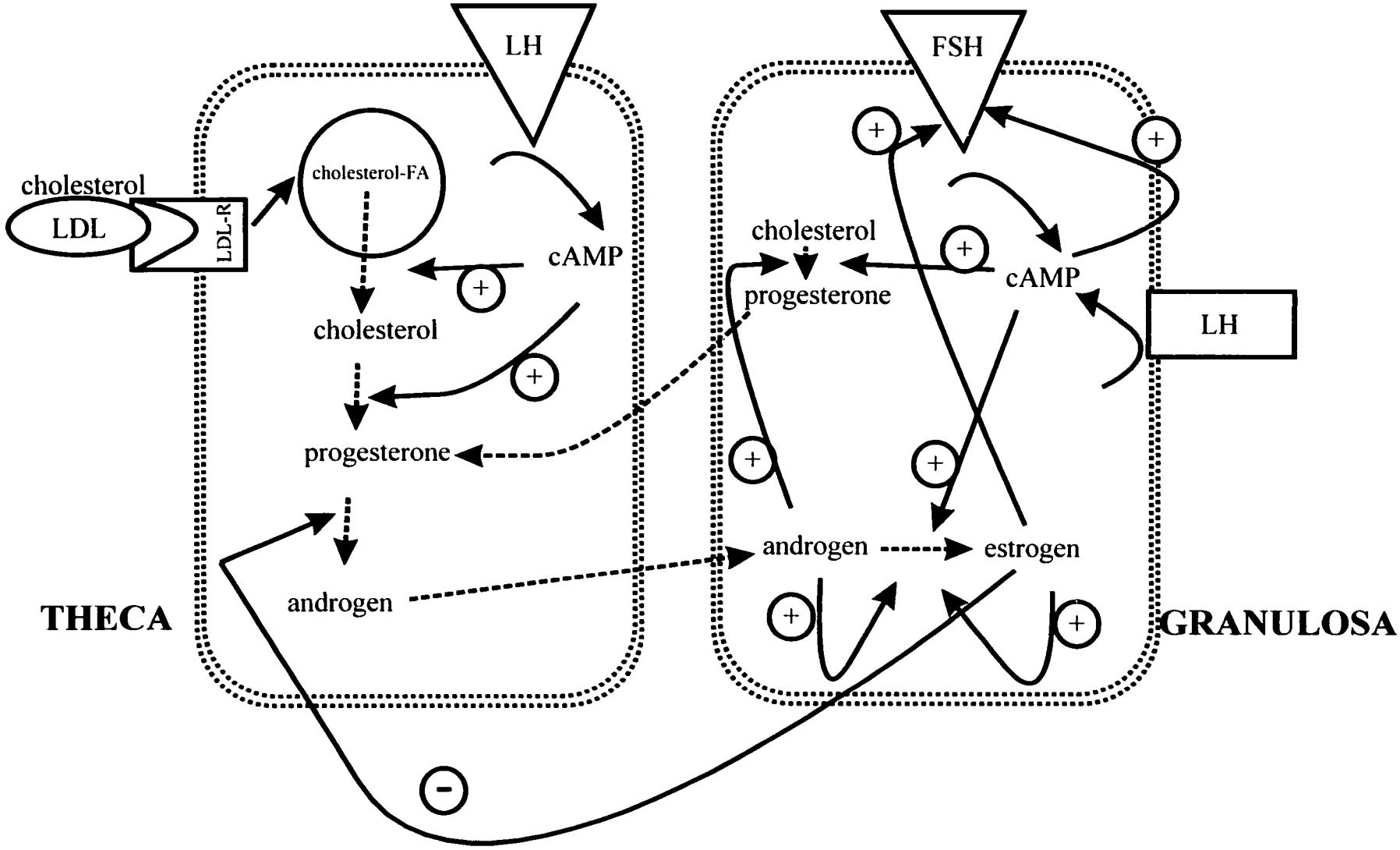
Although the role of gonadotropin in the regulation of early follicle development remains unresolved, it is clear that FSH is essential for the growth of follicles beyond the secondary stage of development. The granulosa cells in preantral follicles are essentially undifferentiated. They do not produce follicular fluid components, lack the ability to synthesize estradiol, and do not possess receptors for LH (Gore-Langton and Armstrong 1988; Lipner 1988). However, these cells do express the receptor for FSH and, after exposure to gonadotropin, acquire the ability to produce estradiol, follicular fluid and LH receptor (Hirshfield 1991). Granulosa cells are exposed to FSH during their development but only undergo FSH-induced differentiation once they have acquired the receptor and intracellular components necessary to respond to FSH. Thus, FSH is a permissive signal for granulosa cell maturation, while the granulosa cell determines the nature of its response. FSH initiates the final stages of follicular

development, designed to render the follicle capable of ovulation and luteinization in response to the LH surge. A variety of experimental conditions, however, will cause the granulosa to luteinize including: removal of the oocyte, pure FSH, cGMP, or placement in culture (Hirshfield 1991). These experimental inductions of luteinization underscore the fact that it is the programming in the granulosa cells, rather than the nature of the inducing signal, that directs their final maturation.

1.1.6 Gonadotropins, steroidogenesis and follicular development

The onset of antrum formation is indicative of granulosa cell differentiation. This is also reflected in the onset of steroidogenesis by these cells (fig. 2). Estrogen produced by the follicle exerts a negative feedback on FSH-release from the pituitary. In addition, estrogen has local effects in the follicle to induce FSH receptors (fig. 2). The combination of reduced circulating FSH and increased local induction of FSH receptors results in the selection of those follicles that produce sufficient estrogen (Hsueh et al 1984). FSH receptors are only found on granulosa cells, while LH receptors are found on granulosa cells of periovulatory follicles and on theca cells of all follicles (fig. 2). These two gonadotropins are the major regulatory hormones for the synthesis of steroids in the ovary. They both act in their respective target cells by the activation of adenylate cyclase and subsequent production of cAMP (Gore-Langton and Armstrong 1988). Estrogen is derived from androgens by the aromatization of

Figure 2: The "two-cell two-gonadotropin" regulation of steroidogenesis in the ovarian follicle. Cholesterol is predominantly acquired through the theca cell lipoprotein receptor (LDL-R), which binds and internalizes circulating cholesterol-lipoprotein complexes. Cholesterol, stored in lipid droplets, is esterified to fatty acids (FA). Binding of LH to its receptor stimulates cAMP production. This second messenger activates the de-esterification of cholesterol, its conversion into progesterone, and then androgen. Androgen leaves the theca and enters the granulosa cell, where it is converted to estrogen by the action of the enzyme aromatase. FSH binding to its receptor on the granulosa cell stimulates cAMP production, which activates the aromatase enzyme. Aromatase is also upregulated by estrogen and androgen. Granulosa cells can produce their own progesterone which can be converted to androgen in the theca. Both androgen and cAMP increase the production of progesterone by granulosa cells. Estrogen and cAMP both increase the sensitivity of granulosa cells to FSH by increasing the number of FSH receptors. Estrogen can feedback on the theca cell to decrease the production of androgens. In more mature follicles, granulosa cells have LH receptors which stimulate steroidogenesis by increasing cAMP production. The major pathway for the production of estrogen is through the conversion of cholesterol to androgen in the theca cell, and the subsequent aromatization of androgen in the granulosa cell. The conversion of cholesterol to progesterone is the rate-limiting step in androgen production, while the availability of androgen is the rate-limiting step in estrogen production.


LH
FSH
cholesterol
LDL
LDL-R
cholesterol-FA
cAMP
cholesterol
progesterone
androgen
cholesterol
progesterone
cAMP
LH
androgen
estrogen
THECA
GRANULOSA

testosterone by aromatase, or by the aromatization and dehydrogenation of androstenedione. These precursors are derived from progesterone or pregnenolone, which is produced by the action of P450 side-chain cleavage on cholesterol (Gore-Langton and Armstrong 1988). The major effect of FSH on granulosa cells early in follicular development is to induce aromatase activity, thus enabling the synthesis of estrogen by these cells. Later in follicular development, FSH also induces the activity of P450 side-chain cleavage enzyme in these cells to enhance the synthesis of progesterone. Upon stimulation by LH, the main steroidogenic function of theca cells is the synthesis of androgens which are the rate-limiting substrates for granulosa cell estrogen production (Gore-Langton and Armstrong 1988). The combined action of FSH on granulosa cells and LH on theca cells is therefore necessary, throughout most of the development of the follicle, for the production of estrogens. This interaction is known as the "two-cell, two-gonadotropin" theory for the control of estrogen production (fig. 2; Gore-Langton and Armstrong 1988). In addition to acting as a substrate for estrogen biosynthesis, androgens act on granulosa cells to enhance the activation of aromatase, as do estrogens, while progesterone inhibits aromatase. Both estrogen and androgen receptors are present in granulosa cells. Estrogens also have paracrine effects on theca cells to inhibit androgen production, thus acting as a potential negative feedback for estrogen production within the follicle (fig. 2). Healthy follicles have high estrogen to androgen ratios, but this is reversed in atretic follicles (Greenwald and Terranova 1988; Gore-Langton and Armstrong 1988).

1.1.7 Intraovarian regulators of follicular development

In addition to the combined effects of FSH and LH, and the interaction of estrogens and androgens, there are a variety of growth factors and cytokines that mediate follicle development and granulosa cell differentiation. Insulin-like growth factor (IGF-1) enhances gonadotropin-stimulated granulosa cell differentiation and is present at high levels in the ovary (Adashi et al 1985). Receptors for IGF-1 have also been identified in granulosa cells (Davoren et al 1986). FSH and estrogen stimulate IGF-1 production and the upregulation of IGF-1 receptor mRNA (Hsu and Hammond 1987). The actions of IGF-1 are inhibited by binding proteins (IGFBP's) that are also produced in the ovary, and FSH inhibits the release of these binding proteins (Adashi et al 1990). Gonadotropin releasing hormone (GnRH), which inhibits differentiation in follicles, stimulates IGFBP production and inhibits the FSH-induced suppression of IGFBP's (Erickson et al 1994). Growth hormone (GH) receptor has been identified in granulosa cells, and GH also increases the production of IGF-1 in the ovary (Davoren and Hsueh 1986).

FSH also induces the expression of epidermal growth factor (EGF) receptor in granulosa cells and this is augmented by GH (Hattori et al 1994; Fujinaga et al 1994). EGF acts to inhibit FSH-induced aromatase and estrogen synthesis, and suppresses the FSH induction of FSH receptor and androgen production by theca cells (Gore-Langton

and Armstrong 1988; Tilly et al 1992c). Similarly, basic fibroblast growth factor (bFGF) receptors are induced by FSH (Shikone et al 1992). bFGF suppresses aromatase and FSH receptor induction by FSH (Tilly et al 1992c), as well as androgen synthesis by theca cells (Hurwitz et al 1990).

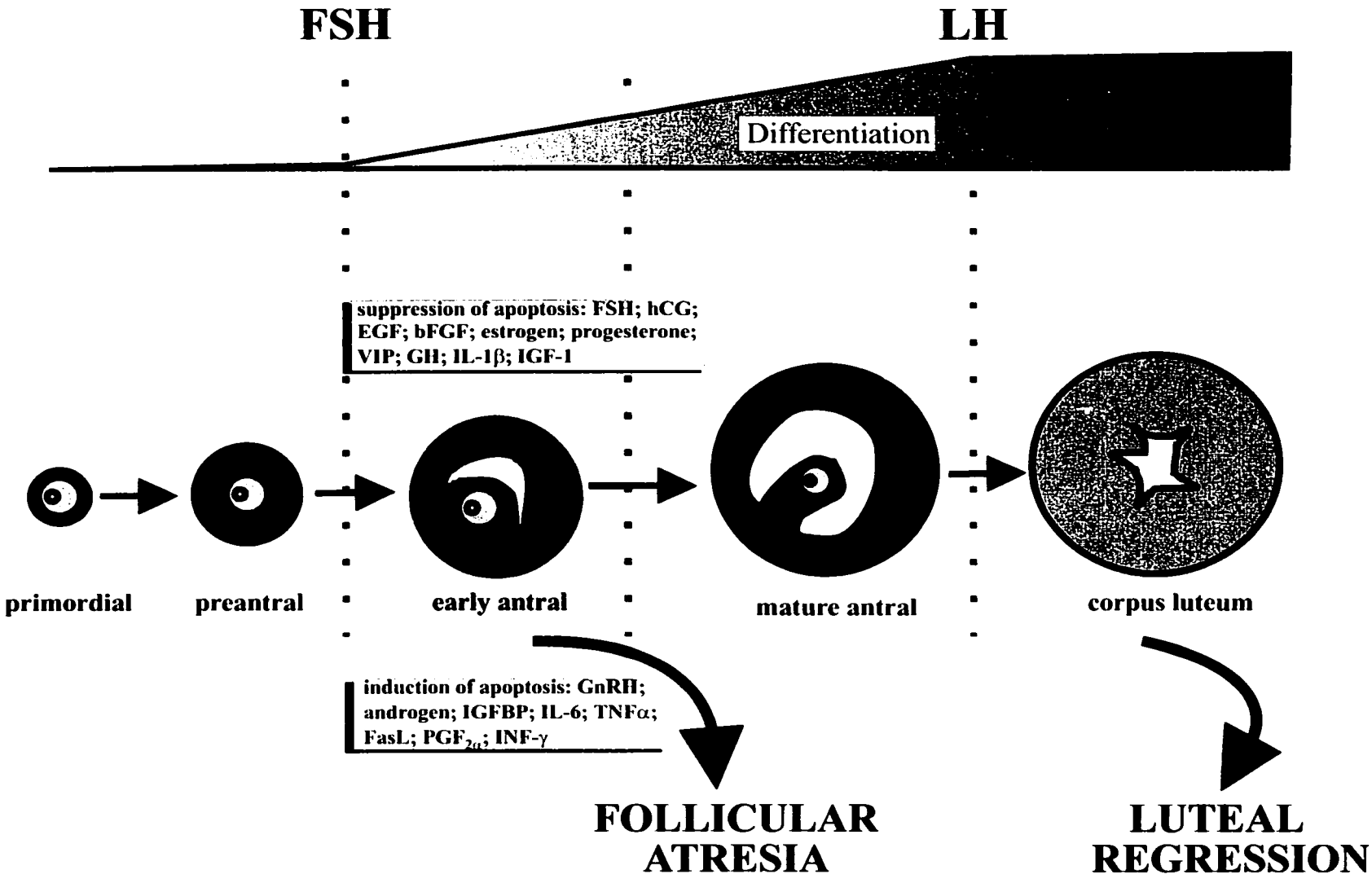
Cytokines and vasoactive intestinal peptide (VIP) are also important local regulators of ovarian cell differentiation. Interleukin-6 (IL-6) is produced by granulosa cells in response to FSH (Gorospe and Spangelo 1993a). Both IL-6 and interleukin-1β suppress granulosa cell differentiation and steroidogenesis (Gorospe et al 1992; Hurwitz et al 1991). VIP-containing nerve fibres are present in the ovary. VIP acts through receptors on granulosa cells to elevate cAMP and increase estrogen production, especially in granulosa cells from immature follicles (Ahmed et al 1986).

It is clear that gonadotropins are the principal hormones that regulate follicle development and granulosa cell differentiation, but the effects of these hormones are influenced by a variety of other local factors that are produced by, and mediate the effects of, gonadotropin stimulation. These intraovarian factors may be particularly important for the early development of follicles before the complete differentiation of granulosa cells renders them more resistant to apoptosis.

1.1.8 Follicular atresia

Granulosa cells that have responded to FSH and undertaken the process of differentiation become dependent on continued exposure to FSH. Preantral follicles less than 300 μm containing undifferentiated granulosa cells are rarely seen to undergo atresia (Hirshfield 1991; Hirshfield 1988; Hsueh et al 1994). However, the early penultimate stage of final follicular development (300-400 μm diameter follicles), when granulosa cells begin to differentiate and produce an antrum, is characterized by increased susceptibility to atresia (fig. 3; Hirshfield 1991; Hsueh et al 1994). FSH is present at low levels in immature rats but does not increase in a cyclic pattern, which results in the atresia of all follicles at the early antral stage. Why follicles are particularly susceptible to atresia at this stage of development is not known. However, it is clear that, in mature cycling animals, only those follicles that begin the transition from the preantral to the antral state during the cyclic elevation of FSH will survive the transition period and have the opportunity to reach the preovulatory state. FSH induces the differentiation and progression of follicles toward maturity and ovulation, but most follicles die by the process of atresia. The onset of atresia has been characterized by the appearance of pyknotic nuclei in granulosa cells of the follicle. The number of pyknotic nuclei indicative of atresia has been estimated to be 5% and can be as high as 20% in the initial stages of the process (Byskov 1978; Hirshfield 1991). Most of the atresia in adult ovaries occurs at the transitional stage between preantral follicle

Figure 3: Ovarian follicular development, atresia and luteal regression. Preantral follicles are essentially undifferentiated until exposed to FSH, after which they begin to form an antrum and produce significant amounts of steroids. The LH surge induces final maturation of the follicle, ovulation and luteinization. Follicular atresia is predominantly observed in early antral follicles. A variety of agents may be responsible for the suppression or induction of apoptosis at this penultimate stage. The corpus luteum, formed by luteinization of the ovulated follicle, undergoes apoptosis after exposure to $PGF_{2\alpha}$ and possibly other factors including INFγ and TNFα.


FSH
LH
Differentiation
suppression of apoptosis: FSH; hCG; EGF; bFGF; estrogen; progesterone; VIP; GH; IL-1β; IGF-1
primordial
preantral
early antral
mature antral
corpus luteum
induction of apoptosis: GnRH; androgen; IGFBP; IL-6; TNFα; FasL; $PGF_{2\alpha}$; INF-γ
FOLLICULAR ATRESIA
LUTEAL REGRESSION

growth and the onset of antrum formation, but the process can be initiated at any stage of follicular development. In preantral follicles, atresia appears to be initiated in the oocyte and spreads to the granulosa cells subsequent to oocyte death (Greenwald and Terranova 1988). In more mature follicles, it is the granulosa cells that degenerate first while the oocyte appears to resist the onset of death before eventually regressing. Theca cells do not die at the same rate as granulosa cells; instead they undergo hypertrophy and accumulate lipid droplets. The theca interstitial layer remains a source of androgen, which may contribute to the demise of granulosa cells in atretic follicles.

1.2 CORPUS LUTEUM

Follicles that successfully mature and ovulate in response to LH form the corpus luteum, a transient endocrine organ that secretes progesterone. The most significant action of progesterone is to prepare the uterus for pregnancy, but this steroid also acts on other reproductive tissues, including the oviduct, to ensure delivery of the conceptus to the uterus; the mammary gland, to induce lobuloalveolar development; and the hypothalamus and pituitary to influence release of gonadotropins (Niswender and Nett 1988). Some effects of progesterone, especially those on the uterus, require prior exposure to estrogen, which induces expression of the progesterone receptor. The corpus luteum begins to form following the preovulatory surge of LH, which induces morphological and biochemical changes in the theca interna and granulosa cells.

Granulosa cells hypertrophy and the basement membrane breaks down as blood vessels from the theca interna invade the granulosa layer in the ruptured follicle. The corpus luteum is comprised of at least two distinct steroidogenic cell types, large and small luteal cells, that may be derived from granulosa and theca cells respectively (Alila and Hansel 1984). Although both cell types are steroidogenic, the large cells secrete significantly more progesterone than small cells. LH stimulates progesterone secretion from both large and small cells, but the small cells are more responsive due to their lower basal secretion of steroid (Hansel et al 1991). Progesterone from the corpus luteum maintains pregnancy for various lengths of time in different species.

1.2.1 Luteal regression

At some point during gestation, signals from the pregnant uterus become necessary for the continued survival and progesterone production by the corpus luteum. If pregnancy does not occur, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released from the uterus induces regression of the corpus luteum in most species (Niswender and Nett 1988). The luteolytic factor in humans and monkeys is not of uterine origin, as hysterectomy does not prolong the lifespan of the CL in these species (Niswender and Nett 1988). $PGF_{2\alpha}$ acts on the CL to disrupt LH-stimulated cAMP production and cAMP-induced steroidogenesis (Thomas et al 1978). Although the luteolysin in the primate CL has not been identified, CL regression in this species is also associated with dissociation of LH from the

stimulation of progesterone. The cessation of LH-stimulated and basal progesterone production signifies functional CL regression. Structural regression of the CL occurs after functional regression and is characterized by cellular accumulation of lipid droplets, cytoplasmic shrinkage and phagocytosis by macrophages.

1.3 APOPTOSIS

1.3.1 Morphological features of apoptosis

Apoptosis is a physiological form of cell death with distinct morphological and biochemical characteristics (Wyllie et al 1980; Schwartzman and Cidlowski 1993). Early morphological signs of apoptosis include the shrinkage of the cell and the loss of microvilli as the cell acquires a smooth exterior and dissociates from its neighbouring cells. Cell shrinkage is accompanied by shrinkage of the nucleus and condensation of chromatin around the nuclear periphery. Progression of cell death leads to continued shrinkage of the cytoplasm and blebbing of the plasma membrane. These blebs may contain organelles which generally remain intact throughout the process. Nuclear blebbing also occurs and the nucleus breaks up into smaller bodies. Nuclear and cytoplasmic components are contained within membranes during cell death, and are called apoptotic bodies. These bodies may be phagocytosed by resident macrophages or neighbouring cells. The morphological features of apoptosis are distinct from

necrosis, wherein the cell swells, cytoplasmic contents escape, and there is general destruction and lysis of the organelles. Necrotic cell death is often accompanied by inflammation as the disrupted cell contents elicit a potent immune response. Conversely, apoptosis occurs without an inflammatory response, because the cell contents are never released from plasma membrane-enclosed vesicles. Necrosis often involves large tracts of cells, since each dying cell can cause damage in surrounding tissue as the inflammatory reaction propagates, but apoptosis is a solitary event and effects on neighbouring cells are restricted to phagocytosis of the apoptotic bodies (Wyllie et al 1980).

1.3.2 Biochemical features of apoptosis

1.3.2.1 DNA degradation

The morphological identification of apoptosis as a distinct form of cell death led to the search for biochemical events underlying these characteristic processes. One of the first biochemical events discovered associated with apoptosis was the degradation of genomic DNA into discrete fragments. Wyllie (1980) reported that thymocytes treated with glucocorticoids underwent apoptosis and degraded their DNA into 180-200 bp multiples. These oligonucleosome fragments could be visualized following agarose gel electrophoresis, as a ladder pattern of degraded DNA. This ladder pattern had

previously been described in studies of chromatin structure, wherein nucleases were used to cleave intact chromatin in isolated nuclei, to demonstrate the organization of DNA around nuclear histones (Hewish and Burgoyne 1973). It was found that Ca^{2+}/Mg^{2+}-dependent endonuclease activity was present in thymocyte nuclei, and that this endogenous endonuclease was responsible for the DNA laddering during apoptosis. Attempts to discover the endonuclease responsible for DNA degradation have led investigators to implicate known enzymes, such as deoxyribonuclease I (DNase I; Boone et al 1997a; Mannherz et al 1995), and deoxyribonuclease II (DNase II; Barry and Eastman 1993), as well as some novel proteins (Pandey et al 1997) in the process of apoptosis (Table 1).

Early reports by Wyllie and colleagues found that proteolysis did not occur during nuclear destruction and that exogenous micrococcal nuclease could reproduce the morphological features of apoptosis in isolated nuclei (Arends et al 1990). DNA degradation was found to precede cell death by hours, and agents that inhibited DNA cleavage, such as aurintricarboxylic acid (AUR) or Zn^{2+}, also inhibited cell death (Cohen and Duke 1984; McConkey et al 1989a; Sunderman 1995). Initially, the degradation of DNA into ladders was considered a universal "biochemical hallmark" of apoptosis, but examples of programmed cell death without apparent DNA ladders suggested that DNA degradation might be a dispensable consequence of apoptosis, rather than a precipitating event (Ucker et al 1992; Ormerod et al 1994). Whether this

Table 1: Endonucleases Implicated in Apoptosis

Endonuclease	Tissue/cell type	m.w. (kDa)	unique features	references
DNase I	ovarian follicle; corpus luteum; prostate; renal distal tubule and collecting ducts; stratified epithelium; gut enterocytes; embryonic lens cells; thymocytes; HL-60 cells; lymph node cells; fibroblasts.	31-36	inhibited by G-actin; depolymerises F-actin; optimal at neutral pH; Ca^{2+}/Mg^{2+}-dependent	Boone et al 1995; Peitsch et al 1993; Polzar et al 1994; Bacher et al 1993; Lipskaia 1994; Torriglia et al 1995.
Nuc18	thymocytes; germinal centre B lymphocytes	18-22	cyclophilin; Ca^{2+}/Mg^{2+}-dependent	Compton & Cidlowski 1987; Gaido & Cidlowski 1991; Montague et al 1994; Lindhout et al 1995.
DNase II	CHO cells; embryonic lens cells; HL-60 cells	27	optimal at acidic pH; Ca^{2+}/Mg^{2+} - independent	Barry & Eastman 1993; Aarruti et al 1995; Torriglia et al 1995; Perez-Sala et al 1995.
unidentified	spleen	27	Ca^{2+}/Mg^{2+}-dependent	Ribeiro & Carson 1993.
unidentified	renal proximal tubule	15		Ueda et al 1995.
unidentified	CTLL2 cell line	40, 58	Ca^{2+}/Mg^{2+}-dependent (58kDa); Mg^{2+}-dependent (40 kDa)	Deng & Podack 1995.
unidentified	hepatoma, pheochromocytoma, MCF7 breast and prostatic carcinoma cells	97		Pandey et al 1994.

is indeed the case remains to be determined, as it is now apparent that DNA is also degraded into large molecular weight fragments that are not detectable by conventional agarose gel electrophoresis, and this type of DNA cleavage may be occurring in those few incidents of cell death where ladder patterns are not found (Oberhammer et al 1993a & 1993b; Walker et al 1994).

1.3.2.2 The role of DNase I in apoptosis

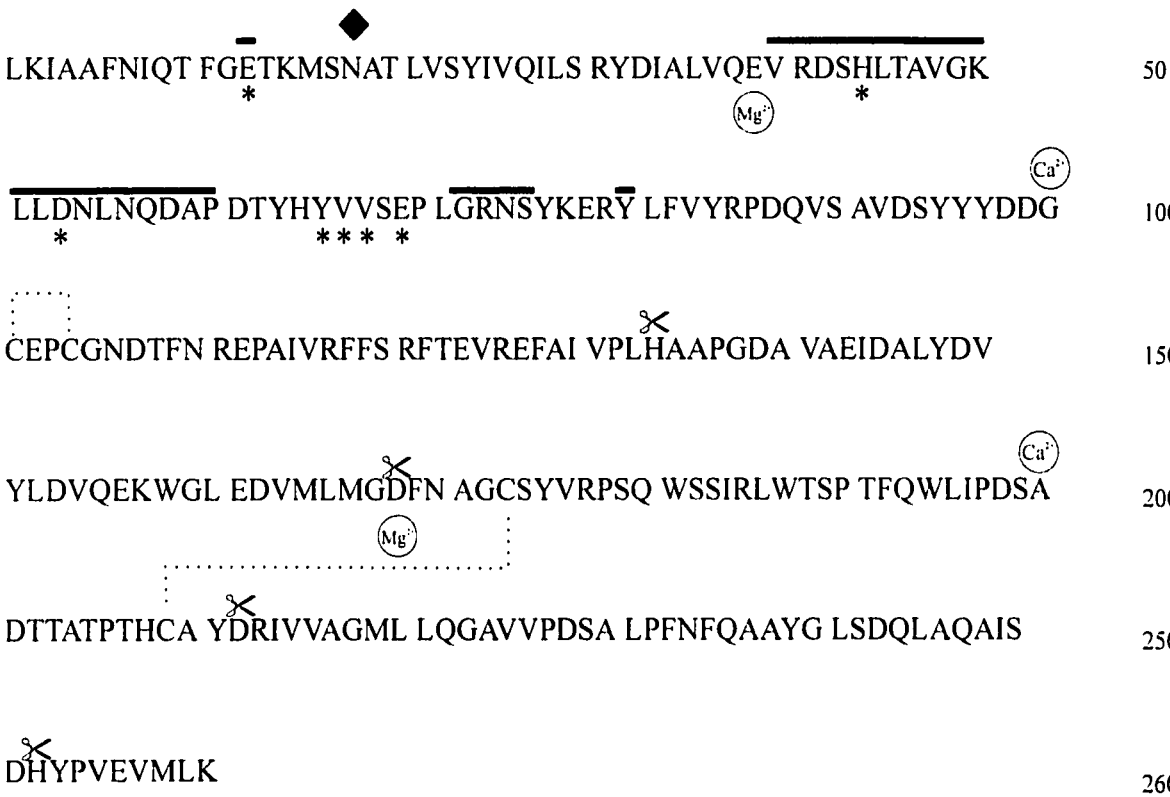
1.3.2.2.1 Distribution of DNase I

DNase I has been implicated as the enzyme responsible for apoptotic DNA degradation in several non-ovarian cell types (Peitsch et al 1993; Polzar et al 1993; Polzar et al 1994; Bacher et al 1993). Secretory forms of DNase I involved in digestion have been isolated from the pancreas (Kunitz 1948; Kreuder et al 1984) and parotid gland (Kreuder et al 1984), but DNase I has also been identified in cell types with no digestive function including the heart, lung, kidney, prostate, and thymus, which suggests that the enzyme has a role in cellular functions (Polzar et al 1994; Lacks 1981).

1.3.2.2.2 Structure and biochemistry of DNase I

All currently available information about the structure of DNase I (fig. 4) is based on studies of the bovine pancreatic enzyme and the human recombinant DNase I that has been used to reduce the viscosity of sputum in patients with cystic fibrosis (Shak et al 1990.) DNase I was first isolated from the bovine pancreas by Kunitz (1948). The enzyme has a pH optimum of 7.5 and requires divalent cations at millimolar concentrations for full activity. The enzyme degrades DNA in the combined presence of Ca^{2+} and Mg^{2+} or in the presence of Mn^{2+} alone. It is inhibited by Zn^{2+}, reducing agents, chelators or G-actin. DNase I displays no base or sequence specificity but preferentially cleaves at the 5' end of pyrimidines (Mannherz et al 1995). The protein is 260 residues in length but has isoforms based on variations in the number and length of attached carbohydrates at the asparagine residue N18. The three dimensional structure of DNase I, first determined by Suck (1982), showed that the enzyme has a core of six-stranded β-pleated sheets forming a sandwich type structure surrounded by eight α-helices and extensive loop regions. The extensive hydrophobic interactions along the β-pleated sheets give the enzyme thermal stability and the ability to regain full activity after treatment with chaotropic agents or detergents. There are two disulphide bridges in DNase I, between C101 and C104, and between C173 and C209. The disulphide bridge between C170-C206 is essential for DNase activity. Two tightly bound Ca^{2+} molecules, at A200 and G100, protect the enzyme from both proteolysis

Figure 4: Amino acid sequence of human DNase I. Residues involved in binding to actin are marked with an asterisk (the strong binding at Y65-V66-V67 and additional contacts at E13, H44, D53 and E69). Disulphide bridges (C101-C104 and C173-C209) are shown as dotted lines. Solid lines indicate the residues involved in DNA binding (E13, V39-P59, G72-S75 and Y79). Scissors indicate residues essential for DNA cleavage (H134, D168, D212, and H252) which are in close proximity to the scissile phosphate of the catalytic site. Cations bind at residues E39, G100, D212 and A200. A third Ca^{2+} also interacts with the catalytic site, but is not shown in this two dimensional representation. DNase I has an approximate molecular weight of 31 kDa based on its amino acid sequence, but isoforms due to the varied glycosylation at N18 (diamond) can produce a wide range of apparent molecular weights for the enzyme.


LKIAAFNIQT FGETKMSNAT LVSYIVQILS RYDIALVQEV RDSHLTAVGK
Mg2+
LLDNLNQDAP DTYHYVVSEP LGRNSYKERY LFVYRPDQVS AVDSYYYDDG
Ca2+
CEPCGNDTFN REPAIVRFFS RFTEVREFAI VPLHAAPGDA VAEIDALYDV
YLDVQEKWGL EDVMLMGDFN AGCSYVRPSQ WSSIRLWTSP TFQWLIPDSA
Ca2+
Mg2+
DTTATPTHCA YDRIVVAGML LQGAVVPDSA LPFNFQAAYG LSDQLAQAIS
DHYPVEVMLK

and from reduction of the essential disulphide bridge. Crystallization of bovine DNase I-oligonucleotide complexes demonstrated that six residues are in close proximity to the scissile phosphate of the catalytic site. Site-directed mutagenesis demonstrated that four of these residues (H134, D168, D212 and H252) were each essential to the catalytic activity of DNase I (Jones et al 1996). One of these residues, D168, was proposed as a ligand for Mg^{2+} , distinct from the Mg^{2+}-binding residue at E39. A third Ca^{2+} ion binds near the catalytic site of the enzyme and is likely involved in DNA hydrolysis. The loop from residues 70-74 and the helix from V48 to P60 bind tightly to the minor groove of DNA. Binding to DNA also involves strong Van Der Waals forces from residues E13 and Y79 (Weston and Suck 1993).

1.3.2.2.3 DNase I interaction with actin

Actin binds and inhibits the activity of DNase I. The main interaction between actin and DNase I occurs at residues Y65-V66-V67 of DNase I and minor contacts occur at residues E13, H44, E69 and D53 (Kabsch et al 1990; Ulmer et al 1996). DNase I binding to DNA involves E13 and interaction with actin at this residue sterically hinders the DNase I-DNA interaction. The residues on actin involved in binding to DNase I (fig. 5) are G42, V43 and M44 for the strong interaction, as well as T203 and E205 for the minor contacts (Kabsch et al 1990; Mannherz 1992).

Figure 5: Amino acid sequence of actin. (*) residues involved in binding to DNase I. Strong interaction with DNase I occurs at G41-V42-M43 and additional binding is provided by residues T202 and E204. Potential cleavage sites for caspase are indicated by arrowheads. Intact actin has an approximate molecular weight of 42 kDa, while the cleavage sites shown will produce products of 41 kDa and 30 kDa.

▼ * * *

MDDDIAALVV DNGSAMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK 50

DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEKIWHHTFY NELRVAPEEH 100

PVLLTEAPLN PKANREKMTQ IMFETFNTPA MYVAIQAVLS LYASGRTTGI 150

▼

VMDSGDGVTH TVPIYEGYAL PHAILRLDLA GRDLTDYLMK ILTERGYSFT 200

* *

TTAEREIVRD IKEKLCYVAL DFEQEMATAA SSSSLEKSYE LPDGQVITIG 250

NERFRCPEALF QPSFLGMESCG IHETTFNSIM KCDVDIRKDL YANTVLSGGT 300

TMYPGIADRMQ KEITALAPST MKIKIIAPPE RKYSVWIGGS ILASLSTFQQ 350

MWISKQEYDE SGPSIVHRKC F 371

1.3.2.3 The role of caspases in apoptosis

Initial studies suggested that proteolysis was not involved in apoptosis and did not accompany the morphological features of nuclear degradation (Arends et al 1990). Genetic studies in the nematode *C. elegans*, identified three genes as essential regulators of cell death. Two of these genes, ced-3 and ced-4, induced cell death and the other, ced-9, suppressed cell death (Hengartner et al 1992; Yuan and Horvitz 1990). Cloning of ced-3 demonstrated that it was homologous to interleukin 1β-converting enzyme (ICE), a mammalian cysteine protease (Yuan et al 1993). ICE was known to convert proIL1β into the active IL1β by cleaving the inactive precursor at two Asp residues (Cerratti et al 1992). Overexpression of ICE caused apoptosis in fibroblasts and HELA cells, and a cow pox virus cytokine response modifier (CrmA), that inhibited ICE *in vitro*, also inhibited apoptosis when expressed in a variety of cells (Miura et al 1993; Martin and Green 1995). These studies demonstrated a role for proteases in apoptosis but the importance of ICE has been negated by findings of normal apoptosis after transgenic deletion of the ICE gene (Li et al 1995). Thymocytes from ICE knockout mice died by apoptosis when exposed to glucocorticoids or radiation, which demonstrated that ICE was not required for the process of apoptosis (Kuida et al 1995). A variety of ICE-related proteases (caspases) have been identified and grouped into a family of proteins called "caspases" (Table 2; Alnemri et al 1996). These enzymes are all aspartate specific cysteine proteases, have some homology to

Table 2: Caspase names for the ICE/Ced-3 protease family

New Name	Original Names
caspase-1	ICE
caspase-2	ICH-1
caspase-3	CPP32, apopain, Yama
caspase-4	TX, ICH-2, ICE_{rel}-II
caspase-5	ICE_{rel}-III, TY
caspase-6	Mch-2
caspase-7	Mch3, ICE-LAP3, CMH-1
caspase-8	FLICE, MACH, Mch5
caspase-9	ICE-LAP6, Mch-6
caspase-10	Mch4

ced-3, and exist as inactive precursors which are activated by cleavage of aspartate residues within the enzyme. It is believed that caspase-3 is the most important mediator of apoptosis in mammalian cells because of its wide distribution and its ability to cleave aspartate residues in inactive forms of other caspases such as caspase-6 or caspase-7 (Alnemri 1997). A limited number of proteins are known to be cleaved by caspases during apoptosis including poly(ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), U1-70 kDa small nuclear riboprotein (U1-70 sNRP), and lamins A and B (Rosen and Casciola-Rosen 1997). All of these substrates are structural or catalytic nuclear proteins. Cleavage products of these proteins are found within apoptotic bodies, suggesting that they are degraded within the nucleus (Rosen and Casciola-Rosen 1997). PARP and DNA-PK are necessary for the repair of double-strand DNA breaks, while U1-70 sNRP is essential for mRNA splicing. Cleavage of these proteins would therefore disrupt normal homeostasis in the cell. The cleavage sites for caspase-3 on all these substrates have the motif -DXXD- (Rosen and Casciola-Rosen 1997). Cleavage at this motif separates important functional domains of the substrates from the rest of the protein, so that caspase-3 action inactivates PARP, DNA-PK and U1-sNRP. The other identified substrate of caspases in apoptosis, lamins, are important structural components of the nuclear membrane, and their degradation by caspase-6 might be responsible for nuclear disintegration into small apoptotic bodies. Other proteins cleaved during apoptosis by putative caspase activity include the cytoskeletal proteins actin (Kayalar et al 1996), fodrin (which

forms the membrane cytoskeleton; Kaufmann 1989), and Gas2 (involved in regulation of microfilament dynamics; Brancolini et al 1995).The signalling mechanisms that activate caspases during apoptosis have not been identified, although there is evidence that these enzymes are controlled by a cascade of proteases (Liu et al 1996a). All caspases are known substrates for other caspases, but the order in which they might be activated during cell death has not been established (Rosen and Casciola-Rosen 1997; Yuan 1997).

Caspase-3 cleavage can serve to activate some substrates, such as the sterol regulatory element binding protein (SREBP; Wang et al 1996). Cleavage of membrane bound SREBP releases the N-terminal portion of the protein, which then enters the nucleus and acts as a transcription factor to activate production of LDL-receptor and cholesterol-synthesizing enzymes (Brown and Goldstein 1997). Thus, cleavage of SREBP results in both increased cholesterol uptake and synthesis, leading to increased cellular levels of cholesterol esters. Cellular sterol levels regulate the activity of the proteases that cleave SREBP, but this protein can also be cleaved by caspase-3 during apoptosis induced by a variety of conditions (Wang et al 1996). Whether there is a function of SREBP cleavage, and thus cholesterol accumulation, in apoptosis is not known. It remains to be determined if the SREBP transcription factor released by caspase-3 might activate other genes involved in cell death.

1.3.2.4 The role of oncogenes in apoptosis

The death repressor gene in *C.elegans*, ced-9, was found to be homologous to the mammalian oncogene bcl-2 (B-cell lymphoma/leukemia-2 gene) which codes for a 25-26 kDa protein (Hengartner et al 1992). This gene was first identified in malignancies of B-cells; wherein a chromosomal translocation juxtaposes the gene with enhancer elements for an immunoglobulin heavy chain locus (IgH), resulting in overexpression of bcl-2 mRNA and protein (Pegararo et al 1984). Unlike other oncogenes, bcl-2 does not induce proliferation, but instead prevents cell death (Reed 1994). Vaux et al (1988) demonstrated the ability of bcl-2 to prolong cell survival in hemopoetic cells. Korsmeyer and colleagues subsequently showed this was due to the ability of bcl-2 to prevent apoptosis (Hockenbery et al 1990). The mechanism of bcl-2 action remains unresolved. The 25-26 kDa protein includes a hydrophobic stretch of amino acids near the carboxy terminus which presumably anchors the protein in membranes. This membrane localization is essential for the function of bcl-2. Early work suggested that bcl-2 was a mitochondrial membrane protein, but localization in the nuclear envelope and endoplasmic reticulum has since been demonstrated (Krajewski et al 1993). The mechanism of bcl-2 prevention of cell death has not been determined, but the oncogene has been demonstrated to block a wide variety of death-inducing signals. Apoptosis induction by chemotherapeutic drugs, ionizing radiation, hydrogen peroxide, growth factor deprivation, calcium, tumour necrosis factor, and Fas in a

variety of cell types are all blocked by bcl-2 (Reed 1994; Oltvai and Korsmeyer 1994). In addition, bcl-2 blocks very early morphological and biochemical events of apoptosis. These observations suggest that bcl-2 blocks a final common pathway to apoptosis in cells, however, the biochemical or mechanistic details of this block remain unclear. The bcl-2 protein can scavenge free radicals and lipid peroxides which have been implicated in some forms of apoptosis (Hockenbery et al 1993). Additionally, bcl-2 appears to play some role in the regulation of intracellular Ca^{2+} stores, as it prevents the accumulation of mitochondrial Ca^{2+} in cytokine deprivation-induced apoptosis of cells (Baffy et al 1993). A family of bcl-2 related proteins has been identified based on the presence of bcl-2 homology-1 (BH-1) and BH-2 domains (Oltvai and Korsmeyer 1994). These domains are involved in the dimerization of bcl-2 related proteins. The family includes the death repressor proteins bcl-x_{long} and bcl-2 and the death inducers bax and bad. The relative levels of these proteins and their homo- and heterodimerization play a role in deciding the fate of a cell. Korsmeyer has suggested that these proteins interact to determine the fate of the cell, and the relative levels of death suppressor vs. death inducer genes is like a switch or "rheostat" that can be adjusted by signals or conditions which act on the cell (Oltvai and Korsmeyer 1994). Excess bax and bad would induce, whereas excess bcl-2 or bcl-x_{long} would suppress, apoptosis. Recently the structure of bcl-2 proteins has been related to diphtheria toxin channel and there is new evidence that bcl-2 and related proteins act as ion channels in lipid membranes (Minn et al 1997). Whether the state of hetero- or

homodimerization of these bcl-2 proteins can create channels with distinct properties has not yet been determined.

1.3.2.5 Activation of proteolysis

A variety of stimuli can induce apoptosis but there are relatively few factors that act primarily as inducers of cell death. These include the cytokines tumour necrosis factor (TNFα) and Fas ligand (FasL). These cytokines are synthesized as type II membrane proteins but can be cleaved, and released in soluble form, by a membrane-associated metalloproteinase. TNFα and FasL act through specific type I membrane receptors (TNFR and Fas respectively) to induce apoptosis in their target cells. These receptors are part of a superfamily that includes receptors for NGF, lymphotoxin-β, CD40, CD27, and the human DR3/Wsl-1. All members of the family have extracellular cysteine-rich repeats. Certain members also share specific homology within their intracellular domains. Receptors within this family that are capable of inducing apoptosis all contain a homologous domain (80 amino acids) that transduces the death signal (Yuan 1997). This death domain functions when the extracellular region of the receptor is trimerized, but not when dimerized, indicating that the trimer of death domains is necessary to transduce the signal from the receptor. Death domains recruit cytoplasmic proteins that also contain death domains. Fas-associating protein with death domain (FADD/MORT1) was first identified as a 23 kDa protein that associated

with the death domain of activated Fas (Chinnaiyan et al 1995). FADD/MORT1 also transduces death signals of the TNFR (Chinnaiyan et al 1996a). Other transducing proteins such as TRADD (TNFR associated death domain protein) have also been identified (Yuan 1997). FADD/MORT1 has a death domain at the carboxy terminus and a death effector domain (DED) at the N-terminus. The DED functions to recruit the ICE-related protease caspase-8 (Yuan 1997). In the case of Fas, binding of the FasL trimer causes trimerization of the receptor and cytosolic recruitment of FADD, which then recruits caspase-8 to initiate a protease cascade within the cell, cleaving and activating caspases. Ligand binding to the TNFR also leads to the activation of caspase-8 but there can also be concurrent recruitment by the TNFR of signals leading to activation of the transcription factor NF-κB, which suppresses TNFα-induced cell death (Hsu et al 1995; Beg and Baltimore 1996; Van Antwerp et al 1996).

1.3.2.6 Regulation of proteolysis by oncogenes

The interaction of bcl-2 related proteins and caspases adds further complexity to the regulation of apoptosis. Activation of caspase-3 in response to the chemotherapeutic agent etoposide, the second messenger ceramide, or cytokines such as Fas and TNFα is inhibited by bcl-2 (Chinnaiyan et al 1996b). Proteolysis is thought to be an irreversible commitment to apoptosis, but Thompson and colleagues have recently demonstrated that bcl-x_{long} can prevent apoptosis even in cells that have previously

undergone Fas-induced protease activation (Boise and Thompson 1997). This suggests that protease activation may not be a final commitment to death, and that bcl-2 related proteins can inhibit several levels of the protease cascade leading to cell death.

1.4 APOPTOSIS IN THE OVARY

1.4.1 Occurrence of apoptosis in the ovary

Apoptosis was first observed in atretic follicles of the rabbit ovary more than 100 years ago by Flemming, who referred to the process as "chromatolysis" (Tilly 1996). Hay et al (1976) and O'Shea et al (1978) described the death of ovarian cells in atretic sheep follicles as apoptosis, based on ultrastructural evidence obtained by electron microscopy. Zeleznik et al (1989) demonstrated that Ca^{2+}/Mg^{2+}-dependent endonuclease activity was present in rat ovarian nuclei. The role of apoptosis in the ovary received scant attention until two reports in 1991 described the occurrence of internucleosomal DNA fragmentation during atresia in rat, porcine and hen ovaries (Hughes and Gorospe 1991; Tilly et al 1991). Apoptosis has since been implicated as the cellular mechanism of follicular atresia and luteal regression in every species where these processes occur (Hsueh et al 1994; Tilly 1996). Morphological and biochemical indices of apoptosis demonstrated that this form of cell death was primarily found in the granulosa, but not theca cells of atretic follicles, although theca

cell apoptosis was reported in grossly atretic follicles of sheep (O'Shea et al 1978). More recently it has become evident that the death of oocytes in prenatal and perinatal ovaries also occurs by apoptosis (Coucouvanis et al 1993; Pesce and DeFelici 1994). Apoptosis was also found to be the cellular mechanism of luteal cell death during regression of the corpus luteum in cows (Juengal et al 1993) and other species (Tilly 1996).

1.4.2 Regulation of apoptosis in the ovary

1.4.2.1 Cell death suppressors

1.4.2.1.1 Estrogen, androgen and GnRH

The demonstration that apoptosis occurred in granulosa cells of atretic follicles resulted in renewed and vigourous research into the cellular and molecular mechanisms of atresia. Hsueh and colleagues found that the controversial atretogenic actions of androgens and GnRH could be proven by the study of apoptosis in hypophysectomized rats treated with diethylstilbestrol (DES) implants (Billig et al 1993; Billig et al 1994). DES, a synthetic estrogen, stimulates preantral follicle growth and granulosa cell division in immature rat ovaries. Hsueh found that withdrawal of DES caused apoptosis in DES-treated rat ovaries *in vivo* and this was prevented by

estrogen and enhanced by androgen (Billig et al 1993). Similarly, DES withdrawal-induced apoptosis was prevented by FSH and enhanced by GnRH treatment (Billig et al 1994). Ovaries from rats subjected to DES withdrawal contained early antral follicles undergoing atresia, indicating that the progression from preantral to early antral stages of follicular development can occur in the absence of FSH stimulation, but that this growth cannot continue without exposure to FSH. The factors capable of stimulating the progression of follicles to the early antral state have not been identified but several factors (i.e. EGF, VIP) or cytokines (i.e. IL1-β, IL-6) are capable of inducing some degree of granulosa cell growth and differentiation. Whatever the case, in the absence of FSH it is clear that early antral follicles undergo atresia following DES withdrawal because the granulosa cells die by apoptosis without gonadotropic support.

1.4.2.1.2 EGF/TGFα and bFGF

Hsueh and colleagues reported that mature antral rat follicles cultured in the absence of serum undergo apoptosis which can be prevented by EGF and bFGF through a tyrosine kinase-dependent mechanism (Tilly et al 1992a). Peluso subsequently found that EGF induced progesterone production by granulosa cells and that part of the apoptosis-suppressing effects of EGF were due to progesterone suppression of intranuclear Ca^{2+} accumulation (Luciano et al 1994). Apoptosis in hen granulosa cells,

cultured in serum free media, was also suppressed by TGFα (Johnson et al 1996; Manchanda et al 1996) and this suppression was found to be partly mediated by TGFα-induced prostaglandin production (Manchanda et al 1996).

1.4.2.1.3 N-Cadherins

Further studies by Peluso and colleagues found that cell-cell contact between granulosa cells enhanced their survival *in vitro*. This led to the finding that homologous adhesion between N-cadherins was also a survival signal for granulosa cells, independent of progesterone production (Peluso et al 1996). A possible mechanism for N-cadherin suppression of apoptosis has recently been suggested by work showing that both bFGF and N-cadherin suppress apoptosis in granulosa cells by causing tyrosine phosphorylation of the FGF receptor (Trolice et al 1997). Phosphorylation of the FGF receptor is part of the activating mechanism leading to the survival promoting effects of this growth factor.

1.4.2.1.4 Vasoactive Intestinal Peptide

Denervation of the ovary leads to disruption of steroidogenesis, ovulation and follicular development, although the precise role of innervation is not known (Lipner 1988). Nerve fibres containing VIP have been identified in developing follicles of

several species and VIP receptors are present on granulosa cells (Ahmed et al 1986). VIP stimulates cAMP production, steroidogenesis and plasminogen activator activity in ovarian cells (Ojeda et al 1990). A potential role for innervation in the survival of follicles was suggested by the fact that VIP suppresses apoptosis in isolated rat and hen follicles (Flaws et al 1995a). This effect of VIP was mimicked by cAMP and inhibited by IGFBP-3, suggesting that VIP suppression of apoptosis is a consequence of IGF-1 secretion mediated by cAMP.

1.4.2.1.5 FSH and intraovarian regulators

FSH prevents apoptosis in cultured follicles and is especially important in survival of early antral follicles *in vitro* (Chun et al 1996) but other factors (fig. 3; Table 3), independently or in response to FSH stimulation, also have suppressive effects on apoptosis in mature antral follicles. These include IGF-1, Il-1β, activin, VIP, GH and LH/hCG (Chun et al 1995, Chun et al 1996; Hsueh et al 1994; Kaipia and Hsueh 1997, Eisenhauer et al 1995). Only FSH completely suppresses the onset of apoptosis in early antral follicles. GH and LH have no suppressive effects on small follicles and IGF-1, activin, VIP, EGF and bFGF can partially but not completely prevent apoptosis in follicles of this size (Chun et al 1996; Kaipia and Hsueh 1997). FSH may act through the stimulation of cAMP to activate PKA or induce transcription of genes controlled by cAMP response elements (CRE). Whether the suppression of granulosa

Table 3: Activators and suppressors of ovarian DNA degradation

Activating	Suppressing
GnRH	FSH
androgen	estrogen
IGFBP	IGF-I
IL-6	bFGF
TNFα	EGF
FasL	VIP
$PGF_{2\alpha}$	progesterone
	GH
	hCG
	IL-1β
	N-cadherin

cell apoptosis by FSH is directly mediated by FSH-induced signals or indirectly by the stimulation of local mediators is not yet known and may depend in part on the stage of follicular development. Initial studies found that FSH did not prevent apoptosis in isolated granulosa cells (Tilly et al 1992a), but subsequent examination revealed that FSH was an important survival signal (Chun et al 1996; Chun et al 1994). FSH prevented apoptosis in whole cultured follicles and this was abrogated by IGFBP-3, which demonstrated that some component of the survival signal provided by FSH was mediated by IGF-1 (Chun et al 1994). Granulosa cells are the primary source of IGF-1 in the rat ovary (Adashi et al 1985). Apoptosis in isolated granulosa cells could not be abrogated by IGF-1, but IGF-1 was effective in preventing cell death in whole isolated follicles (Hsueh et al 1994). These findings suggest that theca cells are important in the action of IGF-1 to prevent apoptosis, and that granulosa-theca cell interaction is essential for follicle survival in a manner similar to the "two cell-two gonadotropin" interaction necessary for steroidogenesis. IGF-1 from the granulosa may bind receptors on the theca to stimulate the release of EGF/TGFα that binds to granulosa cell receptors to promote cell survival.

1.4.2.2 Cell signals of death suppressors

The various agents that can suppress apoptosis in follicles act through different signalling pathways. Gonadotropins and VIP stimulate cAMP production and cAMP

suppresses apoptosis. IGF-1, bFGF, N-cadherin and EGF/TGFα act through receptors with tyrosine kinase activity that recruit signalling proteins to the activated receptor, leading to cellular effectors such as MAP kinase. The signalling system employed by GH is not entirely known but also appears to operate by recruiting signalling kinases to the activated receptor. Finally, the signalling system of the IL1β receptor has not been elucidated, but leads to the production of nitric oxide and generation of cGMP (Chun et al 1995). These varied signals are all capable of inhibiting apoptosis and may converge on a final effector pathway to have this common effect. The control of granulosa cell survival may not be as complex as this however, since many of these death-suppressing hormones can elicit the local production of IGF-1 or EGF. FSH, LH, VIP, and GH all stimulate the local synthesis of IGF-1. In addition, LH, FSH, bFGF and N-cadherin stimulate the production of EGF/TGFα or its receptor. Finally, IGF-1 stimulates EGF/TGFα production locally in the ovary. Thus, the complex signals may converge at the level of production of one or two local mediators or their receptors. This model does not account for the observation by Chun et al (1996) that the suppressive effects of FSH in early antral follicles cannot be mimicked by IGF-1 or EGF/TGFα alone. However, there are no studies of the combined effects of IGF-1 and EGF/TGFα. FSH may have additional effects not mediated by release of local mediators, including the cAMP-induced stimulation of receptors for those mediators, or the induction of genes for different components of the signal transduction cascade for EGF/TGFα or IGF-1. FSH may therefore, act to stimulate EGF/TGFα and IGF-1

release while simultaneously priming the cell to be more responsive to these factors. If the effects of gonadotropin are mediated by these factors, it remains to be determined what signals elicited by EGF/TGFα and IGF-1 are responsible for inhibition of apoptosis in granulosa cells.

1.4.2.3 Direct effects of FSH

1.4.2.3.1 FSH and bcl-2

FSH may have direct anti-apoptotic effects on granulosa cells via the regulation of cell death genes known to regulate apoptosis. Treatment of immature rats with gonadotropin maintained the expression of ovarian bcl-2 and bcl-x_{long} mRNA and decreased expression of the bax gene (Tilly et al 1995a), consistent with the rheostatic regulation of these genes towards cell survival (Oltvai and Korsmeyer 1994). Mice with homologous deletions for bcl-2 and bax have been studied to determine the role of these genes in the ovary. Although ovaries of bcl-2 knockout mice displayed significantly fewer primordial follicles and oocytes than wild type mice, there was no apparent effect of bcl-2 ablation on the apoptosis observed in granulosa cells of the adult ovary (Ratts et al 1995). This suggests that bcl-2 has a role in the suppression of cell death during fetal development of the ovary when the normal complement of primordial follicles and oocytes is established. The fact that there is a reduction, but

not a complete loss, of oocytes and primordial follicles in bcl-2 knockouts suggests that this gene is not essential for survival of these cell types. Other genes may be able to compensate for the loss of bcl-2 in this model. The knockout also demonstrates that the bcl-2 gene is not required for survival of granulosa cells in the adult ovary, which suggests that other genes such as bcl-x_{long} may be sufficient for this purpose. Only bcl-x_{long} mRNA is detectable in granulosa cells of the hen ovary, and the levels of bcl-x_{long} correlate to the susceptibility of hen granulosa cells to undergo apoptosis (Johnson et al 1996). Granulosa cells from immature hen follicles die rapidly in culture and express very little bcl-x_{long} transcript, whereas cells from mature follicles are resistant to apoptosis and express high levels of the mRNA for this death suppressor gene. This suggests that granulosa cell survival in the hen is mediated by bcl-x_{long} rather than bcl-2. Targeted overexpression of bcl-2 has demonstrated that this death suppressor functions in the mammalian ovary. Mice overexpressing bcl-2 in their ovaries demonstrated reduced occurrence of apoptotic DNA fragmentation following withdrawal of gonadotropin *in vivo* (Hsu et al 1996). These bcl-2 overexpressing mice had higher rates of ovulation than wild type mice, and produced larger litter sizes when mated. The incidence of benign ovarian tumours was higher in the bcl-2 overexpression mice than in wild type mice. These results demonstrated that the loss of follicles by signals to induce atresia was abrogated by bcl-2, as was the normal loss of follicles to atresia during the induction of follicular development and ovulation (Hsu et al 1996). Although bcl-2 knockouts showed that this oncogene is not essential

to prevent cell death, the targeted overexpression of the gene demonstrates that bcl-2 does function to prevent apoptosis in ovarian follicles.

1.4.2.3.2 FSH and bax

Gonadotropins decrease the level of bax mRNA in the ovary (Tilly et al 1995a). Mice deficient in the gene for bax have demonstrated a role for this protein in ovarian cell death. Follicles in bax knockout mice become grossly misshapen and contain degenerating oocytes similar to those found in the later stages of follicular atresia (Knudson et al 1995), but granulosa cells in these follicles appear to be resistant to apoptosis. This supports the role of bax as a death inducing gene in granulosa cells of the ovary. The exact role of bax, bcl-2, bcl-x_{long} and other bcl-2 related proteins in ovarian cell function and apoptosis awaits the elucidation of their biochemical mechanism of action.

1.4.2.3.3 FSH and caspases

FSH may also directly suppress apoptosis through the regulation of caspases. FSH-induced survival of follicles was associated with decreased expression of the mRNA for caspase-3 and caspase-2, while expression of these caspases was elevated in follicles undergoing apoptosis in serum free culture (Flaws et al 1995b). The gene for

caspase-1 was expressed at very low levels in the ovary, and levels of this gene did not change in response to apoptotic or survival signals, consistent with recent evidence that caspase-1 is not a principal component of the cell death machinery (Li et al 1995). There are a limited number of proteolytic substrates for the caspases including sNRP, PARP, DNA-PK and actin, but to date there are no published studies demonstrating degradation of these proteins during follicular atresia or luteal regression. These proteases appear to be active at some level in all known forms of apoptosis, so the lack of data concerning proteolytic degradation in the ovary likely reflects the fact that these studies have not yet been done.

1.4.2.3.4 FSH and p53

FSH may directly suppress apoptosis through regulation of the p53 gene. Gonadotropin injection to immature rats reduces the level of p53 mRNA expression in granulosa cells, while induction of apoptosis in cultured follicles was associated with elevated p53 mRNA (Tilly et al 1995b). The transcription factor p53 has been known for some time as an anti-oncogenic protein that regulates the DNA synthesis of the cell cycle, and mutations in the p53 gene are commonly found in cancerous cells that have unregulated proliferation (Donehower and Bradley 1993). Normally, p53 is activated by DNA damage and prevents entry into the cell cycle, allowing time for repair before progression with DNA synthesis. Prolonged activation of p53, however, results in

apoptosis in many cell types (Levine 1997). Induction of p53 in immortalized granulosa cells caused apoptosis (Karen-Tal et al 1995). The mechanism of p53 induced death is not known, but recent evidence has demonstrated that p53 can simultaneously induce bax and suppress bcl-2 gene expression, which may therefore alter the balance of cell death genes leading to apoptosis (Miyashita and Reed 1995).

1.4.2.4 Potential cell death inducers

Apoptosis can be induced in follicles at different stages of development by withdrawal of DES or gonadotropin. It is not clear whether such hormone deprivation is sufficient to cause apoptosis or whether this treatment causes the local production of apoptosis inducing factors. Possible inducing factors (fig. 3; Table 3) include androgen (Billig et al 1993) and GnRH (Billig et al 1994), as well as the cytokines IL-6 (Gorospe and Spangelo 1993b), TNFα (Kaipia et al 1996), and FasL (Hakuno et al 1996). The cytokine IL-6 is produced, and induces DNA fragmentation, in cultured granulosa cells (Gorospe et al 1992; Gorospe and Spangelo 1993a & 1993b).

1.4.2.4.1 TNFα

TNFα, at very high doses, induced apoptosis in cultured mouse luteal cells. This was augmented by prior exposure of the cells to interferon-gamma (IFNγ; Jo et al 1995).

Similarly, TNFα or ceramide, the putative second messenger for this cytokine, induced apoptosis in early antral rat follicles cultured in the presence of FSH (Kaipia et al 1996). Johnson and colleagues found that ceramide induced apoptosis in hen granulosa cells (Witty et al 1996). TNFα, sphingomyelinase, and UV radiation, all of which stimulate ceramide release from sphingolipids, also induced apoptosis (Witty et al 1996). Danorubicin, an agent that stimulates *de novo* ceramide synthesis, also induced apoptosis in that study. Thus, ceramide produced by a variety of stimuli was capable of initiating apoptosis, indicating that this second messenger might be a death signal for granulosa cells.

1.4.2.4.2 Fas/FasL

The Fas/FasL system was first implicated in ovarian apoptosis by Guo et al (1994) and Quirk et al (1995) , who found that monoclonal antibodies to Fas, which mimic the natural ligand, induce DNA fragmentation in cultured human granulosa/lutein cells. This effect of the antibody required prior exposure of the cells to IFNγ, presumably to upregulate the Fas receptor (Quirk et al 1995). Fas antibody also induced apoptosis in rat follicles (Hakuno et al 1996). Injection of mice with anti-Fas antibody caused apoptosis in both CL and follicles, indicating a role for the Fas/FasL system in luteal regression and follicular atresia (Sakamaki et al 1997). Mice bearing the mutation *lpr*, resulting in the absence of functional Fas receptor, were found to have ovaries with

excessive numbers of small antral follicles. This may point to a role for Fas in the elimination of follicles at the penultimate stage (fig. 3) of development (Sakamaki et al 1997). Immunohistochemistry localized Fas/FasL in atretic rat follicles and FasL was found in rat oocyte lysate (Hakuno et al 1996) and Fas was increased in cultured granulosa cells exposed to IFNγ. These granulosa cells died by apoptosis when co-cultured with oocytes. This points to a potential paracrine regulation of follicular atresia by oocytes. The physiological role of such a mechanism has not been resolved.

1.4.2.4.3 $PGF_{2\alpha}$

$PGF_{2\alpha}$ induces functional regression of the corpus luteum in most species (Niswender and Nett 1988). Functional regression is characterized by the rapid loss of progesterone synthesis, followed by structural involution of the CL as the luteal cells undergo apoptosis (Juengal et al 1993; Dharmarajan et al 1994) . The signals initiated in luteal cells by $PGF_{2\alpha}$ include decreased cAMP (Thomas et al 1978), increased intracellular Ca^{2+} (Leung et al 1986; Boone et al 1993; Davis et al 1987), and elevations of free radicals and lipid peroxides (Sawada and Carlson 1991). Which, if any, of these signals elicits luteal cell apoptosis is not known. The rapid decline in progesterone associated with luteal regression may also be a signal for apoptosis, since this steroid does have death suppressing effects in some ovarian cells (Luciano et al 1994).

1.4.2.5 Signalling by death inducers

A variety of signals may be initiated by apoptogenic factors in the ovary. Androgen presumably acts through its receptor to influence transcription. GnRH is known to act through the PLC system to induce intracellular Ca^{2+} transients and PKC activation (Billig et al 1994). The IL-6 signalling mechanism is not fully understood, but can induce transcription through IL-6 response elements. TNFα and Fas can stimulate ceramide production leading to the activation of ceramide dependent kinase. Both these cytokines can also activate the caspase cascade to cell death (Chinnaiyan et al 1996a). Luteolysis induced by $PGF_{2\alpha}$ may be mediated by the PLC system or free radical generation. It is difficult to determine which, if any, of these signals is relevant to the induction of apoptosis in ovarian cells. The caspase cascade is likely to mediate the cell death signal of TNFα and Fas (Yuan 1997). Ceramide generation can induce cell death, but ceramide is also induced by the survival factor IL1-β (Santana et al 1996). Similarly, PKC and Ca^{2+} can induce cell death in some systems (Schwartzman and Cidlowski 1993), but these signals are also induced by hormones that stimulate differentiation and steroidogenesis in the ovary. It may be the case that the induction of cell death in the ovary depends on the relative ability of a signal to activate the caspase cascade in the cell. For example, if caspase-3 is not present in a granulosa cell, then ceramide may be a mediator leading to inhibition of steroidogenesis. Following expression of caspase-3, the same signal that once influenced steroidogenesis might

now initiate apoptosis. Whether this is the case requires further study of the components of the caspase system in ovarian cells. All that is known of caspase 3 in the ovary is that the mRNA encoding this protein is suppressed by gonadotropin and induced following gonadotropin withdrawal (Flaws et al 1995b).

1.5 MODELS TO STUDY FOLLICULAR ATRESIA

1.5.1 *In vitro*

Initial studies of apoptosis in the ovary demonstrated that atretic follicles contained apoptotic granulosa cells. *In vitro* models to induce apoptosis in follicles cultured in serum-free media demonstrated the suppression of apoptosis in granulosa cells by a variety of growth factors, intraovarian regulators, and FSH. These *in vitro* models have deficiencies, including the potential effects of changes in the temperature and oxygen supply during tissue collection. In addition, they involve the study of follicles as isolated compartments, whereas the normal environment for the follicle may be influenced by the presence of the ovarian stroma, CL, and other follicles in a range of developmental states. The use of *in vitro* models allows for the specific study of individual growth factors, intraovarian regulators, and gonadotropin. This approach provides useful results, but conclusions must be tempered by the fact that potential interactions between factors are not addressed by such *in vitro* models. Nonetheless, *in*

vitro models of follicular atresia provide timed, predictable onset of apoptosis and have been used to implicate oncogenes, caspases, and other biochemical phenomena in the early events of granulosa cell apoptosis.

1.5.2 *In vivo*

To fully elucidate the role and regulation of apoptosis in follicular atresia, models for the *in vivo* induction of ovarian apoptosis are necessary. Greenwald and Terranova (1988) have pointed out that it is not feasible to determine the primary events of apoptosis in follicles by studying the spontaneous onset of atresia *in vivo* because of the difficulty in pinpointing the moment when degeneration begins and in determining the criteria for this early step. Once a follicle has degenerated to be sufficiently identified as atretic, it is too late to establish causation. Experimental models in which degeneration occurs as a timed, predictable event are necessary to study the early, crucial events of granulosa cell apoptosis and thus follicular atresia. Apoptosis in mature preovulatory follicles *in vivo* has been examined, using the model of gonadotropin withdrawal by metabolic clearance after a single injection of eCG to immature rats (Hughes and Gorospe 1991), or by hypophysectomy on the day of proestrus (Nahum et al 1996). The onset of apoptosis in the single eCG injection model occurs slowly 3-5 days after the injection because of the long biological half life of eCG (Hughes and Gorospe 1991), while the earliest signs of apoptosis are observed

8 h after hypophysectomy (Nahum et al 1996). Since these models rely on the metabolic clearance of gonadotropin, which is subject to considerable variation between animals, they do not facilitate precise *in vivo* study of very early events in apoptosis following gonadotropin withdrawal. Additionally, these models involve the induction of atresia and apoptosis in mature preovulatory follicles which do not typically undergo atresia in normal cycling rats (Byskov 1978; Greenwald and Terranova 1988; Hirshfield 1991; Hirshfield 1988). Atresia predominantly occurs (under normal physiological conditions) in small antral follicles beginning the process of granulosa cell differentiation (Hsueh et al 1994; Hirshfield 1991), suggesting that this stage is a turning point in the determination of the fate of the follicle. The currently available *in vivo* models to study apoptosis do not focus on this critical penultimate stage of follicular development (fig. 3). A model for follicular atresia in the hamster that uses anti-eCG antiserum to neutralize circulating gonadotropin has been reported (Bill and Greenwald 1981). Atresia was induced rapidly in this model, as serum estradiol levels declined 55% within one hour and histological signs of atresia were evident 4 h after treatment (Bill and Greenwald 1981; Greenwald and Terranova 1988). This model has not been used in other species and has not been investigated in terms of the onset and occurrence of apoptosis.

1.6 SUMMARY OF INTRODUCTION

The essential function of the ovary is to produce oocytes and support pregnancy by the process of follicular development, ovulation and luteinization. Atresia is the most common fate of follicles and regression is the fate of all CL. Granulosa cells and luteal cells die by apoptosis during follicular atresia and luteal regression, respectively. The cellular and molecular mechanisms that regulate apoptosis are not completely understood.

Follicles are composed of an oocyte surrounded by granulosa and theca cells in distinct compartments separated by a basement membrane. To varying degrees during development, the theca layer is invested with blood vessels, nerves and connective tissue. The granulosa cells and oocyte have no direct blood supply and acquire nutrients filtered through the basement membrane. Theca cells are derived from the mesoderm, while granulosa cells are epithelial in origin. Follicle development can be described as distinct stages. Preantral stages of development include primordial, primary and secondary follicles. Primordial follicles are recruited and become primary and then secondary follicles as granulosa cells divide. The formation of a fluid-filled antrum marks the entry of the follicle into the tertiary stage. The final maturation into the Graafian follicle is characterized by increased production of estrogen and a dramatic increase in size due to the accumulation of antral fluid. This growth

necessitates extensive remodelling of the basement membrane. The LH surge induces ovulation and luteinization during which there is a loss of the compartmentalization that existed in the follicle. Luteinization is also characterized by cellular hypertrophy, vascularization and a shift in steroidogenesis from estrogen to progesterone. The hormonal control of early preantral follicular development is not fully understood. FSH is not essential for follicle development at this stage, but gonadotropins can stimulate recruitment and growth of preantral follicles. FSH is essential for follicle development beyond the preantral stage. The transition from preantral to antral follicle is a reflection of the differentiation of the granulosa cells. Differentiated granulosa cells produce steroids and follicular fluid and eventually express the LH receptor.

Granulosa and theca cells cooperate to produce steroids. LH stimulates androgen production by theca cells which is aromatized to estrogens by granulosa cells stimulated by FSH. In addition to the effects of steroids on the reproductive system, both estrogen and androgen have local effects to regulate gene expression in granulosa and theca cells. Healthy follicles have high estrogen to androgen ratios but this is reversed in atretic follicles. Excess androgens may be a signal to induce follicular atresia. Numerous intraovarian factors regulate follicular development including IGF-1, EGF/TGFα, GnRH, GH, bFGF, VIP, and cytokines. Production of these local factors is often regulated by gonadotropins and, in turn, these factors mediate many of the effects of gonadotropins in the ovary. The transition from preantral to antral

follicle also defines a period when follicles become particularly susceptible to atresia. Most follicles do not develop to ovulate, but instead degenerate by atresia. This period of susceptibility to atresia, during early to mid-sized antral growth, is called the penultimate stage of development. Atresia can sometimes occur in preantral follicles and is initiated by the demise of the oocyte, while atresia in antral follicles begins in the granulosa cells. Oocytes in antral follicles resist cell death until the late stages of atresia. An ovulated follicle luteinizes to form a corpus luteum which produces progesterone in response to LH, but eventually undergoes regression. The luteolytic factor ($PGF_{2\alpha}$ in many species) causes cessation of LH induced steroidogenesis, followed by the structural demise of the CL.

Apoptosis, the physiological form of cell death that underlies follicular atresia and luteal regression, has distinct morphological and biochemical features. Morphological signs of apoptosis include the physical detachment of the cell from its surroundings, followed by cytoplasmic and nuclear shrinkage, fragmentation and phagocytosis. Biochemical characteristics of apoptosis include DNA fragmentation and proteolysis under the control of unique cell death genes. DNA degradation results from Ca^{2+}/Mg^{2+}-dependent endonuclease activity and produces a characteristic "ladder" pattern of fragments when resolved by electrophoresis. The "ladder" pattern of low molecular weight DNA degradation is actually the final step in a process that includes the initial production of very-high and high molecular weight DNA fragments. DNase I is a

Ca^{2+}/Mg^{2+}-dependent endonuclease that has been implicated in apoptosis in the thymus, prostate, intestine and other tissues. This enzyme has pH and cation requirements, as well as sensitivity to inhibitors, that are consistent with those of apoptosis. In addition, DNase I is inhibited by actin and by ADP-ribosylation. Other putative apoptosis endonucleases include DNase II, cyclophillin and various unidentified proteins. Another characteristic biochemical feature of apoptosis is the activation of caspases, a unique set of aspartate specific cysteine proteases that cleave a limited number of substrates. Although there is a "cascade" of proteolysis, in which caspases are sequentially cleaved and activated by other caspases, the ultimate trigger for cell death is caspase-3. Caspase-3 cleaves and inactivates the enzymatic activity of PARP, sNRP and DNA-PK, eliminates the ability of actin to inhibit DNase I, and releases a transcription factor from the SREBP. Apoptosis is controlled by a unique set of oncogenes that encode bcl-2 and related proteins. A wide variety of cell death-inducing signals are blocked by bcl-2; however, the biochemical mechanism of cell death suppression by bcl-2 remains unknown. The bcl-2 protein dimerizes with itself, or with a number of related proteins including bcl-x_{long} and bax, which suppress or induce cell death, respectively. Caspase-3 can be activated by TNFα or FasL receptors via a series of protein-protein interactions at peptide sequences called death domains. The activation of caspase-8 by death domain signals is the initial step in the protease cascade leading to caspase-3 cleavage and activation. This cascade of protease activation is regulated by bcl-2 and related proteins.

In all species studied to date, apoptosis has been identified in granulosa cells of atretic follicles and luteal cells of regressing CL. Hormones known to induce or suppress follicular atresia have been found to induce or suppress apoptosis. Steroids, growth factors cytokines or other intraovarian regulators can suppress apoptosis, to varying degrees, during follicular development. FSH is an especially important survival factor in early follicular development. The effects of FSH, to suppress granulosa cell death, may be mediated by IGF-1 and other intraovarian regulators known to suppress apoptosis. A variety of cellular signalling systems are activated by these different survival factors in the ovary, yet they all act to produce the same result. It is possible that the variety of death suppressing signals of the numerous ovarian survival factors converge at the level of one or two local mediators such as EGF/TGFα or IGF-1. FSH may also have anti-apoptotic effects that are not mediated by other factors. The proteins bcl-2 and bcl-x_{long} suppress, while bax induces, granulosa cell apoptosis. Gonadotropin suppresses the levels of bax mRNA in ovarian cells while maintaining the expression of bcl-2 and bcl-x_{long}. Protease inhibitors have been used to demonstrate that caspases are involved in granulosa cell apoptosis and FSH suppresses the expression of caspase-3 mRNA in ovarian follicles. Finally, the transcription factor p53 has been implicated in follicular apoptosis and gonadotropin suppresses p53 mRNA levels in granulosa cells. Withdrawal of gonadotropin or survival factors can induce apoptosis in ovarian follicles but apoptosis can also be induced by several agents. Androgens, GnRH, $PGF_{2\alpha}$ and the cytokines IL-6, TNFα and FasL have all

been implicated as cell death inducers in the ovary. As with the cell death suppressors, the signalling mechanisms of cell death inducing factors are diverse and may depend on the developmental state of the ovarian cell. Cell death signals in the ovary presumably converge at some level to activate caspase-3 and the endonuclease responsible for apoptosis.

In vitro and *in vivo* models have been used to study the regulation of apoptosis in ovarian follicles. Previous *in vivo* studies have focused on atresia in large preovulatory follicles following hypophysectomy or metabolic clearance of injected eCG. These models do not allow for the study of early cellular events in atretic follicles at the penultimate stage of development, when atresia is most commonly observed.

1.7 OBJECTIVES

The nuclei of differentiated granulosa cells and luteal cells, but not undifferentiated granulosa cells, contain a Ca^{2+}/Mg^{2+}-dependent endonuclease activity (Zeleznik et al 1989) and atresia is most commonly observed in early-antral, but not preantral, follicles (Hirshfield 1991). This suggests that the capacity of ovarian cells to undergo apoptosis is developmentally regulated. Hormones, growth factors, cytokines and other intraovarian factors that suppress or induce apoptosis in ovarian cells have all been found to induce or suppress DNA "ladders". These factors must therefore regulate the

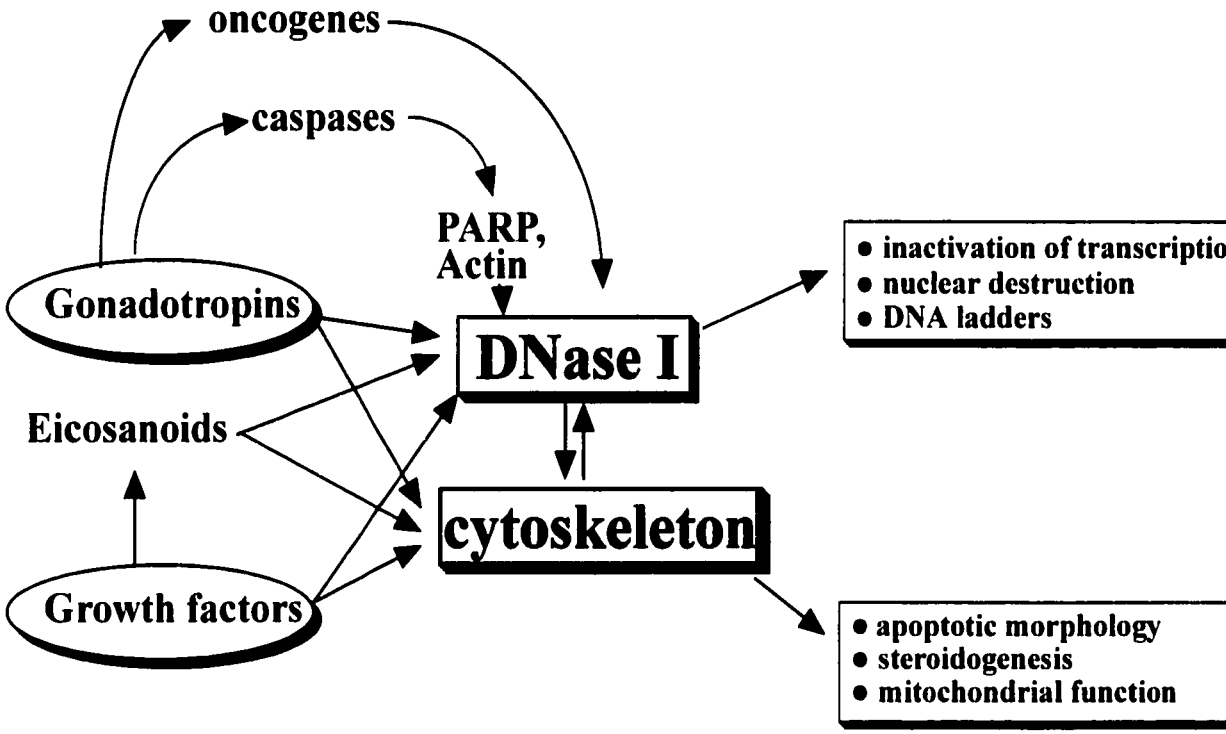
activity of the Ca^{2+}/Mg^{2+}-dependent endonuclease as part of their survival or cell death-inducing signal. In addition, the developmental pattern of endonuclease expression suggests that factors which regulate expression of the endonuclease might also regulate the susceptibility of ovarian cells to apoptosis. In order to fully determine the role and regulation of apoptosis and DNA degradation during follicular atresia and luteal regression, the mechanisms regulating the endonuclease must be elucidated. Unfortunately, the endonuclease involved in ovarian apoptosis has not been identified. The present work was conducted to: (i) establish the presence of an ovarian endonuclease in rat granulosa and luteal cells, (ii) examine its activity relative to the developmental state of ovarian cells, (iii) study its biochemical properties relevant to apoptosis, (iv) identify the endonuclease involved in ovarian cell apoptosis, (v) establish an *in vivo* model for the induction of apoptosis at the penultimate stage of follicular development, and (vi) study the possible regulation of the endonuclease during follicular atresia and luteal regression.

1.8 HYPOTHESIS

DNase I is the endonuclease responsible for apoptotic DNA degradation during ovarian follicular atresia and luteal regression. The penultimate stage of follicular development coincides with the expression of DNase I in granulosa cells. The acquisition of this endonuclease renders the granulosa cells susceptible to apoptosis.

Apoptosis during follicular atresia or luteal regression involves the activation of caspase-3. Actin and/or PARP inhibit DNase I, but these proteins are cleaved by caspase-3 during apoptosis, resulting in the derepression of DNase I and degradation of the genome (fig. 6).

Figure 6: The potential role of DNase I in follicular atresia or luteal regression. This model illustrates the possible regulation of DNase I and the cytoskeleton by gonadotropins, growth factors and eicosanoids, as well as the interaction of DNase I with the cytoskeleton to elicit apoptosis in ovarian cells. Gonadotropins suppress caspase mRNA in the rat ovary, and withdrawal of gonadotropin results in increased expression of these proteases in granulosa cells. Caspases cleave and inactivate actin and PARP, two proteins known to inhibit DNase I. Thus, activation of caspases and the cleavage of actin and/or PARP could result in the derepression of DNase I leading to degradation of the genome. Oncogenes such as *bcl-2* are also upregulated by gonadotropin and inhibit endonuclease activity in isolated nuclei.


oncogenes
caspases
PARP,
Actin
Gonadotropins
DNase I
Eicosanoids
cytoskeleton
Growth factors
• inactivation of transcriptio
• nuclear destruction
• DNA ladders
• apoptotic morphology
• steroidogenesis
• mitochondrial function

2.0 METHODS AND MATERIALS

2.1 MATERIALS

Electrophoresis grade-Tris, acrylamide, N,N-methylenebis-acrylamide, glycine, dtt, SDS, ammonium persulfate, prestained low-range molecular weight markers, peroxidase linked goat anti-rabbit IgG and DC Protein Assay kit were purchased from Bio-Rad Laboratories (Richmond, CA). Magnesium chloride, calcium chloride, sodium chloride, zinc chloride, sodium azide, boric acid, acetic acid, IAA, ethanol, 10% neutral buffered formalin, paraffin, hydrogen peroxide, 3-amino-propyltriethoxysilane (AES), sodium citrate and toluene were obtained from BDH (Toronto, ON). AUR, SAM, agarose, ethidium bromide, Hoechst 33248, acridine orange, EGTA, EDTA, Nonidet P-40, Triton X-100, bromophenol blue, Coomassie blue, calf thymus DNA, phenylmethylsulfonylfluoride (PMSF), ATP, leupeptin, aprotinin, sucrose, DES, eCG, hCG, BSA fraction V, goat serum, rabbit serum, protease K, bovine pancreatic DNase I, rabbit skeletal muscle G-actin, 3,3-diaminobenzidene tetrahydrochloride (DAB), PBS and PBS with 0.05% Tween-20 were purchased from Sigma Chemical Co. (St. Louis, MO). Peroxidase-conjugated chicken anti-rabbit IgG and anti-bovine pancreatic DNase I antibody were from Chemicon (Temecula CA). Goat anti-rat CPP32 antibody was from Research Diagnostics Inc. (Flanders, NJ) and the anti-goat IgG detection system was from Santa

Cruz (Santa Cruz, CA). Immobilon P membranes were from Millipore (Nepean ON, Canada). The enhanced chemiluminescence (ECL) kit, [^{32}P]dCTP, [^{32}P]dideoxy ATP, terminal transferase and klenow enzyme were from Amersham (Arlington Heights, IL). All electron microscopy supplies and chemicals were from Marivac Ltd (Halifax, Nova Scotia). Medium M199, fetal calf serum (FCS) and normal rabbit serum were from Gibco/BRL (Burlington, ON). The anti-eCG antibody initially used in the present studies was a gift from Dr. D. Johnson (Department of Obstetrics and Gynecology, University of Kansas Medical Center, Kansas City, Kansas). Anti-rat DNase I antiserum was a gift from Dr. K. Kishi and Dr. T.Yasuda (Department of Legal medicine, Fukui Medical School, Fukui, Japan).

2.2 ANIMAL TREATMENTS

Sprague-Dawley rats and New Zealand White Rabbits were maintained on 12 h light dark cycles and given food and water ad libitum. Rats were sacrificed by cervical dislocation and rabbits sacrificed by Nembutol overdose. All procedures were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals, Canadian Council on Animal Care and approved by the Ottawa Civic Hospital Animal Care Committee.

2.3 PRODUCTION OF ANTI-eCG ANTISERUM

2.3.1 Injection of rabbits

Male rabbits (approximately 2kg body weight) were injected intramuscularly with 3,000 IU eCG in complete Freund's adjuvant (total 0.5 ml, at two sites) followed 3 weeks later by the same dose of eCG in incomplete Freund's adjuvant. Six weeks thereafter, the rabbits were injected as described with 1,000 IU eCG in incomplete Freund's adjuvant and blood (7 ml per kg body weight) was drawn from the ear vein. The rabbits were then injected with 1,000 IU eCG in incomplete Freund's adjuvant as described and blood was collected 2 weeks and 4 weeks later. The rabbits were then anaesthetized with Nembutol, bled completely and sacrificed by Nembutol overdose.

2.3.2 Anti-eCG IgG ELISA

The rabbit blood was allowed to clot overnight and centrifuged (1000 × g; 5 min) to collect serum which was then stored at -70°C. The titer of each bleed was determined by the Enzyme-Linked-Immunosorbant-Assay (ELISA) as described hereafter. Corning flat bottomed plates were coated (16 h room temp) with 100 µl/well of eCG solution [1 µg/ml carbonate buffer (0.016M sodium carbonate, 0.034M sodium bicarbonate, pH 9.6)], washed 5 times with PBS-Tween, incubated (37°C, 1 h) with

200 µl/well blocking solution (10% FCS in carbonate buffer) and washed as described. Plates were then incubated (37°C, 1 h) with 100 µl/well of the rabbit serum serially diluted in dilution buffer (PBS-Tween, 10% FCS), washed, incubated (37°C, 1 h) with 100 µl/well HRP-conjugated anti-rabbit IgG antibody (1:5000 in dilution buffer) and washed. The plates were incubated in a dark-box (0.5 h, 20°C) with 100 µl/well OPD solution [5mg OPD tablet in 30 ml citrate buffer (0.02M citric acid, 0.03 M sodium citrate, pH 5.0) + 0.01% H_2O_2], stopped by the addition of 50 µl 4N H_2SO_4 and the absorbance was read 450 nm to determine peroxidase activity in each well. Each sample was assayed in triplicate on two different plates and titers were calculated based on the formula : [(A_{450} serum sample - A_{450} preimmune serum) / 0.05] × dilution factor.

2.3.3 Anti-eCG antiserum bioassay

For the bioassay of the antiserum, immature female rats injected with 15 IU eCG were injected 24 h later with antiserum or preimmune serum (100 µl diluted in 0.9% saline, i.p.), ovaries were removed 24 h after treatment and weighed to determine the minimum concentration of antiserum capable of preventing eCG-induced ovarian weight gain.

2.4 MODEL FOR FOLLICULAR DEVELOPMENT IN THE RAT OVARY

2.4.1 Hormone treatments and cell collection

Immature female rats at 23 days of age received injections of DES (1mg/day; s.c., twice daily for 5 days); and were either sacrificed [DES group; ovaries enriched in undifferentiated granulosa cells (Zeleznik et al 1989)] or subsequently injected with eCG (25 IU; s.c.). Forty-eight hours later the latter group of animals were either sacrificed (eCG group; ovaries enriched in differentiated granulosa cells) or administered hCG (25 IU; s.c.) and were sacrificed 5 days thereafter (hCG group; ovaries enriched in luteal cells). Injection regimens were staggered such that all rats were sacrificed on the same day. Blood was collected by cardiac puncture at the time of sacrifice, kept on ice for 20 min and serum obtained by centrifugation (1000 × g, 5 min.) was stored at -20° C. Cells were collected as described (Boone et al 1995; Boone et al 1997). Briefly, ovaries were excised with adhering fat removed and homogenized gently in ice-cold PBS containing protease inhibitors (0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin) with a loose fitting glass-teflon homogenizer (4-5 strokes 200 rpm). Homogenates were passed through a 200 μm Nitex filter (Tetko, New York, NY) and cells collected by centrifugation (2500 × g; 10 min; 4°C).

2.4.2 Collection of cells from non-ovarian tissues

Granulosa cells layers were collected from F3-F1 follicles of white leghorn hens as previously described (Li and Tsang 1995). Bovine corpora lutea were removed from ovaries collected from a local slaughterhouse. Human granulosa/lutein cells were donated with patients consent from the IVF laboratories of the Ottawa Civic Hospital. Rat testes were collected from 80-100 day old animals and other non-ovarian tissues were collected from 30 day old female rats. Tissues from these sources were homogenized and their cells collected by centrifugation as described above for rat ovarian cells.

2.5 ENDONUCLEASE ACTIVITY IN ISOLATED OVARIAN NUCLEI

Nuclei were isolated as described (Boone et al 1995; Zeleznik et al 1989). Briefly, ovarian cells collected from the "DES", "eCG" and "hCG" groups were homogenized in Buffer A (10 mM Tris, 0.25 M sucrose, 3 mM $MgCl_2$, 3 mM EGTA) and filtered through Nitex filter (200 μm). Filtrates were centrifuged (800 × g, 15 min, 4° C), resuspended and incubated in Buffer A containing NP-40 (0.5 %; 10 min, 4° C). Nuclei were collected and washed in Buffer A by centrifugation (800 × g, 15 min, 4° C), and resuspended in Buffer B (10 mM Tris, 25 mM NaCl, 0.34 M sucrose) to an approximate concentration of 5×10^6 nuclei/ml. Aliquots of 200 μl (10^6 nuclei) were

incubated (30 min, 37°C) with 2 mM Ca^{2+}, 5 mM Mg^{2+}, 2 mM Ca^{2+} + 5 mM Mg^{2+} or 2 mM Ca^{2+} + 5 mM Mg^{2+} + 2 mM Zn^{2+}. Incubations were terminated by the addition of ice cold EDTA (5 mM) and DNA was isolated and radiolabeled by the method of Tilly and Hsueh (1993). Briefly, total genomic DNA was isolated, phenol/chloroform purified and resuspended in sterile water. The 3'-end labelling reaction was carried out using 250 ng purified DNA, 10 μCi of [α^{32}P]dideoxy-ATP and 25 units of terminal deoxynucleotidyl transferase at 37°C for 60 min. The reaction was stopped by the addition of 5 μl 0.5M EDTA followed by precipitation in 2.5 volumes of ethanol at -20°C. The DNA pellet was resuspended in 40 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH7.6) and the sample was subjected to TAE agarose gel (2%) electrophoresis at 8 V/cm for 2h at room temperature. The gel was then dried in a slab gel drier without heat for 2 h and exposed to x-ray film at -80°C.

2.6 PREPARATION OF NUCLEAR PROTEIN EXTRACTS

All procedures were carried out on ice or under refrigeration (4°C). Protease inhibitors [PMSF (1 mM), aprotinin and leupeptin (10 μg/ml)] were added to buffers, where indicated, immediately before use. Ovarian cells were resuspended in 10 mM $MgCl_2$ with 0.25% Nonidet P-40 and protease inhibitors and mixed on a nutator platform for 15 min. Nuclei were collected by centrifugation (500 × g; 15 min), washed in 10 mM $MgCl_2$, resuspended in extraction buffer (0.35 M NaCl, 1 mM EDTA, 20 mM Tris, pH

7.5) and mixed on a nutator platform for 2 h to extract protein. Chromatin was pelleted (100,000 × g; 60 min) and 0.1 volume of glycerol was added to the supernatant which was then stored at -20°C. Protein content of the extracts was determined with the Bio-rad DC Protein Assay.

2.6.1 Endonuclease activity in nuclear protein extracts

Endonuclease activity was assayed by the plasmid degradation assay as previously described (Boone et al 1995). Nuclear extracts (40 μg) were diluted to 1 ml in Tris buffer (10 mM Tris, pH 7.5) and 10 μl of the resulting solution was mixed with 30 μl Tris buffer containing cations and/or inhibitors and preincubated (10 min, 37 °C) before the addition of 10 μl Bluescript plasmid (10 μg/ml), followed by incubation for 20 min at 37 °C. Aliquots (10 μl) were removed and mixed with 2 μl stop/loading buffer (25 mM EDTA, 30 % glycerol, 0.25 % bromophenol blue) and kept on ice until electrophoresis. DNA was resolved by electrophoresis on TAE-agarose gel (1%) at 8 V/cm for 2h at room temperature, and the gel was subsequently stained with ethidium bromide (0.5 μg/ml TAE) and endonuclease activity, determined as the production of relaxed circular DNA (Boone et al 1995), was quantified by scanning densitometry on a UV transilluminator using the Bio-rad Gel Doc system (Biorad Laboratories, Richmond, CA).

2.6.2 Nuclease zymography of nuclear protein extracts

Nuclease activity of specific nuclear proteins was measured by SDS-PAGE using the method of Rosenthal and Lacks (1977) modified as previously described (Boone et al 1995). Nuclear extracts were loaded onto standard Laemmli gels (12 %) containing calf thymus DNA (250 μg/ml) and resolved by electrophoresis at 4 °C and 23 mA per gel for 1.5 h. Gels were then soaked overnight in several changes of TE buffer (50 mM Tris pH 7.5, 1 mM EDTA, 0.02% NaN_3; 30 °C), incubated in TE with 2 mM Ca^{2+} and 5 mM Mg^{2+} at 37 °C, stained in ethidium bromide (0.5 μg/ml TE), and photographed on a UV transilluminator. Nuclease activity, appearing as dark bands on a brightly staining background of DNA, was measured by scanning densitometry and expressed in terms of Kunitz units of DNase I activity by comparison to a standard curve produced by zymography of bovine DNase I standards. Nuclease activities were distinguished from false positive histone staining by silver staining the DNA in the gel as described (Boone and Tsang 1997c). Briefly, following incubation to develop bands of nuclease activity, gels were washed (3 h, 30 °C) in 5% SDS to remove protein and stained with the BioRad Silver-Staining kit, omitting the acetic acid in the fixation step. This procedure stains DNA remaining in the gel brown, producing clear bands indicative of nuclease activity. For some experiments, G-actin or BSA was added to the gel to a concentration of 10 μg/ml before polymerization and, following electrophoresis the gel was incubated in TE buffer with cations and 0.05 mM ATP.

2.6.3 Immunodetection of DNase I in nuclear protein extracts

2.6.3.1 Western analysis

Nuclear extracts (40 μg) were resolved by SDS-PAGE as described in section 2.6.2 and electrotransfered (250 mA, 0.5 h) to Immobilon P membranes. The membranes were dried overnight, incubated for 1 h with anti-DNase I antibody [diluted 1:100 in TBS with 5% BSA (fraction V)], washed (3×5 min) in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) and then incubated for 0.5 h with peroxidase-conjugated goat anti-rabbit IgG (diluted 1:2000 in TBS with 5% BSA). Peroxidase activity was visualized using the ECL kit as per the manufacturer's instructions.

2.6.3.2 Immunoprecipitation

Nuclear extracts (40 μg) were diluted in 500 μl phosphate buffered saline (PBS) containing 1% Triton X-100, PMSF (1 mM), aprotinin and leupeptin (10 μg/ml) and 5 μl anti- rat DNase I antiserum or normal rabbit serum (control), then incubated at 4°C for 1h. 75 μl of protein A sepharose (10% w/v in PBS) was then added and the mixture incubated at 4°C for 1h on a nutator platform. The protein A sepharose-antiserum immunoprecipitates were washed in PBS-Triton X-100 (1%) 5 times by centrifugation (2000×g; 1 min), solubilized in sample buffer (20 % glycerol, 5 % SDS

and 100 mM Tris pH 7.5) for 10 min at 60°C and analyzed by zymography, as described in section 2.6.2.

2.6.3.3 Immunodepletion of endonuclease activity from eCG extracts

Nuclei were isolated from DES -treated rats as described (Boone et al 1995, Zeleznik et al 1989) and resuspended in Buffer B (10 mM Tris, 25 mM NaCl, 0.34 M sucrose, pH 7.5) to an approximate concentration of 5×10^6 nuclei/ml. Nuclear extracts (100 μg) from the eCG-treated group were immunoprecipitated with anti-DNase I antibody, anti-IgG antibody or Buffer B, as described above. Sepharose beads were removed by centrifugation, the supernatent was collected and immunoprecipitation was repeated. Ca^{2+} (4 mM) and Mg^{2+} (10 mM) were added to the final supernatent, which was then combined 1:1 with the DES nuclei and incubated (37°C) for 1 h. DNA was then isolated with the Qiagen Blood Amp kit, 3'-end-labelled with [^{32}P]dCTP and klenow enzyme as described (Boone et al 1997b) and resolved by agarose gel (2%) electrophoresis. The gel was then dried (2 h, no heat) and exposed to a Bio-Rad phosphoimager screen (BioRad Laboratories, Richmond CA) to quantify low molecular weight DNA (< 15 Kbp) densitometrically and subsequently to x-ray film at -80°C.

2.7 IMMUNOHISTOCHEMISTRY

Tissues were fixed in 10% neutral buffered formalin, dehydrated in a graded series of alcohol (70-100%), cleared in xylene and embedded in paraffin. Tissues were sectioned (5 μm), mounted on AES slides, deparaffined in xylene and rehydrated through a graded series of ethanol (100-70 %) to PBS.

2.7.1 DNase I

Antigen retrieval was performed as described by Shi et al (1995). Briefly, slides were placed in plastic Coplin jars filled with 0.1 M sodium citrate (pH 1.0), microwaved (2×3 min, 5 min apart), cooled to room temperature and then permeabilized in PBS with 0.2% Triton (30 min). Sections were blocked in 2% BSA (fraction V) for 10 min, incubated 4 h with rabbit anti-rat DNase I antiserum (1:20 in 2% BSA with 0.01 % Tween) or normal rabbit serum (control; 1:20 in 2% BSA with 0.01%Tween) and then washed (3×10 min) in PBS-Tween (0.05%). Sections were then reblocked in 2% BSA, incubated in peroxidase conjugated chicken anti-rabbit IgG antibody (1:200 in 2% BSA with 0.01% Tween) for 30 min, washed as before, stained with DAB/$CuSO_4$ and mounted for examination.

2.7.2 Caspase-3

Rehydrated sections were blocked in 5% BSA for 10 min, and then incubated (3 h, 37°C) in 5% BSA containing 5 μg/ml goat anti-rat CPP32 antibody or 5 μg/ml normal goat serum (control). Slides were then washed in PBS (3 × 10 min) and immunostaining was visualized using the Santa Cruz anti-goat IgG biotin-streptavidin-HRP detection system as per manufacturer's instructions. Sections were not counterstained, and were dehydrated through a graded series of ethanol (70-100%) to xylene, then mounted for examination.

2.8 ANALYSIS OF DNase I mRNA BY RT-PCR

Total RNA was isolated from ovarian cell pellets with the Qiagen RNEasy kit according to manufacturers instructions and quantified by spectrophotometric absorbance at 260 nm. Amplification of DNase I mRNA was performed as described (Liu et al, in press). Briefly, total RNA (5 μg) per 40 μl assay was reverse transcribed (37°C, 1 h) by Superscript reverse transcriptase with 1 μg oligo-dT primer. After heat denaturation (90°C, 5 min), 5 μl of the first-strand cDNA was used for PCR analysis using the sense primer: 5' GTTGCTGTTGGGAAGCTACTGG 3', and antisense primer: 5' CACCTCCACTGGGTAATGGTC 3', which span 639 bp of the coding region of rat DNase I cDNA. The PCR was performed using one denaturation cycle

(94°C, 1 min) followed by 35 amplification cycles (94°C, 30 sec; 60°C, 1 min; 72°C, 50 sec) and a final post-elongation cycle (72°C, 5 min). Amplification products were resolved by agarose (2.5%) gel electrophoresis, stained with ethidium bromide (0.5 μg/ml) and quantified densitometrically by fluorescence under UV transillumination.

2.9 MODELS FOR FOLLICULAR ATRESIA AND LUTEAL REGRESSION IN THE RAT OVARY

Immature female Sprague-Dawley rats at 23 days of age were injected with eCG (15 IU, s.c.) and 24 h later with normal (preimmune) rabbit serum (1:10 in saline; i.p.) or 100 μl anti-eCG antibody (1:10 in saline; i.p.). This protocol has been used to neutralize 25 IU of injected gonadotropin and induce atresia in the hamster (Bill and Greenwald 1981; Greenwald and Terranova 1988), and has now been adopted in the rat (Boone et al 1997b). Luteal regression was induced as described (Sawada and Carlson 1991). Briefly, rats were injected with eCG (25 IU, s.c.) followed 48 h later by hCG (25 IU, s.c.) to induce superovulation and formation of corpora lutea. Seven days after hCG administration, rats were injected at multiple sites along the dorsal hind region with saline (control) or Lutalyse (Upjohn, Kalamazoo; 0.5 mg/rat, s.c.). This protocol for Lutalyse injection induces functional luteal regression in rats 20 min after treatment (Sawada and Carlson 1991).

At the indicated times after serum, anti-eCG antibody, saline or Lutalyse injection, ovaries were excised, cleared of adhering fat, weighed, and either fixed in 10 % neutral buffered formalin for histology or placed in medium M199 for cell viability staining and granulosa cell collection. For ultrastructural studies, ovaries were fixed in 1.6% glutaraldehyde in 0.1 M Na-cacodylate buffer.

2.10 HISTOLOGY AND TUNEL LABELLING

Ovaries fixed in 10% neutral buffered formalin were dehydrated through a graded series of ethanol (70-100%), cleared in toluene, embedded in paraffin and sectioned (4 μm). The sections were mounted on AES-coated slides, cleared in toluene and then rehydrated through a graded series of ethanol (100-70%) to PBS. For routine histology, slides were stained with hematoxylin/phloxine/saffron, observed and photographed by light microscopy. To identify cell death using an *in situ* cell death detection kit, slides were treated (20 min, 37°C) with protease K (10 μg/ml of 20 mM Tris, 2 mM $CaCl_2$, pH 7.5), washed three times in PBS and then incubated (60 min, 37°C) with terminal transferase in the presence of FITC-conjugated dUTP, according to the manufacturer's instructions. The stained sections were then photographed on a Zeiss microscope equipped with epifluorescent optics. As a positive control for *in situ* detection, slides were preincubated (20 min, 37°C) with bovine pancreatic DNase I (0.2 μg/ml of 20 mM Tris, 2 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.5) before the terminal transferase reaction.

2.11 IDENTIFICATION OF CELL DEATH

2.11.1 Viable staining

Ovaries were stained in acridine orange (5 μg/ml of M199) for 5 min at 37°C, washed in M199 (3 × 3min) and photographed with a Zeiss microscope using FITC optics (Delic et al 1991).

2.11.2 Nuclear staining

Granulosa cells were collected by follicle puncture as described (Farookhi 1982), fixed (4% formalin in PBS; 10 min, RT) and washed in PBS. The cells were then resuspended in Hoechst staining solution (0.1 μg Hoechst 33248/ml PBS, 10 min), washed again and spotted onto slides for microscopy. Nuclear staining was observed and photographed using a Zeiss fluorescence microscope. Apoptotic cells were identified by typical nuclear morphology, then counted using randomly selected fields on numbered photographic slides to avoid bias during counting.

2.11.3 Quantification of DNA ladders

Granulosa cells were collected by follicular puncture, pelleted, snap frozen, and stored at -70°C until analysis. Cells were resuspended in 200 µL PBS and the DNA extracted using the Qiagen Blood Amp kit, according to manufacturer's instructions. DNA was stained with ethidium bromide and quantified by fluorescence intensity using the Bio-Rad Gel Doc system. DNA (0.5 µg) was 3'-end labelled as described (Rosl 1992) by incubating (20 min, RT) with Klenow enzyme (2 U in 10 mM Tris, 5 mM $MgCl_2$) and 0.1 µCi [^{32}P]dCTP. Unincorporated nucleotides were removed with the Qiagen Nucleotide Removal kit and the samples were resolved by Tris-acetate-EDTA agarose (1.8%) gel electrophoresis. The gel was then dried (2 h, no heat) and exposed to a Bio-Rad phosphoimager screen to quantify low molecular weight DNA (< 15 Kbp) densitometrically, and subsequently exposed to x-ray film at -80°C.

2.11.4 Electron microscopy

Ovaries were cut into 3 mm cubes, fixed in 1.6% glutaraldehyde in 0.1 M Na-cacodylate buffer, stored at 4°C and processed for electron microscopic examination by standard techniques. Briefly, ovaries were postfixed with 1% osmium tetroxide, washed in dH_2O, followed by "en bloc" saturated aqueous uranyl acetate staining. Specimens were then dehydrated through a graded series of ethanol (70-100%) to

acetone, followed by a graded series of Spurr's resin in acetone (20-100%), and finally embedded in Spurr's Resin. Ultrathin sections were cut, mounted on grids, stained with lead citrate and viewed with a Hitachi 7100 electron microscope.

2.12 WESTERN ANALYSIS

Protein extracts resolved by 9% SDS-PAGE were electrotransferred (30 V, 16 h) to nitrocellulose membranes. The membranes were then blocked (RT, 1 h) with TBS-T with 5% milk powder, then incubated (RT, 2 h) with antibody, washed in TBS-T (3 × 5 min), incubated with HRP-conjugated secondary antibody (1:10,000) in TBS-T with 5% milk powder and washed again in TBS-T. Peroxidase activity was visualized with the ECL kit, as per manufacturer's instructions.

2.13 STATISTICAL ANALYSIS

All experiments were performed at least three times. Data were subjected to analysis of variance and subsequently to Tukey's test. Where indicated, linearity was determined by the χ^2 test. Statistical significance was inferred at $p<0.05$.

3.0 RESULTS

3.1 ENDONUCLEASE ACTIVITY IN ISOLATED NUCLEI

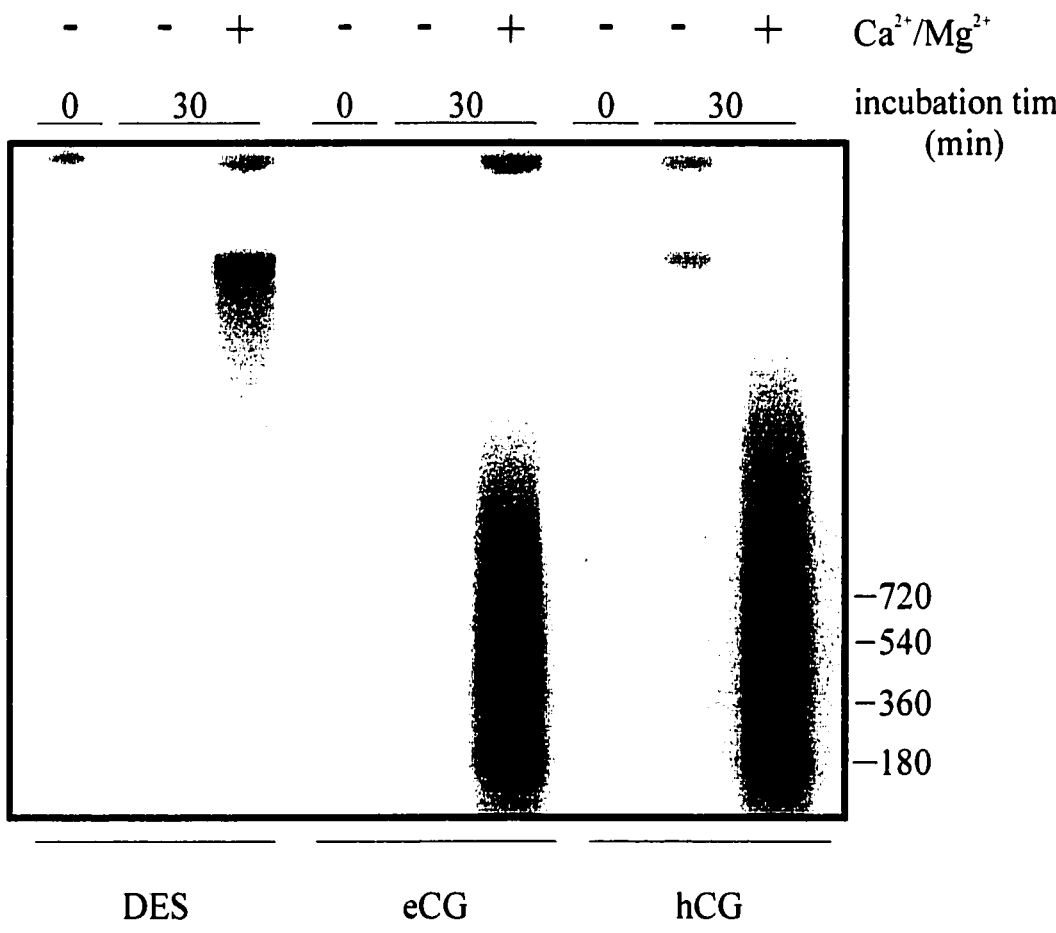
Nuclei isolated from cells of the "DES", "eCG" and "hCG" groups were exposed to the indicated combinations of Ca^{2+} (2 mM), Mg^{2+} (5 mM) or Zn^{2+} (2 mM) *in vitro* for 30 min. DNA was isolated, labelled on 3' ends and resolved by electrophoresis to demonstrate internucleosomal DNA cleavage by endogenous nuclear endonuclease activity (fig. 7). DNA degradation was evident in DES, eCG and hCG groups but significantly more endonuclease activity was present in the nuclei of eCG and hCG groups than in the DES group (fig. 8). The endogenous endonuclease activity present in nuclei of eCG-treated rat ovaries was dependent on both Ca^{2+} and Mg^{2+} and inhibited by Zn^{2+} (fig. 9).

3.2 ENDONUCLEASE ACTIVITY IN NUCLEAR PROTEIN EXTRACTS

3.2.1 Plasmid degradation assay

An example of complete supercoiled DNA degradation in the presence of excess nuclear protein extract is shown in figure 10. Supercoiled plasmid is degraded by nuclear extracts to form first relaxed open circular and subsequently linear DNA.

Figure 7: Developmental pattern of endonuclease activity in isolated rat ovarian nuclei. Ovarian nuclei isolated from rats in the "DES", "eCG" and "hCG" experimental groups were incubated (37°C) in buffer B, or buffer B with Ca^{2+}(2 mM)/Mg^{2+}(5 mM). DNA was extracted, 3'-end labelled with [^{32}P]-ddATP (250 ng DNA/reaction), resolved by agarose (2%) gel electrophoresis and analyzed by autoradiography.



Experimental groups

Figure 8: Densitometric quantification of DNA ladders (>15 kbp) from nuclei exposed to Ca^{2+} and Mg^{2+} *in vitro* as described in figure 7. "Control" represents nuclei incubated in the absence of cations. Data represent the mean ± SEM of three separate experiments. Different letters above bars represent significantly different values ($p<0.05$).

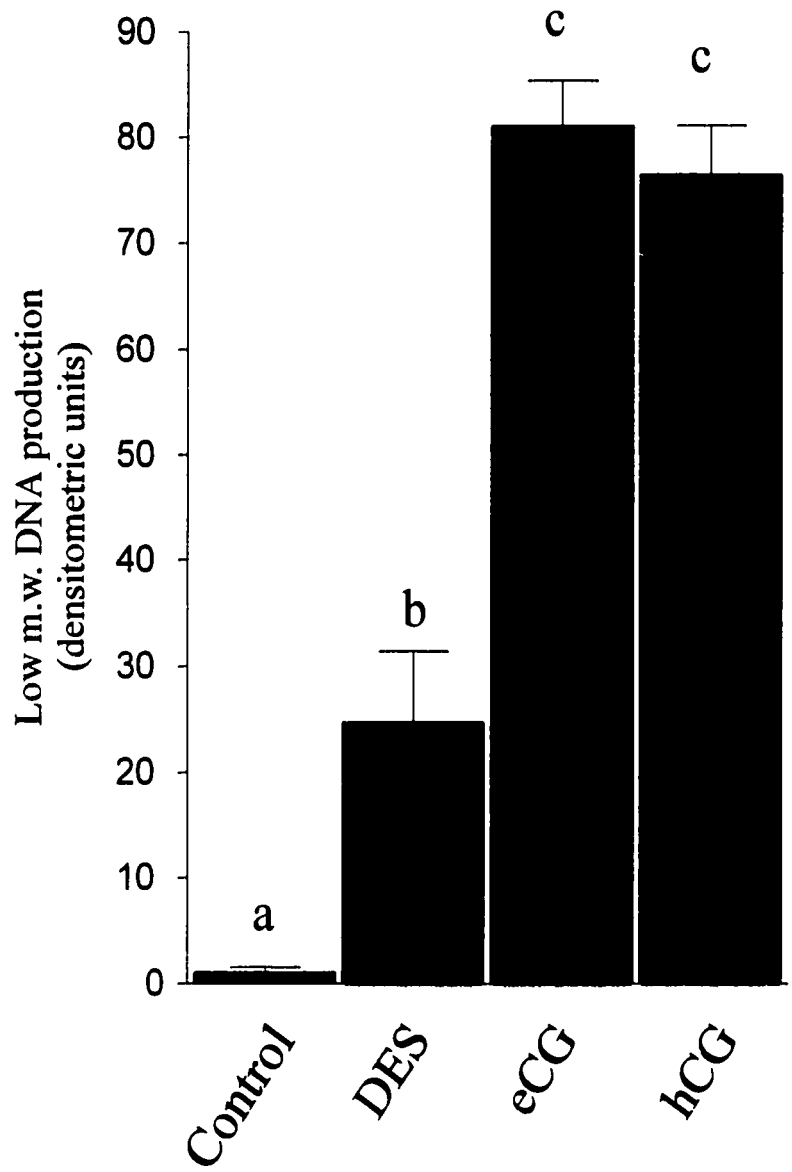

Low m.w. DNA production
(densitometric units)
90
80
70
60
50
40
30
20
10
0
a
b
c
c
Control
DES
eCG
hCG

Figure 9: Cation dependency of endonuclease activity in isolated rat ovarian nuclei. Ovarian nuclei from the "eCG" group were incubated (37°C) in Buffer B with or without Ca^{2+} (2 mM), Mg^{2+} (5 mM), and Zn^{2+} (2 mM), as indicated. DNA was extracted, 3'-end labelled with [^{32}P]-ddATP (250 ng DNA/reaction), resolved by agarose (2%) gel electrophoresis and analyzed by autoradiography.

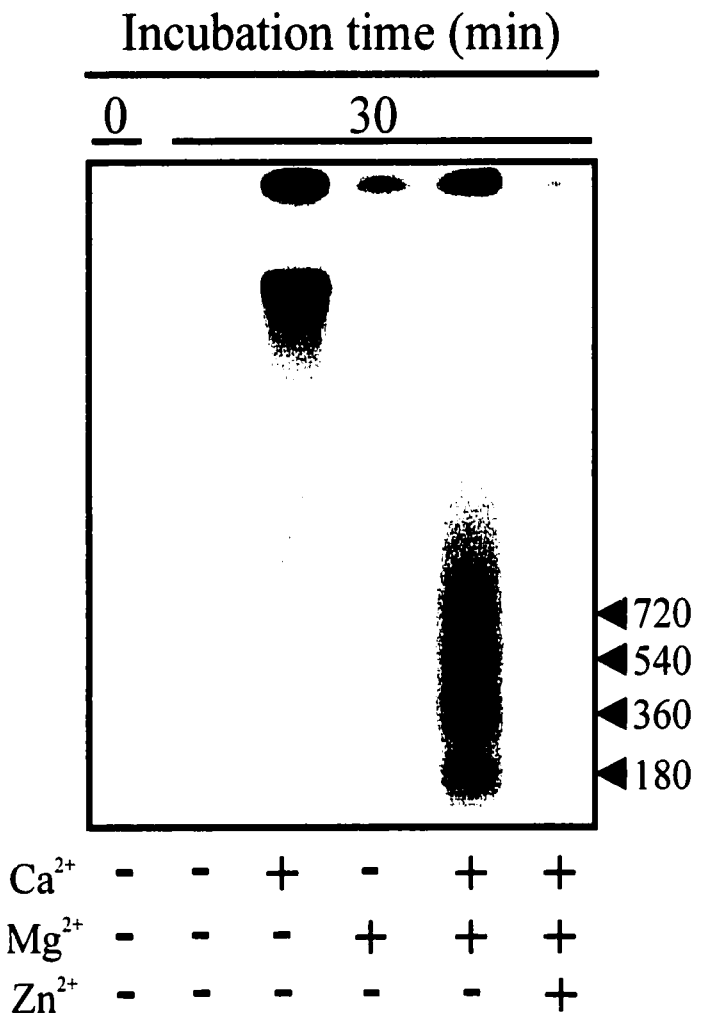
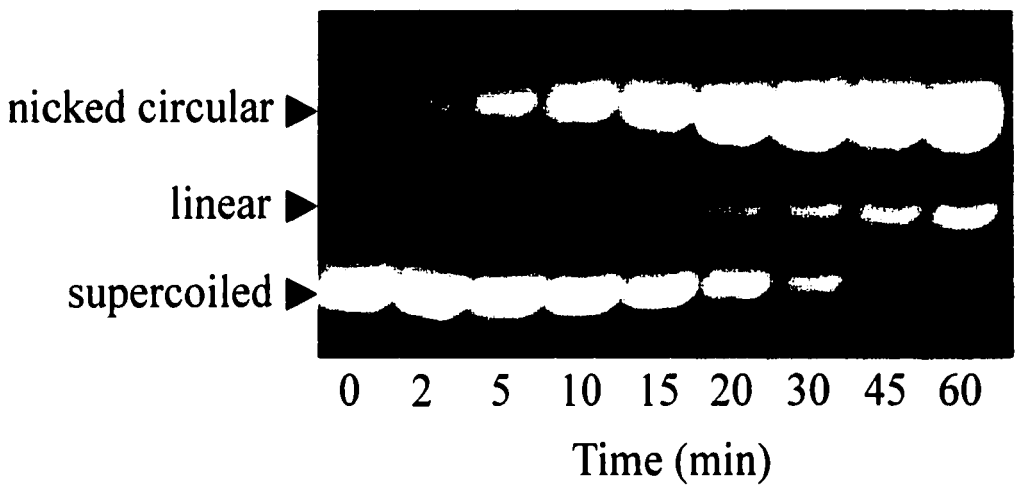

Incubation time (min)
0
30
720
540
360
180
Ca2+ - - + - + +
Mg2+ - - - + + +
Zn2+ - - - - - +

Figure 10: Endonuclease activity in rat ovarian nuclear protein extract. The degradation of plasmid DNA incubated (37°C) with 2.0 μg of ovarian nuclear protein extract from the "eCG" group was resolved by 1% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed on a UV transilluminator. To compare treatment effects, endonuclease activity is measured by densitometrically scanning the ethidium bromide stained gel to monitor the production of nicked circular DNA.


nicked circular
linear
supercoiled
0 2 5 10 15 20 30 45 60
Time (min)

Aliquots of nuclear protein extracts were adjusted so that the production of relaxed circular DNA could be monitored as an index of endonuclease activity as described in the methods section. As shown in figure 11 this assay for endonuclease activity is linear with respect to the amount of protein extract present in the assay at 10, 20 or 30 min incubation (r^2= 0.9615, r^2=0.9652, r^2=0.9708, respectively). Statistical analysis demonstrated linearity as the Chi Square for the results was not significantly different from 1.0.

3.2.2 Endonuclease activity in nuclear protein extracts

Nuclear proteins extracted with 0.35 M NaCl from each treatment group were tested for endonuclease activity by monitoring the degradation of supercoiled plasmid DNA in the presence of divalent cations. Consistent with results obtained with intact nuclei, nuclear extracts from "eCG" and "hCG", but not "DES" groups degraded plasmid DNA in the presence of Ca^{2+} and Mg^{2+} after 10 min (fig. 12). The "DES" group displayed significant endonuclease activity after 30 min incubation, but this was significantly less than the activity present in the "eCG" or "hCG" groups. This extracted endonuclease activity was dependent on both Ca^{2+} (2 mM) and Mg^{2+} (5 mM) and was inhibited by Zn^{2+} (2 mM) (fig. 13).

Figure 11: Endonuclease activity in different amounts of eCG-nuclear protein extract. Activity was measured as described in figure 10 after 10 (squares), 20 (triangles) or 30 (circles) minutes incubation. Data are mean ± SEM of three separate assays.

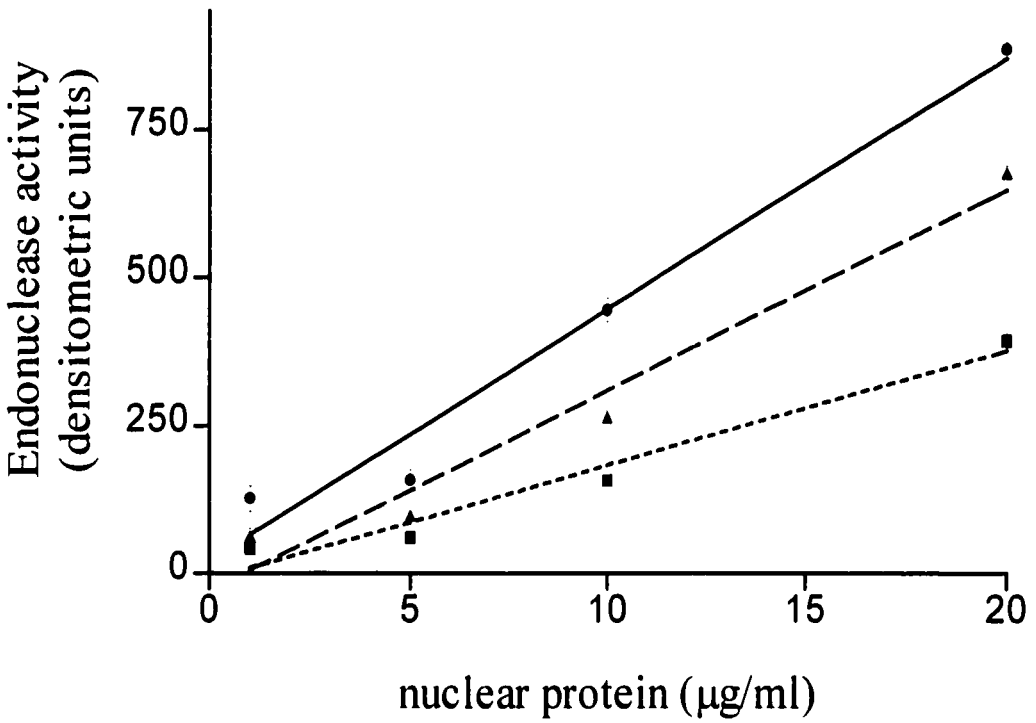

Endonuclease activity
(densitometric units)
750
500
250
0
0
5
10
15
20
nuclear protein (μg/ml)

Figure 12: Developmental pattern of endonuclease activity in ovarian nuclear protein extracts. Aliquots of nuclear protein extract (0.4 μg) from DES (triangle), eCG (diamond) and hCG (circle) groups were incubated (37°C) with plasmid DNA in the presence of Ca^{2+} (2 mM)/Mg^{2+} (5 mM). (square: control, no protein extract). Endonuclease activity was measured as described and data are mean ± SEM of three experiments. (** $P<0.001$ compared to control; * $P<0.05$ compared to control).

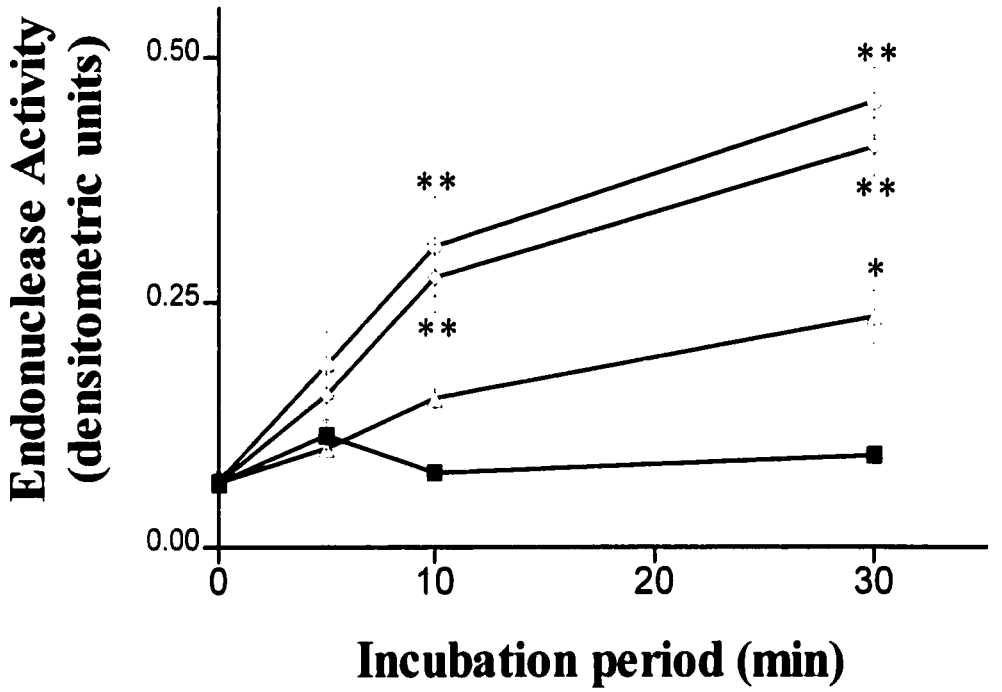
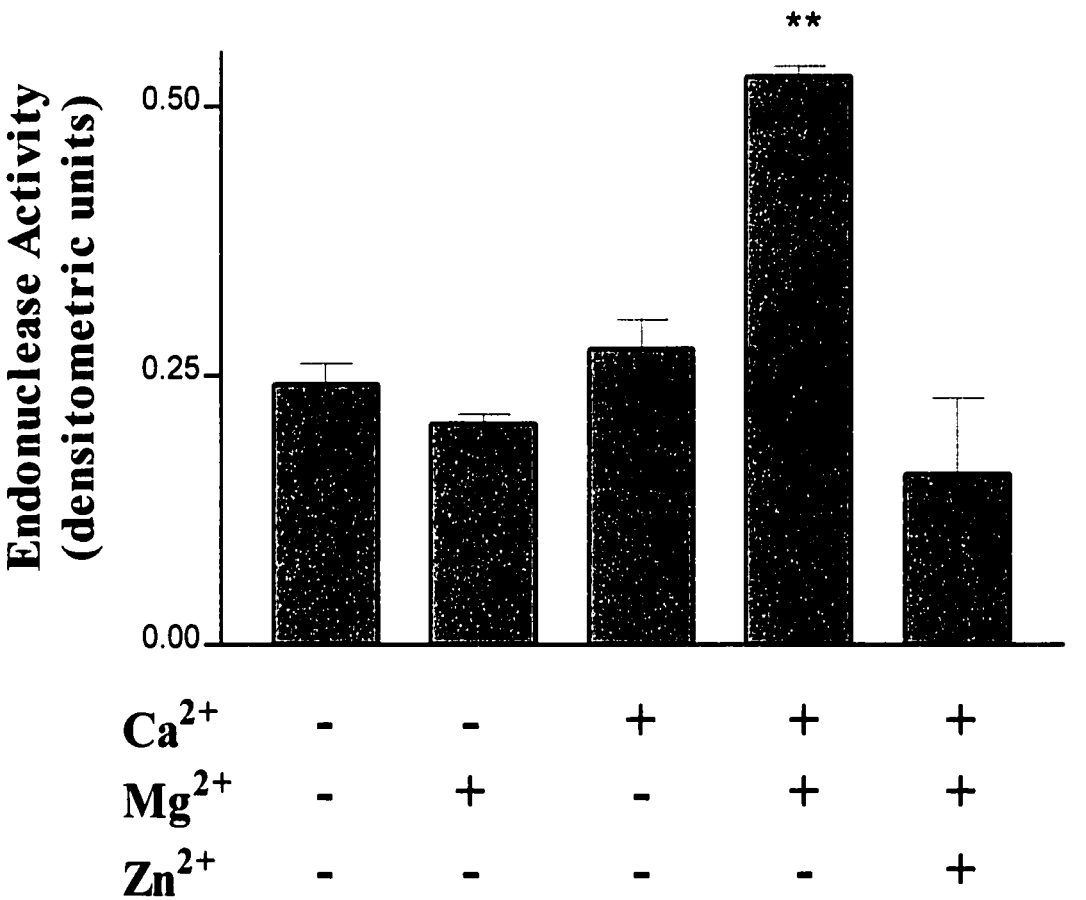

Endonuclease Activity
(densitometric units)
0.50
0.25
0.00
0
10
20
30
**
**
**
*
Incubation period (min)

Figure 13: Cation dependency of endonuclease activity in ovarian nuclear protein extracts. Aliquots of protein extract (0.4 μg) from eCG-treated rat ovaries incubated (37°C, 15 min) with plasmid DNA in the absence and presence of Ca^{2+} (2 mM), Mg^{2+} (5 mM) and Zn^{2+} (2 mM). Endonuclease activity was measured as described and the data presented represent the mean ± SEM of three experiments. (** $P<0.001$ compared to control).


**
Endonuclease Activity
(densitometric units)
0.50
0.25
0.00
Ca2+ - - + + +
Mg2+ - + - + +
Zn2+ - - - - +

3.3 IDENTIFICATION OF NUCLEASES

3.3.1 Zymographic assay

3.3.1.1 Optimization

The method of Rosenthal and Lacks (1977) used 10 μg/ml DNA in the resolving gel but the present findings show that higher concentrations of DNA result in more sensitive detection of nuclease activity. Figure 14 demonstrates that increasing concentrations of DNA in the gel (up to 250 μg/ml DNA) resulted in detection of smaller amounts of bovine DNase I standards. For this reason gels were prepared with 250 μg/ml DNA in all subsequent zymographic analyses.

3.3.1.2 Quantification

Zymographic gels could be scanned densitometrically to quantify the amount of nuclease activity (fig. 15). Although as little as 1.0 ng of DNase I could be measured after 24 h incubation, the assay was much more sensitive if allowed to proceed for 48 or 72 h. These longer time points allowed the routine measurement of as little as 10 or 50 pg of DNase I (fig. 15) The nuclease assay was linear with respect to increasing DNase I at 24, 48 and 72 h incubation (r^2=0.9914, r^2=0.9965, r^2=0.9849, respectively) as the Chi square was not significantly different from 1.0.

Figure 14: Sensitivity of nuclease detection in zymographic gels containing different amounts of DNA. Bovine pancreatic DNase I was resolved by electrophoresis in 12% acrylamide gels co-polymerized with (A) 10, (B) 50, (C) 100, and (D) 250 μg/ml calf thymus DNA, allowed to renature and then incubated (37 °C, 72 h) in the presence of Ca^{2+} (2 mM) and Mg^{2+} (5 mM). Gels were stained with ethidium bromide in order to visualize and photograph the DNA under ultraviolet transillumination.

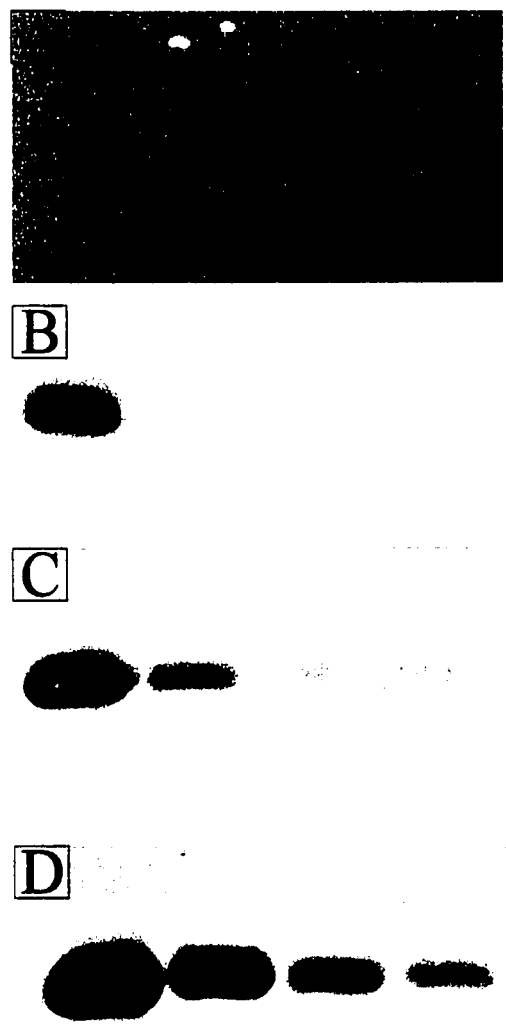
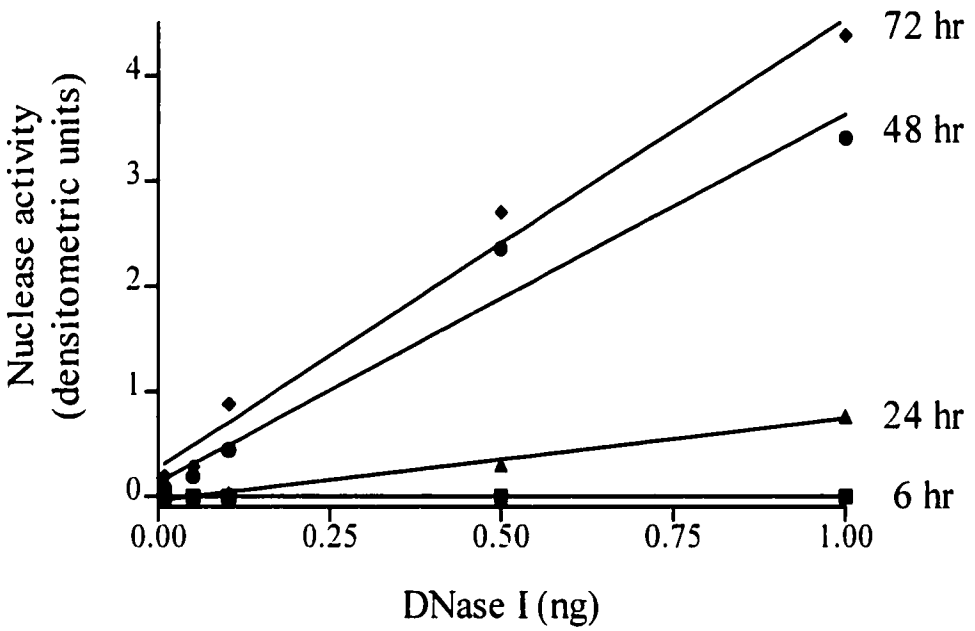

B
C
D
10 5 1 0.5
DNase I (ng)

Figure 15: Nuclease activity of different amounts of bovine DNase I measured by zymography. Gels were incubated as described and photographed after 6 (squares), 24 (triangles), 48 (circles) and 72 (diamonds) h incubation, and nuclease activity of the cleared area on the gel was measured densitometrically.


Nuclease activity
(densitometric units)
4
3
2
1
0
0.00
0.25
0.50
0.75
1.00
DNase I (ng)
72 hr
48 hr
24 hr
6 hr

3.3.2 Zymographic analysis of nuclear protein extracts

Three distinct nuclease activities in the protein extracts of ovarian nuclei were found by zymographic analysis (fig. 16). Two of these activities, with apparent molecular weights of 32 and 34 kDa, had a developmental pattern of expression consistent with the pattern of endonuclease activity observed in intact nuclei. A third nuclease activity (approximately 27 kDa) did not have a developmental pattern of expression and was present in all three treatment groups. The appearance of these nuclease activities was dependent on the conditions of protein separation by SDS-PAGE. The 27 kDa activity was maximal when completely denaturing conditions were used in the SDS-PAGE (dtt in gel loading buffer) and single stranded DNA substrate was present in the gel (fig. 16 A). It was not observed if double stranded DNA was used as substrate (fig.16 C) and was reduced substantially if the SDS-PAGE was performed with semi-denaturing conditions (no dtt in gel loading buffer; fig. 16 B). In contrast, the 32 and 34 kDa bands of activity were most evident when the SDS-PAGE was semi-denaturing and the gel substrate was double stranded DNA (fig. 16 D). The intensities of these bands were diminished if completely denaturing conditions were used during electrophoresis (fig. 16 C) but were not affected dramatically by the type of DNA substrate (fig. 16 B&D). Consistent with the observed cation effects on endogenous endonuclease activity in intact nuclei and protein extracts, the 32 and 34 kDa activities were Ca^{2+}/Mg^{2+} -

Figure 16: Zymographic analysis of nuclease activity in rat ovarian nuclear extracts. Bovine pancreatic DNase I (1ng) or 40 μg of rat ovarian nuclear protein extract from DES, eCG and hCG groups were subjected to electrophoresis in SDS-PAGE (12%) gels containing 250 μg/ml of either single stranded DNA (panels A&B) or double stranded DNA (panels C&D). Electrophoresis was carried out under complete denaturing conditions (panels A&C) or semi-denaturing conditions (panels B&D). After incubation (37°C, 72 h) in the presence of Ca^{2+} (2 mM)/Mg^{2+} (5 mM), nucleases were identified by staining the DNA remaining in the gel with ethidium bromide and photography under UV transillumination. The arrows on the right indicate the positions of the 27, 32 and 34 kDa nuclease activities in the nuclear extracts.

A
B
D
DNase I
DES
eCG
hCG
DNase I
DES
eCG
hCG

dependent and inhibited by Zn^{2+} (Table 4). The 27 kDa activity required only Mg^{2+} for full activity and was also inhibited by zinc (Table 4). The 32 kDa and 34 kDa activity bands had identical cation (Table 4) and substrate requirements and a similar apparent molecular weight when compared to the DNase I from bovine pancreas (approx. 33 kDa) and rat serum (approx. 36 kDa) (fig. 17).

3.3.3 Confirmation of nucleases by silver staining

Nuclear histone proteins can produce false positive nuclease activity in zymography (Boone et al 1997c; Alnemri and Litwack 1989; Baxter et al 1989) because the histones bind to the DNA in the gel and prevent ethidium bromide staining (fig. 18 A, lane 1). Such false positive activity was distinguished from true nuclease activity based on the time-dependent appearance of true nuclease activity (fig. 18 A). In addition, when the DNA in the gel was stained with silver, true clearing of the DNA representing nuclease activity were readily distinguished from dark histone staining (fig. 18 B). Silver staining was found to be as sensitive as ethidium bromide staining for the detection of DNA digestion in the gel (fig. 19). Silver staining of the zymographic gel, shown in figure 16 D, demonstrates that the 32/34 kDa doublet is a true nuclease activity (fig. 20).

Table 4: Effects of cations on the activity of each nuclease compared to bovine DNase I

Treatment	Nuclease activty			
	bovine DNase I	34 kDa	32 kDa	27 kDa
Ca^{2+}(2mM)	-	-	-	-
Mg^{2+}(5mM)	-	-	-	+
Ca^{2+}(2mM)+Mg^{2+}(5mM)	+	+	+	+
Ca^{2+}(2mM)+Mg^{2+}(5mM)+Zn^{2+}(2mM)	-	-	-	-

Figure 17: Zymographic comparison of DNase I to nuclease activity in nuclear protein extract. Electrophoresis and identification of nuclease activity was carried out as described, using semi-denaturing conditions and double stranded DNA in the gel. Lane 1: bovine pancreatic DNase I, 10 ng; lane 2: nuclear protein extract from eCG group, 10 μg; lane 3, rat serum, 40 μg.

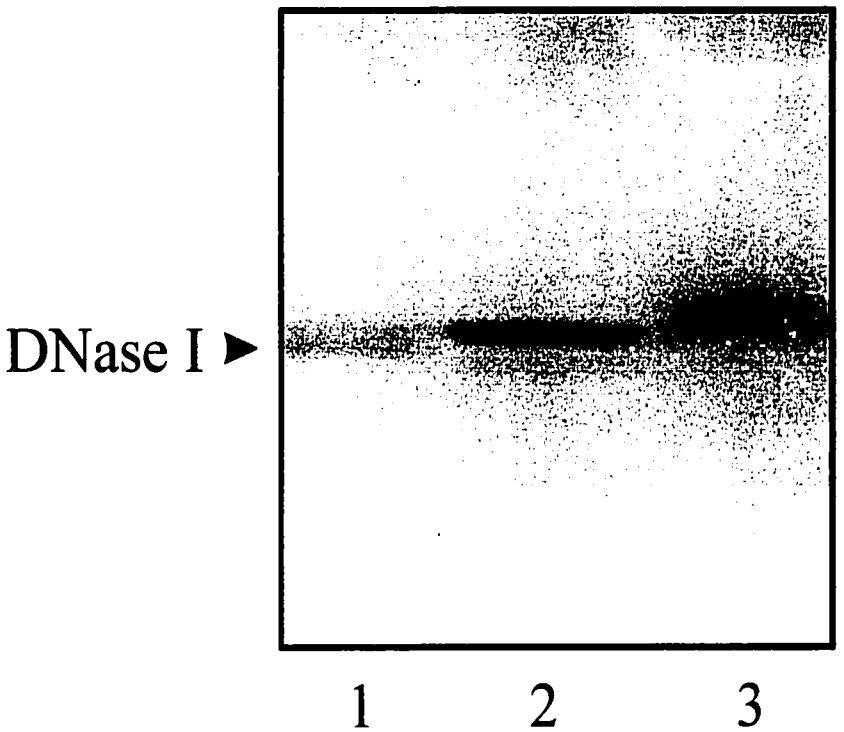

DNase I ►
1
2
3

Figure 18: Nuclease activity is distinct from false positive histone staining. Zymographic analysis was conducted using electrophoresis conditions as previously described. High salt (1.0 M NaCl) nuclear protein extract (40 μg) was used to demonstrate false positive histone (H) staining (lane 1) as compared to true nuclease activity (indicated by 34) in nuclear extract from eCG group (10 μg; lane 2). Ethidium bromide stained gels (panel A) were photographed under UV transillumination before (0 h) and after (24 h) incubation as described. Gels were then washed in SDS (5%, 1 h) and DNA in the gel was identified by silver staining (panel B) to distinguish false positive histone (H) (lane 1) from true nuclease activity (lane 2).

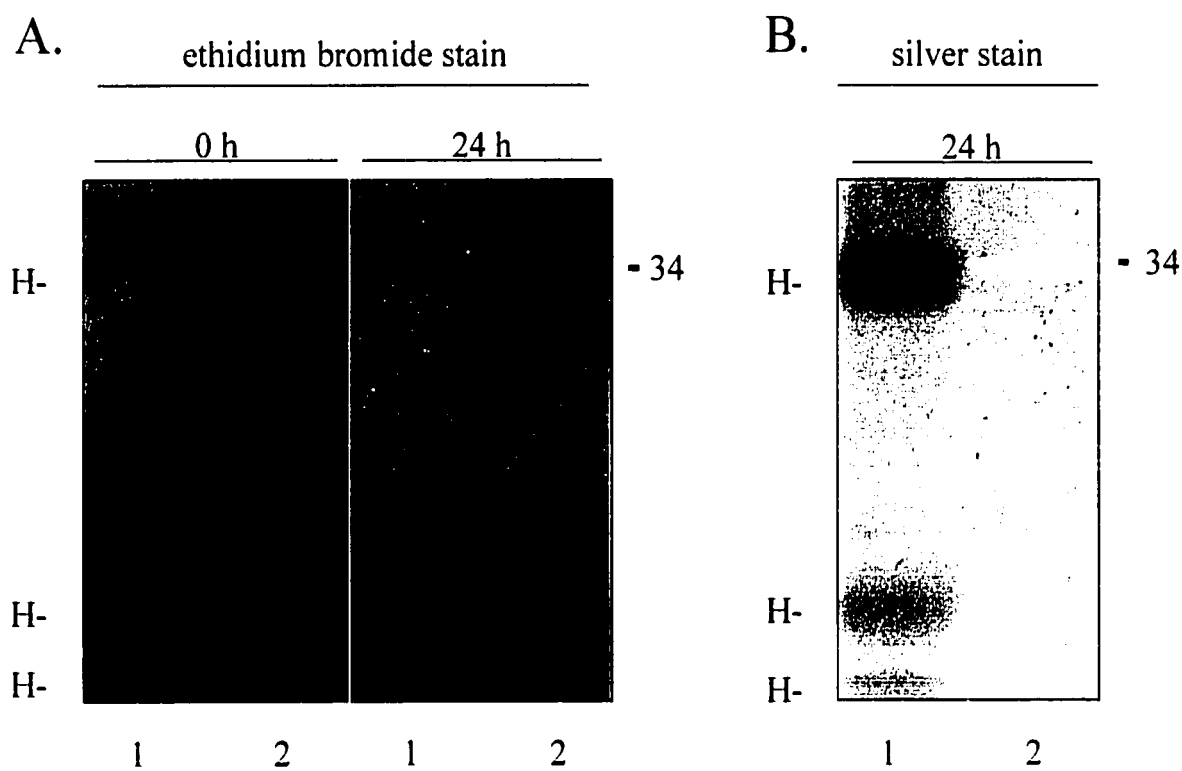

A.
ethidium bromide stain
0 h
24 h
H-
H-
H-
34
1
2
1
2
B.
silver stain
24 h
H-
H-
H-
34
1
2

Figure 19: Comparison of silver (A) and ethidium bromide (B) staining of zymographic gel. DNase I (500,100, 50 and 10 pg bovine pancreatic DNase I) activity was detected with equal sensitivity using silver (A) or ethidium bromide (B) staining .

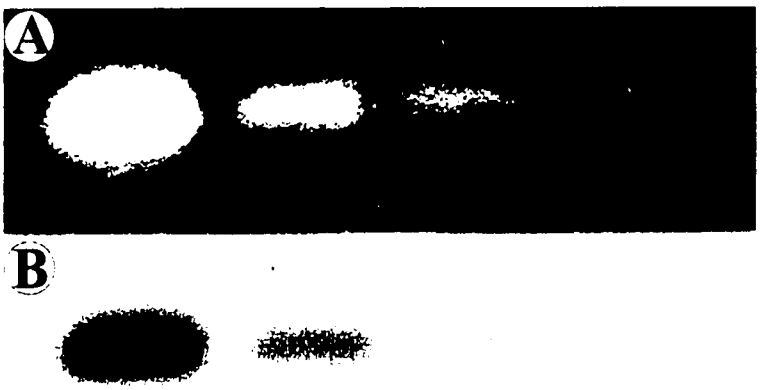
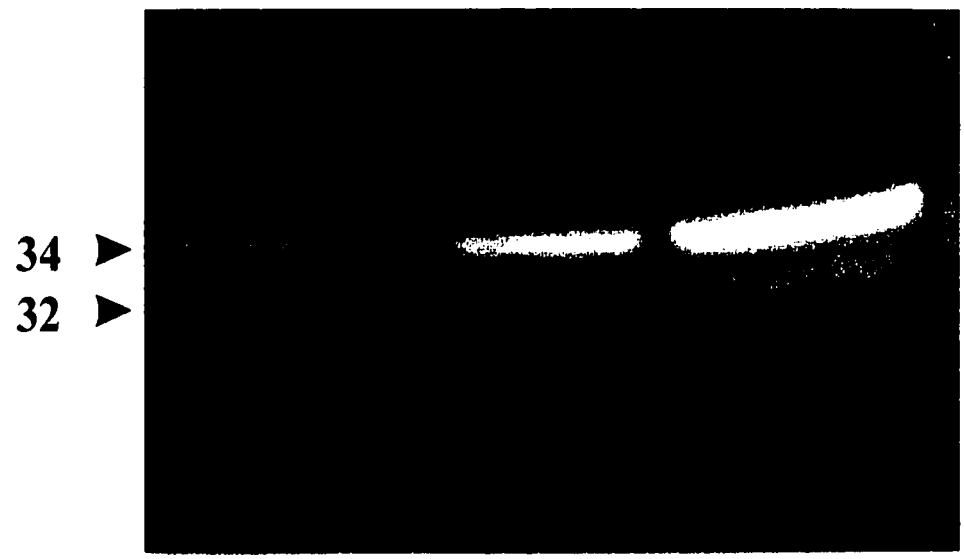

A
B
1
2
3
4

Figure 20: Silver staining of zymographic gel showing the nuclease activities in nuclear protein extracts of DES, eCG and hCG groups. Proteins were resolved by electrophoresis and incubated 72 h, as described. Molecular weights (kDa) are shown on the left.


34
32
DES
eCG
hCG

3.4 BIOCHEMICAL CHARACTERISTICS OF ENDONUCLEASE

3.4.1 Cation and pH requirements

Nuclear protein extracts from rat ovaries of the eCG group were used to analyze the endonuclease activity by both plasmid degradation and zymographic assays (fig. 21). The activity was found to be dependent on both Ca^{2+} and Mg^{2+} at micromolar to millimolar concentrations. Higher concentrations of either cation (up to 10 mM) had slight but insignificant inhibitory effects on the endonuclease activity (fig. 21 B). In addition, Mn^{2+} (2 mM) alone was sufficient to activate the endonuclease (fig. 21 A). The endonuclease was found to have optimal activity at neutral pH and was inactive at pH $\leq$6 or pH 9 (fig. 22).

3.4.2 Sensitivity to inhibitors

The sensitivity of the endonuclease activity to a variety of inhibitors that inhibit DNase I or apoptosis in other cell types was tested (fig. 23). Endonuclease activity was inhibited by Zn^{2+} at concentrations of 0.1 and 1.0 mM, but not 0.01 mM. The reducing agent dtt, which breaks disulfide links in proteins, inhibited the endonuclease activity. Finally, the nuclease inhibitor AUR suppressed the endonuclease activity in the ovarian extracts at all concentrations tested (0.01-1.0 mM). Iodoacetic acid (IAA) and

Figure 21: Zymographic (A) and plasmid degradation (B) analysis of cation requirements of nuclease activity in eCG-nuclear extract. (A) Zymographic analysis of cation requirements for Ca^{2+} (2 mM), Mg^{2+} (5 mM) or Mn^{2+} (2 mM). (B) Plasmid degradation analysis (mean ± SEM of 4 experiments) of endonuclease requirements for Ca^{2+} [in the presence of 5 mM Mg^{2+} (open squares)], and for Mg^{2+} [in the presence of 2 mM Ca^{2+} (closed triangles)].

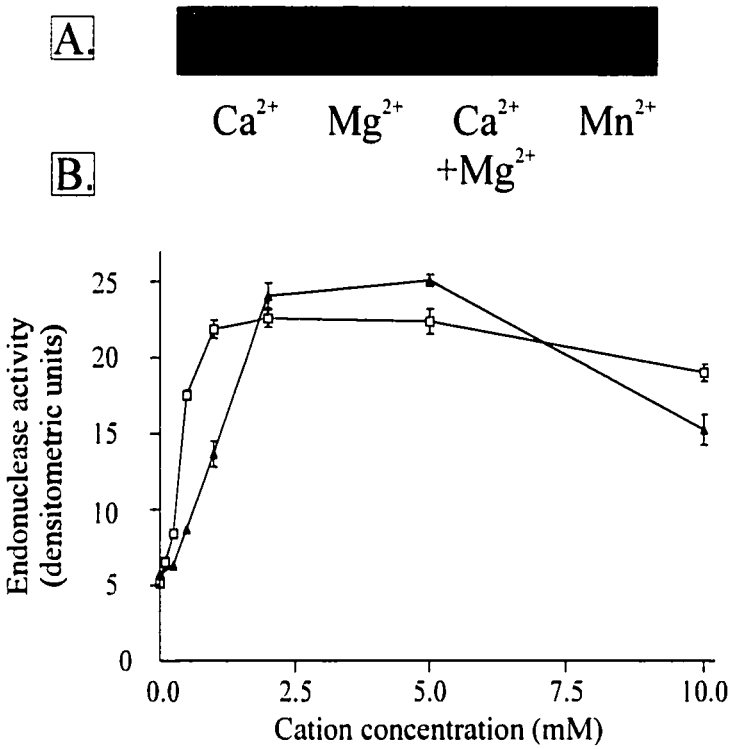

A.
Ca2+
Mg2+
Ca2+
+Mg2+
Mn2+
B.
Endonuclease activity
(densitometric units)
25
20
15
10
5
0
0.0
2.5
5.0
7.5
10.0
Cation concentration (mM)

Figure 22: Zymographic analysis of pH requirements of nuclease activity in eCG-nuclear extract. (A) a representative gel showing nuclease activity in eCG-nuclear extract from three experiments, and (B) the results of three experiments (mean ± SEM), respectively.

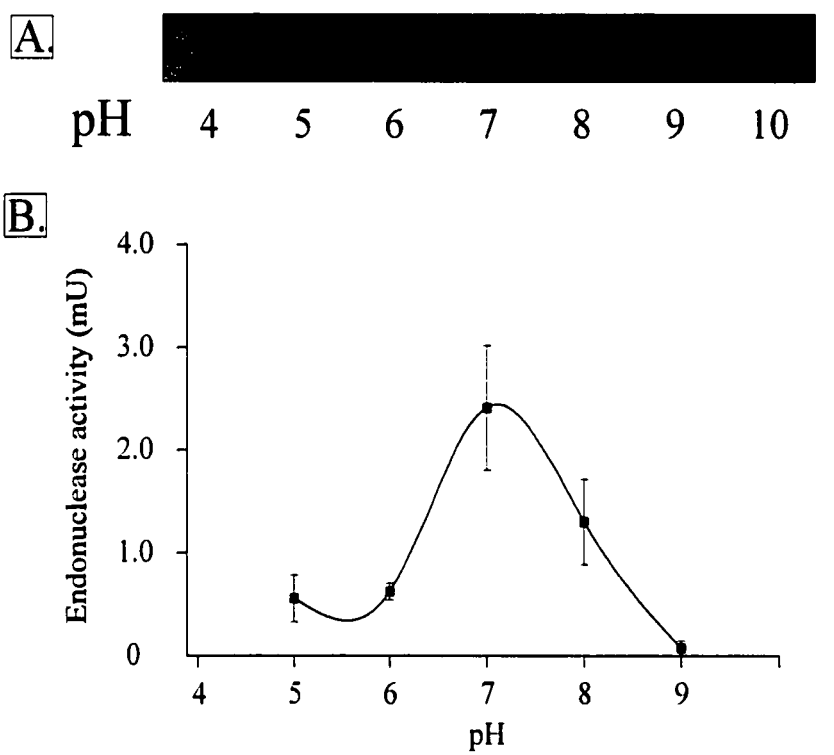
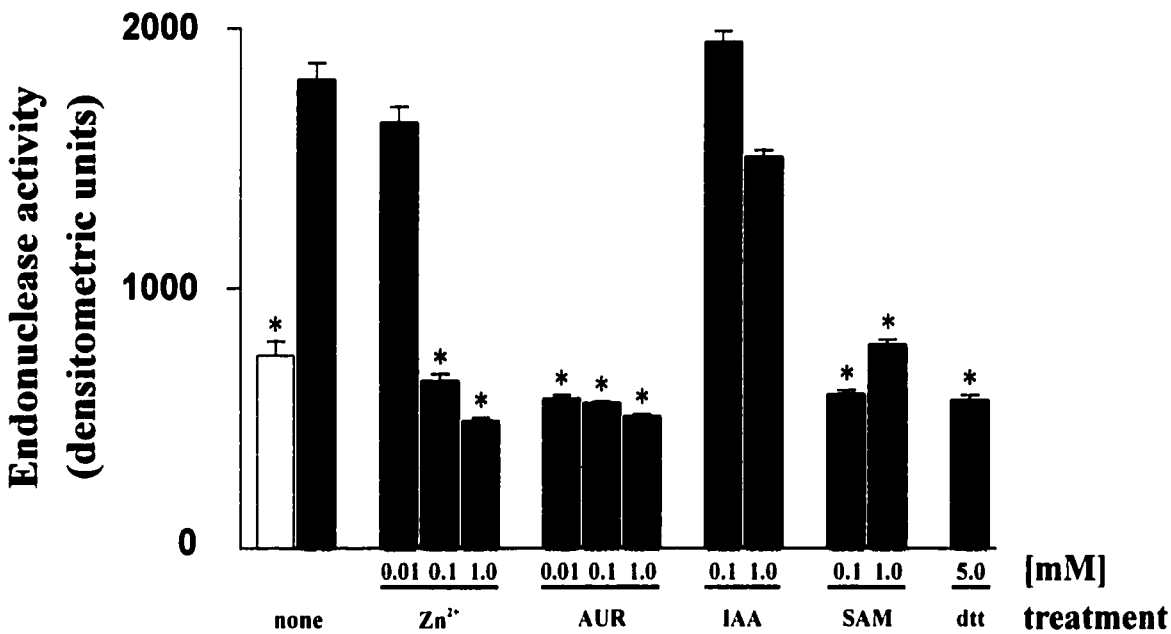

A.
pH 4 5 6 7 8 9 10
B.
Endonuclease activity (mU)
4.0
3.0
2.0
1.0
0
4 5 6 7 8 9
pH

Figure 23: The influence of Zn^{2+}, AUR, SAM, dtt and IAA on endonuclease activity in ovarian nuclear extract. Endonuclease activity was measured in the absence (open bar) or presence (solid bar) of Ca^{2+} (2 mM) and Mg^{2+} (5 mM), as determined by plasmid degradation assay. Results represent mean ± SEM of 4 experiments (* $p<0.05$ when compared to activity in the presence of Ca^{2+}/Mg^{2+} but in the absence of exogenous inhibitors).


Endonuclease activity
(densitometric units)
2000
1000
0
*
0.01 0.1 1.0
0.01 0.1 1.0
0.1 1.0
0.1 1.0
5.0
[mM]
none
Zn2+
AUR
IAA
SAM
dtt
treatment

sodium aurothiomaleate (SAM), two caspase inhibitors previously demonstrated to inhibit DNA degradation in isolated rat follicles (Flaws et al 1995b), were tested for their ability to modulate the endonuclease activity. SAM, but not IAA, was found to be an effective inhibitor (fig. 23).

3.5 IMMUNOLOGICAL AND FUNCTIONAL CHARACTERISTICS OF ENDONUCLEASE

3.5.1 Western analysis and immunoprecipitation

As previously demonstrated in our laboratory, a DNase I-like endonuclease activity was present in the rat ovarian nuclei of eCG and hCG but not DES treatment groups (fig. 24 A). Western analysis using an anti-DNase I antibody identified DNase I immunoreactivity in bands with corresponding molecular weight and developmental pattern similar to those of the endonuclease activity found by zymography (fig. 24 B), suggesting that the DNase I immunoreactive band and the band of nuclease activity were one and the same. To verify this, the anti-DNase I antibody was used to immunoprecipitate DNase I activity from nuclear extracts. As shown in figure 25, the antibody precipitated the band of rat ovarian nuclease and urine DNase I activities corresponding to the ovarian DNase I-like activity and to the bovine pancreas DNase I standard thus immunologically identifying the activity as DNase I. Some nuclease

Figure 24: Zymographic (A) and immunoblot (B) analysis of DNase I in nuclear protein extracts. Nuclear protein extract (40μg) was resolved by electrophoresis in 12% acrylamide gels, then either (A) analyzed for nuclease activity by zymography as described, or (B) electrotransferred (200 mAmp, 1 h) to Immobilon P membrane, and immunoblotted with anti-DNase I antibody as described. Representative results from 3 experiments.

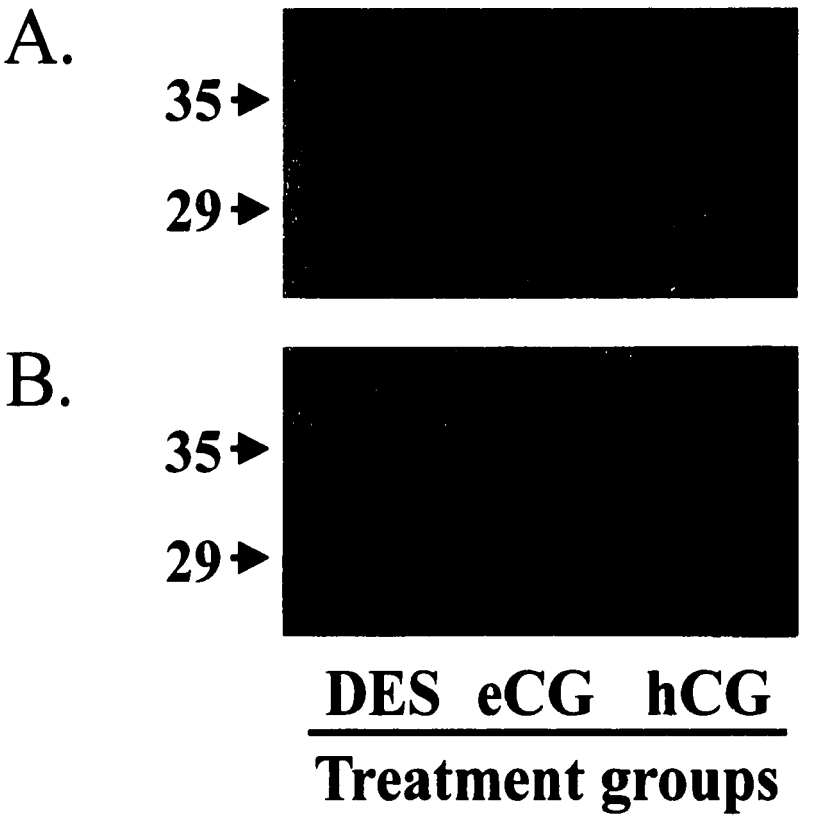
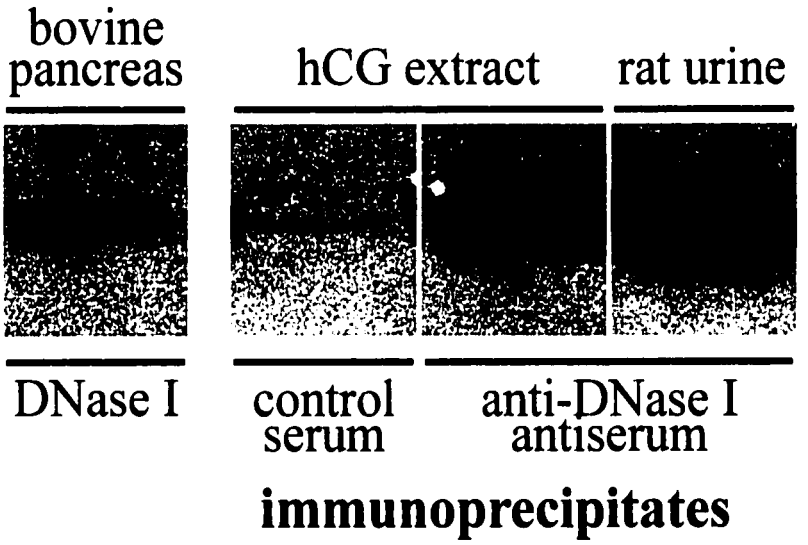

A.
35
29
B.
35
29
DES eCG hCG
Treatment groups

Figure 25: Zymographic analysis of nuclease activity in anti-DNase I antibody immunoprecipitates. Bovine DNase I standard (0.05 ng) immunoprecipitates of hCG nuclear extract with normal rabbit serum or immunoprecipitates of hCG nuclear extract and rat urine with anti-DNase I antibody were analyzed by zymography as described. Representative results of 3 experiments.


bovine pancreas
hCG extract
rat urine
DNase I
control serum
anti-DNase I antiserum
immunoprecipitates

activity was occasionally evident in samples immunoprecipitated with normal rabbit serum. This was due to the presence of DNase I in serum, which cannot be completely removed by washing the sepharose beads used in the precipitation steps.

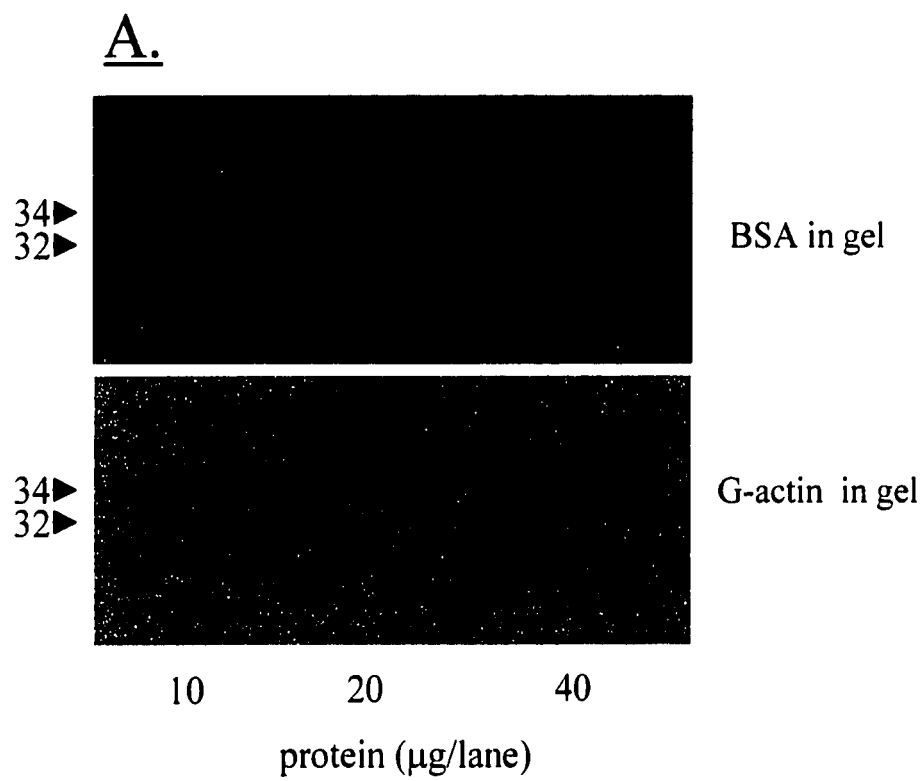
3.5.2 Sensitivity to actin

Since inhibition by actin is a highly characteristic functional feature of DNase I, we determined whether the 32/34 kDa DNase I - like endonuclease activity was sensitive to G-actin. As shown in figure 26, both the 32 and 34 kDa activities were inhibited ($p<0.01$) when G-actin was present in the zymographic gel.

3.6 ROLE OF DNase I IN APOPTOTIC DNA DEGRADATION

It has been demonstrated (Boone et al 1995; Zeleznik et al 1989) that rat ovarian nuclei from the DES group do not degrade their DNA in the presence of Ca^{2+}/Mg^{2+} *in vitro*. For this reason, nuclei from the DES group were used as substrates to demonstrate that DNase I, present in nuclear extracts from eCG-treated rat ovaries, degrades nuclear DNA. As shown in figure 27, nuclei from the DES group did not degrade their DNA in the presence of Ca^{2+}/Mg^{2+} *in vitro*. The addition of eCG extract to DES nuclei produced DNA ladders, demonstrating the presence of the endonuclease in the eCG extract. Immunodepletion of DNase I from the eCG extract significantly

Figure 26: Zymographic analysis of nuclease activity in the presence of G-actin. (A) Representative zymograph in the presence of BSA (control; 100 μg/ml) or G-actin (100 μg/ml). (B) Quantification of DNase I in three zymographic gels (mean ± SEM; **$p<0.01$).


A.
34
32
BSA in gel
34
32
G-actin in gel
10
20
40
protein (µg/lane)

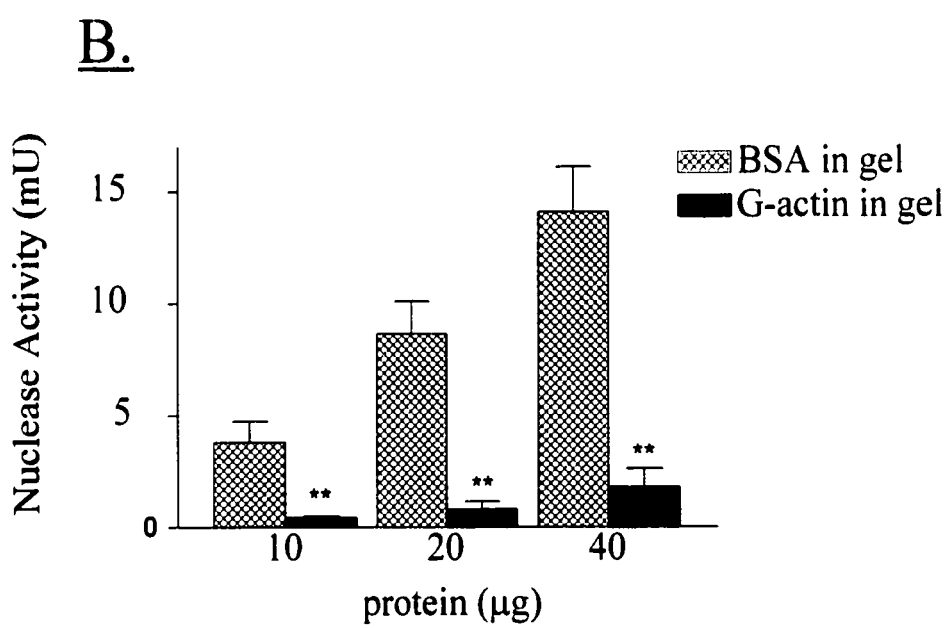
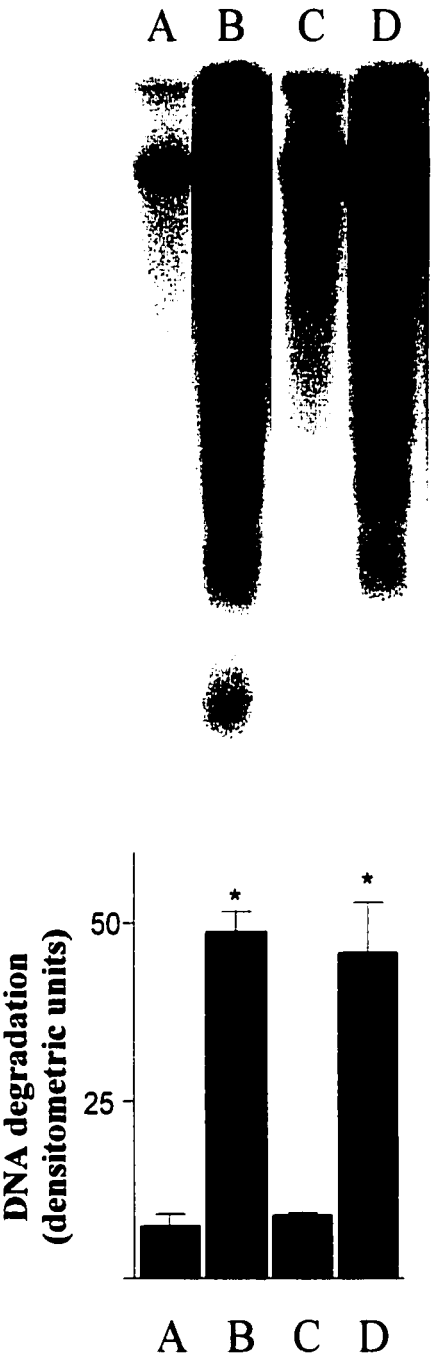

B.
Nuclease Activity (mU)
15
10
5
0
BSA in gel
G-actin in gel
**
**
**
10
20
40
protein (µg)

Figure 27: Effect of immunodepletion with anti-DNase I antibody on endonuclease activity in eCG nuclear protein extract. Electrophoretic analysis of 3'-end labelled DNA isolated from rat ovarian nuclei of the DES group incubated in the presence of: (A) Ca^{2+}/Mg^{2+} (2 mM / 5 mM); (B) Ca^{2+}/Mg^{2+} (2 mM / 5 mM) and eCG nuclear protein extract; (C) Ca^{2+}/Mg^{2+} (2 mM / 5 mM) and eCG extract previously treated with anti-DNase I antibody (for DNase I immunodepletion); or (D) Ca^{2+}/Mg^{2+} (2 mM / 5 mM) and eCG extract previously treated with anti-IgG antibody (control). Upper panel: representative autoradiogram; lower panel: quantification of DNA ladders (mean ± SEM) from 3 different experiments. (* indicates significant difference from lane A, $p<0.001$)


A B C D
DNA degradation (densitometric units)
50
25
*
*
A B C D

reduced the ability of the extract to produce ladders in DES nuclei. Control immunodepletions with anti-IgG antibody did not significantly reduce ability of the eCG extract to produce ladders in DES nuclei.

3.7 IMMUNOLOCALIZATION OF DNase I DURING OVARIAN FOLLICULAR DEVELOPMENT AND ATRESIA

Since the endonuclease was indistinguishable from DNase I, the anti-DNase I antibody was used in the localization of DNase I in rat tissues (fig. 28). DNase I immunoreactivity was present in the nuclei of luteal cells (fig. 28 i) and granulosa cells of antral (fig. 28 f,g,h) but not preantral (fig. 28 b,d) follicles, consistent with the developmental pattern of DNase I activity found by zymography and Western analysis. Not all granulosa or luteal cell nuclei contained DNase I immunoreactivity, and some cytoplasmic staining was also evident in the luteal cells. Theca cells typically exhibited only peripheral nuclear staining for DNase I, but some also displayed nuclear staining (fig. 28 g,h). Interestingly, granulosa cells in a morphologically atretic preantral follicle also showed positive staining for DNase I (fig. 28 c), which suggests that the presence of DNase I may correlate with atresia in this follicle type. Oocytes in many preantral follicles were positively stained (fig. 28 b,c,d) while oocytes did not stain for DNase I in antral follicles (fig. 28 g). Oocyte staining often appeared to be restricted to the ooplasm and was excluded from the germinal vesicle and the zona pellucida (fig.

Figure 28: Immunohistochemical staining of rat tissues with anti-DNase I antibody. Sections were incubated with anti-DNase I antiserum (1:20), washed, exposed to peroxidase-conjugated chicken anti-rabbit IgG antibody (1:500), washed, and developed with DAB/$CuSO_4$. Positive DNase I staining appears black. Preantral follicles (b,c,d); Antral follicles (f,g,h); Luteal tissue (i); Adult rat testes (j). An atretic preantral follicle (c) displayed DNase I staining in granulosa and theca cells, whereas these cells did not stain in healthy preantral follicles (b,d). Oocytes in preantral (b,d) but not antral (g) follicles stained for DNase I. In antral follicles (f,h) most nuclei of granulosa cells but not theca cells stained for DNase I. In adult rat testes (j) spermatagonia (arrow) stained for DNase I, whereas spermatocytes (arrowheads) did not. For controls (a,e) primary antibody was replaced with 1:20 normal rabbit serum and some non-specific staining (e) was evident in theca cells. [f: 100×] [a,e,j: 160×] [b,c,d,g,h,i: 250×] A: antrum, CL: corpus luteum, E: epithelial cells, G: granulosa cells, O: oocyte, T: theca cells, ST: seminiferous tubule.

28 d). Ovarian epithelial cells were also observed to stain positively for DNase I (fig. 28 d). The same pattern of staining for DNase I was obtained using anti-bovine DNase I antibody (Chemicon, Temecula CA) (data not shown). In the adult rat testes (fig. 28 j), DNase I staining was evident in spermatogonia (arrow), but not spermatocytes (arrowheads),within seminiferous tubules.

3.8 NUCLEASE ACTIVITY IN NON-OVARIAN TISSUES AND OVARIES OF DIFFERENT SPECIES

Zymography of nuclear extracts from different tissues of an immature (30 day old) female rat (fig. 29) identified large amounts of activity, corresponding in molecular weight to DNase I, in the liver, kidney and spleen. Extracts from brain, fat and uterus as well as from adult rat (80 day old) testes did not contain detectable levels of nuclease. The 32 kDa protein was more evident in the kidney and spleen while the 34 kDa activity was predominant in the ovary and liver. Zymographic analysis of nuclear extracts from cow, human and chicken ovaries (fig. 30) identified bands of nuclease activity ranging from 30-34 kDa.

Figure 29: Zymographic analysis of DNase I activity in non-ovarian rat tissues. Nuclear extract (40 μg) from different rat tissues, compared to DNase I from bovine pancreas (0.05 ng). A representative result of three experiments is shown.

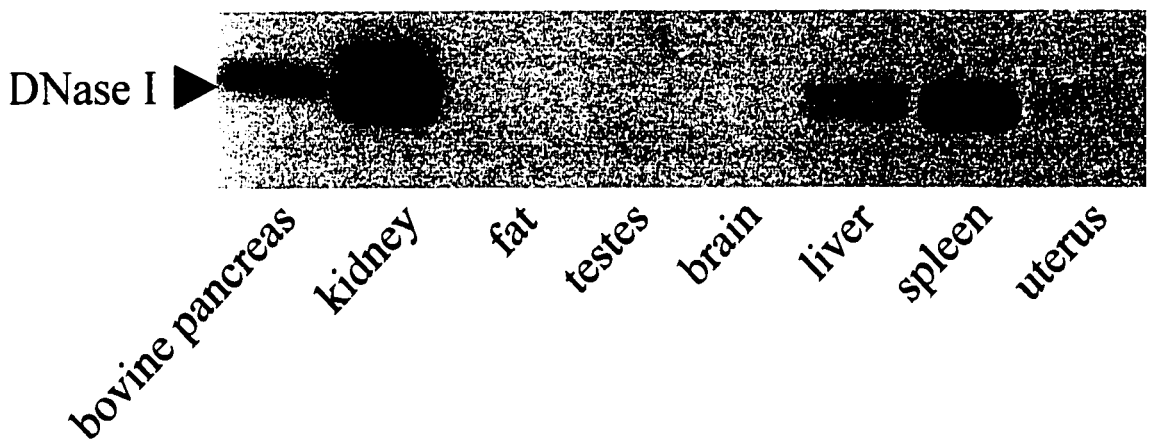
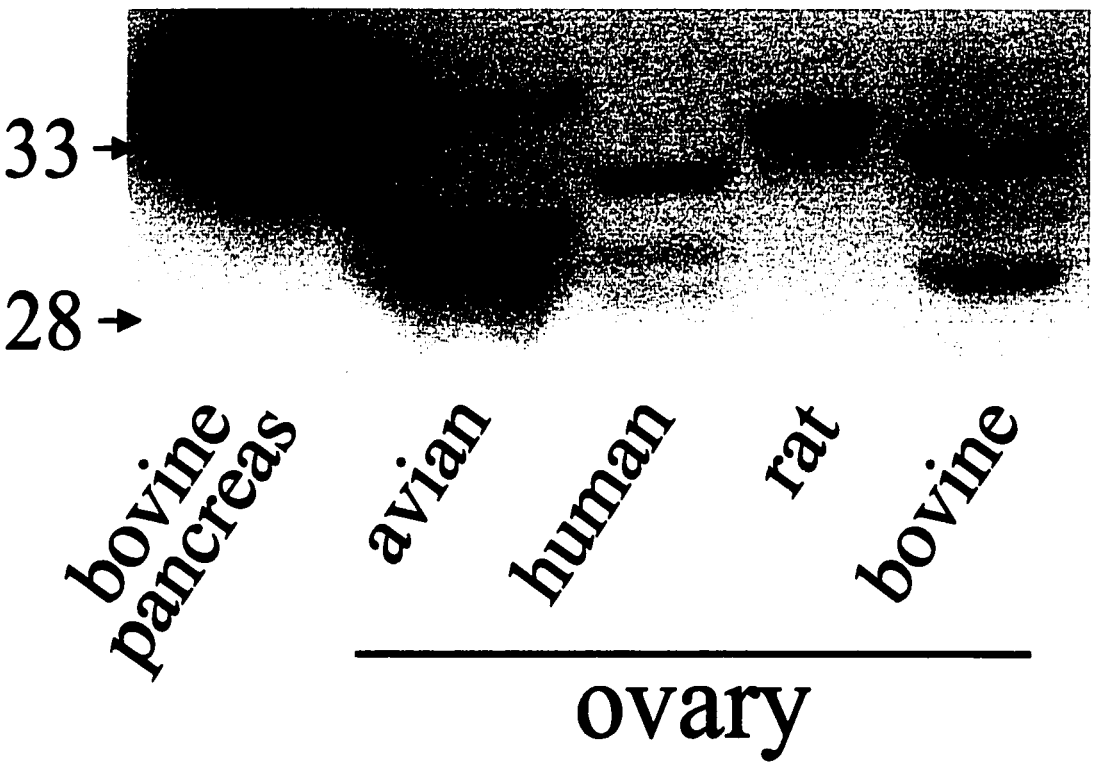

DNase I
bovine pancreas
kidney
fat
testes
brain
liver
spleen
uterus

Figure 30: Zymographic analysis of endonuclease activity in 10 μg of nuclear extract from ovaries of different species. The "bovine pancreas" lane contains bovine pancreatic DNase I (0.5 ng). Molecular weights (kDa) are indicated on the left.


33
28
bovine pancreas
avian
human
rat
bovine
ovary

3.9 DNase I mRNA EXPRESSION

The mRNA encoding DNase I was present in rat ovarian cells, as demonstrated by RT-PCR with DNase I specific oligonucleotide primers (fig. 31). DNase I transcripts could be amplified from both DES and eCG-treated rat ovaries but there was a developmental pattern of DNase I expression. The eCG group had significantly higher levels of DNase I mRNA than the DES group.

3.10 INDUCTION OF FOLLICULAR ATRESIA BY ANTI-eCG ANTIBODY

3.10.1 Anti-eCG IgG titer

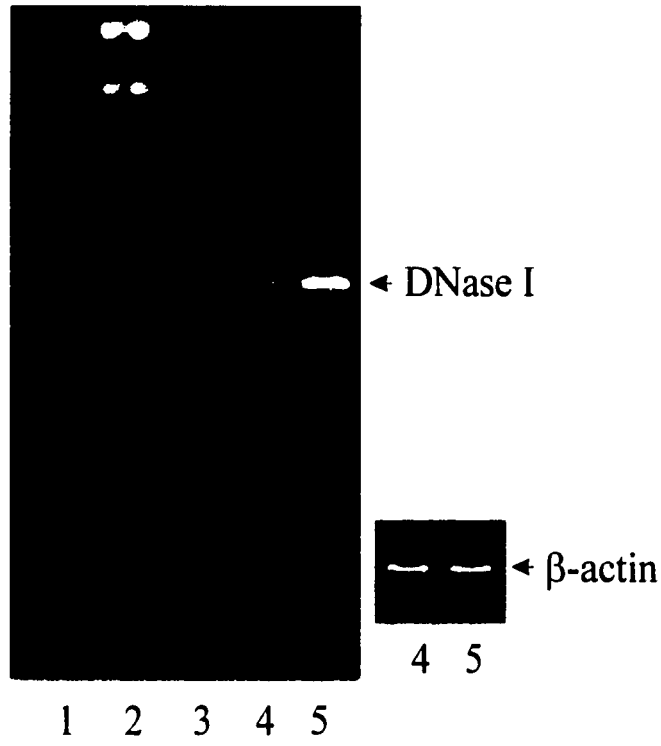
The anti-eCG titer in serum from multiple bleeds of two immunized rabbits was analyzed by end-point ELISA. All titers fell within the range of $1.6\text{-}1.9 \times 10^6$ and, since there was no significant difference between bleeds or rabbits in the titer of anti-eCG IgG, the serum was pooled for subsequent studies.

3.10.2 Anti-eCG bioassay

As shown in figure 32, a significant gain in ovarian weight occurred between 24 and 48 h after injection of eCG to immature rats. Ovaries removed 48 h after eCG

Figure 31: Developmental pattern of DNase I mRNA in the rat ovary. (A) Total RNA (1μg) extracted from rat ovaries of DES (lane 4) and eCG (lane 5) groups was reverse transcribed by incubation (42°C, 1 h) with AMV-RT and then amplified with rat DNase I specific primers in the presence of Taq polymerase, resolved by electrophoresis and visualized by ethidium bromide staining and UV transillumination as described. Lane 1: control (no RNA); lane 2: 0.5μg ϕ174 HaeIII digest (mw markers); lane 3: PCR product of rat DNase I cDNA. Inset shows the PCR product from β-actin specific primers of the DES (lane 4) and eCG (lane 5) samples, used for the normalization of the densitometric analysis of DNase I PCR product. One representative of five experiments is shown. (B) Densitometric quantification of DNase I level in DES- and eCG-treated rat ovarian mRNA amplified by RT-PCR. (bars represent mean ± SEM of five experiments; * represents significant difference $p<0.05$).

A.


DNase I
β-actin
4 5
1 2 3 4 5

B.

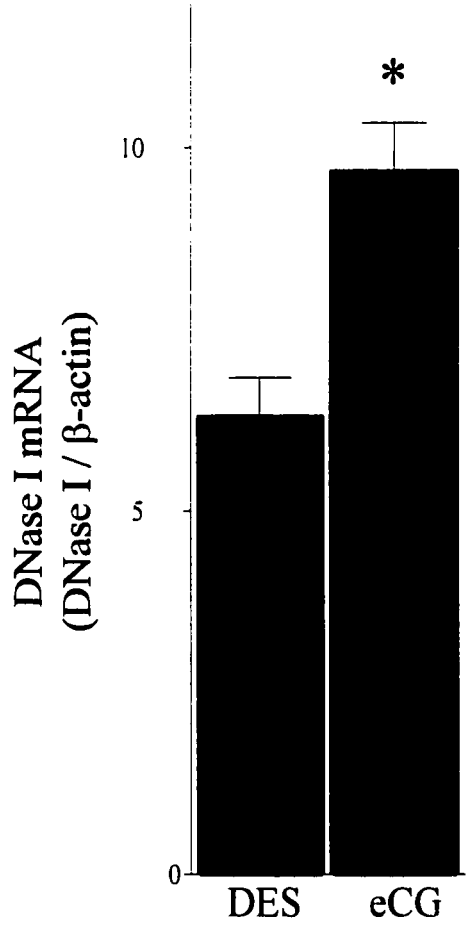
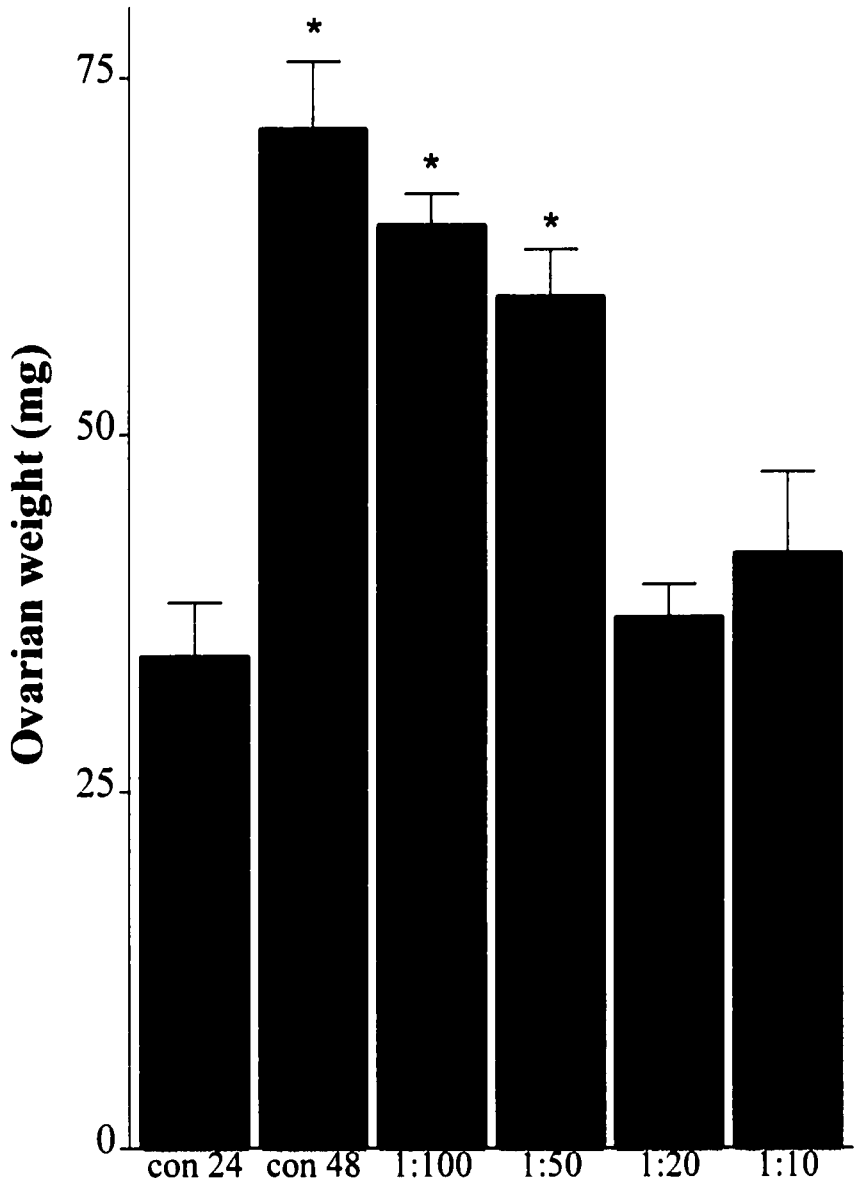

*
10
5
0
DNase I mRNA
(DNase I / β-actin)
DES
eCG

Figure 32: The effect of anti-eCG antiserum on ovarian weight in eCG-treated rats. Rats injected with eCG were sacrificed 24 h later (con 24) or subsequently injected with normal rabbit serum (con 48) or anti-eCG antiserum (at indicated dilutions) and ovaries removed 24 h later. Bars represent mean ± SEM of five separate experiments. (*significant difference from con 24 ($p<0.05$)).

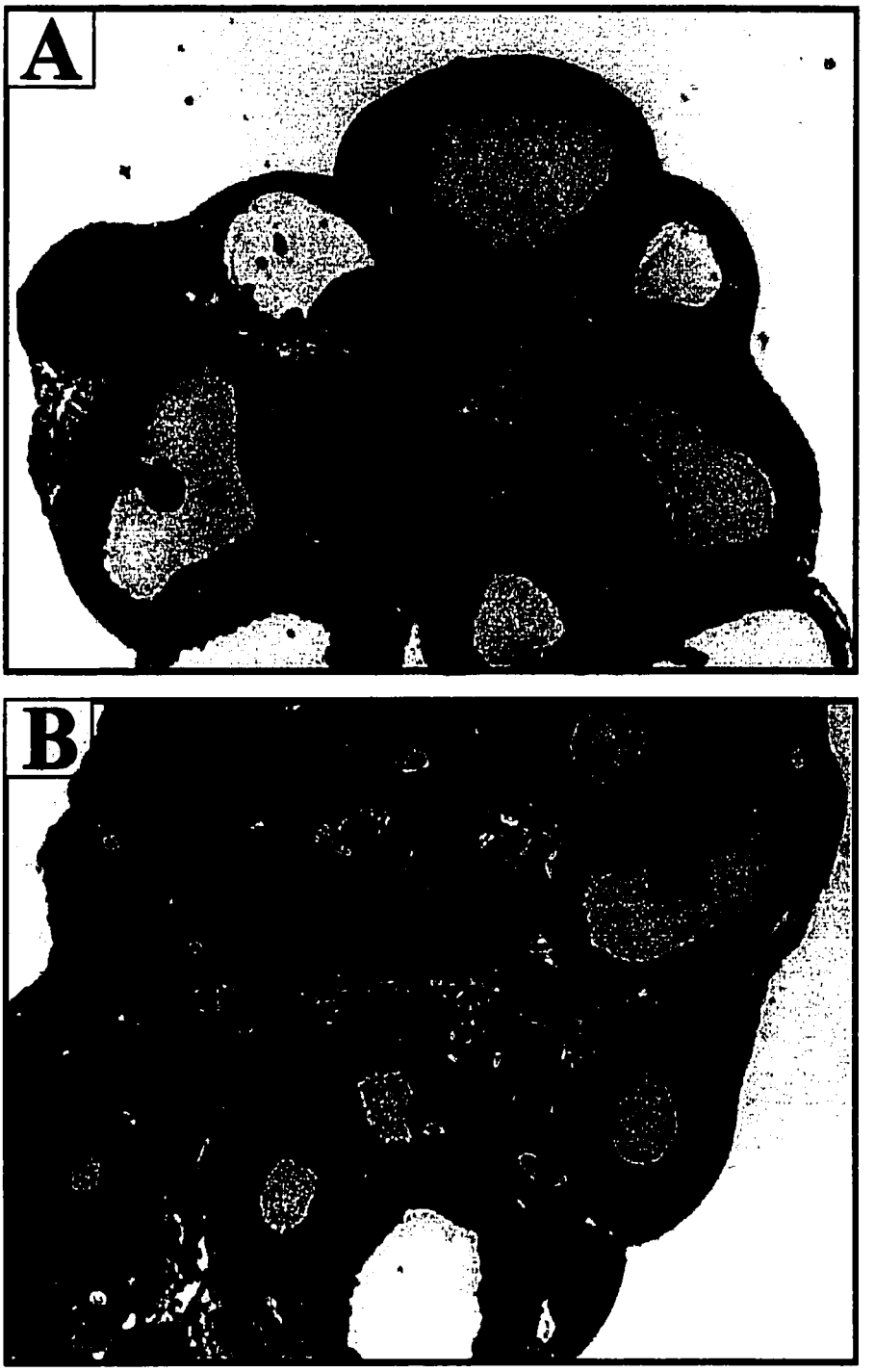

Ovarian weight (mg)
75
50
25
0
*
*
*
con 24
con 48
1:100
1:50
1:20
1:10

treatment contained numerous mature, fluid-filled follicles. Intraperitoneal injection of 100 µl of 1:10 or 1:20 dilutions of anti-eCG antiserum prevented the gain in ovarian weight observed between 24 and 48 h in eCG-treated rats (fig. 32). Large fluid filled follicles were less apparent in the ovaries of rats treated with 1:10 or 1:20 dilutions of the antiserum.

3.10.3 Histology

Rats were injected with eCG and 24 h later with an anti-eCG antibody or normal rabbit serum (control) and ovaries were removed 24 h after treatment for histological examination. Figure 33 shows a typical result of anti-eCG antibody treatment on gross ovarian morphology. Control ovaries contained numerous large antral follicles at the preovulatory stage (fig. 33 A). Several layers of granulosa and theca cells were evident and healthy oocytes (arrowhead) were often found within the discus proligerous. In contrast, ovaries from antibody-treated animals contained numerous small to medium sized follicles and few larger follicles (fig. 33 B). Granulosa cell layers were uneven, occasionally detached from the basal lamina, and loose granulosa cells could sometimes be seen within the antral cavity.

Figure 33: Photomicrographs (100×) of histological sections of rat ovaries. Rats were injected with eCG (15 IU, s.c) and 24 h later with normal rabbit serum (A) or anti-eCG antibody (B). Ovaries removed 24 h after treatment were processed and stained with hematoxylin/phloxine/saffron. Arrows indicate examples of atretic follicles. Arrowhead indicates a healthy oocyte within the discus proligerous.


A
B

3.10.4 Localization of cell death in the ovary

3.10.4.1 Viable staining

Figure 34 shows whole ovaries removed 24 h after treatments and stained with acridine orange (Delic et al 1991) and viewed by fluorescence microscopy to visualize dying cells, which are identified by brightly staining DNA. Cell death was not observed in control ovaries (fig. 34 B) but was apparent in the granulosa, but not the theca cell layers, of small to medium sized antral follicles of anti-eCG treated rat ovaries (fig. 34 C).

3.10.4.2 TUNEL

The observations of granulosa cell death in the anti-eCG treated rats were confirmed with TUNEL labelling of histological sections. Figure 35 shows a typical result comparing the incidence of DNA fragmentation in follicles from control and antibody-treated rats 24 h after treatment. DNA fragmentation, identified as 3'-end labelling with FITC-conjugated dUTP in the presence of terminal transferase, was evident in granulosa cells in small to medium sized antral follicles of the antibody-treated group (fig. 35, A&B). Although some larger antral follicles in the antibody-treated group showed cells with fragmented DNA (fig. 35, D), most follicles of that size did not

Figure 34: Photomicrographs of viable staining for cell death in rat ovaries. Control (A: 40×; B: 160×) and anti-eCG antibody-treated (C: 160×) ovaries were stained with acridine orange (5μg/ml; 5 min) and visualized with fluorescence microscopy using an FITC filter. Nuclear material in dead cells stains brightly compared to background. Representative result of five experiments.

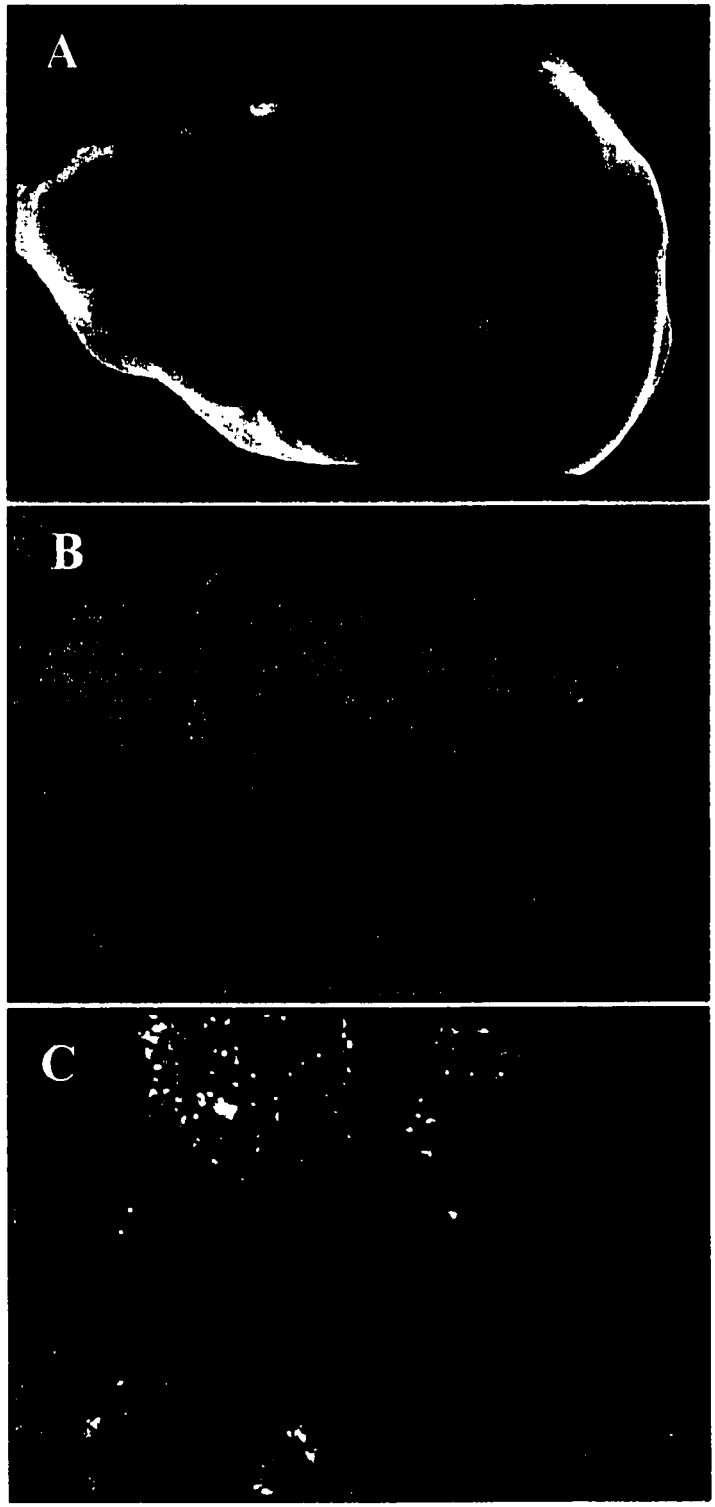
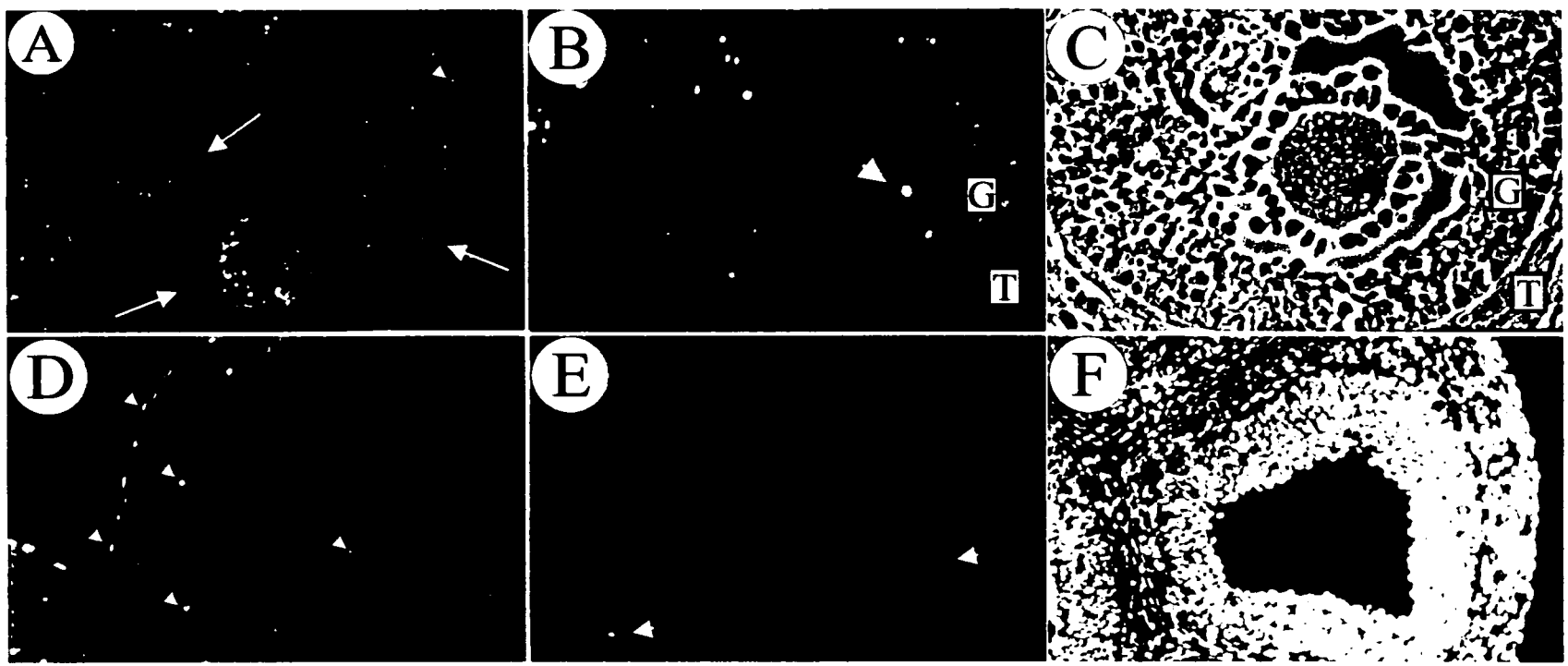

A
B
C

Figure 35: Photomicrographs identifying cell death in rat ovarian follicles. Fluorescent TUNEL staining of anti-eCG antibody-treated (A-D) and control (E) rat ovaries removed 24 h after treatment. A higher magnification (400×) of an atretic follicle from an antibody-treated rat (B: TUNEL; C: phase contrast) demonstrated the occurrence of cell death in granulosa (G) but not theca (T) cells. Granulosa cell death was prevalent in small and medium sized follicles of antibody-treated rats (A&B) and was less frequently observed in large follicles of antibody-treated (D) or control (E) rat ovaries. Note the occurrence of cell death in the ovarian surface epithelial cells (D&E). A positive control (F) for TUNEL , produced by treating the ovarian section with DNase I, showed fluorescence in all cell nuclei. Arrowheads indicate dead cells. Arrows indicate follicles containing dead cells.


A
B
G
T
C
G
T
D
E
F

show DNA fragmentation. In smaller follicles of antibody-treated rats, DNA fragmentation was apparent in granulosa cells throughout the follicle (fig. 35, A&B). Occasionally, cell death was observed in follicles in the control ovaries (fig. 35, E), but to a much lesser extent than in the antibody-treated group. Cell death was rarely observed in theca cells of either treatment group. Interestingly, surface epithelial cells displayed fragmented DNA; this was seen in both control and treated ovaries (fig. 35, D).

3.10.5 Induction of granulosa cell apoptosis by anti-eCG antibody

3.10.5.1 Hoechst staining

Granulosa cells isolated from eCG-treated rats 24 h after injection with normal rabbit serum or anti-eCG antibody were stained for examination of nuclear morphology or frozen for determination of apoptotic DNA production. Morphological indices of apoptotic nuclei (Wyllie et al 1980; Arends et al 1990), including nuclear blebbing, chromatin condensation and increased fluorescence intensity (fig. 36), were evident in some nuclei of granulosa cells isolated from control ovaries (2%, fig. 37 B) but significantly more apoptotic nuclei were observed in granulosa cells from antibody-treated rats (12 %, fig. 37 B; fig. 36 B, arrowheads).

Figure 36: Photomicrograph (400×) of Hoechst stained granulosa cells. Granulosa cells from normal rabbit serum- (control; A) and anti-eCG antibody- (treated; B) rats were stained with Hoechst 33248 (0.1 μg/ml) to visualize nuclear DNA with fluorescence microscopy. Arrowheads indicate apoptotic nuclei. Representative result of five experiments.

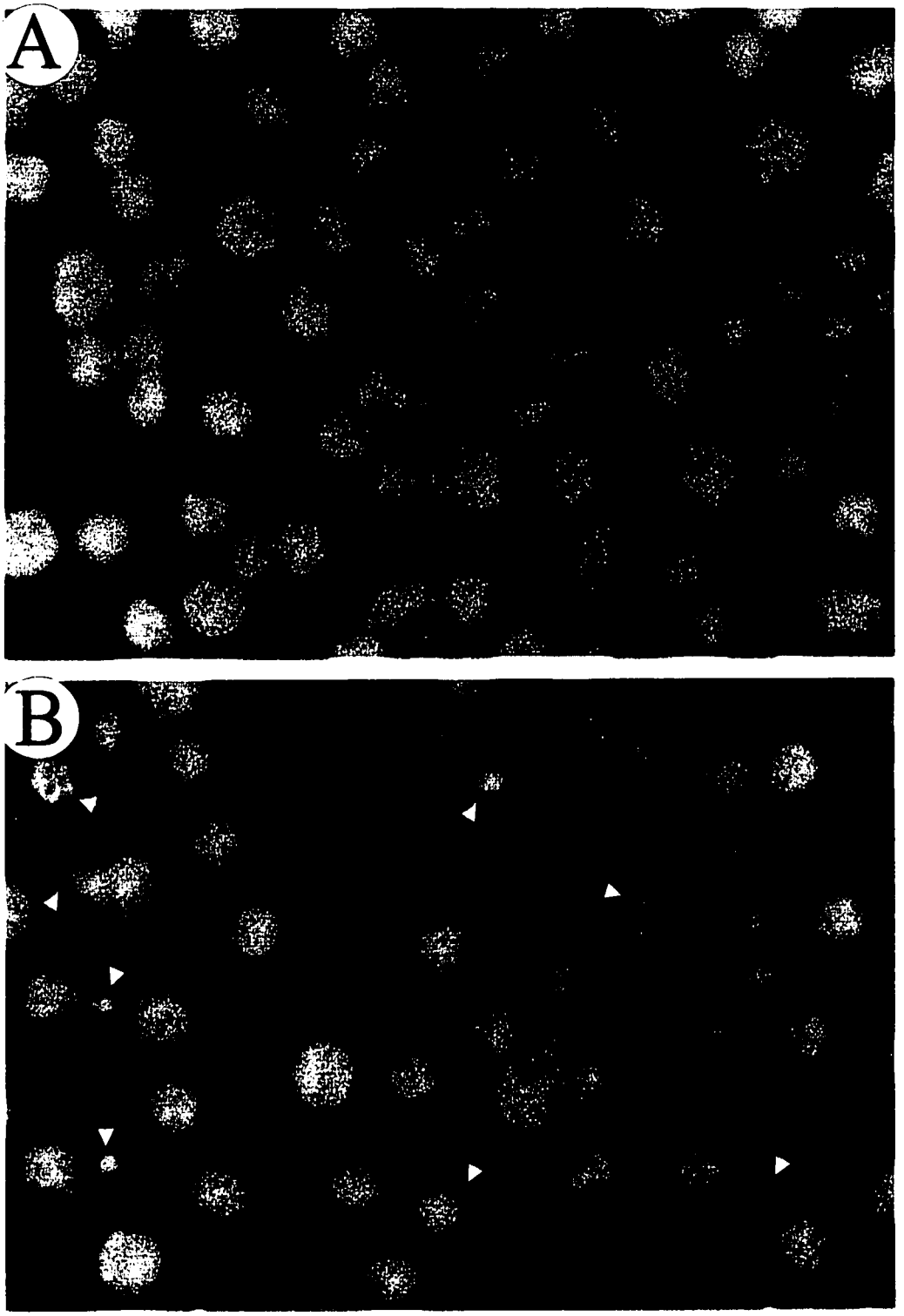
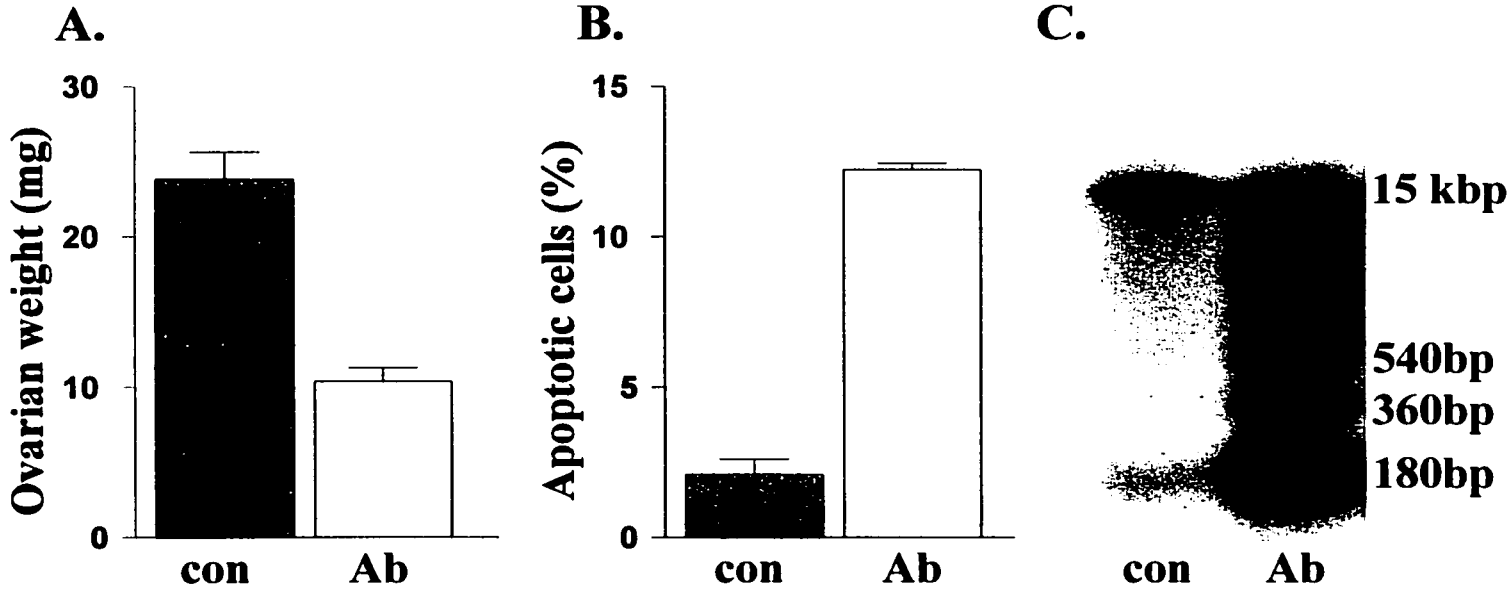

A
B

Figure 37: Physical (A), morphological (B) and biochemical (C) indications of atresia and apoptosis in anti-eCG antibody-treated rat ovaries. Ovaries removed 24 h after treatment with normal rabbit serum (con) or antibody (Ab) were examined for ovarian weight (A), Hoechst-stained apoptotic cell number (B), and apoptosis by DNA ladders (C). A significant difference ($p<0.05$) was found between (Ab) and (con) in (A) and (B). Mean ± SEM of five experiments.


A.
Ovarian weight (mg)
30
20
10
0
con
Ab
B.
Apoptotic cells (%)
15
10
5
0
con
Ab
C.
15 kbp
540bp
360bp
180bp
con
Ab

3.10.5.2 DNA ladders

Electrophoresis followed by autoradiography revealed that the DNA from antibody-treated, but not control, rat granulosa cells displayed the characteristic fragmentation ladder pattern indicative of apoptosis (fig. 37 C). A time course of low molecular weight (<15 Kbp) DNA production in granulosa cells from control and antibody-treated rats indicated that increased low molecular weight DNA production could be observed by 1 h after anti-eCG antibody injection. DNA ladders were consistently observed to be increased after 6 and 24 h in the antibody-treated group (fig. 38). Compared to that observed after 1 h, significantly more low molecular weight DNA was observed 6 and 24 h after treatment ($p<0.05$).

3.10.5.3 Electron microscopy

3.10.5.3.1 Granulosa cells

Granulosa cells with the typical morphological features of apoptosis described by Wyllie et al (1980) were evident in ovaries 1 and 24 h after antibody treatment. Evidence of apoptosis, as shown in figure 39, included increased irregularity of nuclear outline, chromatin condensation, nuclear and cytoplasmic fragmentation and

Figure 38: Time course (1-24 h) of induction of DNA ladders in granulosa cells by anti-eCG antibody. Granulosa cell DNA from control (circles) and antibody-treated (triangles) rats was 3'-end labelled and analyzed by agarose gel electrophoresis and densitometry on low molecular weight (<15 Kbp) DNA. (*$p<0.05$, **$p<0.01$, significantly different from control group at 1 h). Mean ± SEM of five experiments.

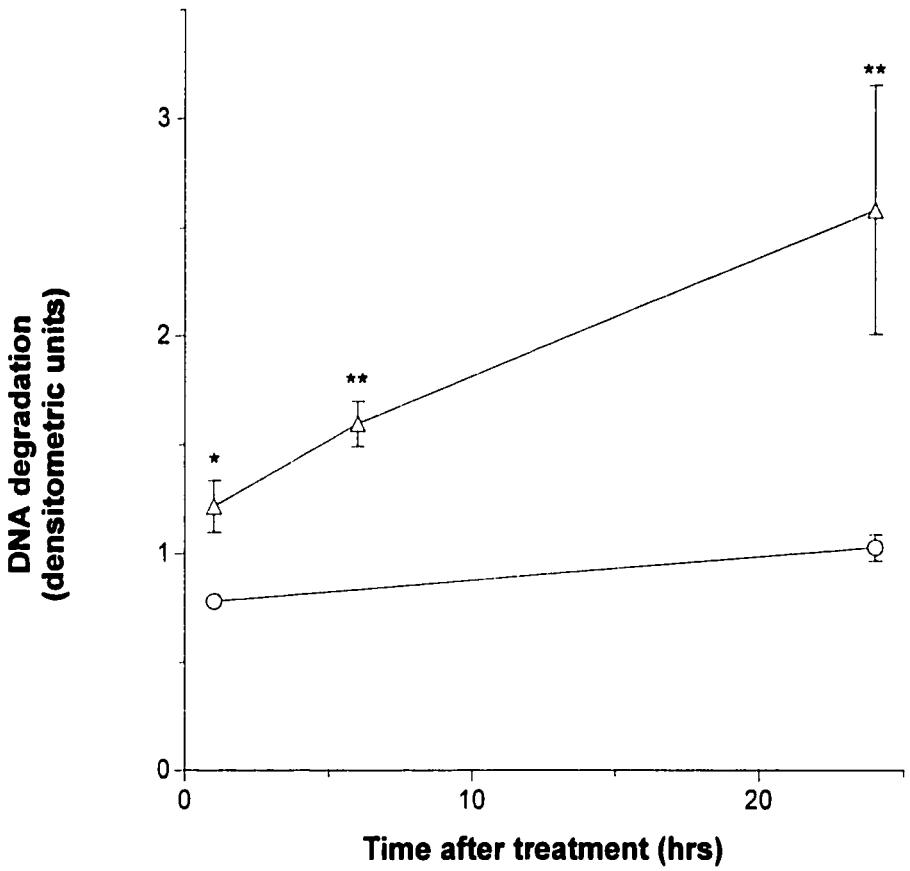
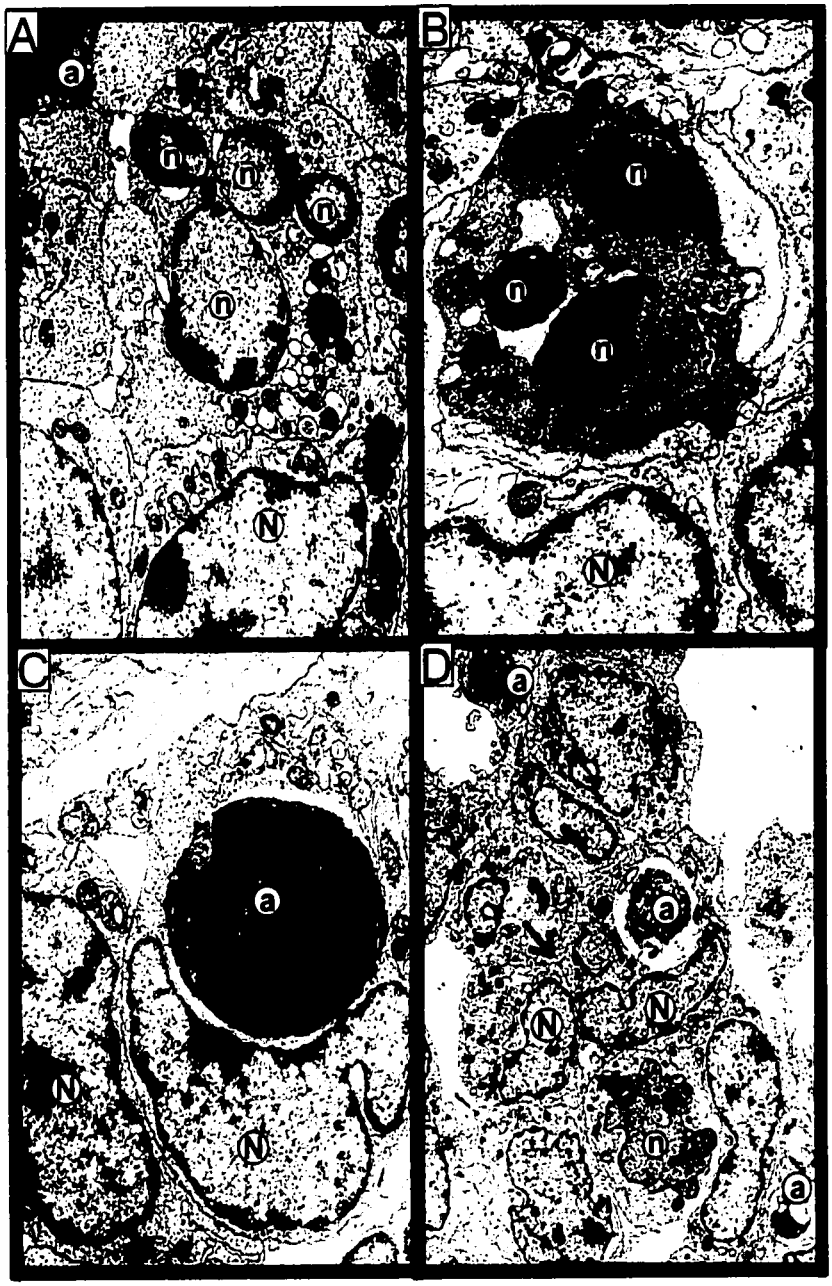

DNA degradation
(densitometric units)
3
2
1
0
0
10
20
Time after treatment (hrs)
*
**
**

Figure 39: Electron micrographs of granulosa cells from anti-eCG antibody treated rat ovaries. (A) Four nuclear fragments (n) can be seen within a single cell from an ovary 1 h after antibody treatment. A healthy nucleus (N) is present in a neighbouring cell. (B) Extensive chromatin condensation (n) within a nucleus from an ovarian cell 24 h after antibody treatment. The condensing cytoplasm appears darker than that of surrounding cells. (C) A large apoptotic body (a) has been engulfed by a neighbouring cell, causing the nucleus (N) in the engulfing cell to deform compared to the nucleus in the neighbouring cell. (D) Numerous apoptotic bodies (a) are evident among granulosa cells 24 h after antibody treatment. A fragmenting nucleus (n) can be seen within a rounded granulosa cell, which is disassociating from surrounding cells (arrowhead), and does not make the same flattened cell-cell contact (arrow) as observed between other granulosa cells. (panel A-C: 600×; panel D: 2000×).


A
a
n
n
n
n
N
B
n
n
n
N
C
a
N
N
D
a
a
N
N
n
a

phagocytosis of apoptotic fragments. Occasionally, granulosa cells with alterations in the appearance of their cell-cell contact could be observed in the antibody treated group (fig. 39 D). Phagocytic bodies were more prevalent in ovaries 24 h after antibody treatment than in any other treatment groups (fig. 39 C&D).

3.10.5.3.2 Basement membrane and theca cells

We did not observe apoptosis in theca cells, although signs of follicle atresia, including increased cytoplasmic lipid droplets and rounded cell shape, were evident in this cell type after antibody treatment (fig. 40 A). The smooth basement membrane-granulosa cell contact found in control ovaries (fig. 40 B) was ruffled in appearance in antibody-treated animals (fig. 40 C). In addition, the lamina densa displayed a lack of regular organization in the antibody-treated group; instead, the basement membrane contained numerous fibrils crisscrossing on the theca side of the membrane (fig. 40 C).

3.11 CASPASE-3 IN THE OVARY

3.11.1 Actin and PARP cleavage during apoptosis in the ovary

Apoptosis was evident as an increase in the extent of DNA ladders in ovarian follicles treated with anti-eCG antiserum, or in luteal tissue from rats treated with $PGF_{2\alpha}$ (fig.

Figure 40: Ultrastructural characteristics of atretic follicles after anti-eCG treatment. (A) Theca cells (T) do not appear apoptotic but do contain numerous lipid droplets not seen in control ovaries. (B) The basement membrane (b) in a control ovary contains parallel fibres (lamina densa) and a smooth granulosa cell (G) - basement membrane interface. (C) The basement membrane (b) in a follicle from the antibody-treated group contains crisscrossing fibrils on the theca (T) side of the membrane. The granulosa cell (G) - basement membrane interface is no longer smooth. (A: 2500×; B&C: 12000×).

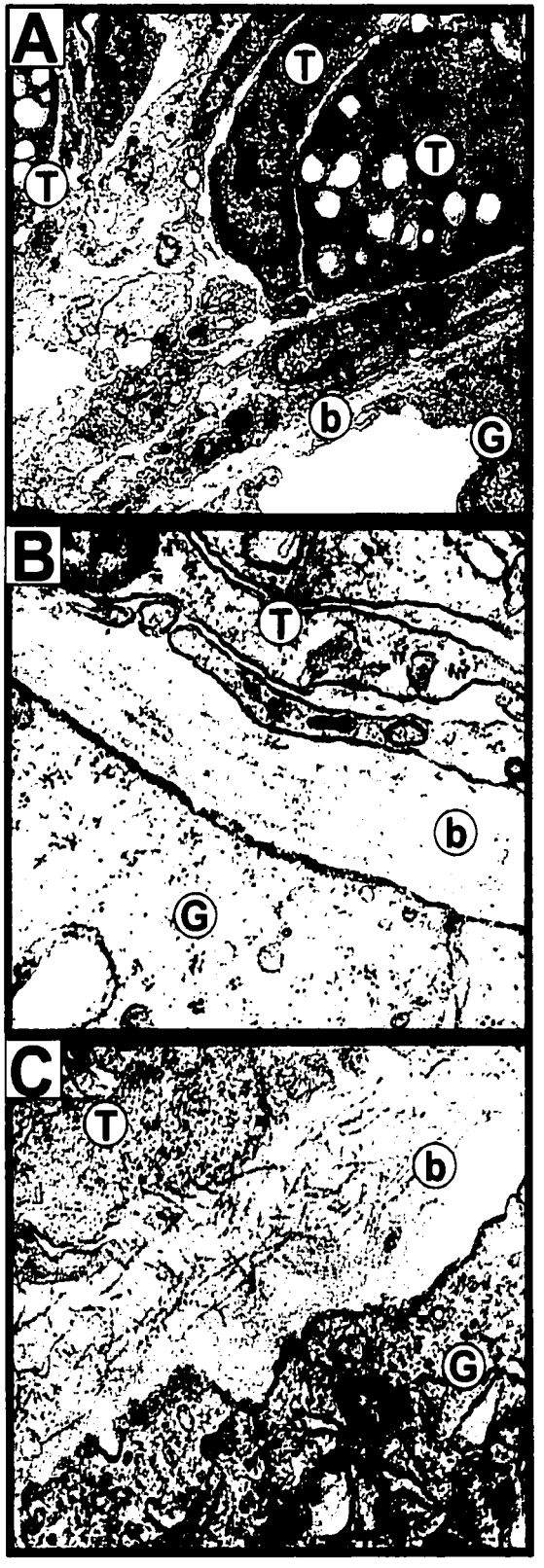
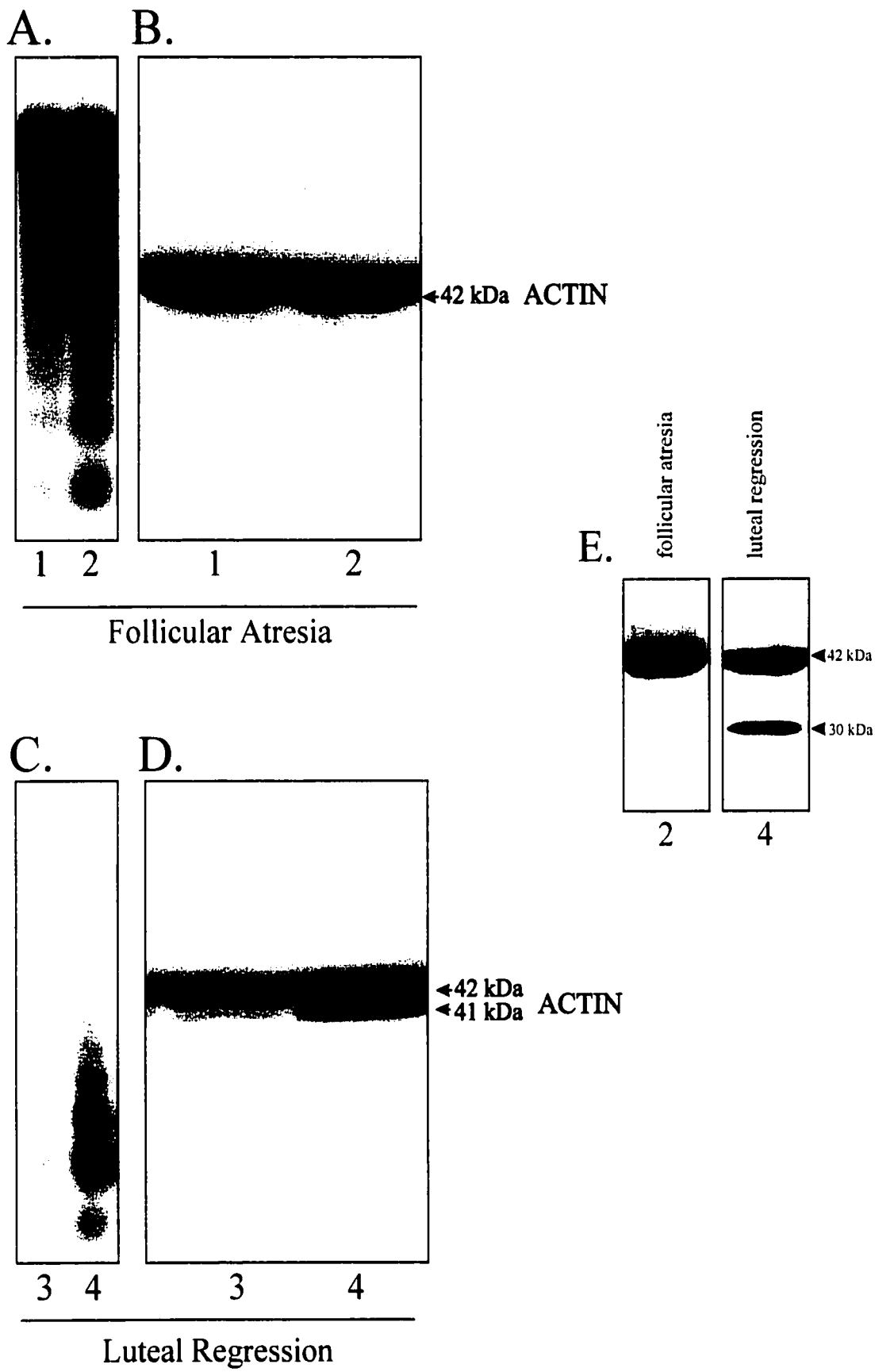

A
T
T
T
b
G
B
T
b
G
C
T
b
G

Figure 41: Low molecular weight DNA degradation and actin cleavage during induced follicular atresia and luteal regression. Follicular atresia following injection of normal serum (lane 1) or anti-eCG antibody (lane 2). Luteal regression following injection of saline (lane 3) or $PGF_{2\alpha}$ (lane 4) as described in the materials and methods. DNA was isolated, end-labelled with [^{32}P]-dCTP and resolved by electrophoresis (panels A&C). Total cell protein was extracted and 40 μg was resolved by 9% SDS-PAGE, electrotransferred to nitrocellulose, immunoblotted with anti-actin antibody and visualized by ECL, as described in materials and methods (panels B, D&E). Apoptosis is indicated by an increase in the intensity of DNA ladders (panels A&C). Actin degradation is indicated by the production of a 41 kDa fragment from the intact 42 kDa actin (panels B&D), and also by the production of a 30 kDa fragment visualized after longer exposure of the ECL-treated membrane to x-ray film (panel E). Molecular weights for the actin degradation analysis are shown on the right side of panels B, D&E. Representative results of three experiments.


A.
B.
42 kDa ACTIN
1 2 1 2
Follicular Atresia
E.
follicular atresia
luteal regression
42 kDa
30 kDa
2 4
C.
D.
42 kDa
41 kDa ACTIN
3 4 3 4
Luteal Regression

41 A&C). Actin cleavage to produce the 41 kDa product from the intact 42 kDa form, was evident in protein extracts from apoptotic luteal tissue (fig. 41 D), but not follicular granulosa cells (fig. 41 B). In addition, a cleavage product of approximately 30 kDa could be observed in the extracts from regressing luteal, but not atretic follicular cells (fig. 41 E). Similarly, cleavage of PARP from the intact 116 kDa protein to produce a 85 kDa fragment, was not evident in anti-eCG treated rat ovaries (fig. 42 A), but the 85 kDa fragment was observed in protein extracts from rats treated with $PGF_{2\alpha}$ (fig. 42 B).

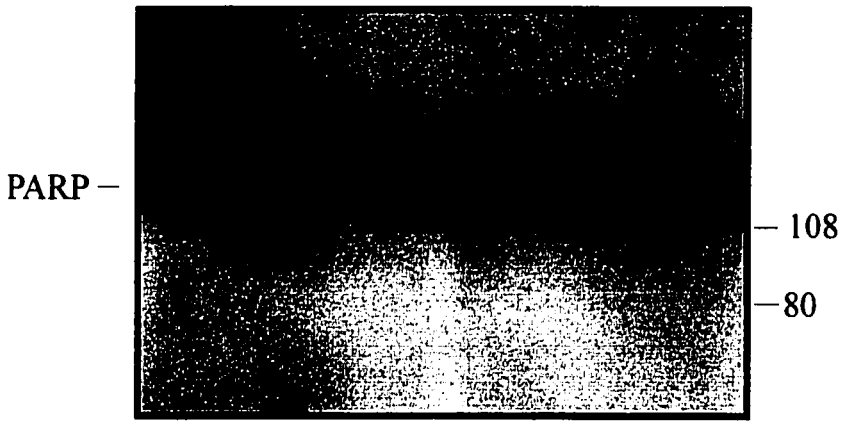
3.11.2 Localization of caspase-3 and apoptosis in the ovary

Caspase-3 was localized in follicles and luteal tissue of ovaries from the eCG and hCG groups (fig. 43). Positive immunostaining was observed in luteal cells of the CL and in the theca and oocytes of follicles (fig. 43 B,C,E&F). Immunostaining for caspase-3 was weak or absent in granulosa cells and interstitial tissue. In the intensely staining theca and luteal cells, caspase-3 was localized in the cytoplasm while the nucleus contained little or no staining for this enzyme (fig. 43 C&F).

Caspase-3 and apoptosis were localized in ovarian follicles of rats treated with anti-eCG antiserum, or in luteal tissue from rats treated with $PGF_{2\alpha}$ (fig. 44). As previously observed, the CL exhibited intense staining for caspase-3 (fig. 44 A), and there was no

Figure 42: Western analysis of PARP cleavage during induced follicular atresia and luteal regression. (A) Follicular atresia following injection of normal serum (control) or anti-eCG antibody. Luteal regression following injection of saline (control) or $PGF_{2\alpha}$ as described in the materials and methods. Protein extracts (40μg) were resolved by electrophoresis, electrotransferred to nitrocellulose membranes and immunoblotted with anti-PARP antibody, as described. PARP cleavage is indicated by the production of the 85 kDa fragment (arrowhead) from the intact 116 kDa protein. Representative result of 3 experiments.

A: Follicular atresia



B: Luteal regression

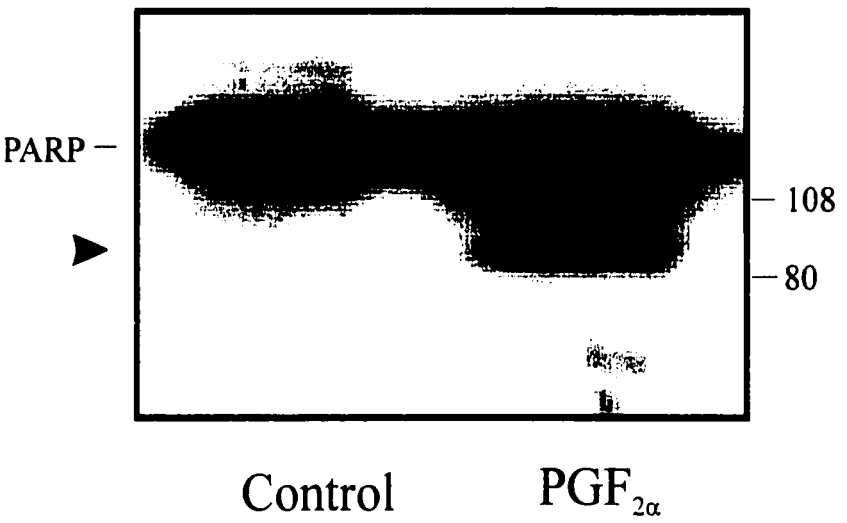


Figure 43: Localization of caspase-3 in the rat ovary. Ovaries removed from rats 48 h after eCG injection (ovaries enriched in antral follicles), or 7 days after eCG+hCG injection (ovaries enriched in CL) were processed for histology as described in materials and methods. Cleared and rehydrated sections were incubated with normal rabbit serum (control; A&D) or rabbit anti-CPP32 antibody (5 μg/ml; B, C, E&F), washed and incubated with biotinylated goat anti-rabbit IgG. The sections were again washed, incubated with avidin-biotin-HRP and developed with DAB/$CuSO_4$. Positive immunostaining (black) was observed in the theca cells (t) and oocytes (arrowhead) of follicles (B, C&E) and in luteal cells (lc) of the CL (E&F). Immunostaining for caspase-3 was weak or absent from granulosa cells (gc; B&C) and interstitial tissue (I; B&E). In positively stained cells, caspase-3 was localized in the cytoplasm (c) while the nucleus (n) contained little or no staining for this enzyme (F). [A, B, D&E: 100×] [C&F: 640×].

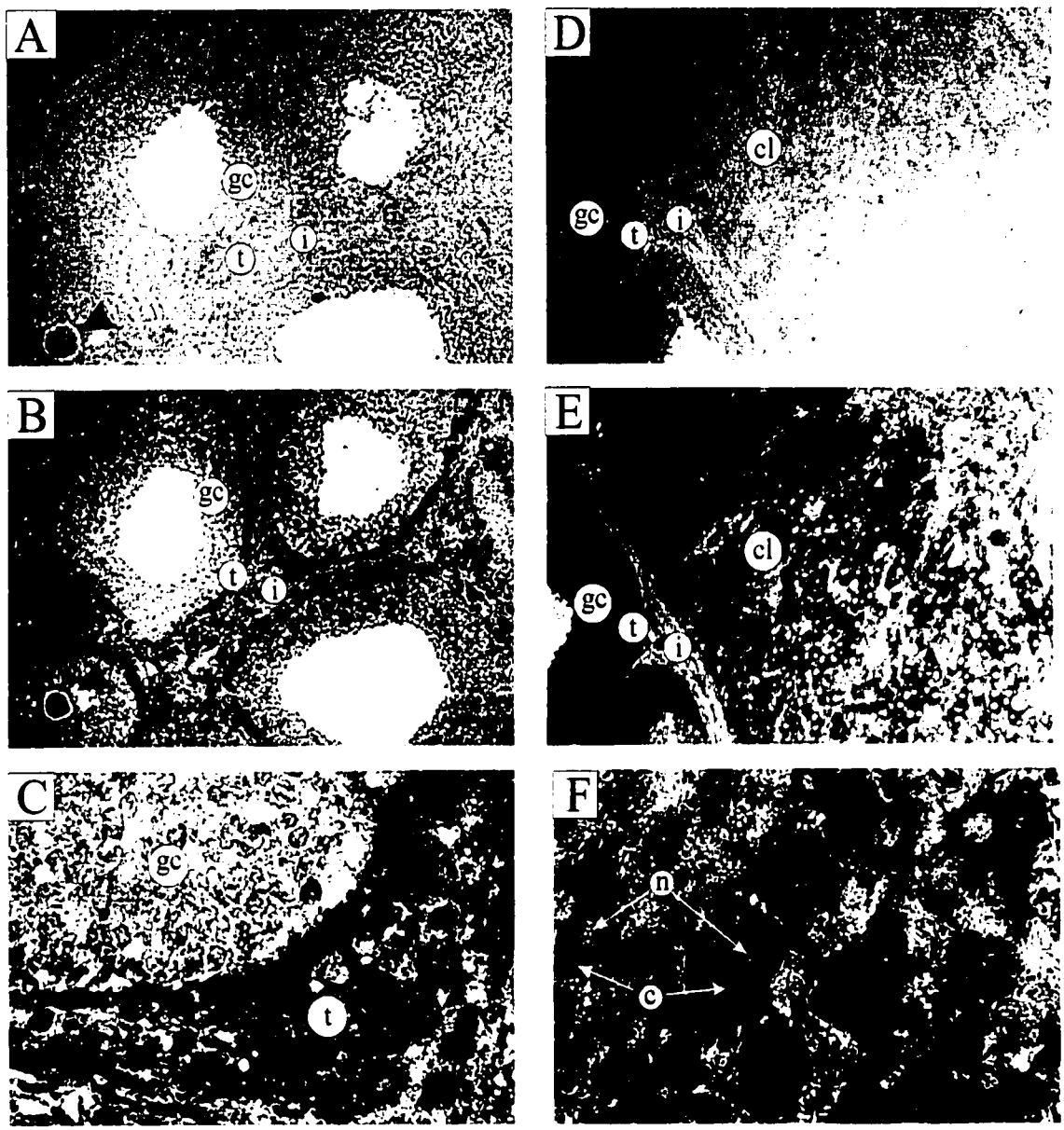
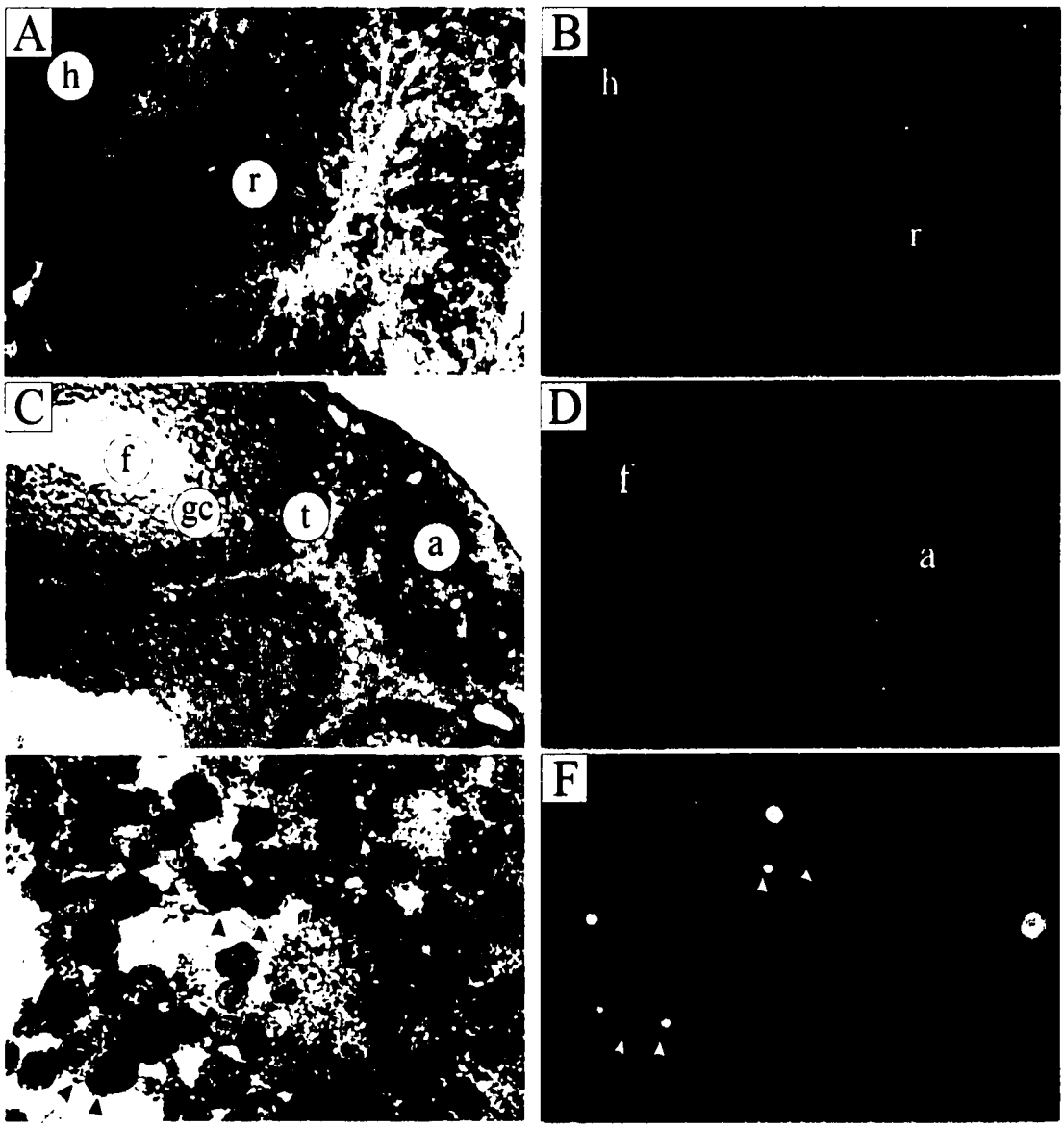

A
gc
t
i
D
cl
gc
t
i
B
gc
t
i
E
cl
gc
t
i
C
gc
t
F
n
e

Figure 44: Localization of caspase-3 and apoptosis in atretic follicles and regressing luteal tissue. Caspase-3 (A, C&E) and apoptosis (B, D&F) were localized in histological sections, with the anti-CPP32 antibody and TUNEL respectively, as described in materials and methods. (A) There was no difference in caspase-3 staining in healthy (h) vs. regressing (r) CL which both displayed intense caspase-3 immunoreactivity. (B) In an adjacent section, the same regressing CL (r) contained numerous TUNEL-positive cells which were not evident in the healthy CL (h). (C) Healthy follicles (f) displayed typical caspase-3 staining in theca (t) but not granulosa (gc) cells. However, an atretic follicle (a) contained scattered caspase-3-positive granulosa cells. (D) In an adjacent section, scattered TUNEL-positive granulosa cells were evident in the atretic (a) but not the healthy (f) follicles. (E) In an atretic follicle, positive caspase-3 staining was evident in some granulosa cells (arrowhead) while others did not display caspase-3 immunoreactivity (arrow). (F) In an adjacent section, some granulosa cells were TUNEL-positive (arrowhead) while others were not (arrow). [A-D: 100×] [E&F: 640×]


A
h
r
B
h
r
C
f
gc
t
a
D
f
a
F

apparent difference in the extent of caspase-3 immunostaining in CL that were positive or negative for apoptosis identified by TUNEL (fig. 44 B). Conversely, follicles that contained TUNEL-positive apoptotic granulosa cells (fig. 44 D&F) displayed immunostaining for caspase-3 in both the granulosa and theca layers (fig. 44 C&E), while healthy follicles exhibited no caspase-3 in granulosa cells (fig. 44 C). TUNEL and caspase-3 localization were done in adjacent sections, so it is not possible to assess the colocalization of caspase-3 and apoptosis. However, atretic follicles contained scattered apoptotic granulosa cells (fig. 44 F) and these follicles displayed scattered caspase-3 staining, in a similar pattern, in the adjacent section (fig. 44 E).

4.0 DISCUSSION

4.1 IDENTIFICATION OF ENDONUCLEASES IN THE OVARY

4.1.1 DNase I-like endonuclease

Zymographic analysis with double stranded DNA as substrate indicated the presence of a doublet of DNase I - like endonuclease (34 and 32 kDa) in ovarian cell nuclei. Similar doublets of DNase I have also been demonstrated in homogenates of rat kidney, lymph node and small intestine (Lacks 1981). These doublets may represent differences in carbohydrate side chains similar to two forms of DNase I isolated from bovine pancreas (Lacks 1981). Alternatively, they may be the result of differences in protein processing or products of multiple genes, although Northern analysis of DNase I mRNA in a variety of rat tissues (Polzar et al 1994) and the ovaries of DES and eCG groups revealed only a single transcript. It is possible that, in the ovary, the lower molecular weight 32 kDa band may be a proteolytic product of the larger 34 kDa form. It is intriguing to speculate that proteases may activate a latent form of DNase I to induce apoptosis by converting an inactive precursor to a smaller activated form(s). Although both 32 and 34 kDa forms of the DNase I-like endonuclease are active in zymographic gels, only one of these forms might degrade genomic DNA *in vivo*. No biochemical characteristics that distinguish the 32 and 34 kDa enzymes from each

other were found in the present studies.

4.1.2 Other endonucleases

4.1.2.1 Nuc18

Endonucleases have been identified and implicated in apoptosis in various tissue and cell types. Nuc18 was first demonstrated as an endogenous endonuclease in thymocytes. Increased levels of this enzyme were found in nuclear extracts during glucocorticoid-induced apoptosis (Compton and Cidlowski 1987). Although suspected to be histone 2B (Alnemri and Litwack 1989; Baxter et al 1989), nuc18 was subsequently found to be a legitimate nuclease (Gaido and Cidlowski 1991) with sequence similarity to cyclophilins (Montague et al 1994), a group of proteins with high affinity binding for the immunosuppressive drug cyclosporin A. Interestingly, recombinant cyclophilins also have nuclease activity that is activated by cyclosporin A. It is possible that the immunosuppressive effects of cyclosporin A may be mediated by the endonucleolytic activity of cyclophilins in thymocytes (Montague et al 1994). Cyclophilins have been found in a wide range of organisms and in every mammalian tissue studied, and are present in the cytoplasm, nucleus, ER, or secretory pathway of cells (Montague et al 1994). Cyclophilins are likely present in the ovary since cyclosporins have been shown to increase estradiol levels in rats (Esquifino et al 1995)

and to decrease progesterone levels and numbers of corpora lutea in gonadotropin-primed mice (Husein and Pringle 1992). Cyclophilin mRNA is routinely used as a housekeeping gene for Northern analysis in many tissue types including the ovary (Michel et al 1992). Although no evidence for nuc18 in ovarian nuclear extracts was found in the present study, cyclophilins may be primarily localized in other cellular compartments in ovarian cells including the cytoplasm, endoplasmic reticulum and Golgi (Montague et al 1994).

4.1.2.2 DNase II

The possible role of DNase II in apoptosis has also been examined. DNase II is a 27 kDa, Ca^{2+}/Mg^{2+} - independent nuclease. It is active at pH below 6.0 but does not produce 3'-OH ends that are characteristic of apoptotic DNA. While unable to detect significant Ca^{2+}/Mg^{2+}-dependent endonuclease activity in Chinese Hamster Ovary cells, Barry and Eastman (1993) successfully identified a cation independent, acidic endonuclease believed to be DNase II. Similar observations have been reported in bladder cancer T24 cells (Shemtov et al 1995). Polymorphonuclear leukocytes and differentiated CD34+ cells are believed to contain both Ca^{2+}/Mg^{2+}-dependent endonuclease and DNase II-like activities (Anzai et al 1995). DNase II has been immunolocalized and implicated in apoptosis during chicken lens cell differentiation (Aarruti et al 1995; Torriglia et al 1995). This chicken embryo DNase II-like nuclease

exists in three forms (18, 23 and 60 kDa), and can produce the DNA ladder pattern of apoptosis. Decreased pH has been shown to be associated with apoptotic signals and sufficient to induce apoptosis in bladder cancer and HL-60 cells (Shemtov et al 1995; Perez-Sala et al 1995). DNase II may be involved in apoptosis in certain cell types, but the present work did not detect any cation independent, acidic endonuclease activity in ovarian cell nuclear extracts, which indicates that DNase II may not be important for apoptotic DNA degradation in the ovary.

4.1.2.3 Unidentified enzymes

Various unidentified and unique endonuclease activities with apparent molecular weights of 40, 58, and 97 kDa (Deng and Podack 1995; Pandey et al 1994) have been demonstrated in different cell lines. Hypoxia/reoxygenation injury in rat renal proximal tubules was correlated with increased levels of a 15 kDa endonuclease activity (Ueda et al 1995). A 27 kDa activity purified from the nuclei of human spleen produced apoptotic DNA degradation in isolated nuclei *in vitro* (Ribeiro and Carson 1993). There are several reports of high molecular weight DNA fragmentation in the absence of low molecular weight DNA production, suggesting the presence of functionally different pools of endonuclease activity in the nucleus (Pandey et al 1994; Walker et al 1994; Earnshaw 1995; Cohen et al 1994). These studies often use intercalating dyes such as ethidium bromide for comparison of the abundance of low

(100-1000 base pairs) and high (50-300 kilobase pairs) molecular weight DNA species. Since such dyes will stain the larger DNA with 50-3000 times more intensity than oligonucleosomes, the relative production of high and low molecular weight DNA fragments during apoptosis must be studied with caution. The differential sensitivity of endonucleases and the apoptotic process to various inhibitors, as well as their differences in cation requirements, supports the theory that distinct nucleases are responsible for the high and low molecular weight production of DNA during apoptosis (Pandey et al 1994; Weaver et al 1993). The high molecular weight DNA is apparently produced by a Mg^{2+}-dependent activity which is insensitive to zinc and may involve the action of proteases. More recently, Sikorska and colleagues have described a 97 kDa Ca^{2+}/Mg^{2+}-dependent endonuclease capable of internucleosomal DNA cleavage (Pandey et al 1997). This endonuclease activity was easily extracted from nuclei, leaving a Mg^{2+}-dependent activity within nuclei that was capable of high molecular weight, but not internucleosomal DNA cleavage. The 97 kDa activity had the same cation requirements and sensitivities as DNase I, but was distinguished from this enzyme based on its molecular weight and sensitivity to denaturing conditions during electrophoresis. Extracts from several cell lines contained the 97 kDa activity, as did nuclear extracts from rat kidney, liver, brain and thymus. Although the cell lines did not contain any apparent DNase I-like activity, extracts from rat tissues did display a DNase I-like activity that was not further characterized in that study.

4.2 CHARACTERIZATION OF OVARIAN ENDONUCLEASE

4.2.1 Evidence of DNase I

Apoptotic DNA degradation is a consistent feature of ovarian follicular atresia and luteal regression, although the endonuclease responsible for this process has not been identified. Since initial studies demonstrated a DNase I - like endonuclease to be present in nuclei of differentiated rat granulosa and luteal cell nuclei, comparison of the enzyme with DNase I was undertaken. The ovarian endonuclease was found to be biochemically and immunologically indistinguishable from DNase I, as evident by: (i) presence of an activity doublet with a molecular weight of 32/34 kDa similar to DNase I described in other reports (Lacks 1981; Peitsch et al 1995); (ii) dependence on Ca^{2+} and Mg^{2+}, active at neutral pH and stimulated by Mn^{2+} alone, characteristics common to DNase I and endonucleases implicated in apoptosis in a variety of cell types (Schwartzman and Cidlowski 1993; Bortner et al 1995); (iii) sensitivity to Zn^{2+}, AUR and dtt as well as G-actin (Kreuder et al 1984; Lacks 1981); (iv) immunoprecipitation and identification with an anti-DNase I antibody of the immunoreactive bands with the same molecular weight and developmental pattern as the 32/34 kDa nuclease activity and (v) depletion of the ability of the eCG extract to produce DNA ladders in target DES nuclei following immunoprecipitation with anti-DNase I antibody. While these studies strongly suggest that the endonuclease is DNase I, further studies employing

antisense DNase I knockouts in ovarian cell cultures following gonadotropin stimulation would provide further support for this contention.

4.2.1.1 Tissue distribution

The predicted molecular weight of DNase I based on its amino acid sequence is 31 kDa but post-translational modifications can account for a wide variation in apparent molecular weight of the enzyme (Lacks 1981; Yasuda et al 1993) . The 32/34 kDa doublet observed is consistent with other reports of DNase I measurement by zymography and may represent isoforms containing different degrees of sialic acid modification (Lacks 1981). The kidney and spleen displayed primarily the 32 kDa band of activity while the 34 kDa band was more evident in the nuclei of the ovary and liver. The occurrence of DNase in nuclear extracts of the liver is consistent with the classic work of Hewish and Burgoyne (1973) which showed that nuclei isolated from the liver degrade nuclear DNA into nucleosomal length fragments when exposed *in vitro* to Ca^{2+}/Mg^{2+}. DNase I like endonuclease activities ranging from 30-34 kDa were found in nuclear extracts from cow, chicken and human ovarian cells, consistent with reports of apoptosis in the ovaries of these species (Hsueh et al 1994; Palumbo and Yeh 1995). Interestingly, the bovine pancreatic DNase I had an apparent molecular weight several kilodaltons larger than the bovine ovarian enzyme. This may reflect alternate processing of transcripts between the two tissues or different post-

translational modifications, since the pancreatic form is a secreted digestive enzyme and the ovarian form is localized in the nucleus and not involved in digestion. The significance of such modifications on the activity of DNase I is not known, but biochemical differences between the 32 and 34 kDa enzymes present in the rat ovary have not been found. This does not exclude the possibility that differences in the activity or function of the 32 and 34 kDa isoforms may exist *in vivo*. Detectable levels of DNase I were not found in the uterus and testes, although apoptosis has been reported to occur in these tissues (Billig et al 1995; Moulton 1994; Tapanainen et al 1993). Apoptosis in the uterus is associated with decidual tissue (Moulton 1994) or the shedding of the endometrium during the cycle. These tissue types would not have been present in the immature females used in the present study and endothelial cells may have been diluted out by the other nuclei collected from the whole tissue. A similar dilution effect could account for the lack of detectable DNase I in the adult rat testes, where apoptosis occurs primarily in the spermatogonia around the inner periphery, but not other testicular cells, of the seminiferous tubules (Billig et al 1995; Tapanainen et al 1993). The present work demonstrated that spermatogonia, but not other testicular cells, stain positively for DNase I. This is consistent with a potential role of DNase I in apoptosis in the rat testes.

Autoimmune mice (MRL-lpr/lpr) and patients with systemic lupus erythematosus (SLE) are known to produce antinuclear autoantibodies (Casiono and Tan 1996; Rosen

and Casciola-Rosen 1997). Antibodies that also react with DNase I have recently been identified in serum from mice and patients with SLE (Puccetti et al 1995). These anti - DNase I antibodies inhibited the activity of DNase I *in vitro* and some of these autoantibodies entered and localized within the nuclei of Jurkat and HL60 cells (Madaio et al 1996). Once internalized, these autoantibodies bound and inhibited DNase I and rendered the cells resistant to apoptosis. Both DNA degradation and nuclear fragmentation were inhibited by these autoantibodies. These results demonstrated a key role for DNase I in apoptosis and a possible role in autoimmune disease (Madaio et al 1996). Administration of the autoantibodies to normal mice resulted in their nuclear localization in the kidney, liver and other organs. This was associated with glomerular hypercellularity in the kidney likely due to the inhibition of apoptosis in those cells (Vlahakos et al 1992). These results, and the present finding of DNase I - like endonuclease in the kidney, suggest that apoptosis is part of the normal glomerular cell turnover in this organ and that DNase I is directly involved in that turnover. Further, the finding of anti-DNase I autoantibodies in SLE indicates that DNase I may be a substrate for proteases involved in apoptosis, since other protease substrates such as PARP and DNA-dependent kinase generate autoantibodies in SLE (Rosen and Casciola-Rosen 1997; Casiano and Tan 1996).

4.2.1.2 Sensitivity to inhibitors

Consistent with DNase I, the ovarian endonuclease was inhibited by Zn^{2+}, AUR and dtt. Inhibition by dtt reflects the fact that DNase I has two disulphide bridges that are essential for normal function of the enzyme (Suck et al 1986; Mannherz et al 1995). Both Zn^{2+} and AUR have been shown to inhibit apoptotic DNA degradation in other cell types (Schwartzman and Cidlowski 1993). Sensitivity to apoptosis was correlated to intracellular Zn^{2+} levels in thymocytes and leukemic cell lines (Adebodun and Post 1995; Sunderman 1995), and apoptosis was implicated in Zn^{2+} deficient mice (Sunderman 1995). AUR is known to inhibit apoptosis in thymocytes, neurons and other cells (McConkey et al 1989a, Batistatou and Greene 1991). The protease calpain (Posner et al 1995) and topoisomerase II (Catchpoole and Stewart 1994), two proteins implicated in apoptosis, are inhibited by AUR, indicating that the anti-apoptotic actions of this compound may be at multiple sites. Similarly, the recent observation that Zn^{2+} inhibits cleavage of PARP and caspase-3 in addition to endonuclease activity (Wolf et al 1997) indicates that the anti-apoptotic actions of this cation are likely to be multifarious.

4.2.1.3 Chromatin degradation in apoptosis

The role of DNA degradation in apoptosis remains to be determined. Studies have shown that low molecular weight DNA degradation does not always occur during cell death (Ucker et al 1992; Oberhammer et al 1993b; Earnshaw 1995). However, DNA degradation also includes the production of high molecular weight fragments from intact chromatin (Brown et al 1993; Weaver et al 1993; Walker et al 1995; Montague and Cidlowski 1996). The necessity of high molecular weight DNA degradation in apoptosis has not been determined, but no evidence of apoptosis in the absence of high molecular weight DNA degradation has been found to date. The process of DNA degradation can be divided into three stages, which may occur sequentially or simultaneously during cell death (Walker et al 1995). These stages reflect the higher order structure of chromatin resulting from the organized coiling of DNA and its interaction with the nuclear architecture. First, chromatin is cleaved at rare sites where the DNA interacts with structural components of the nucleus. These sites are thought to contain stretches of single stranded DNA. This type of cleavage is dependent on Mg^{2+} but not Ca^{2+}, and would presumably render the cell incapable of normal division, as the physical control over chromosome reorganization and movement would likely be lost. Second, chromatin is cleaved to produce high molecular weight fragments 300 to 50 Kbp. This type of cleavage is dependent on Mg^{2+} and very low concentrations of Ca^{2+}, and results from double strand breaks in the DNA. Finally, double stranded

Ca^{2+}/Mg^{2+}-dependent cleavage at the internucleosomal regions produces the well known ladders of apoptotic DNA. This type of cleavage is dependent on higher concentrations of Ca^{2+} than that needed for the high molecular weight degradation, and proteolysis may also be necessary for this stage to proceed (Walker et al 1995; Weaver et al 1993). Although different endonucleases may be responsible for these stages of chromatin degradation, Sikorska and colleagues have suggested that a single DNase I like enzyme could account for each type of chromatin cleavage (Walker et al 1995; Walker and Sikorska 1994). DNase I has double hit kinetics in the presence of Mg^{2+} alone. This means that the enzyme will only cut one strand of DNA and must release and reattach to chromatin before making a second cut. This type of activity would account for the Mg^{2+}-dependent single strand breaks at rare sites characteristic of stage I of chromatin degradation. In the presence of low concentrations of Ca^{2+}, DNase I acts with single hit kinetics, such that it remains bound and cuts both strands of the DNA. This type of activity would be necessary for the characteristic degradation of chromatin described above as stage II. Higher concentrations of Ca^{2+} are necessary for DNase I to act on internucleosomal DNA to produce the ladders seen during most forms of apoptosis. Thus all three stages of chromatin degradation could conceivably be produced by DNase I or a DNase I-like endonuclease. The DNA double-strand breaks in apoptotic, but not necrotic, cells were found to be consistent with a DNase I type of activity (Didenko and Hornsby 1996). The extent to which low molecular weight ladders occur during apoptosis may depend on the ability of the cell to

accumulate sufficient intranuclear Ca^{2+} and possibly to activate required proteases (Weaver et al 1993; Liu et al 1997). The absence of DNA ladders in some forms of apoptosis should not be considered an indication that DNA degradation is not important to cell death, but rather that complete degradation of the genome is not always required.

DNase I could be the endonuclease responsible for all stages of chromatin cleavage and studies of the regulation of this enzyme are necessary to completely understand the process of apoptosis in the ovary. Alternately, more than one endonuclease may be involved in the different stages of chromatin cleavage. Other endonucleases with biochemical features of DNase I have been described, including a 97 kDa enzyme that becomes more difficult to extract from the nucleus when cells become apoptotic (Pandey et al 1997). No evidence of this enzyme in the ovary has been found, but another endogenous nuclear enzyme with an approximate molecular weight of 27 kDa was identified. This enzyme was Mg^{2+}-dependent and active on single stranded-, but not double stranded-DNA. These properties make this enzyme a possible candidate for the production of rare high molecular weight chromatin breaks described as stage I.

4.3 DEVELOPMENTAL PATTERN OF DNase I IN THE OVARY

4.3.1 Gonadotropins and follicle development

The present studies show that DNase I protein content in the ovary is regulated by the developmental state of the follicle. DNase I was present at high levels in antral follicles and luteal tissue but low or negligible levels in preantral follicles. Preantral follicles contain mitotically active, undifferentiated granulosa cells which express FSH receptors. As the granulosa cells differentiate under the influence of FSH, an antrum is formed in the follicle and steroid production is increased. These events are thought to be initiated and maintained by FSH. It is during the initial antrum formation, the penultimate stage of follicular development, that atresia is most likely to occur (Hsueh et al 1994; Hirshfield 1991). This increased susceptibility to atresia may be due to the fact that, as shown in the present studies, antrum formation corresponds to the appearance of DNase I in the nuclei of the granulosa cells. FSH is a survival factor in early antral follicles (Chun et al 1996), and the present work suggests that FSH suppresses the activation of DNase I.

4.3.2 Gonadotropin suppression of DNase I

4.3.2.1 Bcl-2

The inhibition of apoptosis in the rat ovary by FSH is believed to be associated with the maintenance of bcl-2 and bcl-x_{long} mRNA expression (Tilly et al 1995a). Oncogenes such as bcl-2 are known to suppress endonuclease activity in fibroblast nuclei (Arends et al 1993; Fernandez et al 1995; McConkey et al 1995). Suppression of DNase I activity in the nuclei of ovarian cells by bcl-2 or related oncogene products has not been investigated, but this may be one means by which FSH suppresses the activity of DNase I in antral follicles.

4.3.2.2 Caspases

FSH suppression of DNase I may also be mediated by caspases since gonadotropin has been shown to down-regulate the expression of ICE-related proteases (caspases) in the rat ovary (Flaws et al 1995b). Caspases induce apoptosis when transfected into fibroblasts (Miura et al 1993). One of the substrates for caspases is PARP (Lazebnik et al 1994), which has been shown to inhibit endonucleases in a variety of cell types (Carson et al 1986; Nelipovitch et al 1988; Ray et al 1992; Rice et al 1992) and to inhibit the Ca^{2+}/Mg^{2+}- dependent endonuclease in bull seminal plasma (Tanaka et al

1984). Although the bull seminal plasma endonuclease has not been identified, DNase I is the endonuclease found in human (Sawazaki et al 1992; Yasuda et al 1993) and rabbit (Takeshita et al 1994) seminal plasma. Alternatively, the suppression of DNase I by FSH via caspases may be mediated by actin. It has recently been demonstrated that actin is a substrate for caspases and that this proteolytic degradation of actin eliminates its ability to inhibit DNase I (Kayalar et al 1996; Mashima et al 1997). Although actin is a cytosolic protein, it is also highly abundant in the nucleus (Amankwah and DeBoni 1994; Parfenov et al 1995). It is conceivable that the down-regulation of caspases in the ovary by FSH maintains an active pool of PARP or actin which inhibits DNase I in granulosa cells. Conversely, removal of FSH could up-regulate caspase expression and cause the degradation of PARP or actin and the subsequent derepression of DNase I, thus leading to the DNA degradation associated with follicular atresia. The potential interaction of FSH, caspases and DNase I has not been examined, but other studies have demonstrated that the protease inhibitors IAA (Flaws et al 1995b) and SAM (Flaws et al 1995b; Kaipia et al 1996) suppressed the formation of apoptotic DNA during ovarian follicular atresia *in vitro*. IAA and SAM were used in those studies as caspase inhibitors, but it was not clear whether the inhibition of DNA degradation was a direct effect on the endonuclease. In the present study, SAM directly inhibited the endonuclease in nuclear extracts which may explain how SAM inhibited the onset of DNA degradation in the whole follicles. However, IAA did not have direct inhibitory effects on the endonuclease, indicating that the inhibition of

DNA degradation in whole follicles by IAA (Flaws et al 1995b) may have resulted from inhibition of caspases. Although SAM directly inhibited the endonuclease, it is also possible that it prevents apoptotic DNA degradation in intact follicles via its inhibition of caspases.

4.3.3 Other ovarian cell types and development

Although hormone treatments used in this work produce ovaries enriched in granulosa and luteal cells, nuclei from other ovarian cells and cells of the immune system are likely to be present in these preparations. Macrophages, granulocytes and T-lymphocytes have been localized in the rat follicle and corpus luteum . Although endonucleases have not been identified in any of these cell types, T-lymphocyte precursor cells (thymocytes) have been shown to contain an endogenous DNase I (Peitsch et al 1993). Very few T-lymphocytes are present in the rat ovary, however, and the number of T-lymphocytes does not appear to vary during follicular development or the lifespan of the corpus luteum (Brannstrom et al 1993; Brannstrom et al 1994). Endonuclease activities have not been described for other ovarian cell types, but apoptotic DNA degradation has been demonstrated to be limited to granulosa and luteal cells, suggesting that these cells are the source of the Ca^{2+}/Mg^{2+}-dependent endonuclease. Immunocytochemical study of DNase I in the rat ovary was therefore necessary to confirm the cellular distribution of the enzyme during follicular

development.

4.3.4 Immunohistochemistry of DNase I during development

4.3.4.1 Follicle and corpus luteum

Consistent with the biochemical identification of DNase I in ovarian nuclear extracts, the present studies localized the enzyme in nuclei of luteal and granulosa cells. However, DNase I was not evident in nuclei of granulosa cells in healthy preantral follicles. The granulosa cells in smaller immature follicles are not commonly observed to undergo apoptosis (Hirshfield 1991). The present results suggest that this is due, at least in part, to the absence of DNase I. The present findings that differentiation of the granulosa cell is correlated to the localization of DNase I in the granulosa cell nucleus is consistent with other work describing the expression of the endonuclease relative to the differentiation of intestinal enterocytes (Polzar et al 1994). That study also showed that, while many enterocytes expressed DNase I, only a few near the villar tip underwent apoptosis (Polzar et al 1994). Similarly, the present work found DNase I to be expressed in differentiated granulosa and luteal cells but did not find these cells to be undergoing cell death. This suggests that, once an antrum forms, the granulosa cell has the ability to undergo apoptosis and requires only a signal to initiate the process, while granulosa cells in preantral follicles do not have the necessary endonuclease to

degrade endogenous DNA, and thus require the synthesis of the enzyme before apoptosis can occur. There are no studies to date on the requirement for new protein synthesis during apoptosis of granulosa cells from preantral follicles, but the present results suggest that the synthesis of DNase I would be required to initiate DNA degradation in preantral follicles. Consistent with this hypothesis, morphologically atretic preantral follicles also displayed strong immunostaining for DNase I. It is possible that the synthesis of DNase I in immature follicles is sufficient to initiate apoptosis in the granulosa cells and thus follicular atresia. The finding of DNase I localization primarily in the nuclei of granulosa and not theca cells is consistent with the reports that apoptosis occurs primarily in the granulosa cells and not the theca cells of ovarian follicles (Tilly 1996; Tilly et al 1991).

4.3.4.2 Oocyte

Atresia in preantral follicles is observed to start in the oocyte and to subsequently occur in the surrounding granulosa cells (Greenwald and Terranova 1988; Hirshfield 1991). Strong staining for DNase I was found in oocytes of preantral follicles within which the granulosa cells did not stain for DNase I. The presence of DNase I in the oocytes of healthy preantral follicles could account for the observation that atresia is initiated in the oocytes of this follicle type. In contrast, oocytes in antral follicles often do not stain for DNase I. This supports the observation that oocytes are often found to

resist degeneration for some time after the rest of the follicle becomes atretic.

4.4 THE DNase I GENE AND ITS POTENTIAL REGULATION

The cDNA sequences for human (Shak et al 1990; Yasuda et al 1995), rat (Polzar and Mannherz 1990) and mouse (Peitsch et al 1995) DNase I are now known. GC content and CpG/GpC dinucleotide ratios in the 5' regions of the human DNase I gene suggest that DNase I is not a housekeeping gene, but is subject to potential regulation (Yasuda et al 1995). Basic transcriptional elements in the 5' region of the human DNase I gene include putative CAAT and TATA box-like sequences and consensus binding elements for the transcription factor Sp1. There are additional putative regulatory elements, including three Pan1 motifs (transcription activators in pancreatic acinar cells; Meister et al 1989), a consensus sequence for TRE (thyroid hormone response element), two sequences similar to GRE (glucocorticoid response element), a sequence for AP-2 (cAMP or phorbol ester signals for growth control), two GATA-1 motifs, an IL-6 response element and a putative AP-1 recognition sequence (Yasuda et al 1995). The pan1 motifs probably reflect the high level of expression of DNase I in the human pancreas where the enzyme is synthesized for use in alimentary digestion. The GRE's and AP-2 sites are interesting, as endonuclease expression and apoptosis are known to be regulated by glucocorticoids, cAMP and phorbol ester in different cell types

(McConkey et al 1990; McConkey et al 1989b and 1989c; Aharoni et al 1995).

4.4.1 DNase I gene and the ovary

4.4.1.1 FSH, cAMP and IL-6

FSH stimulates cAMP (Gore-Langton and Armstrong 1988) and IL-6 (Gorospe et al 1992) production in granulosa cells and initiates differentiation. The present studies found that granulosa cell differentiation was correlated with the presence of DNase I in nuclear extracts. Stimulation of granulosa cells with high doses of cAMP or IL-6 has been shown to induce apoptosis (Aharoni et al 1995; Gorospe and Spangelo 1993b), but it is not known if endonucleases are upregulated by this second messenger. The presence of an IL-6 response element may account for the observation that IL-6 induces apoptosis in granulosa cells. In the present work, the level of ovarian DNase I mRNA in immature rats increased with follicle development after gonadotropin treatment. This may have been due to the FSH-stimulated production of cAMP or IL-6 in granulosa cells. Gonadotropin treatment suppresses apoptosis, which suggests that cAMP or IL-6 induction of DNase I expression is accompanied by the induction of some factor that suppressed the activity of the enzyme. In this regard, gonadotropin is known to maintain bcl-2 and suppress bax expression (Tilly et al 1995a), and also to suppress expression of the death protease caspase-3 (Flaws et al 1995b). The

regulation of DNase I may involve a variety of transcription factors, some of which can be implicated in apoptosis in ovarian cells. It must be emphasized however that the 5' region of the rat ovarian DNase I gene may differ from that of the human leukocyte DNase I gene (Yasuda et al 1995), and that the mere presence of consensus motifs for transcription factors in the 5' region of the gene is suggestive, but not proof, of such factors being involved in the regulation of the gene in any given cell type.

4.4.1.2 Estrogen and androgen

There are no studies to date on the role of new gene activity or protein synthesis in ovarian apoptosis. The requirement of new protein synthesis for apoptosis appears to be dependent on the cell type or apoptogenic signal (Cohen and Duke 1984; Schwartzman and Cidlowski 1993; Duke et al 1983; Waring 1990; Martin et al 1988). Hsueh and coworkers reported that DES withdrawal induced apoptosis in the ovarian follicles of DES-treated rats, which was enhanced by administration of androgen after DES withdrawal (Billig et al 1993). Since DNase I was not observed in the nuclei of DES-treated rats, it is possible that estrogen suppresses the production of DNase I in these cells. Estrogen withdrawal would allow the production of DNase I to proceed in the absence of gonadotropic support, resulting in apoptosis in the follicle. It is not known whether estrogen and androgen regulate the expression of DNase I in the ovary, but androgen ablation does upregulate DNase I mRNA in the prostate (Bacher et al

1993; Rauch et al 1997).

4.4.2 DNase I mRNA expression in the ovary

The expression of the DNase I gene was investigated, using RT-PCR to amplify the mRNA from DES and eCG treated rat ovaries. There was a developmental pattern of DNase I mRNA in rat granulosa cells. DNase I transcripts were present in DES-, but were significantly higher in granulosa cells from eCG-treated rats. DNase I protein was consistently and significantly higher in the eCG group and was only marginally detectable in the DES-treated ovary. The levels of DNase I mRNA detected with RT-PCR must be viewed with some caution, as this technique provides only semi-quantitative results and requires further analysis by Northern or RNase protection assays to accurately quantify transcript levels. However, the current findings with RT-PCR suggest that DNase I mRNA levels may not exactly reflect DNase I protein levels in this tissue. Similarly, Sikorska and colleagues found very high levels of mRNA transcript for DNase I in the rat brain (Liu et al, in press), while the current work found no detectable DNase I in the nuclear extracts from that tissue. It is possible that the mRNA detected in the DES group was from oocytes, since other results showed intense oocyte staining for DNase I in preantral follicles. Although oocytes account for a relatively small number of the cells in DES-treated ovaries, it is possible that these very large cells contain significant DNase I transcripts. It is also possible that the

discrepancy in message and protein reflects the fact that the current studies examined the protein from nuclear extracts. If the protein was excluded from the nucleus in the undifferentiated granulosa cells of the DES group, then it would not be collected in the extraction procedure. This seems unlikely, since immunohistochemistry did not reveal strong immunostaining for DNase I in the cytosol of DES granulosa cells. It remains to be determined how the production of DNase I protein in the ovary is regulated. The protein might be produced but rapidly degraded, or production of the protein might be regulated at the level of translation. Translational regulation of DNase I has been suggested by studies of DNase I mRNA and protein levels in the rat intestine (Zanotti et al 1995) and prostate (Rauch et al 1997). In intestinal crypts, *in situ* hybridization revealed that epithelial cells expressed DNase I mRNA long before they became immunopositive for DNase I protein, or underwent apoptosis (Zanotti et al 1995). Similarly, prostatic cells in adult rats which expressed DNase I mRNA, but not protein, were found to contain both DNase I protein and mRNA following androgen withdrawal (Rauch et al 1997). In our studies it is possible that DES might act to suppress DNase I at the level of translation. The cleavage and inactivation of U1-70 sNRP, an enzyme necessary for mRNA splicing, is an early event in apoptosis (Rosen and Casciola-Rosen 1997) which would result in the loss of functional mRNA production. Translational regulation of DNase I might, therefore, be necessary for the continued production of the endonuclease despite the early proteolytic events of apoptosis. A final possibility is that the protein being examined in this study is not

DNase I, but some closely related enzyme that is the product of a different gene. While the present results cannot discount this, it seems an unlikely explanation, given the breadth of the immunological and biochemical characterization consistent with the identification of DNase I. In addition, the mRNA for DNase I is certainly present in the ovary. If the protein being studied is not DNase I, then it would remain to be answered why DNase I mRNA is present in granulosa cells, and where the DNase I protein coded by that mRNA has gone.

4.4.3 Possible regulation of DNase I production in the ovary

It is not yet known how the production of DNase I is regulated in the ovary. Billig et al (1993) have demonstrated that estrogen withdrawal from hypophysectomized rats induces apoptosis in ovarian follicles. Whether DNase I is produced in granulosa cells following estrogen withdrawal is not known, but clearly an endonuclease activity is activated by such treatment, since intense DNA ladders are found in these rat ovaries. Other nuclease activities were not detected in DES rat ovaries, suggesting that for DNA degradation to occur following DES withdrawal, DNase I must first be produced. The mRNA for DNase I was found in DES granulosa cells, so these cells might have the capacity to rapidly begin production of DNase I in order to complete the apoptotic program following DES withdrawal. The work by Billig et al (1993) was conducted with hypophysectomized rats, which lack endogenous gonadotropin,

indicating that endonuclease production did not require FSH-induced differentiation of the granulosa cells. Histological observation of ovaries from rats subjected to DES withdrawal indicates that many follicles progress to the early antral stage of development in this model. This means that gonadotropin exposure is not requisite for at least some measure of granulosa cell differentiation. It is possible that a local factor or cytokine is responsible for the small amount of differentiation that can be observed following DES withdrawal. Thus, some amount of granulosa cell differentiation and production of DNase I could occur following DES withdrawal, rendering the cell capable of complete execution of the apoptotic program, but how this might be regulated is not known. Estrogens inhibit androgen production and enhance estrogen production, so the withdrawal of DES might cause a shift in the ratio of estrogen and androgen levels in follicles. Androgens enhance apoptosis, but whether this is mediated by the regulation of DNase I expression in the ovary is not known. In the prostate, androgen withdrawal was correlated with increased expression of DNase I (Bacher et al 1993; Rauch et al 1997). Other local factors that could influence DNase I expression or activity include GnRH or agents produced by the oocyte. In this context, oocytes were found to express FasL and induced apoptosis when cocultured with Fas-expressing granulosa cells (Hakuno et al 1996).

4.5 POSSIBLE ROLE AND REGULATION OF DNase I IN THE OVARY

4.5.1 Estrogen and androgen

It has been demonstrated that apoptosis occurs in the preantral follicles of DES-treated rats following DES withdrawal, and that this cell death process is inhibited by estrogens but enhanced by androgens (Billig et al 1993). The present findings raise the possibility that estrogens suppress apoptosis by preventing the expression of DNase I in preantral granulosa cells. Alternatively, androgens might act as inducers of DNase I expression and thus apoptosis in undifferentiated granulosa cells. It is possible that DNase I is expressed at all stages of follicular development but only becomes localized in the nuclei during granulosa cell differentiation. Studies of DNase I in other differentiating cell types suggest that the presence of DNase I in the nucleus reflects a change in its expression and not a change in its subcellular distribution (Peitsch et al 1993; Polzar et al 1994). Immunohistochemical localization of DNase I in antral but not preantral granulosa cells in the present work also supports the conclusion that the enzyme is not present in the cytoplasm of undifferentiated granulosa cells. Further studies are needed to determine whether DNase I expression in preantral granulosa cells is modulated by estrogen and androgen in a pattern consistent with the known effects of these steroids on follicular atresia and apoptosis.

4.5.2 EGF/TGFα and bFGF

Tilly et al (1992a) demonstrated that eCG-primed rat follicles undergo a spontaneous onset of apoptotic DNA fragmentation when cultured in serum-free media. This spontaneous activation of endonuclease was found to be suppressed by EGF/TGFα, bFGF or genistein (a tyrosine kinase inhibitor). The present studies indicate that endogenous DNase I activity is present but inactive in granulosa cell nuclei of eCG-primed rats. Growth factors such as EGF/TGFα or bFGF may be responsible for the suppression of DNase I activity in differentiated granulosa cells. This might be mediated by tyrosine phosphorylation of DNase I or other proteins that regulate DNase I activity such as G-actin (Lazarides and Lindberg 1974; Maliska-Blaszkiewicz and Roth 1993). It is not presently known whether these proteins are directly regulated by tyrosine phosphorylation, but EGF/TGFα is known to regulate actin remodelling (Peppelenbosch et al 1993) and possibly to regulate actin binding proteins such as gelsolin or profilin via phospholipase C (Pollard and Cooper 1986). Gelsolin and profilin are among the actin binding proteins known to be bound to membrane PIP_2 and released by the action of PLC on the phospholipid.

4.5.3 $PGF_{2\alpha}$

Apoptosis has also been implicated in luteal regression (Juengal et al 1993; Dharmarajan et al 1994). Functional luteolysis, indicated by cessation of luteal progesterone production, precedes structural luteolysis. Apoptosis is believed to be primarily involved in the structural phase of luteolysis (Palumbo and Yeh 1995). The current results suggest that luteal apoptosis involves activation of an endogenous DNase I. Prostaglandin $F_{2\alpha}$, the luteolysin in most species (Horton and Poyser 1976), has been shown to induce prolonged increases in luteal cell calcium concentrations (Leung et al 1986; Davis et al 1987), which may be important in the activation of the nuclear endonuclease. Alternatively, the activity of DNase I may be suppressed by genes controlled by progesterone, and the loss of this steroid during luteolysis could initiate apoptotic DNA degradation.

Prostaglandins may also play a role in suppression of apoptosis in granulosa cells. Prostaglandins secretion in hen granulosa cells is stimulated by TGFα (Li et al 1994) and can augment or partially mimic the the mitogenic response of hen granulosa cells to TGFα (Li and Tsang 1995). TGFα can suppress serum withdrawal-induced apoptosis in hen granulosa cells (Johnson et al 1996) and inhibitors of phospholipase A_2 and cyclooxygenase reverse this effect (Manchanda et al 1996). The signals elicited by prostaglandins that suppress apoptosis are not known, but these eicosanoids can

activate cAMP, PKC and cytosolic Ca^{2+} signals in different cell types (Boone et al 1993).

4.5.4 Ca^{2+}

The role of an endogenous Ca^{2+}/Mg^{2+} - dependent endonuclease in apoptosis has been known for some time. The cation requirements of the enzyme suggest that elevated intracellular Ca^{2+} observed during apoptosis in thymocytes (McConkey et al 1989a, 1989c; Kizaki et al 1989) could be a common initiating signal for apoptosis in all cell types. Reports of apoptosis in the absence of elevated intracellular Ca^{2+} levels, protection from apoptosis by calcium ionophore, and an increase in intracellular Ca^{2+} concentration by growth promoting signals suggest that the signals to initiate apoptosis may not be universal but may instead be dependent on the species and cell type in question (Thompson 1994). The role of intracellular calcium in ovarian cell apoptosis has not been elucidated. Luciano et al (1994) showed that EGF/TGFα inhibits apoptosis in rat granulosa cells by stimulating progesterone synthesis which in turn modulates the intracellular distribution of calcium. Endogenous endonuclease activity has been shown to require calcium concentrations far higher than the resting levels normally measured within healthy cells (Schwartzman and Cidlowski 1993). However, other factors such as ATP can increase nuclear uptake of calcium and reduce the minimum calcium level required to activate endogenous endonuclease activity (Jones

et al 1989). In addition, it is possible that other post-translational modifications or enzyme cofactors might increase the sensitivity of the endonuclease to calcium, resulting in its activation by cytosolic concentrations of the cation, similar to the effect diacylglycerol has on the sensitivity of protein kinase C to calcium.

The present studies also show that zinc inhibits the 32 and 34 kDa endonuclease activity in nuclear extracts and the ladder pattern of DNA degradation in isolated ovarian nuclei *in vitro*. Whether this divalent cation inhibits apoptosis in ovarian cells remains to be determined. In this context, zinc has been shown to inhibit both DNA degradation and apoptosis in thymocytes (Cohen et al 1994; Duke et al 1983; Cohen and Duke 1984; Fleiger et al 1989). Chelation of intracellular zinc induced apoptosis in thymocytes (McCabe et al 1993) and fluorescent measurements of intracellular zinc revealed that elevations in the level of this cation correlated to resistance to apoptosis while decreased intracellular zinc was correlated to cell death (Zalewski et al 1993). Zinc is essential for normal cell function, and zinc deficiency is teratogenic (Rogers et al 1995). Embryos from pregnant mice maintained on a zinc free diet displayed excess cell death after as little 4 days of maternal zinc deficiency, suggesting that part of the essential function of zinc is due to its ability to inhibit apoptosis (Rogers et al 1995). Conversely, zinc can induce apoptosis in thymocytes in a manner that is dependent on new protein synthesis (Telford and Fraker 1995). These opposite effects of zinc are dose-dependent, such that high concentrations (75-600 μM) of the cation inhibit

apoptosis, while low concentrations (7.5-15 μM) can induce apoptosis (Provinciali et al 1995). These effects of zinc on apoptosis in thymocytes are consistent with the effects of zinc on DNase I. High concentrations of zinc inhibit, while low concentrations of this cation can stimulate DNase I (Campbell and Jackson 1980) or other endonucleases (Pandey et al 1997). In addition to actions at the level of the endonuclease, zinc can inhibit the cleavage of caspases and dephosphorylation of the retinoblastoma susceptibility protein, two early events in apoptosis (Wolf et al 1997) demonstrating that the anti-apoptotic actions of zinc are complex.

4.5.5 Cytoskeleton

The extent to which an ovarian cell is differentiated may determine whether it is capable of undergoing apoptosis, or at least DNA degradation by an endogenous endonuclease. There are examples of apoptosis in the absence of low molecular weight DNA degradation or in which DNA degradation occurs some time after cell death (Oberhammer et al 1993a & 1993b; Omerod et al 1994; Ucker et al 1992). Morphological apoptosis can be induced in mechanically enucleated cells, and bcl-2 suppresses the "cytosolic" apoptosis of such cells (Jacobson et al 1994). DNase I may have roles in apoptosis independent of its DNA degrading activity. As stated earlier, a characteristic feature of DNase I is its ability to bind and be inhibited by G-actin (Hitchcock 1980; Lazerides and Lindberg 1974; Kreuder et al 1984). Indeed, the

crystallization and atomic modelling of G-actin required that it be bound to DNase I (Kabsch et al 1990; Mannherz 1992). The polymerization of G-actin (42 kDa) to F-actin is inhibited by DNase I and F-actin can be depolymerized by DNase I (Mannherz et al 1975). The actin-DNase I interaction occurs naturally in cells (Maliska-Blaszkiewicz and Roth 1993), although isoforms of the enzyme differ in their affinity for actin (Kreuder et al 1984; Lacks 1981; Polzar et al 1994). Clearly, cytoskeletal changes occur during apoptosis, as reflected in cytosolic and nuclear condensation and fragmentation of the cell. DNase I may participate in the morphology of apoptosis by its interaction with G-actin or F-actin. The cytoskeleton is more than just a structural element as it participates in the regulation of diverse cellular functions including steroidogenesis and responses to growth factors (Peppelenbosch et al 1993). Agents that disrupt the cytoskeleton also interfere with steroidogenesis in ovarian granulosa (Carnegie and Tsang 1987; Carnegie and Tsang 1988) and luteal (Gwynne and Condon 1982) cells. DNase I delivered to adrenal cells in liposomes disrupted steroid production (Osawa et al 1984), which was attributed to cytoskeletal changes induced by DNase I. The induction of cytosolic apoptosis in enucleated cells (Jacobson et al 1994) might therefore not exclude a central role of the endonuclease in apoptosis, but point to another function of DNase I in the disruption of cellular activity at the level of the cytoskeleton. Degradation of the genome may be a secondary or incidental function of DNase I.

4.6 INDUCTION OF APOPTOSIS IN THE OVARY

In vitro studies of preovulatory follicles and isolated ovarian cells have shown that gonadotropins suppress apoptosis in the ovary (Billig et al 1994; Chun et al 1994; Tilly et al 1995a; Tilly and Tilly 1995; Chun et al 1996). Preantral follicles isolated from DES-treated rats and cultured in the absence of serum undergo apoptosis that can be prevented by FSH (Chun et al 1996), indicating that this gonadotropin is an important survival factor in early follicle selection and development. Gonadotropin withdrawal can be induced very rapidly at any time in eCG-primed hamsters with a single injection of anti-eCG antibody (Bill and Greenwald 1981). The present findings demonstrate that this is also a good *in vivo* model to study apoptosis in early antral follicles in the rat.

4.6.1 Comparison to other *in vivo* models

The present study demonstrates that anti-eCG antibody induced apoptosis *in vivo* in small to medium sized antral follicles of eCG-primed rat ovaries. Apoptosis was detectable by 1 h after antibody injection as a significant increase in low molecular weight DNA that could be visualized as DNA ladders on electrophoretic gels. This model of eCG withdrawal should facilitate the study of early apoptotic features of atresia in follicles at the penultimate stage of development that cannot be addressed

using other models of eCG withdrawal. Atresia can be induced by a single injection of eCG to immature rats resulting in mature preovulatory follicles that degenerate 3-5 days later (Hughes and Gorospe 1991; Greenwald and Terranova 1988). This degeneration occurs at a slow and irregular pace, as gonadotropin is metabolically cleared from the circulation. The anti-eCG antibody allows for the rapid, synchronous removal of gonadotropin support from eCG-primed rat follicles *in vivo* at any chosen time after initial injection of gonadotropin. Rapid removal of gonadotropin can be achieved by hypophysectomy, leading to signs of apoptosis in preovulatory follicles 8 h later (Nahum et al 1996). This model also requires the metabolic clearance of existing gonadotropin in the circulation and, like the single eCG injection model, involves the study of apoptosis in large preovulatory follicles. Although atresia can occur any time during follicular development, normal atresia is most commonly observed in smaller follicles beginning the process of antrum formation and not in large preovulatory follicles. The anti-eCG antibody model induces atresia in these smaller follicles, and may more closely mimic the physiological incidence of atresia. Finally, the administration of anti-eCG antibody is technically simpler than hypophysectomy and removes gonadotropin, but not other pituitary hormones, from the circulation.

4.6.2 Ovarian apoptosis during eCG withdrawal

4.6.2.1 Morphology

Dead cells in small to medium sized antral follicles were identified by both *in situ* TUNEL labelling and acridine orange staining. Although TUNEL has been employed for this purpose in other studies (Billig et al 1994; Tilly and Tilly 1995), this is the first report of acridine orange staining to detect cell death in the ovary. The technique is simple, provides rapid results, and avoids any possible artifacts that may arise from fixation and processing of tissues. One caveat of TUNEL and acridine orange staining however, is that these methods do not distinguish between necrotic and apoptotic cell death. It was therefore necessary to confirm the occurrence of cell death due to apoptosis by morphological analysis of Hoechst stained granulosa cells from antibody-treated rats. Typical morphological signs of apoptosis were observed in these cells. In addition, necrotic DNA degradation, which results in a smear of DNA during electrophoretic analysis, was not observed in these studies. Hoechst staining of granulosa cells identified apoptosis in approximately 12% of the population in antibody-treated rats (c.f. 2% in controls), consistent with the identification of atresia by pyknotic nuclei described by Byskov (Byskov 1974; Byskov 1978). Although electron microscopic observation demonstrated the presence of granulosa cells with classic apoptotic morphology, most of the apoptosis presented as apoptotic bodies

already enclosed within other granulosa cells. This may reflect the rapid nature of this process once a cell has entered the apoptotic pathway. In addition, the absence of vasculature within the granulosa compartment may account for the accumulation of these phagocytic bodies within neighbouring cells.

4.6.2.2 Loss of cell-cell contact

Granulosa cells in anti-eCG antibody treated rat ovaries were occasionally observed, by electron microscopy, to be undergoing an apparent loss of normal cell-cell contact concurrent with early signs of nuclear fragmentation. The observed ultrastructural difference in membrane contact between an apoptotic granulosa cell and its surrounding cells is consistent with reports of suppressed apoptosis by cell-cell contacts in cultured granulosa cells (Peluso et al 1996; Trolice et al 1997). The rounding of the cell membrane and loss of flattened contact with neighbouring cells likely reflects a change in gap junctions and possibly cell adhesion molecules on the apoptotic granulosa cell. In serum-free culture, single granulosa cells died by apoptosis while cells in clumps remained viable (Peluso et al 1996). It was determined that this effect was due to homologous binding of N-cadherins between cells, and not due to gap junctions. The present findings suggest that part of the survival effect of gonadotropin is mediated by maintenance of N-cadherin or cell adhesion mediated signalling and that, following eCG withdrawal, the loss of cell-cell interaction is one

of the early events in granulosa cell apoptosis. In the mouse ovary, estrogen stimulated an increase in N-cadherin mRNA (MacCalman et al 1995). The rapid decline in estrogen associated with gonadotropin withdrawal could lead to decreased N-cadherin levels in the cell and the loss of N-cadherin mediated survival signals. The timing of this loss in cell-cell contact, relative to other biochemical markers of cell death, has not been determined.

4.6.2.3 Theca

Ultrastructural features of apoptosis have been observed in the theca of atretic sheep follicles (O'Shea et al 1978), but the current studies did not frequently demonstrate ultrastructural apoptotic morphology in the rat theca layer despite changes indicative of atresia, such as lipid droplet accumulation and cell rounding. This might represent a species difference or could be the result of different stages of follicle atresia being studied.

4.6.2.4 Basement membrane

Dramatic changes were seen in the basement membrane following antibody treatment. In particular, the interface of the granulosa-basement membrane changed from smooth with parallel filaments of collagen in the lamina densa, to a convoluted interface with

collagen fibrils crisscrossing on the thecal side of the basement membrane. The basement membrane acts as a molecular sieve to exclude large serum proteins from the inside of the follicle. Bavagandoss et al (1983) reported a loss of the lamina densa in atretic follicles, but the present report is the first to show the apparent degradation of collagen in the lamina densa at the ultrastructural level. Farookhi has suggested that inappropriate stimuli could lead to changes in basement membrane permeability leading to infiltration of serum proteins, such as complement, and subsequent atresia of the follicle (Farookhi 1980). The results indicate that gonadotropin withdrawal may lead to disrupted basement membrane integrity, and possibly allow access of serum proteins to the follicle. The nature of the serum proteins that might invade the follicle and the role of such factors in the initiation or progression of apoptosis is not known.

4.6.2.5 Activation of the endonuclease

The rapid onset of apoptosis is a key feature of this model. Previous work in hamsters has shown that serum estradiol levels decline by 55% within 1 h of anti-eCG treatment (Bill and Greenwald 1981). The finding that apoptotic DNA degradation (endonuclease activation) is evident within 1 h of antibody treatment is consistent with the reported rapid decline in estrogen levels, although it is not yet known, whether a drop in estrogen levels is a cause or an effect of granulosa cell apoptosis. The preferential cleavage of transcriptionally active chromatin may be one function of

endonuclease activity during apoptosis (Arends et al 1990), and selective decreases in aromatase mRNA have been found in atretic porcine follicles (Tilly et al 1992b). Estrogen suppresses apoptosis in immature follicles *in vivo* and androgen enhances apoptosis after estrogen withdrawal (Billig et al 1993). The cessation of aromatase gene transcription after endonuclease activation could conceivably cause estrogen levels to decline and androgen levels to increase, but it is not known whether one or both effects on steroid production mediate the progression of apoptosis in granulosa cells.

4.6.3 Withdrawal of FSH vs. LH activity

Both FSH and LH activity are present in eCG and therefore the induction of apoptosis by eCG withdrawal could reflect the cessation of either FSH or LH stimulation, or both. In small follicles, FSH binds exclusively to granulosa cells while LH binding is restricted to the theca (Camp et al 1991; Bortolussi et al 1979). Apoptosis occurred in the granulosa but not the theca after eCG withdrawal. FSH but not LH is a dominant survival factor in cultured early antral follicles (Chun et al 1996) and apoptosis was observed primarily in follicles of this size after antibody treatment *in vivo*, suggesting that it was the withdrawal of FSH stimulation that induced apoptosis after antibody treatment. Although FSH withdrawal can induce apoptosis in preovulatory follicles *in vitro* (Chun et al 1994; Tilly et al 1995a; Tilly and Tilly 1995; Chun et al 1996), this

was not observed in larger follicles after eCG withdrawal *in vivo*. Complete removal of FSH stimulation is possible *in vitro* by culturing follicles in serum free media, but this is not the case *in vivo*. It is probable that during antibody treatment the levels of eCG decreased to a level below that required for small antral follicle survival but sufficient amounts of the gonadotropin remained to stimulate larger follicles, which have more receptors and are more responsive to small amounts of gonadotropin.

Gonadotropin withdrawal by the anti-eCG antibody is an excellent model since it is technically simple and induces apoptosis in follicles commonly seen to undergo atresia under normal physiological conditions. The ability to induce atresia in small to medium sized follicles in a controlled fashion and the rapid onset of apoptosis following gonadotropin withdrawal make this model ideal for the study of signalling mechanisms and early events in granulosa cell apoptosis and follicle atresia *in vivo*.

4.7 REGULATION OF DNase I DURING APOPTOSIS AFTER eCG WITHDRAWAL

4.7.1 Gonadotropins

DNA degradation, not always observed during apoptosis in other systems (Omerod et al 1994; Bortner et al 1995), is a consistent feature of follicular atresia and luteal

regression. A complete understanding of the regulation of DNA ladders in ovarian cells by hormones, growth factors and cytokines requires the identification of the endonuclease. To that end, the present work has shown that the enzyme responsible for apoptotic DNA degradation is biochemically, immunologically and functionally indistinguishable from DNase I. The model, shown in figure 6, shows how gonadotropins and intraovarian factors could possibly regulate DNase I or the cytoskeleton to mediate both biochemical and morphological features of apoptosis as well as ovarian steroidogenesis. FSH might suppress the activity of DNase I via the bcl-2 or caspase-mediated pathways. The interaction of G-actin and DNase I may serve to suppress apoptotic DNA degradation in the ovary. Conversely, DNase I binding to G-actin may mediate cytoskeletal changes associated with apoptosis. Studies of the regulation of DNase I expression during follicular development and DNase I activation during apoptosis will help to elucidate the cellular mechanisms of ovarian follicular atresia and luteal regression.

4.7.2 Bcl-2

The mechanism of bcl-2 action to inhibit apoptosis has not been resolved but several studies of bcl-2 overexpression have given insight into possible biochemical functions of this oncogene product (Reed 1994). These studies have focused on effects of bcl-2 overexpression on mitochondrial, nuclear and ER functions, since these are the normal

subcellular localizations for this protein. In all these studies, bcl-2 overexpression conferred resistance to the induction of cell death. Overproduction of bcl-2 was found to have no effect on mitochondrial oxidative phosphorylation as measured by ATP levels and oxygen consumption in PC12 cells (Mah et al 1993; Zhong et al 1993). Electron microscopic studies of bcl-2 in the nucleus demonstrated that the protein distributes in patches reminiscent of nuclear pore complexes (Krajewski et al 1993), but the colocalization of bcl-2 and nuclear pores has not been systematically studied. If bcl-2 does interact with the nuclear pore complex, it might function to influence nuclear transport, pore formation or nuclear membrane assembly (Reed 1994). The localization of bcl-2 in the ER raised the possibility that it may regulate apoptosis through effects on intracellular Ca^{2+} signalling. Although bcl-2 could inhibit apoptosis induced by Ca^{2+} ionophores, the overexpression of bcl-2 did not prevent rises in intracellular Ca^{2+} (Zhong et al 1993). The partitioning of Ca^{2+} was affected by bcl-2 in cytokine dependent cells (Baffy et al 1993; Marin et al 1996), such that redistribution of Ca^{2+} from the ER to the mitochondria during apoptosis was prevented by bcl-2 overexpression. These results show that bcl-2 can have effects on the distribution of intracellular Ca^{2+} but not on Ca^{2+} signalling systems. A similar effect on the redistribution of Ca^{2+} to the nucleus could conceivably have direct effects on the ability of cells to activate endonucleases like DNase I during apoptosis. It remains to be determined if bcl-2 effects on Ca^{2+} partitioning are relevant to apoptosis. Both partitioning of Ca^{2+} in the nucleus and apoptosis of rat granulosa cells was suppressed

by progesterone (Luciano et al 1994). Regulation of Ca^{2+} partitioning and suppression of apoptosis are also functions of bcl-2, which raises the possibility that progesterone suppression of apoptosis may be mediated by bcl-2.

4.7.3 Caspases and bcl-2

Liu et al (1996b) have provided a possible link between bcl-2 and the activation of caspase-3. bcl-2 overexpression prevents the activation of caspase 3 by a variety of apoptotic signals. The major portion of bcl-2 in cells is found on the outer mitochondrial membrane suggesting a role for mitochondria in apoptosis. Liu and colleagues found that cytochrome c (from mitochondria) is a necessary component for the activation of caspase-3 (Liu et al 1996b). In cells overexpressing bcl-2, cytochrome c release from mitochondria, in response to apoptotic stimuli is blocked, preventing the activation of caspase-3 (Kluck et al 1997; Yang et al 1997). Whether this represents the main function of bcl-2 to suppress apoptosis, and the mechanism whereby bcl-2 prevents cytochrome c release is not known.

4.7.4 Caspases

Activation of caspases triggers apoptosis in cells. The most extensively studied caspase is caspase-3. This enzyme is activated by a variety of apoptotic signals

including serum withdrawal, Fas activation and radiation (Chinnaiyan et al 1996a; Erhardt and Cooper 1996; Hasegawa et al 1996; Schlegal et al 1996; Enari et al 1996). Transgenic deletion of caspase-3 results in mice with excessive neuronal cells due to lack of apoptosis in the brain (Kuida et al 1996). Caspase-3 treatment initiates the apoptotic program in normal cytosol (Enari et al 1996). Although caspase-3 cleaves several different protein substrates including PARP, DNA-PK, and sNRP, a causal relationship between degradation of these proteins and initiation of DNA degradation has not been established. Caspase-3 alone is not able to induce DNA degradation in isolated nuclei (Liu et al 1997; Yang et al 1997).

4.7.4.1 PARP cleavage

PARP cleavage was not detected following anti-eCG antibody induced apoptosis in rat follicles *in vivo*. Several recent lines of evidence suggest that PARP cleavage by caspase-3 is not essential for apoptosis or DNA degradation. Work by Liu et al (1997) has shown that DNA degradation in isolated nuclei can proceed in the absence of PARP cleavage. Transgenic mice deficient in the gene for PARP display no defect in the normal process of apoptosis (Wang et al 1995). In the present studies, induction of apoptosis by anti-eCG antibody in rat ovarian follicles was not accompanied by measurable PARP cleavage. It is unclear why PARP cleavage was not detected, since DNA degradation was clearly evident in this model. The recent evidence that PARP

cleavage is not essential for DNA degradation indicates that the possible interaction of PARP and DNase I is not involved in the regulation of apoptosis in the ovary. Although the absence of PARP degradation in the present study is surprising, it is not inconceivable, given the recent evidence that PARP degradation is a dispensable consequence of cell death.

4.7.4.2 Actin cleavage

DNase I is inhibited by actin, and this interaction is known to occur in intact cells (Lazerides and Lindberg 1974; Maliska-Blaszkiewicz and Roth 1993). The inhibition of DNase I by actin awaits a physiological explanation. It has been suggested that actin inhibition serves to protect the genome during mitosis (Lazerides and Lindberg 1974). Others have suggested that the function of actin inhibition is to prevent apoptotic DNA degradation by DNase I (Peitsch et al 1993; Polzar et al 1993). The finding that caspases cleave actin, and that cleaved actin does not inhibit DNase I (Kayalar et al 1996), raises the interesting possibility that the actin-DNase I interaction might have the physiological function of preventing apoptosis. Activation of caspases during apoptosis could conceivably lead to the cleavage of actin, removing inhibition of DNase I and causing DNA degradation. Actin cleavage was demonstrated during apoptosis in PC12 pheochromocytoma cells and in extracts of U937 cells (Kayalar et al 1996; Mashima et al 1997). Conversely, Lavin and colleagues showed that actin

cleavage did not occur during the induction of apoptosis in four different human cell lines (Song et al 1997). The lack of actin cleavage was not due to the absence of activated proteases, since DNA-PK was degraded during apoptosis, and actin was cleaved by extracts from each of the cell lines. Thus, the cleavage of actin by proteases *in vitro* did not necessarily reflect the proteolysis of actin in intact cells. The degradation of actin during apoptosis in the ovary was examined. Actin was cleaved in a manner consistent with the action of caspase activity during luteal regression induced by $PGF_{2\alpha}$, but actin cleavage was not observed during atresia induced by anti-eCG antibody. DNA degradation was observed during both luteal regression and follicular atresia, thus indicating that actin cleavage is not essential for apoptotic DNA degradation. It remains to be determined whether the inhibition of DNase I by actin is regulated by some other mechanism, or whether the actin-DNase I interaction has some other cellular function not involved in apoptotic DNA degradation or apoptosis.

These negative findings of actin and PARP cleavage as mechanisms for the regulation of DNase I activity and, therefore, of DNA degradation suggest that some other mechanism should exist for the inhibition of DNase I. An alternate explanation is that DNase I is not the endonuclease responsible for the degradation of DNA in ovarian nuclei. While this is possible, it does not account for the fact that the endonuclease activity could be removed from nuclear extracts with an anti-DNase I antibody, as shown by the reconstitution experiments with nuclei from DES-treated rats. Extracts

from eCG -treated rat ovarian nuclei contained an activity able to produce DNA ladders in ovarian cell nuclei from DES-treated rats, but immunoprecipitation of the eCG extract with anti-DNase I antibody removed this activity.

4.7.4.3 DNA fragmentation factor

Recent experiments by Wang and coworkers, with reconstituted nuclei and cytosol, have begun to determine which proteolytic events are involved in the DNA degradation associated with apoptosis, and have identified a novel protein that triggers DNA fragmentation after being cleaved by caspase-3 (Liu et al 1997). This protein, called DNA fragmentation factor (DFF), is a heterodimer of 40 and 45 kDa. The latter subunit is cleaved by caspase-3 *in vitro* and during apoptosis. Purified DFF did not induce DNA degradation in nuclei until it was cleaved by caspase-3. Although caspase-3 alone was able to cause cleavage of PARP and lamins in isolated nuclei, neither of these events were associated with DNA fragmentation. When cleaved DFF was added to nuclei in the presence of caspase-3 inhibitors, there was no cleavage of PARP or lamins, but DNA degradation was observed (Liu et al 1997). These results demonstrate that DFF may be the link between proteolysis and DNA degradation during apoptosis. In addition, these findings suggest that PARP and lamin cleavage are not part of the caspase-3 mechanism that causes DNA degradation. The nature of the DFF action that leads to DNA degradation has not been determined. DFF was not able

to cleave naked DNA and is therefore not a nuclease itself, but rather some sort of nuclease activator (Liu et al 1997). The present results indicate that DNase I is the endonuclease responsible for DNA ladders in ovarian cells. This suggests that DFF somehow activates the DNase I that is endogenously present in ovarian nuclei. Whether this involves a direct interaction of DFF with DNase I, or activation of a signal transduction pathway that activates DNase I, remains to be determined.

4.7.5 Caspase-3 in the ovary

The immunolocalization of caspase-3 in the rat ovary revealed that the enzyme is present in the theca and oocytes of healthy follicles and luteal cells of the CL. Little or no staining was present in granulosa cells of healthy follicles or interstitial tissue. In atretic follicles following gonadotropin withdrawal some granulosa cells were positive for caspase-3. The scattered pattern of positive staining for caspase-3 in atretic follicles was similar to the scattered pattern of cell death identified by *in situ* end-labelling. This suggests that caspase-3 is not normally present in granulosa cells but is expressed as part of the process of apoptosis. Consistent with this hypothesis, Flaws et al (1995b) showed that rat ovarian mRNA levels for caspase-3 are suppressed by gonadotropin treatment, and increased in follicles undergoing apoptosis. Reed and colleagues have recently published an exhaustive study of caspase-3 localization in a variety of human tissues (Krajewska et al 1997). They reported that caspase-3 is

present in most tissues, but there are dramatic differences in the level of the protein between different cell types. In the human ovary, intense caspase-3 staining was found in the theca cells of secondary follicles and in the corpus luteum. Relatively little staining was found in granulosa cells from secondary follicles. It was suggested that the low levels of caspase-3 in some tissues did not necessarily preclude a role for this enzyme in cell death, but rather that cells expressing large amounts of caspase-3 would require only activation of the enzyme for death to proceed, whereas cells not containing caspase-3 would need to synthesize the protein before undergoing apoptosis (Krajewska et al 1997). Proteolysis of PARP and actin was evident in CL during $PGF_{2\alpha}$-induced regression, but not in gonadotropin withdrawal-induced follicular atresia. This is consistent with the relatively high levels of caspase-3 in CL observed in the present study and reported by Krajewska et al (1997). Caspase-3 may be active in the gonadotropin deprived granulosa cell, but at levels not detected using PARP or actin as indicators. Presumably the caspase-3 would be active enough to cleave sufficient DFF to initiate detectable levels of DNA ladders, but not enough to detect general proteolysis of PARP or actin. Since the expression of caspase-3 is apparently limited to cells undergoing apoptosis (approximately 12 % in this study) the proteolysis of PARP or actin might be masked by the larger numbers of granulosa cells not expressing the enzyme. In the CL, PARP or actin degradation would be more widespread since larger numbers of these cells contain caspase-3. The present immunohistochemical observations of caspase-3 do not provide information about the

activity of the enzyme. Presumably, the enzyme is not active in healthy CL or theca cells of the follicle. The work of Liu et al (1997) demonstrates that apoptosis requires the presence of DFF, caspase-3 and an endogenous nuclear endonuclease. There are no studies, thus far, of the distribution of DFF in the ovary. It is possible that the caspase-3 is active in healthy CL or theca cells, but this does not lead to apoptosis due to the absence of DFF in these cell types. In addition, the present work has demonstrated that theca cells may not undergo apoptosis because they lack the DNase I found in susceptible granulosa cells. Caspase-3 may have other functions unrelated to apoptosis in cells such as the cleavage of SREBP (Wang et al 1996), which is a normal response of cells deficient in sterols. Cleavage of SREBP releases a transcription factor which induces genes that encode the receptor for LDL and enzymes involved in cholesterol biosynthesis, resulting in the accumulation of cholesterol in the cell (Brown and Goldstein 1997). Whether this is a function of caspase-3 in the ovary is not known, but both theca cells and luteal cells are the principal sites of cholesterol uptake in the ovary (Gore-Langton and Armstrong 1988). Under normal circumstances, low sterol levels activate the proteolysis of SREBP and higher cellular levels of sterols stop the proteolysis. Goldstein, Brown and colleagues produced truncated forms of SREBP containing only the portion responsible for transcriptional activation and found that cultured cells expressing this protein filled up with large amounts of cholesteryl esters (Brown and Goldstein 1997). In theca cells, the activation of caspase-3 and cleavage of SREBP might account for the hypertrophy and accumulation of lipid droplets observed

following gonadotropin withdrawal with the anti-eCG antiserum. It is possible that lipid droplets full of esterified cholesterol could serve to allow the theca to continue to produce large amounts of androgens to induce, or augment, apoptosis in the granulosa cells during follicular atresia. Similarly, luteal cells accumulate cholesterol in lipid droplets, which allows for the production of large amounts of progesterone, and this may be due to the activity of caspase-3. DNase I was present in healthy luteal cells, suggesting that if caspase-3 is active in this cell type there must not be the requisite DFF to cause apoptosis. In addition, there may be other proteases that respond to sterol levels in the cell by cleaving SREBP (Brown and Goldstein 1997) while caspase-3 might only be active in luteal cells during apoptosis.

4.8 SUMMARY OF DISCUSSION

This work has established the presence of endogenous endonuclease activity in rat ovarian cells and demonstrated that a 32/34 kDa activity had a developmental pattern of expression that correlated with the ability of ovarian nuclei to undergo apoptosis. Biochemical characterization of the 32/34 kDa endonuclease showed that it had cation, pH and sensitivity to inhibitors that matched the biochemical features of apoptosis. The 32/34 kDa activity was identified as DNase I, based on its inhibition by G-actin, and identification and immunoprecipitation with anti-DNase I antibody. The role of DNase I in the production of low molecular weight DNA (DNA "ladders") was

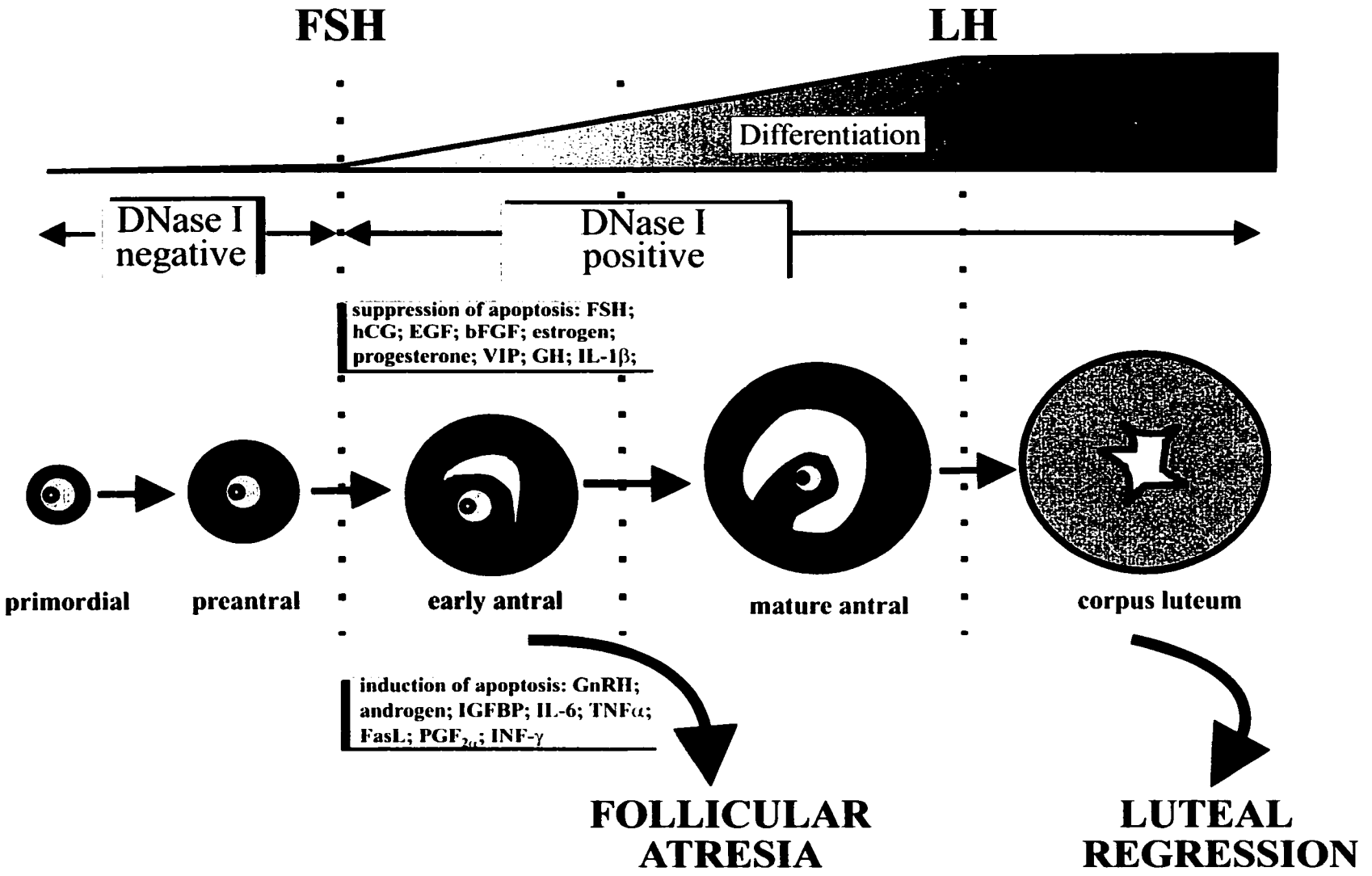
demonstrated by the fact that immunodepletion of DNase I from nuclear protein extracts removed the ability of these extracts to produce ladders in isolated nuclei. There was a developmental pattern of DNase I mRNA in ovarian cells, but the pattern suggested that DNase I production in the ovary may be regulated at the level of translation. Immunohistochemical analysis of DNase I in the rat ovary also suggested a role for this enzyme in apoptosis. DNase I was localized in the nuclei of granulosa cells in antral follicles, luteal cells and oocytes of preantral follicles which are all prone to apoptosis. Granulosa cells in preantral follicles, oocytes of antral follicles and theca cells did not display DNase I immunoreactivity. To study the potential regulation of DNase I during apoptosis in the ovary, an experimental system was developed wherein immunodepletion of gonadotropin *in vivo* resulted in apoptosis of rat ovarian follicles at the penultimate stage of development. Using this system, and an established technique for the induction of apoptosis in the CL, the possible regulation of DNase I by cleavage of actin or PARP was investigated. Actin and PARP were cleaved during apoptosis associated with luteal regression, but not follicular atresia, suggesting that cleavage of these two proteins is not associated with the activation of DNase I. Caspase-3 was not detected in granulosa cells of healthy follicles, which may explain why extensive PARP and actin cleavage were not observed during apoptosis in that tissue. The PARP and actin cleavage observed during CL regression was consistent with the intense caspase-3 immunostaining in that tissue. In atretic follicles, caspase-3 was localized in granulosa cells in a pattern consistent with the occurrence

of apoptosis, which suggests that although caspase-3 is not widespread in granulosa cells, it may be expressed during granulosa cell apoptosis. Although actin and PARP cleavage are not involved in DNase I activation, caspase-3 may activate DNase I by cleaving the recently discovered DFF. Theca cells contain substantial caspase-3, but do not die by apoptosis possibly because they do not have DNase I in their nuclei.

4.9 CONCLUSION

These studies lead to the conclusion that DNase I is the endonuclease responsible for low molecular weight DNA degradation in the ovary, and provide a basis for a model of the expression and activation of DNase I during follicular development and atresia. As shown in figure 45, follicles undergo a transition from the preantral to antral stage, accompanied by differentiation of the granulosa cells. This transition marks the entry of the follicle into the penultimate stage of development when the fate of the follicle, to progress toward ovulation or die by atresia, is determined by the actions and interactions of gonadotropins and intraovarian factors. The transition from preantral to antral development also marks the point when granulosa cells acquire DNase I, and thus the ability to die in response to the survival or death signals provided by gonadotropins and intraovarian factors. Once the granulosa cells acquire DNase I, a signal is sufficient to cause them to undergo apoptosis. This signal may be due to the loss of a survival signal such as IGF-1 or EGF/TGFα, or due to the presence of a death

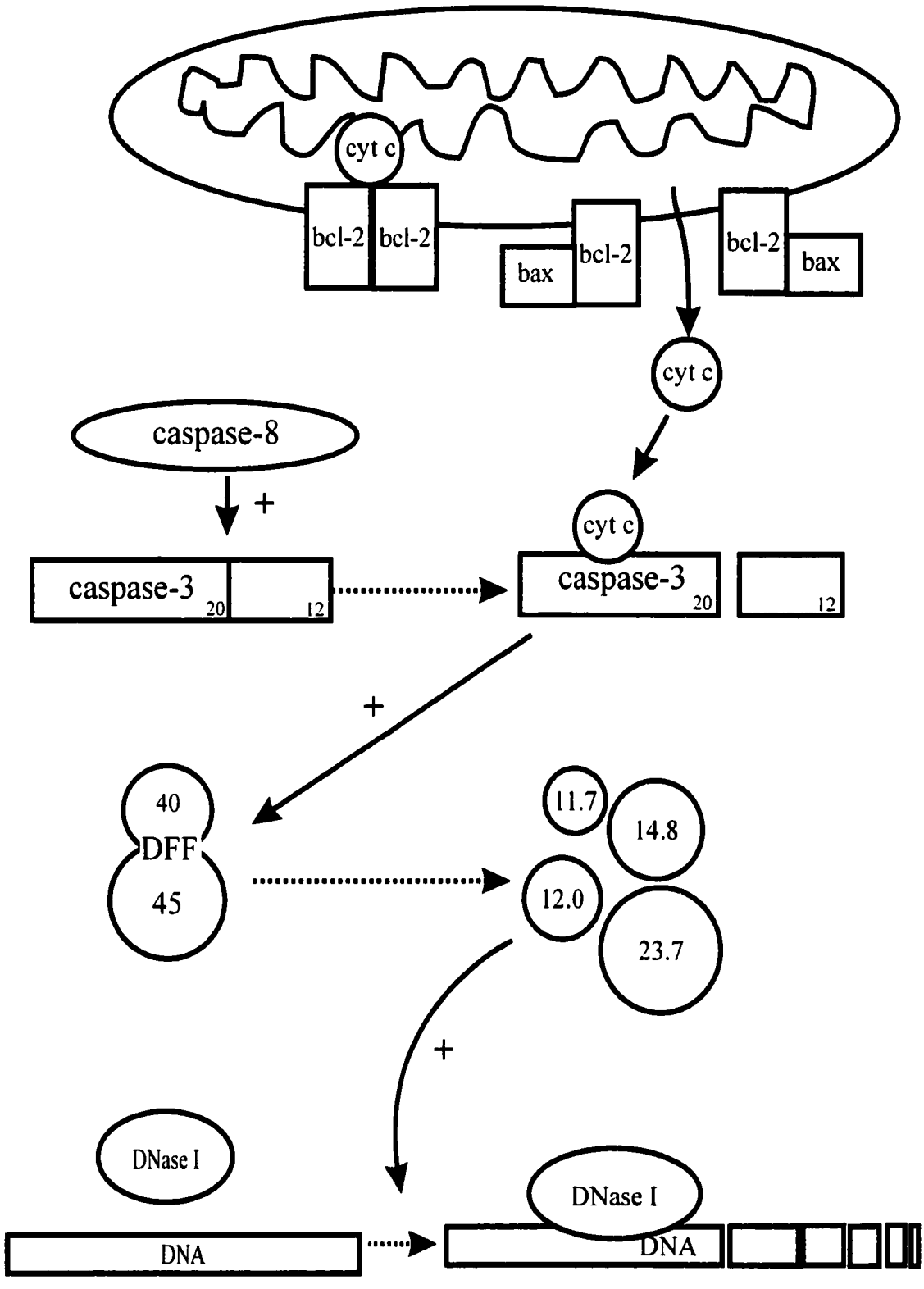
Figure 45: DNase I expression during follicular development, atresia and luteal regression. DNase I is not present in preantral follicles but is expressed in antral follicles after the initiation of granulosa cell differentiation by FSH. The occurrence of DNase I in granulosa cells correlates with the penultimate stage of development when follicles are most susceptible to atresia. Factors that induce or suppress apoptosis in granulosa and luteal cells must act, in part, to activate or suppress the endogenous DNase I activity.


FSH
LH
Differentiation
DNase I negative
DNase I positive
suppression of apoptosis: FSH; hCG; EGF; bFGF; estrogen; progesterone; VIP; GH; IL-1β;
primordial
preantral
early antral
mature antral
corpus luteum
induction of apoptosis: GnRH; androgen; IGFBP; IL-6; TNFα; FasL; PGF2α; INF-γ
FOLLICULAR ATRESIA
LUTEAL REGRESSION

signal, such as FasL or TNFα. The activation of DNase I does not appear to involve actin or PARP but may be mediated by caspase-3 (fig. 46). The degradation of nuclear DNA occurs when caspase-3-cleaved DFF is mixed with isolated nuclei. Thus only caspase-3, DFF and DNase I may be necessary for the low molecular weight degradation of DNA. A model for the regulation of DNase I, involving caspase-3 and DFF, is shown in figure 46. Caspase-8 is activated by a death signal and cleaves caspase-3. In addition to cleavage, caspase-3 requires mitochondrial cytochrome-c for activity. Release of this molecule is regulated by the dimerization state of bcl-2 and related proteins. Cleaved caspase-3, in the presence of cytochrome-c, acts on DFF to degrade the 45 kDa subunit of this protein. The cleaved 45 kDa subunit acts on the nucleus, or directly on DNase I, to induce DNA fragmentation. The mechanism whereby DFF might interact with DNase I is not yet known.

It seems likely that the main function of DNase I in ovarian cells is to degrade DNA during apoptosis. The role of DNA fragmentation in ovarian apoptosis is not completely understood. It has been suggested that DNA degradation ensures that genetic information in a potentially harmful cell is destroyed before being released to neighbouring cells. However, ovarian cells that die by apoptosis are not malignant or virulent; therefore, this seems to be an unlikely function of DNA degradation in this cell type. DNA fragmentation, even at the earliest high molecular weight stages of the process, would render the cell incapable of the necessary chromatin reorganization that

Figure 46: Revised model for the regulation of DNase I and apoptosis in ovarian cells. Three components are necessary for the induction of DNA degradation in the nucleus: activated caspase-3, DNA fragmentation factor (DFF), and DNase I. Caspase-3 cleaves the 45 kDa subunit of DFF and renders it capable of activating latent DNase I in the nucleus. Caspase-3 is inactive until proteolytically cleaved by caspase-8 and requires cytochrome c for full activity. Cytochrome c is normally retained in the mitochondria by the action of homodimeric bcl-2. Cell death inducing gene products (such as bax) heterodimerize with bcl-2 to allow cytochrome c release from the mitochondria which can activate caspase-3. Actin and PARP cleavage are not necessary for activation of DNase I, although these two proteins may be cleaved by caspases during apoptosis. Gonadotropins suppress apoptosis, in part, by maintaining high levels of bcl-2, and low levels of bax and caspase-3 in granulosa cells.


cyt c
bcl-2
bcl-2
bax
bcl-2
bcl-2
bax
cyt c
caspase-8
+
caspase-3
20
12
cyt c
caspase-3
20
12
+
40
DFF
45
11.7
14.8
12.0
23.7
+
DNase I
DNA
DNase I
DNA

accompanies cell division. Thus, if a cell committed to death subsequently received a signal to undergo mitosis, it would not be able to respond to that signal. This may be important in the ovary where follicles do not develop in isolation, but rather amongst other follicles in a range of developmental states within an ovary containing various local and systemic hormones, growth factors and cytokines. Finally, one important feature of apoptosis is the absence of widespread inflammation in the tissue. This is accomplished, in part, by the shrinkage and encapsulation of cellular components in membrane-enclosed vesicles. In order to remove the nucleus, it is necessary to physically reduce it to the smallest components possible. In addition, entire tissues must be removed by the process of apoptosis in the ovary; for this to occur, they must be physically broken down in a controlled fashion. This controlled reduction in tissue size may be one of the important functions of DNA fragmentation during apoptosis. Indeed, the continuous growth and development of follicles and CL during the reproductive lifespan of the female would make such a function essential.

Whatever the function of DNA degradation in the ovary, it is now clear that low molecular weight DNA degradation is a consistent feature of ovarian follicular atresia and luteal regression in diverse species. Further study of DNase I, the enzyme responsible for that DNA degradation, will provide insight into the cellular and molecular mechanisms of ovarian follicular atresia and luteal regression.

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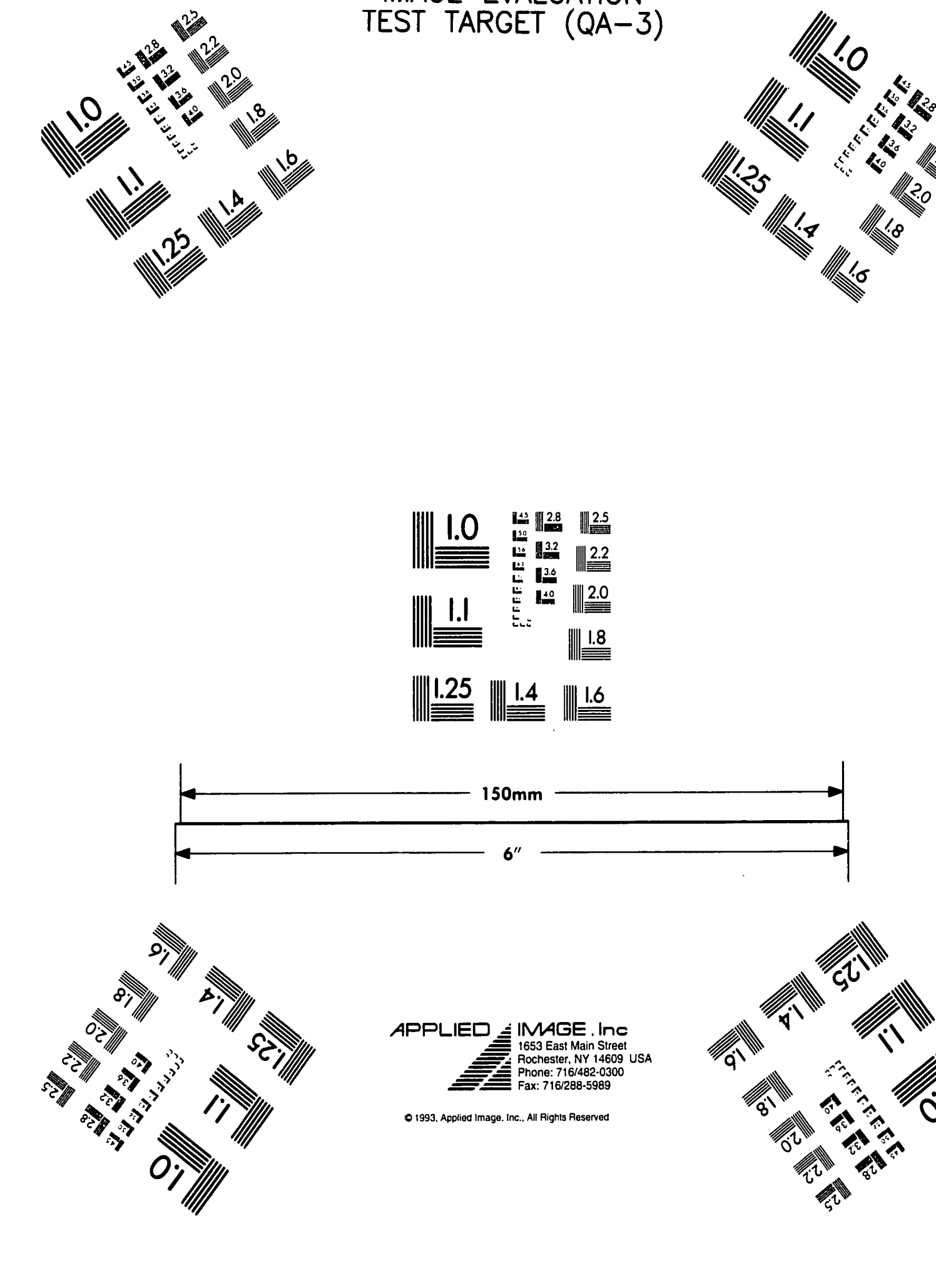
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