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Acknowledgments

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<tr>
<td>ACTH</td>
<td>adrenal corticotrophin hormone</td>
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<td>CAPK</td>
<td>ceramide activated protein kinase</td>
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<tr>
<td>CAPP</td>
<td>ceramide activated protein phosphatase</td>
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<tr>
<td>Cer</td>
<td>ceramide</td>
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<tr>
<td>dbcAMP</td>
<td>dibutyryl cyclic adenosine monophosphate</td>
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<tr>
<td>dhCeramide</td>
<td>dihydroceramide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>FasL</td>
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<td>FasR</td>
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<td>follicle stimulating hormone</td>
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<td>hCG</td>
<td>human chorionic gonadotrophin</td>
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<td>IL-1</td>
<td>interleukin-1</td>
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<td>kD</td>
<td>kiloDalton</td>
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<tr>
<td>LC/ESI/MS</td>
<td>liquid chromatography interphased electrospray ionizing mass spectrometry</td>
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<td>LH</td>
<td>leuteinizing hormone</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>Abbreviation</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>NL</td>
<td>neutral lipids</td>
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<tr>
<td>OSE</td>
<td>ovarian surface epithelium</td>
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<td>PC</td>
<td>phosphatidylcholine</td>
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<td>PE</td>
<td>phosphatidylethanolamine</td>
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<td>SIP</td>
<td>steroidogenesis inducing protein</td>
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<td>SM</td>
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<td>TGF-α</td>
<td>transforming growth factor alpha</td>
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<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
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Growth and Apoptosis in Surface Epithelial Ovarian Cancer

Manda Ghahremani

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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Abstract

Ovarian cancer is the leading cause of death from gynecologic malignancy. 80-90% of ovarian cancers in the adult originate from the ovarian surface epithelium (OSE). The control of cell number of the OSE, as in other cell types, is determined by a balance between cell proliferation and cell death. In examining factors which may regulate the growth of OSE, steroidogenesis inducing protein (SIP) was identified as a potent mitogen for several ovarian cancer cell lines derived from surface epithelium. We hypothesize that the majority of ovarian cancers originate from OSE lined inclusion cysts which fail to be eliminated in the stroma. To identify the naturally occurring ligands that are involved in activating the mechanisms by which inclusion cysts are normally eliminated, we examined the ability of anti-Fas mAb, known to activate the Fas receptor, to induce apoptosis in two cell lines, HEY and Caov-3, derived from ovarian carcinomas of surface epithelial origin. Treatment of each cell line with anti-Fas mAb caused chromatin condensation, nuclear segmentation and apoptotic body formation, morphological changes indicative of apoptosis. Furthermore, we have shown that anti-Fas mAb activates the sphingomyelin-ceramide signal transduction pathway. Treatment of Caov-3 cells for 30 min caused a 40% decrease in the total sphingomyelin content. The six most abundant sphingomyelin species identified in Caov-3 cells were 34:1, 36:1, 40:1, 41:1, 42:1, and 42:2. Specifically three of these species, 34:1, 40:1 and 42:2, were significantly reduced upon treatment with anti-Fas mAb. Treatment of these cell lines with ceramide, a product of sphingomyelin hydrolysis, using a cell permeable analogue C2-ceramide, also caused the above cells to undergo apoptosis. The ability of anti-Fas mAb and C2-ceramide to induce apoptosis was also observed in ovarian cancer cells derived from ascites fluid of a patient with ovarian cancer. Therefore, the Fas ligand/receptor system acting through the sphingomyelin-ceramide pathway, provides a mechanism by which ovarian surface epithelial cancer cells can be induced to undergo apoptosis. Thus, the lack of proper functioning of this apoptotic mechanism can lead to ovarian tumorigenesis.
CHAPTER I:

INTRODUCTION
Ovarian Cancer:

Cancer of the ovary is the leading cause of gynecologic cancer death in the Western World (1). Each year approximately 60% of diagnosed cases of ovarian cancer result in fatalities (2). Whereas germ cell teratomas and granulosa-thecal cell tumors are the most frequent ovarian tumors in the prepubertal female, 80-90% of ovarian cancers in the adult originate from the ovarian surface epithelium (3) which consists of a single layer of cuboidal or low columnar epithelial cells. This single layer of epithelial cells covers the ovary and undergoes mitosis following each ovulation to repair the wound on the surface of the ovary caused by the rupture of the follicle. The incidence of ovarian cancer correlates with the number of ovulations that occur during reproductive life (3). This correlation was derived from epidemiological data showing that the interruption of ovulation as a result of pregnancy, lactation, or oral contraceptive use, significantly reduced the risk of developing ovarian cancer (3,4). Conversely, nulliparous women or those who have taken fertility drugs to hyperstimulate the ovary (5,6) are at an increased risk of developing ovarian cancer, presumably due to the increased number of ovulations. Despite the existence of this epidemiological evidence and extensive research on endocrinological, environmental, and genetic factors, the molecular basis of the etiology of ovarian cancer is not understood.
In the past three decades, various hypotheses regarding the etiology of this disease have been formulated.

**“Incessant Ovulation” Hypothesis:**

The “incessant ovulation hypothesis” formulated by Fathalla (7) in 1972, proposed that the localized trauma caused by each ovulation and the breakdown of the ovarian surface epithelium at the site of follicular rupture to release the ovum, may be an important factor in the etiology of ovarian cancer. Current knowledge of tumorigenesis supports this hypothesis since the repetitive injury and repair of the surface epithelium due to successive ovulations, would increase the opportunities for mutations that could compromise the subsequent growth of the cells (8).

**Gonadotrophin Hypothesis:**

This hypothesis suggested that elevated plasma gonadotrophin levels increased the risk of developing ovarian cancer (9). According to this theory the use of oral contraceptives, that abolishes the ovulatory surge of gonadotrophins, offers protection against developing ovarian cancer. However, this theory is inconsistent with the protective effects of pregnancy, since during the period of gestation the levels of hCG are high. Also in women with polycystic ovarian disease (PCO), luteinizing hormone (LH) levels are usually elevated, and yet an increased incidence of ovarian cancer has not been observed in these women. In support of this hypothesis however, the high gonadotrophin levels in the post-menopausal period have been suggested to be a possible significant
factor in the development of ovarian cancer (10), since it is usually during this period that women are diagnosed with this disease.

**Genetic Basis of Ovarian Cancer:**

Overexpression of the HER-2/neu oncogene occurs in approximately 30% of breast and ovarian cancers (118). Mutation of the p53 tumor suppressor gene, with resultant overexpression of mutant p53 protein, occurs in 50% of late stage ovarian cancers and 15% of early stage cases of ovarian cancer (118). Recently, the BRCA1 tumor suppressor gene on chromosome 17q was identified and shown to be responsible for some cases of hereditary breast and ovarian cancer. Families in which mutations in this gene exist are usually characterized by early age of disease onset (118). Presently, it remains unclear what fraction of hereditary ovarian cancers are due to BRCA1 mutations.

**“Inclusion Cyst Formation” Hypothesis:**

In an attempt to derive a link between the etiology of ovarian cancer and the number of ovulations, Cramer and Welch (11) proposed that the first step in tumorigenesis of the surface epithelium is the formation of epithelial inclusion cysts derived from crypts or invaginations of the ovarian surface epithelium. Following each ovulation, the surface epithelial cells proliferate to heal the wound. The healing process can result in the formation of a crypt in the ovarian surface. These crypts can become internalized into the ovarian stroma where they form inclusion cysts lined with ovarian
surface epithelial cells. The formation of the cysts result in the disruption of the underlying connective tissue, and as the cysts are internalized the ovarian surface epithelial cells become in contact with stromal cells (FIG. 1), a microenvironment which may, under certain conditions, be conducive to proliferation.

Once internalized, the sequestered surface epithelium in these inclusion cysts are continually exposed to stromal derived factors which may lead to cellular transformation and neoplasms or the proliferation of existing mutations, unless the OSE lined inclusion cysts are somehow eliminated from the stroma. This is supported by the observation that in patients with early de novo ovarian cancer, persistence of inclusion cysts has been observed (12,13).

Based on the latter observations and hypotheses, it can be concluded that during normal reproductive life, newly generated surface epithelial cells that may contain mutations, are internalized into the stroma in the form of inclusion cysts and are subsequently eliminated. In women who develop ovarian cancer, surface epithelial cells in inclusion cysts are not eliminated, allowing potential sites of tumorigenesis to persist.

The understanding of the etiology of ovarian cancer of surface epithelial origin, centers around the mechanisms that are normally involved in the induction of growth and elimination of the OSE. Looking at the proliferation of OSE in fetal development and in the adult, provides some clues as to how this proliferation may be regulated.
FIG. 1. Following ovulation the wound on the surface of the ovary is repaired by the proliferation of OSE. The healing process can lead to the formation of crypts or closed epithelial lined structures. Penetration of the tunica albuginea results in the formation of inclusion cysts in direct contact with stromal cells. These inclusion cysts are the major sites of origin of ovarian cancer.
The origin of ovarian surface epithelium:

The OSE develops in the fetus, from the invagination of the coelomic mesothelium over the embryonic gonadal ridge (14). It originates from the epithelial lining of the intraembryonic coelum and is an epithelium of mesodermal origin. The Mullerian duct as well as the follicular cells (presumptive granulosa cells) arise from invaginations of the coelomic epithelium into the genital ridge. Thus, the OSE shares a common derivation with the epithelial cells of the oviduct, uterus, and granulosa cells.

Proliferation of OSE during fetal development:

In the human fetus, from 16 to 20 weeks of gestation, the OSE undergoes marked proliferation accompanied by a general loss of epithelial polarity and cellular orientation, severe nuclear irregularity, nucleoli prominence and nuclear infolding and pleomorphism, resulting in a clearly defined surface epithelium made up of multiple cell layers (14). This proliferative activity, resembles that seen in surface epithelial neoplasms of the adult ovary. Gondos (15) noted that this proliferative activity coincided with the appearance of interstitial cells in the ovarian stroma that have the morphological and biochemical characteristics of steroid producing cells. During this period, the OSE are in direct contact with the mesenchymal interstitial cells, due to the incomplete formation of the tunica albuginea that subsequently separates the OSE from the underlying mesenchyme. By 24 weeks of gestation, the underlying tunica albuginea is fully formed and the proliferative activity of the fetal OSE ceases (16). The OSE becomes a single-cell layer, similar in appearance to the surface epithelium of the adult
ovary. The specific growth regulatory factors that initiate and terminate this proliferative phase of the OSE are unknown but their actions appear to be dependent on direct cell-cell interactions between the OSE and the underlying mesenchymal-interstitial cells.

**Proliferation of OSE in the adult:**

The surface epithelium of the normal ovary generally remains quiescent or has a low mitotic activity (17). In the normal adult ovary the proliferative activity of the OSE is dependent on adjacent growing follicles in which androgen secreting thecal cells surround estrogen-secreting granulosa cells (18). LH acts on thecal cells to stimulate the synthesis of androgens (androstenedione, testosterone) from the precursor cholesterol. Androgens are converted to estrogens by granulosa cells through the action of FSH on aromatase (18). During the fertile life of a woman, a dominant follicle is selected on day 4-5 of each 28-day menstrual cycle. As the selected follicle enlarges and approaches the preovulatory stage, it protrudes from the surface of the ovary. The surface epithelium in the area of the growing follicle, undergoes rapid mitosis in order to accommodate the rapid increase in follicular size. Prior to ovulation induced by the LH surge, the OSE cells around the stigmata of the preovulatory follicle become flattened and degenerate (19) likely through the process of apoptosis as reported in ewes by Ackerman and Murdoch (20). After ovulation and release of the ovum from the follicle, the wound created at the rupture site on the OSE is repaired by the rapid proliferation of the epithelium. This is followed by a return to the normal low proliferative or quiescent state of the surface epithelium (5).
**Proliferative/inhibitory stimuli for OSE:**

Attempts to show a correlation between levels of gonadotrophins or steroid hormones and growth of normal OSE or ovarian cancer cell lines, gave inconsistent results (5, 21). However, instead of directly stimulating proliferation, these hormones may regulate the production or release of growth factors and/or their receptors. For example, cellular levels of TGF-α are known to be elevated by estrogens and the 5' flanking region of the TGF-α gene contains estrogen response elements (22). Estrogen stimulated the synthesis of TGF-β by rat granulosa cells (23). Progesterone and estradiol (E2) can significantly increase the release of EGF/TGF-α from ovarian tumor tissue (24).

**TGF-α/EGF:**

TGF-α is a member of the epidermal growth factor (EGF) family and binds to the same receptor as EGF, the EGF receptor (EGFR), which is a specific transmembrane receptor with tyrosine kinase activity (25). In general, the effects of TGF-α are qualitatively similar to EGF. TGF-α is synthesized as a transmembrane glycoprotein precursor. The pro-TGF-α can remain anchored in the plasma membrane and can interact directly with its receptor on adjacent cells or it can be cleaved and released as a soluble peptide to act in an autocrine or paracrine manner (25). TGF-α is a potent mitogen for many cell types including granulosa cells of the developing human follicle (26). TGF-α has been implicated in the regulation of apoptosis and has diverse effects on differentiated functions (27). The presence of TGF-α peptide in both normal OSE and HEY cells (an ovarian cancer cell line of surface epithelial origin) has been
demonstrated by immunocytochemistry (28). EGF and TGF-α stimulate the growth of many normal and malignant cells in vitro, including normal human OSE and human ovarian carcinoma (3, 28). Other human cancer tissues in which TGF-α is found, include breast (29), colon (30) and kidney (31). It is also found in the urine of cancer patients (32).

**Growth Inhibition of OSE:**

**TGF-β:**

Transforming growth factor β (TGF-β) is a structurally unrelated and functionally distinct polypeptide from TGF-α, and belongs to a large family of growth factors which regulate growth, differentiation, motility, tissue remodeling and repair and apoptosis. In mammals it exists in 3 isoforms -β1, -β2, -β3 of which -β1 is the most abundant. Although TGF-β stimulates growth of fibroblastic cells, it is a potent growth inhibitor in the majority of other cell types (33). In most cells, its effects oppose those of TGF-α/EGF. The inhibitory effects of TGF-β on proliferation of normal OSE cells and some ovarian cancer cell lines have been reported. mRNA for TGF-β1 and -β2 (34,35), as well as immunoreactive TGF-β1 and-β2 (36) have been found in normal OSE and ovarian cancer cell lines. Thus both TGF-α and TGF-β are present in the OSE. Loss of responsiveness to TGF-β1 or decreases in its production by free-floating tumor cells obtained from ascites fluid have been observed in 40% of cases (37).
Steroidogenesis Inducing Protein (SIP):

It is hypothesized that EGF/TGF-α that can stimulate OSE proliferation, may promote repair of postovulatary defects in the OSE. At the time of ovulation, approximately 6 ml of follicular fluid rich in growth factors and cytokines spills over the OSE as the follicle ruptures (38). TGF-α, TGF-β, as well as other growth factors and cytokines have been shown to be present in follicular fluid of the developing follicle (38). Recently a factor called steroidogenesis inducing protein (SIP) was isolated from human follicular fluid (39). SIP has a molecular weight of approximately 60 kilodaltons and was originally identified in follicular fluid on the basis of its profound effects on in vitro steroid production by testicular, ovarian and adrenal cells (40). SIP is distinct from LH/hCG in physicochemical, immunological and functional properties. In contrast to LH, SIP stimulates steroid production in early luteal and adrenal cells (40). Furthermore, unlike trophic hormones (LH, hCG, and ACTH), whose actions on steroidogenesis are mediated through cAMP, SIP does not stimulate cAMP production in the target cells, indicating a cAMP independent mechanism (40). It has been shown that SIP signals through the tyrosine kinase pathway (41). SIP was identified as a potent mitogen for immature rat Leydig cells and it was found to interact with TGFβ to stimulate proliferation of rat granulosa cells (42). The follicular fluid released from the follicle at the time of ovulation flows over the surface of the ovary and growth factors including SIP present in the follicular fluid may provide stimuli for the proliferation of surface epithelial cells during the healing process that follows ovulation.
**Dibutyryl cyclic AMP:**

cAMP is a pleiotropic regulator of cell metabolism, growth and differentiation. The gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), act primarily through the generation of cAMP, and the subsequent activation of protein kinase A (43). One of the most prominent effects of cAMP is growth regulation, including both growth stimulation and inhibition (43). Stimulation of cultures of primary rat granulosa cells by high levels of cAMP induced apoptosis in more than 90% of these cells (44). Also, one study reported that elevation of intracellular cAMP markedly inhibited the growth of the hormone refractory prostate cancer cell line PC-3 (43). Interestingly, the former study also showed that cAMP exerts a positive control on the regulation of TGF-β2 gene expression, as well as inducing TGF-β2 secretion by PC-3 cells (43). To date there have been no published reports on the effects of cAMP on normal and malignant OSE.

Since the regulation of proliferation of OSE is essential for the proper functioning of the ovary, it is not surprising that a variety of factors ensure the tight regulation of this process. The mitotic activity of OSE on the surface of the ovary is dictated by the different phases of follicular development and ovulation. While on the surface the OSE are separated from the stroma by a layer of connective tissue (tunica albuginea) (Fig. 1). However, as mentioned previously, following ovulation and formation of OSE lined crypts, these crypts are internalized into the stroma as inclusion cysts. The OSE is now in the stromal microenvironment which is rich in steroids and growth factors which can
modulate this proliferative activity. Thus it is logical that there is a mechanism which normally eliminates OSE lined inclusion cysts from the stroma. Such a mechanism would ensure that any mutations which may have occurred as a result of mitotic divisions of OSE following ovulation, would be eliminated without the risk of propagation. The molecular mechanism by which these inclusion cysts are normally eliminated has not been identified. In this thesis I propose that this is achieved by programmed cell death or apoptosis. If the apoptotic mechanism fails, the inclusion cysts would persist in the stroma where proliferative stimuli could lead to tumor formation. A most important observation in the context of this hypothesis is the persistence of these inclusion cysts in women with early de novo ovarian carcinoma (12, 13).

**APOPTOSIS:**

Apoptosis is an active, genetically governed process whereby cells die by a controlled, intrinsic program designed for their demise, without producing an inflammatory reaction such as that seen in necrosis (FIG. 2). Necrosis is a form of cell death resulting from acute cellular injury, which is typified by rapid cell swelling and lysis. In contrast, apoptosis is characterized by shrinkage of the apoptotic cell, membrane blebbing, chromatin condensation and formation of apoptotic bodies which are membrane bound nuclear fragments that bud off from the cell (45). These apoptotic bodies may subsequently be phagocytosed by neighboring cells. A unique biochemical event associated with apoptosis, is the activation of Ca\(^{2+}/Mg^{2+}\)-dependent endogenous endonucleases (45). These endonucleases specifically cleave DNA at internucleosomal regions resulting in fragments which, when separated by agarose gel electrophoresis,
FIG. 2. Apoptosis vs. Necrosis. Apoptosis is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and formation of apoptotic bodies. In apoptosis, the dying cell maintains its plasma membrane integrity. Necrosis is characterized by rapid cell swelling and lysis. There is an early loss of cell membrane integrity, resulting in leakage of cytoplasmic contents and the induction of an inflammatory response.
form a distinctive ladder pattern composed of DNA oligonucleosomal fragments that are multiples of 180-200 base pairs in length. Although the formation of the DNA ladder is considered a hallmark of apoptosis, the generation of DNA ladders is not a prerequisite step in apoptosis, as many instances of this form of cell death have been observed to occur without detectable DNA fragmentation (46). Intact apoptotic cells may also be identified by changes in cell morphology and chromatin condensation using phase contrast or electron microscopy or by fluorescence microscopy following treatment with DNA binding dyes (47).

The recognition of the role of apoptosis and its regulation in health and disease, has become prominent in all avenues of cell biology, tissue homeostasis and cancer. The Australian pathologist John F. R. Kerr and his Scottish colleagues broke ground in a paper published in 1972, when they contended that the same type of cell death evident during embryonic development, which was documented as early as the 1950’s, also happens in mature organisms and continues throughout life (48). They adopted the word *apoptosis* from classical Greek meaning “dropping off” as in the dropping off of flower petals or falling leaves, to distinguish this kind of cell death from *necrosis* which is Greek for “make dead”. Surprisingly, their profound insights in their 1972 publication, did not gain much attention by researchers for more than a decade.

Suppression of apoptosis increases the susceptibility of an individual to malignancy, whereas uncontrolled, excessive cell death is associated with degenerative
diseases (i.e. AIDS) (45). The control of cell number and tissue homeostasis is
determined by a balance between cell proliferation and cell death. Cell proliferation is a
highly regulated process involving numerous components such as growth factors, proto-
oncogenes and tumor suppressor genes. It is now appreciated that the regulation of cell
death is just as complex as the regulation of cell proliferation and the decision of a cell to
undergo apoptosis is influenced by a wide variety of regulatory stimuli.

**Apoptosis in the ovary:**

Apoptosis is vital to ovarian function. In fact, the maintenance of the health and
the proper functioning of the ovary is characterized by extensive cell death. More than
99% of ovarian follicles endowed at birth are destined to undergo programmed cell death
and the exhaustion of these follicles serves as a “clock” for female reproductive
senescence. Throughout reproductive life, cohorts of follicles are continuously recruited
from the pool of primordial follicles established during fetal life (49). Once a cohort of
follicles is recruited to grow, it is destined to undergo atresia, unless rescued by survival
factors (46). Thus the fate of a follicle, either to ovulate or undergo atresia, is
determined by the balance of signals that granulosa and thecal cells receive. Elimination
of granulosa cells in atretic follicles is a rapid process governed by apoptosis (50).
Thecal cells in atretic follicles persist for a longer period of time than granulosa cells, but
are also eliminated via apoptosis (51). Apoptosis is likewise involved in the process of
structural luteal regression (46) and in the process of dissolution of the follicular wall and
rupture at ovulation (46).
As mentioned previously, TGF-α and TGF-β are naturally occurring ligands that can influence apoptosis. TGF-β has been identified as an important factor in the regulation of programmed cell death in other systems including the liver, immune system and mammary gland where it can either act as an enhancer or inhibitor of apoptosis (33). EGF/TGF-α have been shown to protect rat granulosa cells from undergoing apoptosis (52), whereas in hepatocytes these factors enhance apoptosis (33). Recently our lab demonstrated that treatment of rat thecal cells with the combination of TGF-α and TGF-β caused the cells to undergo apoptosis, whereas in the presence of TGF-α or TGF-β alone the cells remained healthy (51).

Another naturally occurring ligand/receptor complex which regulates apoptosis is the Fas ligand/receptor system. The involvement of the Fas ligand/receptor system in the apoptotic mechanisms by which immune cells are eliminated has been studied extensively. For the last three years our laboratory has explored the possible role of the Fas ligand/receptor system in the regulation of ovarian cell number and the maintenance of tissue homeostasis.

THE FAS DEATH FACTOR:

Fas Antigen:

The Fas antigen is a 50 kD transmembrane protein that is expressed in many neoplastic and normal cells, including several hematopoietic cell lines, lymphoma cells and activated normal T and B lymphocytes (53). Many tissues and cell lines weakly express Fas, but abundant expression was found in mouse thymus, liver, heart, lung,
kidney and ovary (54). In humans the Fas gene is located on the long arm of chromosome 10 (53). Human Fas consists of 325 amino acids with a signal sequence at the NH₂-terminus and a membrane-spanning region in the middle of the molecule, indicating that Fas is a type I membrane protein (53). Fas was first identified in 1989 when a monoclonal antibody (mAb), anti-Fas, was developed that demonstrated cytolytic activity toward several cell lines (55). At approximately the same time, Trauth et al (56) independently identified another mAb, APO-1, that mediated tumor regression in mice by induction of apoptosis. Subsequent studies showed that the anti-Fas mAb also induced cell death by apoptosis (57). Molecular cloning of the cDNAs for Fas and APO-1 showed that they are the same molecule, which has also been designated as CD95 (57).

The structure of Fas indicated that it belonged to the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor family (53). This family includes two TNF receptors (TNF-R1 and TNF-R2), the low-affinity NGF receptor, B cell antigen CD40, T cell antigens OX40, CD 27 and 4-IBB, and Hodgkin’s lymphoma cell surface antigen CD30 (Fig. 3A). The extracellular regions of members of this family consist of three to six cysteine rich domains. The amino acid sequence of the extracellular region is relatively conserved, whereas the cytoplasmic region is not, except for some similarities between Fas and TNF-R1 (53). The cytoplasmic domain conserved between Fas and TNF-R1 is necessary and sufficient for transduction of the apoptotic signal. This domain was therefore designated a death domain (53).
FIG 3. TNF and its receptor family. (A) The TNF receptor family members are schematically shown. The striped regions represent cysteine-rich subdomains; each member of the family contains three to six of them. The death domain (about 80 amino acids) in the cytoplasmic regions of Fas and TNF-R1, which have some similarity, are shown as bold lines. The symbol --- indicates an N-glycosylation site. (B) The TNF family members are schematically shown. These members are type II membrane proteins, except for the α subunit of lymphotoxin which is a secretory protein. The shaded portion of each member is the extracellular region which shows significant similarity (25-30% identity) among the members. The number of the amino acids in the homologous region and the cytoplasmic region are indicated. The symbol ---- indicates an N-glycosylation site [Reproduced from (53), S. Nagata et al.].
Fas Ligand:

In 1994 Fas ligand (FasL) was purified and identified as a protein with a molecular weight of 40 kDa which had cytolytic activity against cells expressing Fas (22). FasL has no signal sequence at the NH$_2$-terminus, but it has a domain of hydrophobic amino acids in the middle of the molecule indicating that it is a type II membrane protein with the COOH-terminal region outside the cell and it belongs to the TNF family (53). A stretch of about 150 amino acids in the extracellular region of the Fas ligand has significant homology to the corresponding region of other members of the TNF family (Fig. 3B).
Sphingomyelin-Ceramide Pathway:

The sphingomyelin pathway is an ubiquitous, evolutionary conserved signaling system analogous to more well defined systems, such as the cAMP and phosphoinositide pathways (59). Historically, sphingomyelin was considered to be only a structural element of the plasma membrane, however experiments in the last decade revealed an alternate function for sphingomyelin in signal transduction (59). The sphingomyelin cycle was initially described by Okazaki et al. in 1989 in human HL-60 leukemia cells, in which sphingomyelin was rapidly and reversibly hydrolyzed in response to vitamin D3 (60). The hydrolysis of sphingomyelin was shown to be accompanied by the concomitant generation of ceramide (61). Sphingomyelin (N-acylsphingosin-1-phosphocholine) is a phospholipid preferentially concentrated in the outer leaflet of the plasma membrane of most mammalian cells and consists of a sphingoid base backbone, a fatty acid, and a phosphocholine head group (62). The sphingomyelin-ceramide signaling pathway is initiated by enzymatic hydrolysis of the phosphodiester bond of sphingomyelin by a specific sphingomyelin-directed phospholipase C, a sphingomyelinase, generating ceramide and phosphocholine (FIG. 4) Many of the receptors that couple to the sphingomyelin signal transduction pathway are members of the TNF receptor superfamily including the TNF receptor, the Fas receptor, and the low affinity nerve growth factor (63). Interleukin-1 (IL-1) and dexamethasone were also shown to induce sphingomyelin hydrolysis in certain cell types (63).
FIG. 4. Sphingomyelin hydrolysis via the action of a sphingomyelinase, generating ceramide and phosphorylcholine. [Reproduced from (62), W.D. Jarvis et al.]
There are two classes of sphingomyelinases, defined by their optimal pHs and subcellular location. An acidic isoform with an optimum pH of 5 is predominantly found in lysosomes. There are two forms of sphingomyelinase with neutral pH optima (64); one of the neutral isoforms is a Mg$^{2+}$-dependent membrane bound enzyme, and the other is a Mg$^{2+}$-independent cytosolic enzyme. Activation of Fas ligand/receptor complex has been linked to activation of both neutral and acidic sphingomyelinases (65). Many unanswered questions still remain regarding the mechanism of sphingomyelinase activation through cell surface receptors. In particular how the receptors link to these enzymes is unknown.

**CERAMIDE:**

Ceramide serves as the second messenger of the sphingomyelin pathway, mediating many of the effects of the extracellular stimuli which activate the pathway to induce growth arrest and programmed cell death (66). Ceramide generation is associated with the postreceptor actions of the TNF receptor superfamily, as well as other unrelated humoral factors such as interleukin 1 and 1,25-dihydroxyvitamin D3 (66). Details of the apoptosis-inducing pathway downstream of ceramide generation have yet to be elucidated, although several immediate targets for ceramide action have been identified. One target of ceramide is a 97-kDa serine/threonine kinase, termed ceramide-activated protein kinase (CAPK), leads to signaling through the mitogen activated protein kinase (MAP kinase) cascade (67). A ceramide activated serine-threonine protein phosphatase (CAPP) has also been identified and has been implicated in the ability of ceramide to
induce apoptosis and downregulate the c-myc protooncogene (67). In addition, ceramide has been shown to activate PKCζ, although the extent and specificity of this activation has not been determined (67). Ceramide is also known to activate the putative guanine nucleotide exchange factor VAV which activates ras (62). Ras proteins like other G proteins, contain bound GTP in the active state and GDP in the inactive state and ras activation involves rapid exchange of GTP for bound GDP. Ras activation has been implicated in the process of Fas induced apoptosis (62), although further studies are needed in different cell systems to confirm this effect.
Formation of Hypothesis:

The control of cell number of the OSE, as in other cell types, is determined by a balance between cell proliferation and cell death. The repetitive injury and repair cycles of the OSE, which are associated with ovulation, may cause mutations in genes involved in growth regulation. We propose that surface epithelial cells generated during the repair process are normally sequestered in inclusion cysts and are eliminated by apoptosis through the Fas ligand/receptor system. A loss or alteration in the apoptotic mechanism could result in the persistence of inclusion cysts, and exposure to proliferative stimuli generated by stromal cells in the microenvironment, could lead to propagation of mutations and the establishment of neoplastic tissue. The study of cell growth and cell death in transformed OSE was undertaken, using human surface epithelial carcinoma cell lines HEY and Caov-3, and to a limited extent OVCAR-3. To examine the possibility that the Fas ligand/receptor system may be involved in the induction of apoptosis in human ovarian surface epithelial cells, we used the HEY and Caov-3 cell lines. Furthermore we explored the possibility that Fas was acting through the sphingomyelin-ceramide pathway to induce apoptosis in these ovarian carcinoma cell lines.
CHAPTER II:

GROWTH STIMULATION/INHIBITION OF SURFACE

EPITHELIAL OVARIAN CANCER CELL LINES
INTRODUCTION:

As discussed in Chapter 1, the proliferation of the OSE is precisely regulated at clearly defined stages of the menstrual cycle. A specific aim of this chapter was to elucidate factors which regulate the proliferation of the OSE. Increased proliferation of OSE may lead to formation of mutations which would subsequently be internalized into the stroma as inclusion cysts, and thus the study of such growth regulating factors is integral to the testing of our hypothesis. The regulation of progression of a cell through mitosis depends on the activity of growth factors and their receptors (68). Local actions of such growth factors as TGF-α and TGF-β are mediated by autocrine and/or paracrine mechanisms and are essential for maintenance of cellular homeostasis. Epithelial cells in culture that respond to EGF/TGF-α with increased mitotic activity, are usually growth-inhibited by TGF-β (3). Cultures of surface epithelial cells from normal human ovaries respond to TGF-α with an increase in DNA synthesis (28), whereas TGF-β attenuates growth (35). These observations have implicated TGF-α and TGF-β in the regulation of the proliferative activity of the OSE during the menstrual cycle. Immunohistochemical studies in our lab have localized TGF-α in the surface epithelium and surface crypts of the normal human ovary (28). Treatment with TGF-α significantly stimulated the synthesis of DNA of primary cultures of normal OSE cells. This is consistent with previous findings that EGF enhanced the growth of normal OSE cells in vitro (21, 69, 70), and the demonstration that they contain TGF-α/EGF receptors (21, 69).
TGF-α mRNA and EGF receptor mRNA have been detected in primary ovarian carcinoma (71) and TGF-α stimulates the growth of some primary ovarian cancer cells and cell lines derived from the OSE (72, 73). mRNA for TGF-β1 and -β2 (35), as well as immunoreactive TGF-β1 and -β2 (36), are found in normal OSE and OSE cancer cell lines.

Thus, the growth of ovarian cancer cell lines HEY, Caov-3 and OVCAR-3 in response to TGF-α and TGF-β were assessed by measuring thymidine incorporation into DNA. As well, the responses of the above cell lines to dibutyryl cyclic AMP (dbcAMP) and steroidogenesis inducing protein (SIP) were assessed. HEY cells were previously studied and characterized extensively by our laboratory and shown to have retained many of the characteristics of normal OSE, such as morphology and expression of growth factors and their receptors (TGF-α and TGF-β), and yet grew very fast in culture and were thus used in our experiments. Caov-3 and OVCAR-3 cell lines were also chosen on the basis of their growth characteristics and doubling time in culture, although Caov-3 cells had a more rapid doubling time than OVCAR-3.

MATERIALS & METHODS

Ovarian Cancer Cell Lines Derived from the Surface Epithelium:

The ovarian epithelial cancer cell line HEY used in these experiments was established by Dr. R. Buick from a disaggregated xenografted human ovarian tumor originally
grown from a peritoneal deposit of a papillary cystadenocarcinoma of the ovary. The origin and growth conditions of this tumor cell line have been described (74). The ovarian cancer cell line Caov-3 deposited in the ATCC by J. Fogh, Memorial Sloan-Kettering Cancer Centre, New York, NY, was derived from a 54 year old Caucasian woman. NIH:OVCAR-3 was established in 1982 by T.C. Hamilton from the malignant ascites of a 60 year old Caucasian patient.

**Culture of Cell Lines:**

The OVCAR-3 cell line was grown to confluence in culture flasks (75 cm²) in culture medium RPMI 1640 (without phenol red) containing 20% fetal bovine serum. HEY and Caov-3 cell lines were both grown to confluence in culture flasks (75 cm²) in culture medium containing 10% heat inactivated calf serum. The culture medium was Eagle's Minimal Essential Medium, with Earle's salts and the following amino acid supplements at a 0.1 mM concentration: L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine, and glycine. The medium also contained 4 mM glutamine, 2.5 g/L NaHCO₃, 1.5 mM HEPES, and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml gentamycin; GIBCO, Grand Island, NY). The medium did not contain phenol red.

**Treatment of Cell Lines:**

Monolayers of cultured HEY, Caov-3 and OVCAR-3 cells were removed from the culture flasks using 0.25% trypsin containing 1 mM EDTA. Cells recovered by
centrifugation were washed twice in culture medium, and plated in serum free medium at a density of $5 \times 10^4$ cells per well (16mm diameter) of a 24-well Nunc plate, allowed to attach, and washed twice a day for 2 days.

After 48 h in culture, the medium was removed, the cells were washed, fresh medium was added, and the cells were treated for 18 h with TGF-α, TGF-β, SIP or dbcAMP. Treatment time (18 h) was chosen on the basis of previous studies in our lab which established that peak incorporation of thymidine into DNA (S phase of cell cycle), occurred at around 18-22 h after treatment. After 18 h of treatment, the medium was then replaced with 0.5 ml culture medium containing 0.5 uCi [3H]thymidine, and the cells were incubated for 4 h. At the end of this period, the medium was aspirated, and the cells were washed with culture medium. Fresh culture medium (0.5 ml) was added in each well, and the cells were disrupted by sonication at 4°C. Aliquots (200 ul) of the sonicate were filtered through Whatman DE81 filter paper discs (Clifton, NJ) on a Millipore system (Bedford, MA). The DNA bound to the filter discs was washed twice with 2.0 ml distilled water. The filters were transferred to the scintillation vials containing 5.0 ml CytoScint (ICN Biochemicals, Inc., Irvine, CA) and counted in a β-counter.

Statistics:

Each experiment measuring [3H]thymidine incorporation into DNA was performed at least 3 times using cells prepared at different times. Figures show the mean
± SD of triplicate or quadruplicate determinations in a representative experiment. The significance of difference was assessed by Student's t-test and ANOVA. P<0.05 was considered to be significant.

RESULTS

GROWTH RESPONSE TO TGF-α

HEY CELLS:

Treatment of HEY cells with TGF-α at a concentration of 2.5 or 10ng/ml significantly stimulated (P<0.05) the incorporation of [3H]thymidine into DNA (FIG.1A).

CAOV-3 CELLS:

Treatment of Caov-3 cells with TGF-α caused a significant decrease (P<0.05) in [3H]thymidine incorporation into DNA at 1.0, 2.5, 5.0 and ng/ml, with the maximal decrease at 5ng/ml (FIG. 1B).

OVCAR-3 CELLS:

Treatment of OVCAR-3 cells with TGF-α caused an increase in [3H]thymidine incorporation into DNA which was significant at 5ng/ml and 10ng/ml (P<0.05) (FIG.1C).
FIG. 1. [\(^{3}\text{H}\)]Thymidine incorporation into A)HEY, B)Caov-3 and C)OV CAR-3 cell DNA after 18 h of treatment with varying concentrations of TGF-\(\alpha\). Control cells were untreated. [\(^{3}\text{H}\)]Thymidine incorporation per well was assayed after a 4 h incubation with 0.5 uCi [\(^{3}\text{H}\)]thymidine. Each value is the mean \(\pm\) SD of three replicate cultures. The data are from single experiments representative of three similar experiments. Asterisks indicate a significant difference from control \((P<0.05)\).
FIG. 1. Morphological features of apoptosis induced in HEY cells by anti-Fas mAb and C\textsubscript{2}-ceramide, including chromatin condensation, nucleoplasmic segmentation and apoptotic body formation. HEY cells were plated to give subconfluent cultures. Cells were treated with C\textsubscript{2}-ceramide or dihydroceramide for 5 h, or with anti-Fas mAb for 30 h. (A) control, (B) anti-Fas mAb (5.0 ug/ml), (C) C\textsubscript{2}-ceramide (12uM) and (D) dihydroceramide (12uM). Cells were stained with 10uM Hoechst 33342 and 4.5 ug/ml propidium iodide. The observed morphological changes induced by anti-Fas mAb and C\textsubscript{2}-ceramide have been reproducible in all the experiments performed throughout this study. (Magnification: x400)
GROWTH RESPONSE TO TGF-β

HEY Cells:

Based on previous experiments performed in our lab on the effects of TGF-β on HEY cells, it is known that TGF-β does not inhibit growth of HEY cells, and in fact at a concentration of 1ng/ml, it stimulates DNA synthesis. This concentration of TGF-β was retested and it confirmed that it caused a significant increase in thymidine incorporation into DNA (p>0.05) (FIG. 2A).

Caov-3 Cells:

Caov-3 cells were treated with varying concentrations of TGF-β, however the results of the triplicate experiments were not consistent. Thus the statistically significant decrease observed in a representative experiment shown in FIG. 2B, does not reliably reflect the effects of TGF-β on these cells, and TGF-β most likely has no significant effect on Caov-3 cells.

OVCAR-3 Cells:

OVCAR-3 cells were treated with TGF-β at concentrations ranging from 0.5 ng to 10 ng/ml. Despite a slight decrease in DNA synthesis at 1ng/ml, this change was found to be statistically insignificant (p>0.05) (FIG. 2C).
FIG. 2. [3H]Thymidine incorporation into A) HEY, B) Caov-3 and C) OVCAR-3 cell DNA after 18 h of treatment with varying concentrations of TGF-β. Control cells were untreated. [3H]Thymidine incorporation per well was assayed after a 4 h incubation with 0.5 uCi [3H]thymidine. Each value is the mean ± SD of three replicate cultures. The data are from single experiments representative of three similar experiments. Asterisks indicate a significant difference from control (P<0.05).
GROWTH RESPONSE TO SIP

The SIP preparation which was used to treat our cells also contains albumin which copurifies with SIP and is needed for SIP's biological activity.

HEY Cells:

HEY cells were treated with preparations of SIP at concentrations of 25, 50 and 100 ug/ml. SIP had a significant stimulatory effect on [³H]thymidine incorporation into HEY cell DNA and increased DNA synthesis over two fold at a concentration of 100 ug/ml (FIG. 3A).

Caov-3 cells:

Caov-3 cells were treated with preparations of SIP of varying concentration with a significant increase in thymidine incorporation into DNA at 100 ug/ml (P<0.05) (FIG. 3B).

OVCAR-3:

OVCAR-3 cells were treated with SIP preparations of varying concentration and SIP was able to induce a significant increase in thymidine incorporation into DNA, at lower concentrations than in Caov-3 cells (FIG. 3C).
FIG. 3. $[^3]$HThymidine incorporation into A)HEY, B)Caov-3 and C)OVCA-3 DNA after 18 h of treatment with varying concentrations of a SIP preparation. Control cultures were not treated. $[^3]$HThymidine incorporation per well was assayed after a 4 h incubation with 0.5 uCi $[^3]$Hthymidine. Each value is the mean ± SD of three replicate cultures. The data are from a single experiment representative of three similar experiments. Asterisks indicate a significant difference from control (P<0.05).
GROWTH RESPONSE TO DIBUTYRYL CYCLIC AMP

Treatment of the three cell lines with dbcAMP (40 μM) for 18 h, caused a statistically significant reduction in thymidine incorporation into DNA in all cell lines (P<0.05) (FIG. 4).
FIG. 4. [3H]Thymidine incorporation into A) HEY, B) Caov-3 and C) OVCAR-3 cell DNA after 18 h of treatment with dbcAMP (40μM). Control cells were untreated. [3H]Thymidine incorporation per well was assayed after a 4 h incubation with 0.5 μCi [3H]thymidine. Each value is the mean ± SD for three replicate cultures. The data are from single experiments representative of three similar experiments. Asterisks indicate significant difference from control (P<0.05).
**DISCUSSION**

As demonstrated by the results, the effects of TGF-α are not consistent in all three cell lines. TGF-α exerted the most significant stimulatory effect on HEY cells which are known to express mRNA for TGF-α that is translated into immunoreactive peptide (28). Furthermore, it is known that in HEY cells TGF-α upregulates its own mRNA, (75). Thus, the stimulatory effect of TGF-α on HEY and OVCAR-3 cells may be mediated through up-regulation of TGF-α mRNA leading to further autocrine and paracrine stimulation. The stimulatory effect of TGF-α on OVCAR-3 cells and its inhibitory effect on Caov-3 cells found in our study, were also reported in another study (76). TGF-α mRNA and EGF receptor mRNA have been detected in primary ovarian carcinomas (71). In a study of 133 patients with ovarian cancer, 88% of ovarian cancers contained TGF-α whereas 27% contained EGF, but the concentrations of TGF-α were 10-fold higher than EGF (77). As previously mentioned, TGF-α has a growth promoting effect on normal OSE that may be involved in promoting mitosis of the OSE during the wound healing process which follows ovulation. It has been suggested that unrestrained growth stimulatory effects of TGF-α may be a factor leading to development of ovarian cancer (78). Ovarian cancer cell lines are in general, less responsive to exogenous TGF-α than normal ovarian epithelial cells (79), suggesting that those cancer cells which do not respond to TGF-α, may be maximally growth stimulated by endogenously produced growth factor.
TGF-β is known to act as an autocrine growth inhibitory factor for normal ovarian surface epithelial cells. Since the majority of ovarian cancer cell lines are resistant to the growth inhibitory effects of TGF-β, it has been suggested that some ovarian cancers may develop in part because of loss of this autocrine inhibitory pathway (80). Despite evidence that some ovarian cancer cell lines are significantly growth inhibited or even induced to undergo apoptosis after treatment with TGF-β (81), two of our cell lines (HEY and OVCAR-3) provide evidence in support of resistance to the growth inhibitory effects of TGF-β. It should be noted here that androgens have been shown by our lab to down regulate TGF-β receptors in HEY cells, an action that would eliminate the inhibitory effects of TGF-β. This points to the significant role which is played by steroids in overlooking and regulating the functions of growth factors. One can speculate that since ovarian cancer predominantly occurs in postmenopausal women in whom the androgen/estrogen ratio has increased, the increased level of androgens may contribute to the loss of the inhibitory effects of TGF-β.

The cAMP analogue used in these studies, dbcAMP, caused a significant inhibition of DNA synthesis in all three cell lines. cAMP serves as an intracellular second messenger that influences cellular functions through the activation of G-proteins and adenyl cyclase (82) and has been shown to exert both stimulatory and inhibitory effects on proliferation depending upon the cell type and the extracellular signals (83). Biological effects of gonadotropins (FSH and LH) on ovarian cells are mediated via cAMP (84); however, there is no evidence of gonadotrophin receptors in OSE. The
identity of hormones or other agents which would raise the intracellular levels of cAMP and inhibit proliferation of ovarian epithelial cells remains unknown. The growth inhibitory effects of cAMP has been observed in primary cultures of granulosa cells that underwent apoptosis after induction of high intracellular levels of cAMP with the use of Forskolin (85). The latter study also demonstrated a possible cross-talk between p-53 and cAMP generated signals in the induction of apoptosis (85). Interestingly, another study reported that dbcAMP induced TGF-β gene expression and growth arrest in the human androgen-independent prostate carcinoma cell line PC-3 (43). These studies point to potential mechanisms by which cAMP can induce its inhibitory effects in our cell lines, however, knowing the effects of cAMP on the proliferation of normal untransformed ovarian epithelial cells is important in evaluating the significance of its observed effects on transformed cells.

The most novel and interesting part of our findings is that SIP which is normally found in follicular fluid, is a potent mitogen for ovarian epithelial cells. Indeed SIP was found to be more potent than TGF-α, hitherto known to be the most potent growth factor along with EGF, for ovarian epithelial cells (86). Our collaborators have confirmed similar effects of SIP on at least four other ovarian cancer cell lines derived from the surface epithelium (38). Since SIP is present in follicular fluid, it is released over the surface of the ovary at the time of ovulation and follicular rupture. It could then serve as a proliferative factor for the OSE to aid in repair of the wound created by rupture at the surface, and may also seep into crypts on the ovarian surface and cause proliferation of
OSE lining the crypts. Thus the presence of high amounts of SIP in follicular fluid (38) and its role as a mitogen for ovarian epithelial cells, may provide a possible link between the number of ovulations and the incidence of ovarian epithelial cancer.
CHAPTER III

ACTIVATION OF Fas LIGAND/RECEPTOR SYSTEM KILLS OVARIAN CANCER CELL LINES BY AN APOPTOTIC MECHANISM.
INTRODUCTION:

As discussed in the formulation of the hypothesis in chapter I, the repetitive injury and repair cycles of the ovarian surface epithelium, which are associated with ovulation, can result in mutations in genes involved in growth regulation. We have proposed that surface epithelial cells generated during the repair process are normally sequestered in inclusion cysts and are eliminated by apoptosis (87).

Since ovarian carcinomas originate from inclusion cysts that have failed to be eliminated, a most fundamental question in understanding their etiology centers around the nature of the apoptotic signals and molecular mechanisms by which the surface epithelial cells within these cysts are normally lost. This rationale formed the basis of the present investigation, in which we have examined the possibility that the Fas ligand/receptor system may be involved in the induction of apoptosis in human ovarian surface epithelial cells.

Our interest in the Fas ligand/receptor system stemmed from the observation that the ovary, in addition to the immune system, thymus, and heart, expresses abundant levels of Fas mRNA (54, 88). Cells expressing Fas can undergo apoptosis upon exposure to either Fas ligand (89) or an agonistic Fas antibody (57, 90). As discussed in Chapter I, Fas ligand/receptor interaction leads to the activation of the enzyme sphingomyelinase (SMase) that hydrolyses sphingomyelin (SM) to phosphocholine (PC) and ceramide, the latter being the proposed intracellular mediator of apoptosis.
The hypothesis to be tested here is the ability of anti-Fas mAb to induce apoptosis in the human ovarian cell lines HEY and Caov-3 which are derived from carcinomas of the surface epithelium. Furthermore, we explored the possibility that Fas was acting through the sphingomyelin-ceramide pathway to induce apoptosis in these ovarian carcinoma cell lines.

**MATERIALS & METHODS**

**Culture of Ovarian Cancer Cell lines Derived From the Surface Epithelium**

The ovarian cancer cell lines HEY and Caov-3 were cultured as described in Chapter II.

**Treatment of Cell Lines.**

Monolayers of cultured HEY and Caov-3 cells were removed from the culture flasks using 0.25% trypsin containing 1 mM EDTA. Cells recovered by centrifugation, were washed twice in culture medium, and plated in serum-free medium to give a sub-confluent density. They were treated with anti-Fas mAb (5.0 ug/ml) (Transduction Laboratories, Kentucky TN), 2 h after plating. Cells grown to subconfluence were also treated with C2-ceramide (N-acetyl-N-sphingosine) (12uM) (Calbiochem, La Jolla, CA) or dihydroceramide (12 uM) (Calbiochem, La Jolla, CA).
Cell Counts

HEY and Caov-3 cells were treated with anti-Fas antibody (5.0 ug/ml) and C2-ceramide (12 uM), and after 30 h of treatment, the culture medium was discarded and cells that remained attached to the culture dish were removed with 0.25% trypsin and 1mM EDTA. When the cells rounded up, they were gently agitated until all cells detached from the plate. Trypsinization was inhibited by the addition of soy bean trypsin inhibitor (Sigma Chemical Company, St.Louis, MO). The cells were centrifuged and resuspended in MEM and stained with trypan blue staining solution (0.4%) (GIBCO). The number of cells that excluded trypan blue was counted in four replicate aliquots from each suspension using a hemocytometer. Experiments were repeated four times using different cell samples.

Apoptosis Assay

Hoechst 33342 (Sigma Chemical Company, St. Louis, MO) is a DNA binding dye which was prepared as a 2mM stock solution in deionized water. Propidium iodide is also a DNA binding dye which (Sigma Chemical Company, St. Louis, MO) was prepared as a stock solution of 50 ug/ml in PBS. HEY and Caov-3 cells were plated on glass cover slips placed in the bottom of wells of 24-multiwell culture dishes. Cells were treated with anti-Fas antibody (5.0 ug/ml) for 30 h and with C2 ceramide for 5 h. The times of treatment were selected based on the times at which morphological changes were observed under the light microscope. The cultures were incubated simultaneously
with 10uM Hoechst 33342 and 4.5 ug/ml propidium iodide for 10 min in a water-bath at 37°C and then placed on ice (47). Normal (non-apoptotic) cells are much less permeable to propidium iodide than apoptotic cells. Hoechst 33342 rapidly enters all cells but has been shown that apoptotic cells are somewhat more permeable to Hoechst 33342. Thus, this differential uptake of dyes between apoptotic and normal cells and the apoptosis associated changes in chromatin organization and cell permeability causes the apoptotic nuclei to stain differently from normal nuclei. Formation of a DNA ladder is also an indication of apoptosis, however our previous experiments with the cell lines treated with C2 ceramide, using ethidium bromide to visualize such a ladder pattern in isolated DNA, gave negative results. It has been noted that a DNA ladder does not always accompany the process of apoptosis (46); however a different techniques such as 32P-end labeling of DNA may be needed to visualize such a DNA ladder pattern.

**Isolation of Phospholipids from Ovarian Cancer Cells Lines**

Ovarian cancer cells cultured in 75 ml flasks, were treated 2 h after plating, with anti-Fas mAb (5.0 ug/ml) for 30 min. Medium from control and treated cultures were collected after 30 min and attached cells were removed with 0.25% trypsin containing 1mM EDTA. Medium and detached cells were pooled together and phospholipids were extracted with chloroform:methanol 2:1 (91).

**Normal Phase High Pressure Liquid Chromatography Interphased With Electrospray Ionizing Mass Spectrometry (LC/ESI/MS) of Phospholipids**
Normal phase HPLC separation of phospholipids was performed on Spherisorb 3 micron columns (100 mm x 4.6 mm ID, Analtech, Deerfield, IL) installed into a Hewlett Packard (Palo Alto, CA) model 1090 Liquid Chromatograph connected to a Hewlett Packard model 5988B Quadrupole mass spectrometer equipped with a nebulizer assisted electrospray interface, ESI (HP 59987A). N-palmitoyl and N-nervonoyl-D sphingomyelins (semi-synthetic from bovine brain cerebrosides) were used as LC/ESI/MS standards. The column was eluted with a linear gradient of 100% solvent A (chloroform/methanol/30% ammonium hydroxide, 80 : 19.5 : 0.5, by volume) to 100% solvent B (chloroform/methanol/water/30% ammonium hydroxide, 60 : 34 : 5.5 : 0.5, by volume) in 14 min, then at 100% B for 10 min (92, 93). Negative ESI spectra were taken in the mass range 400-1100 (93). Selected ion spectra were retrieved from the total ion spectra by computer. The masses in the figures are the actual masses of the ([M+H]) ions. The nominal masses are one unit lower.

Statistical Procedures

Fluorescent microscopy and cell count experiments were performed at least three times, using cells prepared at different times. Figures of cell counts (Fig.3 & Fig.4) show the mean ± standard error of triplicate determinations in a representative experiment. The significance of the differences between different treatments was analyzed using Student’s t test.

RESULTS
Induction of Apoptosis with Anti-Fas mAb

HEY (Fig.1) and Caov-3 (Fig.2) cells were stained with Hoeschst 33342 and propidium iodide in order to visualize morphological changes of the nucleus. Untreated ovarian cancer cells did not show signs of nuclear fragmentation and cytoplasmic segmentation throughout the study (Fig. 1A & 2A). After 30 hours of treatment with anti-Fas mAb (5.0 ug/ml) hallmarks of apoptosis, including nuclear compaction, cytoplasmic blebbing and fragmentation into dense particles called apoptotic bodies were observed (Fig. 1B & 2B). Following these changes, treated HEY and Caov-3 cells rounded up and detached from the culture plate. Performed cell counts showed a significant reduction upon treatment with anti-Fas antibody (5.0 ug/ml) in HEY cells which were reduced by 40% (P<0.01) (FIG.3), and Caov-3 cells which were reduced by 56% (P<0.01), after 30 h of treatment (FIG.4).
**FIG. 1.** Morphological features of apoptosis induced in HEY cells by anti-Fas mAb and C₂-ceramide, including chromatin condensation, nucleoplasmic segmentation and apoptotic body formation. HEY cells were plated to give subconfluent cultures. Cells were treated with C₂-ceramide or dihydroceramide for 5 h, or with anti-Fas mAb for 30 h. (A) control, (B) anti-Fas mAb (5.0 ug/ml), (C) C₂-ceramide (12uM) and (D)dihydroceramide (12uM). Cells were stained with 10uM Hoechst 33342 and 4.5 ug/ml propidium iodide. The observed morphological changes induced by anti-Fas mAb and C₂-ceramide have been reproducible in all the experiments performed throughout this study. (Magnification: x400)
FIG. 2. Morphological features of apoptosis induced in Caov-3 cells by anti-Fas mAb and C2-ceramide, including chromatin condensation, nucleoplasmic segmentation and apoptotic body formation. Caov-3 cells were plated to give subconfluent cultures. Cells were treated with C2-ceramide or dihydroceramide for 5 h, or with anti-Fas mAb for 30 h. (A) control, (B) anti-Fas mAb (5.0 µg/ml), (C) C2-ceramide (12uM) and (D)dihydroceramide (12uM). Cells were stained with 10uM Hoechst 33342 and 4.5 µg/ml propidium iodide. The observed morphological changes induced by anti-Fas mAb and C2-ceramide have been reproducible in all the experiments performed throughout this study. (Magnification: x400)
FIG. 3. Cell counts of viable HEY cells after treatment of cells with 5.0 μg/ml anti-Fas antibody, 12 μM C2-ceramide, or 12 μM dihydroceramide (dhceramide), for 30 h.
FIG. 4. Cell counts of viable Caov-3 cells after treatment of cells with 5.0 ug/ml anti-Fas antibody, 12 uM C2-ceramide, or 12 uM dihydroceramide (dhceramide), for 30 h.
Involvement of Sphingomyelin-Ceramide Cycle in Ovarian Cancer Cell Apoptosis

Hydrolysis of SM, as the result of apoptotic stimuli, is generally a rapid process localized within the plasma membrane (63). Hence, Caov-3 cells were exposed to anti-Fas mAb for 30 minutes and subsequently were analyzed for phospholipid content. The total lipid extracts were resolved by HPLC based on their polarity. The neutral lipids (NL) were eluted first, followed by the more polar phospholipids PE (phosphatidylethanolamine), PC (phosphatidylcholine) and SM (sphingomyelin). SM peak was observed over the 13.807-14.346 min period after the injection of the sample, and was analyzed further by identifying its most abundant molecular species. Structural identification of these molecules was based on the [M+1]−, representing the pseudomolecular ion, and on chromatographic retention time. The most abundant species of SM were identified by number of carbons : number of double bonds, and mass/charge ratio (m/z), as 34:1, d18:1/16:0 (sphingosin base/fatty acid) (703 m/z); 36:1 (d18:1/18:0, 731 m/z); 40:1 (d18:1/22:0, 787 m/z); 41:1 (d18:1/23:0, 801 m/z); 42:1 (d18:1/24:0, 815 m/z); and 42:2 (d18:1/24:1, 813 m/z) (Fig.5).

Levels of identified SM species in Fig. 4 were measured in untreated and anti-Fas mAb treated samples. The total level of SM was reduced by 40% in cells treated with anti-Fas mAb for 30 minutes (Fig.6). These treated cells specifically showed a rapid decrease in the levels of the SM species 34:1 (44% of control value), 40:1(70% of control value), and 42:2 (54% of control value) (Fig.7). There was no significant
FIG. 5. Percentage levels of the most abundant molecular species of sphingomyelin in Caov-3 cells under control conditions 2 h after plating.
**FIG. 6.** Total Sphingomyelin levels 2 h after plating under control conditions, and after treatment with anti-Fas mAb (5.0 µg/ml) for 30 min. Data are presented for a single experiment which is representative of two separate experiments.
FIG. 7. Levels of the most abundant molecular species of sphingomyelin in cultured Caov-3 cells after treatment with anti-Fas mAb (5.0 ug/ml) compared to untreated sample. Data presented are from a single experiment which is representative of two separate experiments.
decrease in the levels of SM species 36:1, 41:0 and 42:1 during the given period of treatment with anti-Fas mAb.

**Induction of apoptosis with ceramide**

Ceramide, the central lipid in the SM metabolic pathway, affects cell differentiation as well as exerting potent anti-proliferative activities accompanied by cytotoxicity (94). Because natural ceramide contains saturated or monosaturated fatty acids chains and is poorly soluble in aqueous solutions, a cell permeable analog, C2-ceramide, synthesized and tested for its ability to induce apoptosis (95), was used in this study. Treatment of HEY and Caov-3 cells for 5 h with 12 μM C2-ceramide resulted in morphological changes indicative of apoptosis, followed by rounding up and detachment of these cells from the culture plate (Fig. 1C & 2C). This cytotoxicity demonstrated significant structural specificity of C2-ceramide, since a closely related stereoisomer dihydroceramide failed to induce apoptosis (Fig. 1D & 2D). The number of HEY and Caov-3 cells that remained attached to the culture dish after 30 h of treatment with C2-ceramide was dramatically reduced, whereas treatment with dihydroceramide showed no significant reduction in the number of viable cells compared to control levels (FIG. 3 & FIG. 4).
DISCUSSION

The observations made in these experiments, give new insight into apoptotic signals and molecular mechanisms that may be involved in the regulation of growth versus death of cells derived from human ovarian surface epithelium. The first novel finding presented in this study was that anti-Fas mAb was capable of inducing apoptosis in ovarian surface epithelial carcinoma cell lines (HEY and Caov-3), as evidenced by chromatin condensation, nucleoplasmic fragmentation, apoptotic body formation and detachment of cells from the culture dish. We then showed that treatment of surface epithelial ovarian cancer cells with anti-Fas mAb which activates the Fas receptor, caused a rapid decrease in the levels of three specific species of sphingomyelin. Further we demonstrated that the apoptotic inducing effects of anti-Fas mAb, are duplicated when a cell permeable ceramide analogue, C2-ceramide, was used to treat the cells.

As mentioned earlier the Fas ligand/receptor system was initially recognized in the immune system where it is involved in down-regulation of immune reactions and in T cell-mediated cytotoxicity (53). A number of recent studies have shown the presence of a functionally active Fas ligand/receptor system in ovarian cell cultures. One study has shown that the interaction between Fas antigen on rat granulosa cells and Fas ligand on oocytes, induces granulosa cell apoptosis (96). The temporal expression of Fas was shown to correlate with the progression of follicular development, suggesting a possible role for Fas in apoptosis in atretic follicles (96). Fas antigen-mediated apoptosis in human granulosa/luteal cells has also been reported and this apoptotic effect of anti-Fas
mAb was increased after pretreatment with interferon γ (97). These results taken together with our observations, suggest that the Fas receptor/ligand system is a potent regulator of cell growth and inducer of cell death in different ovarian cell types, and depends on the presence of Fas antigen on these cells as well as the availability of the Fas ligand.

The second novel demonstration in this study is that activation of Fas can cause a rapid hydrolysis of sphingomyelin in ovarian surface epithelial carcinoma cell lines. Activation of Fas by binding ligand or anti-Fas mAb, is known to cause the hydrolysis of sphingomyelin to phosphocholine and ceramide. This pathway is known to exist in normal rat thecal/interstitial cells, since activation by a combined treatment with transforming growth factor-α plus transforming growth factor-β, or treatment with anti-Fas mAb, caused a rapid hydrolysis of sphingomyelin and subsequent elimination of cells by apoptosis (98). Treatment of Caov-3 cells with anti-Fas mAb caused a rapid and significant increase in sphingomyelin hydrolysis. This was confirmed by measuring the levels of the most abundant species of sphingomyelin in both untreated and treated samples. Anti-Fas mAb caused a rapid reduction in the levels of the sphingomyelin species 34:1, 40:1 and 42:2 after only 30 minutes of treatment. This is the first time that different species of sphingomyelin have been measured in ovarian surface epithelial cells and implicated in ovarian cancer cell apoptosis. It should be noted here that our measurements of sphingomyelin levels represent the total cellular sphingomyelin content including the sphingomyelin found in membrane-bound subcellular organelles. This may serve to explain the rapid and specific reduction of sphingomyelin species 34:1, 38:1 and
42:2, since they may represent the sphingomyelin species found within the cell membrane which are readily accessible for hydrolysis following the activation of Fas. The other sphingomyelin species may be those found within membranes of sub-cellular organelles and thus unaffected by the binding of Fas mAb to the Fas antigen on the plasma membrane. Another possibility is that the sphingomyelin species which are not hydrolyzed are those that play a structural role in the plasma membrane.

The ability of ceramide to induce apoptosis in these cells, supports its involvement in the sphingomyelin-ceramide signal transduction pathway in ovarian cancer cell apoptosis. Dihydroceramide was used to establish the stereospecificity of ceramide in inducing apoptosis. None of the apoptotic changes were observed when dihydroceramide was used to treat HEY and Caov-3 cells. As discussed in Chapter I, recent studies have established that the generation of the lipid messenger ceramide through the sphingomyelin-signaling pathway represents a stimulus involved in the induction of apoptosis by cytotoxic humoral factors of the TNF superfamily (59).

Induction of apoptotic cell death in hen granulosa cells by ceramide has been recently reported (99). C2-ceramide has also been shown to induce apoptosis in cultured rat thecal/interstitial cells (100). This study showed that exogenously administered cell-permeable C2-ceramide was able to induce apoptosis in ovarian cancer cells, thus mimicking the effects of anti-Fas mAb on these cells. This is the first time that the involvement of ceramide in apoptosis of epithelial ovarian cancer cells has been demonstrated.
Before initiating our investigations, we hypothesized that ovarian cancers of the surface epithelium originate from epithelium-lined inclusion cysts which fail to be eliminated in the stroma via apoptosis. Our results now demonstrate that the ovarian surface epithelial cancer cell lines HEY and Caov-3 actually continue to express the Fas antigen, as they were able to respond to the apoptotic stimulation of anti-Fas antibody. Thus, it is possible that the failure of elimination of epithelial lined inclusion cysts, is due to the down-regulation of the Fas ligand gene expression within the stroma, or the loss of the mechanisms required to activate Fas ligand.
CHAPTER IV

A PRELIMINARY STUDY ON THE EFFECTS OF ANTI-Fas mAb & C2-
CERAMIDE ON OVARIAN EPITHELIAL TUMOR CELLS ISOLATED FROM
ASCITES FLUID
INTRODUCTION:

In the search for an understanding of the etiologic factors in ovarian cancer, and the development of more effective therapeutic measures, the use of human ovarian cancer cell lines derived from the surface epithelium has been a common practice among researchers. These cell lines offer the advantage of growing easily, however they may also undergo changes with recurrent passage in culture. Cell lines are the result of a selection occurring during their establishment in culture and may not always reflect the heterogeneity of the different malignancies encountered in vivo. (101).

The culture of primary ovarian surface epithelial cells, either normal or neoplastic, in monolayer systems with liquid media has rarely been pursued, (102, 103) likely because of the difficulties encountered in growing cultures which are not contaminated with stromal elements and the difficulty in obtaining high adhesion and cell growth. The isolation of tumor cells from fresh samples is associated with a number of difficulties. Solid tumor samples require enzymatic digestion and/or mechanical disruption to release the cells (104). The other issue is that it is difficult to separate infiltrating non-malignant cells (e.g. monocytes, fibroblasts, lymphocytes and neutrophils) from malignant cells by physical methods (105).

As previously stated, ovarian surface epithelial lined inclusion cysts are the sites of origin of ovarian cancer. Ovarian cancer eventually breaks through the surface epithelium and spreads through the peritoneum as ascites cells which produce growth factors to promote proliferation of ovarian cancer cells (106). Ascites fluid which contains these surface epithelial cancer cells can be obtained aseptically from patients either at the time of diagnostic laparotomy or after relapse at the time of therapeutic paracentesis (105). During the processing of ascites fluid from patients with surface epithelial ovarian carcinoma it was observed that more than half of the tumor cells were present in the form of clumps whereas the nonmalignant cells were primarily single cells (105). Therefore a simple filtration technique has been developed for isolating these tumor cell clumps from the ascites fluid (105). This technique was used in our studies to
isolate tumor cells from the ascites fluid of a patient. The tumor cells were isolated from the patient’s ascites fluid, cultured, and observed following treatment with anti-Fas mAb and C2-ceramide. The hypothesis which this chapter addresses is whether anti-Fas mAb is capable of inducing apoptosis in ovarian tumor cells derived from the ascites fluid. Studying the effects of anti-Fas mAb on ascites tumor cells provides critical information about the pattern of expression of Fas receptor following tumor formation and the potential therapeutic measures which may be taken to treat clinical cases of surface epithelial ovarian cancer.

MATERIALS & METHODS:

Dr. Hirte kindly provided us with ascites fluid (LEP-2 cells) from a patient with a diagnosis of epithelial ovarian carcinoma which was aseptically obtained and frozen at -70 °C following a procedure for the processing of the obtained ascites fluid, (105).

Isolation of Tumor Cell Clumps

After thawing, the cells were washed once in PBS. The cells were then resuspended in PBS and assessed for cell number and viability by trypan blue exclusion. An aliquot of unfractionated cells was saved and the remaining cell suspension was passed over a 30-um nylon mesh filter (Spectrum Medical Industries, Inc., Los Angeles, CA) in a 55-mm filtration unit (Spectrum). The filter was then washed five times with 10-ml volumes of PBS. Clumps not passing through the filter were then back-washed off the filter, suspended in PBS, and spun at 1000g for 5 min. After centrifuging the supernatant was discarded and the cells were resuspended in 2 ml of DME/F12 (Sigma Chemical Co., St. Louis, MO), supplemented with insulin (5ug/ml), transferrin (10 ug/ml), epidermal growth factor (5ng/ml), phosphoethanolamine (5x10^{-5} M), ethanolamine (5x10^{-5} M), penicillin (100 U/ml), streptomycin (100ug/ml) (all additives were obtained from Sigma Chemical Co., St. Louis, MO) and 3% fetal bovine serum (Gibco, Burlington, Ontario, Canada). The cells (10x10^6) were diluted in 20 ml of the above medium and 0.5 ml aliquots were plated in 24 well Nunc plates or 0.35 ml in 8 well chamber slides. The cultures were washed after 24 h in the above culture medium.
After 48 h the cultures were washed in serum free medium and cultured in this medium during the treatment period.

**Apoptosis Assay:**

Cells were treated with anti-Fas antibody (10.0 μg/ml) for 30 h or with C2 ceramide (15 uM) or dihydroceramide for 5 h. The times of treatment were selected based on the times at which morphological changes were observed under the light microscope. The cultures were incubated simultaneously with 10μM Hoechst 33342 and 4.5 μg/ml propidium iodide for 10 min in a water-bath at 37 C and then placed on ice (47), as described in Chapter 3.

**RESULTS**

**Induction of Apoptosis with anti-Fas mAb and C2-ceramide**

Tumor cells were stained with Hoeschst 33342 and propidium iodide in order to visualize morphological changes of the nucleus. Untreated cells did not show signs of nuclear condensation and fragmentation (Fig. 1A). After 30 h of treatment with anti-Fas mAb (10.0 μg/ml) hallmarks of apoptosis, including nuclear compaction, cytoplasmic blebbing and fragmentation into apoptotic bodies were observed (Fig. 1B). Likewise, treatment of the tumor cells with C2-ceramide duplicated the effects of anti-Fas mAb after treatment with C2-ceramide (15 uM) in 5 hours (Fig. 1C). Treatment with dihydroceramide did not change the morphology of cells and their appearance was the same as control cells (Fig. 1D).
**FIG. 1.** Morphological features of apoptosis induced in LEP-2 cells by anti-Fas mAb and C₂-ceramide, including chromatin condensation, nucleoplasmic segmentation and apoptotic body formation. The arrow in (A) indicates an intact healthy nucleus in green; the arrow in (B) indicates apoptotic body formation and the arrow in (C) indicates nuclear condensation. Untreated cells did not show signs of nuclear condensation and fragmentation (A). Cells were treated with anti-Fas mAb (10.0 ug/ml) for 30 h (B), C₂-ceramide (15 uM) for 5 h (C), or dihydroceramide (15 uM) for 5 h (D).
DISCUSSION

The results obtained in this preliminary study have significant clinical implications. The cells are ovarian surface epithelial tumor cells which have been isolated from the ascites fluid of an ovarian cancer patient. Thus, unlike cell lines which undergo recurrent passages in culture, these cells are cultured only once and may be considered to better represent the in vivo characteristics of ovarian surface epithelial cancers. Moreover, as formation of ascites indicates advancement of disease (106), the apoptotic response of these ascites derived tumor cells to anti-Fas mAb proves to be potentially promising for therapeutic intervention of this disease. However, as the effects of anti-Fas mAb on different normal human tissues in vivo are not known, further studies are needed to be able to assess the risks of using anti-Fas mAb for tumor therapy. Some preliminary studies in this area have been performed which are discussed in the General Discussion (Chapter V).
CHAPTER V

GENERAL DISCUSSION
The investigations presented in this thesis were based on the hypothesis that ovarian surface epithelial cancers originate from epithelium lined inclusion cysts which fail to be eliminated in the stroma via apoptosis. Implicit in our hypothesis was the assumption that repetitive injury and repair cycles to the OSE caused by successive ovulations, could give rise to harmful mutations. In the event that apoptotic mechanisms fail to eliminate the mutations in OSE lined inclusion cysts, this would result in the persistence of inclusion cysts. Exposure of potentially mutated OSE to proliferative factors within the stroma, could cause tumorigenesis. Thus, as supported by epidemiologic evidence, successive ovulations and the subsequent increased proliferation of the OSE to repair the wound, are conditions which lead to the development of OSE lined inclusion cysts which may contain harmful mutations. Therefore, identification of factors which are growth stimulatory for OSE are as important to the development of our hypothesis of ovarian cancer, as growth inhibitory and apoptotic factors.

The significant findings presented here, support and extend our hypothesis of the etiology and progression of ovarian cancer. With regards to the identification of naturally occurring growth factors that regulate growth of ovarian carcinoma cells, we have confirmed that TGF-α and TGF-β have the ability to influence the number of cells that enter the S phase of the cell cycle and synthesize DNA. The mRNA for both of these growth factors are present in normal OSE (28, 35). Our lab has previously demonstrated that TGF-α mRNA is translated into TGF-α immunoreactive peptide in normal OSE and in HEY cells (28). Immunoreactive TGF-β has also been localized in HEY cells (75).

Our most original finding regarding factors which influence growth of ovarian surface epithelial cells, was the identification of SIP as a potent growth stimulator for ovarian cancer cell lines. SIP was more effective than TGF-α, hitherto identified as the most potent growth factor (along with EGF) for ovarian epithelial cells (86). The structure of SIP is not known and therefore we do not know the family of growth factors in which this factor belongs. However the finding that SIP is more potent than TGF-α,
suggests that SIP is distinct from TGF-α. Previous studies in our lab indicated that both EGF and TGF-α stimulate DNA synthesis in rat Leydig cells, however SIP was significantly more potent than TGF-α (41). Furthermore, in immature rat granulosa cells, TGF-α/EGF inhibited SIP plus TGF-β stimulated DNA synthesis identifying SIP as a growth factor that is distinct from EGF/TGF-α. (42). The presence of high amounts of SIP in follicular fluid and its role as a powerful mitogen for ovarian epithelial cells, may provide a possible link between the number of ovulations and the incidence of ovarian cancer of surface epithelial origin.

A most significant finding presented in this thesis was that the cell lines HEY and Caov-3 derived from human carcinoma of surface epithelial origin underwent apoptosis, as evidenced by cell shrinkage, chromatin condensation and apoptotic body formation, when treated with an agonistic monoclonal anti-Fas antibody which like Fas ligand, binds to and activates the Fas receptor (87). These studies showed that Fas is expressed and functional in these human carcinoma cells derived from ovarian surface epithelium. Using immunohistochemical techniques our collaborators (Dr. Katja Teerds and Dr. Hans van der Donk in Holland) have shown that Fas receptor is localized in the surface epithelial cells of sections of normal human ovaries and Fas ligand is localized in the stroma. Further support for the presence of Fas ligand in the stroma comes from studies in the rat. These studies carried out in our lab indicated that thecal/interstitial (stromal) cells isolated from rat ovaries, contained significant levels of mRNA for Fas ligand (as assessed by PCR). Based on these observations, we propose that normal surface epithelial cells express the Fas receptor and remain healthy and viable as long as they are protected from direct contact with the Fas ligand in the stroma. Upon internalization of the crypt and the formation of an inclusion cyst this barrier is disrupted and surface epithelial cells are now in direct contact with stromal cells that synthesize Fas ligand (FIG. 1). This cell-cell contact between the mesenchymal/stromal cells and the epithelial cells, activates the Fas receptor and the signal transduction pathway that causes apoptosis and potentiates the demise of the inclusion cyst. We propose that the Fas ligand/receptor system is involved in the normal elimination of surface epithelial lined inclusion cysts.
FIG. 1. Upon internalization of the crypt and formation of an inclusion cyst, the barrier between Fas receptor (Fas R) and Fas ligand (Fas L) is removed and they can now interact in the stroma to eliminate OSE lined inclusion cysts which are the sites of origin of surface epithelial ovarian cancer.
within the stroma. Subsequently, the failure of the Fas ligand/receptor system to operate, would result in the persistence of inclusion cysts in the stroma and their continuous exposure to stroma-derived factors, which could lead to propagation of mutations, increasing the chance of tumor formation. Since our experimental results indicate that Fas is expressed and functional in human ovarian cancer cells, we speculate that the failure of inclusion cysts to undergo apoptosis, and the subsequent onset of tumorigenesis, is the result of lack of expression of Fas ligand or its improper interaction with Fas receptor.

The Fas ligand/receptor system has been shown to be involved in the induction of apoptosis in a variety of cancer cell lines, including malignant human lymphocyte lines (107). SV40-transformed human epidermal keratinocyte cell line KJD and human skin squamous cell carcinoma (SCC) cell line HSC, which strongly express the Fas antigen, were killed by the anti-Fas mAb by the process of apoptosis (108). One study demonstrated that eight of ten myeloma cell lines were induced to undergo programmed cell death by anti-Fas antibody and of the two myeloma cells which were resistant to anti-Fas mAb treatment, one did not express the Fas antigen (109). The prostate cancer cell line PC-3 which initially did not respond to treatment with anti-Fas antibody, did undergo apoptosis in response to anti-Fas antibody after transfection with the Fas antigen (110).

Our studies also established that activation of Fas by anti-Fas mAb resulted in the hydrolysis of sphingomyelin. Ceramide, the product of sphingomyelin hydrolysis, induced apoptosis in ovarian cancer cell lines HEY and Caov-3. The latter observation identified the sphingomyelin-ceramide pathway as the signal transduction mechanism by which Fas ligand/receptor system causes apoptosis. Fas induced apoptosis through the sphingomyelin-ceramide pathway has also been reported by our lab in rat thecal interstitial cells, however, there were differences in the specific species of sphingomyelin which were reduced in these cells versus cells from the ovarian cancer cell line Caov-3.
To test the relevance of our findings in ovarian cancer cells in vivo, preliminary studies using ovarian cancer cells isolated from the ascites fluid of a patient, established the ability of anti-Fas mAb and C₂ceramide to induce apoptosis in ascites derived cells. This attached significant clinical relevance to our findings using the cell lines. However, although the anti-Fas mAb induced death in a variety of human cancer cells in vitro, its potential in vivo effects are unknown. In humans, Fas antigen is expressed in such areas as the thymus and the immune system, kidney, liver, prostate and the ovary (111), and it is not known what influence the anti-Fas Ab might have on normal tissues. In an animal model, regression of a human B cell tumor in mice was achieved by the intravenous injection of the anti-Fas mAb, and apoptosis induced by the anti Fas mAb was recognized in the regressing tumor (56). On the other hand, mice that underwent intraperitoneal administration of anti-Fas mAb, were killed rapidly (112). Recently however it was reported that when Fas-expressing Yac-1 lymphoma cells were implanted into peritoneal cavity of mice, intraabdominal injection of Fas ligand reduced tumor in mice without systemic toxicity (113). This study suggests that locally applied Fas ligand is capable of killing certain tumor cells efficiently without systemic toxicity and may be used for local tumor treatment. Further investigation in this area would determine the suitability of Fas ligand or anti-Fas mAb for use in combating ovarian cancer in a clinical setting.

The hypothesis on which our studies were based, is consistent with Cramer and Welch’s “inclusion cyst formation” hypothesis, and extends it by proposing a molecular mechanism for the elimination of surface epithelial lined inclusion cysts. Understanding the role of apoptosis and identifying the physiological mechanisms and cellular pathways responsible for induction of apoptosis in ovarian surface epithelial cells is integral to an understanding of the etiology of ovarian cancer. In attempting to understand such physiological mechanisms which regulate cell growth and cell death in the OSE, the role of steroids must not be forgotten. As the chief hormonal products of the ovaries, steroids play a crucial role in regulating normal ovarian physiology and female reproductive functions. In the context of our hypothesis, it is important to note that steroids have been
extensively implicated in the regulation of apoptosis in a number of different tissues and cell systems. Glucocorticoids induce apoptosis in leukocytes (114) and androgen withdrawal or antiandrogens induce apoptosis in prostate and in androgen-dependent prostate carcinomas (114). Removal of progesterone or use of anti-progestins cause apoptosis of the epithelial cells of the uterus (114) and estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis (115). Normal OSE expresses estrogen, androgen and progesterone receptors (ER, AR and PR respectively) and ER, AR and PR are retained in 63%, 69% and 48% of ovarian tumors respectively (116). Steroid levels increase in the serum of patients with ovarian epithelial cancer. These levels decrease with the reduction of tumor volume and increase prior to the recurrence of the tumor (116).

Further, gonadal steroids can regulate growth factor expression or the expression of their receptors. Cellular levels of TGF-α are known to be elevated by estrogens as a portion of the TGF-α gene contains estrogen response elements (22). The synthesis of TGF-β by rat granulosa cells is stimulated by estradiol (23). As previously mentioned, results by our lab have demonstrated that androgens down regulate TGF-β receptors in OSE, removing the inhibitory effect of TGF-β and potentially leading to unrestrained growth. It has been suggested that loss of autocrine growth inhibition by TGF-β might be an early step in the development of some, but not all, ovarian cancers (80). Thus, androgens may cause decreased sensitivity of OSE to the growth inhibitory effects of TGF-β in perimenopausal and postmenopausal women where estrogen to androgen ratio shifts in favor of androgens (117). In direct relevance to our hypothesis, some exciting new results from our lab indicate that dihydrotestosterone (DHT) regulates Fas ligand mRNA in rat thecal interstitial cells. Of course if DHT also regulates the expression of Fas ligand mRNA of human OSE, one can see how disturbances in the levels of androgens such as the elevated androgen levels in post-menopausal women, could lead to persistence of inclusion cysts due to lack of presence of Fas ligand and the break down of the Fas ligand/receptor apoptotic mechanism.
Future Research Directions:

Having discussed the significant role which steroids may potentially play in regulating cell growth and cell death in the OSE, a pertinent question of interest to address experimentally is the role which steroids play in regulating the expression of mRNA for Fas ligand and Fas receptor. The method of PCR may be used to determine whether steroids and in particular androgens and estrogens have a positive or negative effect on mRNA expression for Fas ligand and receptor in normal and transformed OSE.


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