THE INVOLVEMENT OF THE CYCLOOXYGENASE ENZYMES IN THE *GALLUS DOMESTICUS* MODEL OF OVARIAN CANCER

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The main objective of the work presented was to determine if the cyclooxygenase (COX) enzymes are involved in ovarian carcinogenesis in the hen as a model for the human disease. The expression patterns of COX-1 and -2 mRNA and protein in normal hen ovaries and hen ovarian tumors were determined. COX-1 mRNA expression was significantly increased in ovarian tumors of the hen while there was no difference in COX-2 mRNA expression in normal hen ovaries as compared to ovarian tumors. COX-1 protein localized to glandular areas of ovarian tumors and a significant increase in prostaglandin E_2 (PGE_2) in ovarian tumors indicated that the increase in COX-1 mRNA represented a functional increase. The expression of COX-1, COX-2, and vascular endothelial growth factor (VEGF) mRNA and protein were also determined in ascites cells collected from hens with ovarian cancer. VEGF was previously associated with COX-1 expression in human ovarian cancer and is implicated in the etiology of ovarian cancer, including ascites formation. We found a significant increase in VEGF mRNA in ascites cells collected from hens with ovarian cancer as compared to normal hen ovaries and a correlation between ascitic VEGF mRNA expression and ascites volume. VEGF protein was expressed in ascites cells and also localized to glandular areas of ovarian tumors. We found no indication that treatment with COX

inhibitors decreased VEGF expression, but our data show that ascites cell proliferation could be decreased using a nonspecific (aspirin) or a COX-1 specific inhibitor, while equivalent concentrations of a COX-2 specific inhibitor were ineffective at decreasing proliferation. Finally, the effect of feeding hens a diet containing aspirin (a common COX inhibitor) on ovarian cancer incidence and/or progression was determined. We found a significant effect of aspirin treatment on the stage of ovarian cancer, which indicates that inhibition of the COX enzymes may slow the progression of the disease. These combined data suggest that the COX enzymes, in particular COX-1, contribute to ovarian carcinogenesis in the hen, which supports data in women. The hen, therefore, may be the ideal spontaneous *in vivo* model for studies involving the COX enzymes and ovarian cancer.

BIOGRAPHICAL SKETCH

Mary Ellen Urick was born November 7, 1980 and raised in Paxinos, PA. She graduated valedictorian of the class of 1999 from Lourdes Regional High School and continued her education at Bucknell University where she graduated *magna cum laude* with a degree in Animal Behavior in 2003. During her years at Bucknell, she completed an undergraduate honors project studying the recovery of ultrasonic vocalizations after serial lesions to the ventromedial hypothalamus in Syrian hamsters under the guidance of Dr. Owen Floody. Her rewarding undergraduate research experience led her to enter Dr. Patricia Johnson's lab in 2003 in pursuit of a Ph.D. Mary Ellen cherishes the colleagues she's met and memories she's made at Cornell and will begin a postdoctoral position in Dr. Deborah Citrin's laboratory in the Radiation Oncology Branch of the National Cancer Institute in August 2008.

To my family, who taught me the important lessons in life,
especially my grandmother, Margaret Zlotorzynski, who encouraged me to
"Enjoy it while I can"

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LIST OF ABBREVIATIONS

18S	18S ribosomal subunit
3-β-HSD	3-beta-hydroxysteroid dehydrogenase
akt	protein kinase B
ATP	adenosine triphosphate
BAD	Bcl-2- associated death promoter
BIM	Bcl-2- interacting mediator of cell death
CA125	cancer antigen 125
cAMP	cyclic adenosine monophosphate
Caov-3	human ovarian cancer cell line established from an ovarian tumor from a patient with adenocarcinoma
CEA	carcinogenic embryonic antigen
c-myc	myelocytomatosis viral oncogene homolog
COX	cyclooxygenase
EGFR	epidermal growth factor receptor
EOC	epithelial ovarian cancer
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
Erb β-2	epidermal growth factor receptor beta-2; protooncogene
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FIGO	International Federation of Gynecology and Obstetrics
FSH	follicle stimulating hormone
H & E	hematoxylin and eosin
IgG	immunoglobulin G

KO	knock-out
k-ras	k-*ra*t *s*arcoma; oncogene
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
NAG-1	NSAID activated gene-1
NF-κβ	nuclear factor kappa beta
NS-398	selective COX-2 inhibitor; N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide
NSAID	non-steroidal anti-inflammatory drug
OVCAR-3	human ovarian cancer cell line derived from malignant ascites from a patient with poorly differentiated papillary adenocarcinoma of the ovary
OSE	ovarian surface epithelium
p27	protein 27
p53	protein 53
PDGF	platelet-derived growth factor
PCNA	proliferating cell nuclear antigen
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PG	prostaglandin
PI3K	phosphoinositide 3-kinase
PI	propidium iodide
POF	postovulatory follicle
PPARδ	peroxisome proliferator-activated receptor delta
PR	progesterone receptor
Pten	phosphatase and tensin homolog

Rb1	retinoblastoma protein 1
SC-560	selective COX-1 inhibitor; 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole
SKOV-3	human ovarian cancer cell line derived from malignant ascites from a patient with adenocarcinoma of the ovary
SV40 T	simian virus 40 larger tumor antigen
Tag 72	tumor-associated glycoprotein 72
TBST	tris-buffered saline with 0.1% tween-20
TGF-α	transforming growth factor alpha
TxA_2	thromboxane A2
VEGF	vascular endothelial growth factor
VPF	vascular permeability factor
Wnt	“wingless” and “int” drosophila phenotypes combined; protooncogene

CHAPTER 1

LITERATURE REVIEW

Ovarian Cancer

Ovarian cancer is the most fatal gynecologic malignancy in the western world [1]. The high mortality rate associated with ovarian cancer is due in part to vague symptoms associated with early disease, lack of effective screening techniques to detect early cases and inadequate treatment options for patients with advanced stages of the disease. A lack of understanding of the underlying mechanisms behind the development and progression of ovarian adenocarcinoma is the main impediment to decreasing deaths due to ovarian malignancy.

The International Federation of Gynecology and Obstetrics (FIGO) developed a widely used staging system for ovarian cancer, consisting of four stages that describe the advancement of ovarian tumor growth [2, 3]. Stage I describes growth limited to the ovaries. Once growth spreads beyond the ovaries into pelvic organs, the cancer is considered Stage II. Patients with growth of tumors that involves one or both ovaries with peritoneal metastasis beyond the pelvis are classified as Stage III. Finally, Stage IV describes cancers that involve one or both ovaries with distant metastasis beyond the peritoneal cavity. More than 70% of women are initially diagnosed with Stage III or IV ovarian cancer according to the FIGO classification [4].

The five-year survival rate of ovarian cancer patients ranges from 80-90% for FIGO Stage I to 5-20% for FIGO Stage IV [3]. Because survival is strongly inversely correlated with stage, identification of women with early

stages of ovarian cancer is crucial for their survival. Until recently, women with early stages of ovarian cancer were considered asymptomatic, which explains why the majority of early cases are not detected. In 2007, the Gynecologic Cancer Foundation, Society of Gynecologic Oncologists, and American Cancer Society made a consensus statement regarding symptoms of ovarian cancer [5]. This statement defined the symptoms as including bloating, pelvic or abdominal pain, difficulty eating or feeling full quickly, and urinary symptoms including urgency or frequency. Studies have shown that even early stage ovarian cancer can produce these symptoms [5]. The symptoms of ovarian cancer remain relatively vague, however, and many patients may still delay seeking medical attention until the cancer has progressed to a later stage.

Most ovarian tumors can be classified into one of three main categories, divided according to the cell type of origin for the malignancy: sex cord-stromal, germ cell tumors, and surface epithelial-stromal tumors [6, 7]. Sex cord-stromal tumors develop from the transformation of theca, granulosa, or stromal cells, while germ cell tumors develop from primordial germ cells. Surface epithelial-stromal tumors, better known as epithelial ovarian cancers (EOCs), arise from the ovarian surface epithelium (OSE) and are believed to be the source of the majority (> 85%) of human ovarian cancers [7, 8].

The OSE is contiguous with the peritoneal mesothelium [9] and EOCs are characteristically heterogenous in nature. The underlying molecular and genetic events leading to ovarian adenocarcinoma are still largely unknown. OSE cells develop the characteristics of other mullerian duct-derived cells as malignant transformation occurs. Epithelial tumors are therefore divided into five major subtypes based on the characteristics the tumor cells acquire: serous

(fallopian tube-like), mucinous (endocervical-like), endometrioid (endometrium-like), clear cell (resembles endometrial glands in pregnancy), and Brenner tumors (urothelial in appearance) [6, 7, reviewed in [10]]. There are also mixed forms, unclassified forms, and undifferentiated carcinomas [7]. Nearly 80% of all EOCs are categorized as serous [8]. The subtypes of EOC differ not only in morphology but also with respect to occurrence, response to chemotherapy, and survival rates. Generally speaking, survival rates for women with EOC decrease for cancers diagnosed at later stages, at older ages, with larger volume of tumor and for women diagnosed with clear cell or undifferentiated cancers [11-15]. There also appears to be a survival advantage of Hispanic and Filipina women and a survival disadvantage for African-American women as compared to white women [11].

Only 5-10% of ovarian cancers can be explained by genetic predisposition or family history and a number of hypotheses have been developed in an attempt to explain the origin of spontaneous ovarian cancer. Two leading hypotheses are the incessant ovulation hypothesis and the gonadotropin stimulation hypothesis. The incessant ovulation hypothesis was first presented in 1971 by Fathalla [16]. In this hypothesis, it is proposed that the repeated rupture and repair of the OSE with each ovulation stimulates proliferation and permits transformation to occur. Through repeated trauma of OSE cells, it is thought that mutations may be incorporated into the genome, possibly due to exposure of OSE cells to genotoxins that are generated during the ovulatory process [17]. Alternatively, cells of the OSE may be exposed to a novel environment following the incorporation of these cells into the ovarian stroma in the form of inclusion cysts [8]. Inclusion cysts are believed to arise through one of two possible mechanisms (Fig. 1-1). OSE

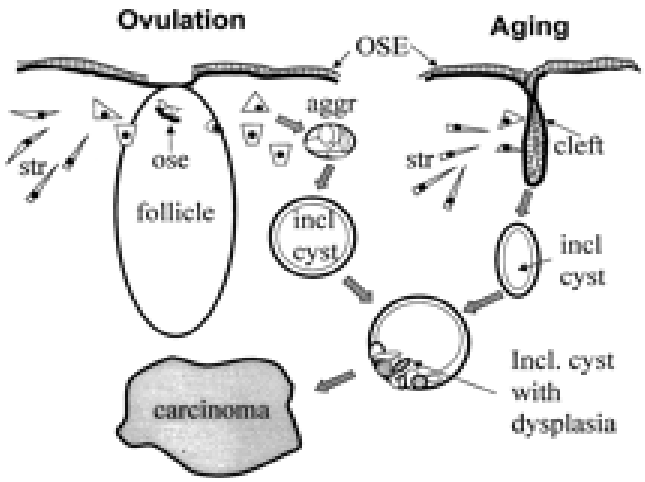

Figure 1-1. The formation of inclusion cysts.

Adapted from Auersperg *et al*. [8].

Inclusion cysts can form through the incorporation of fragments of the OSE into ruptured follicles or through the pinching off of surface clefts [8].

cells may be displaced following ovulation if they become trapped within ruptured follicles. Alternately, OSE cells lining natural clefts on the ovarian surface may be pinched off and incorporated into the stroma of the ovary [8]. The incessant ovulation hypothesis has been supported by the fact that ovarian cancer risk is increased by nulliparity, while a history of one or more full term pregnancies and use of oral contraceptives decreases a woman's risk [18, 19]. Weaknesses in this theory exist, however. For example, progesterone-only contraceptives, which do not completely inhibit ovulation, also decrease a woman's risk of EOC as effectively as ovulation-inhibiting contraceptives, and women with polycystic ovarian syndrome (PCOS), who have decreased ovulatory cycles, are at increased risk of the disease [reviewed in [20]].

The gonadotropin stimulation hypothesis was developed after the incessant ovulation hypothesis, and proposes that over-stimulation by gonadotropins, specifically follicle stimulating hormone (FSH) and luteinizing hormone (LH) leads to malignant transformation of the OSE. This hypothesis was developed after the observation that rodents that underwent bilateral oophorectomy with transplantation of the ovary under the splenic capsule developed ovarian tumors, while intact ovarian function in these animals suppressed tumor formation [reviewed in [21]]. Loss of ovarian function leads to decreased negative feedback to the hypothalamic-pituitary axis and a subsequent increase in gonadotropin release. Indirect evidence for this hypothesis includes the fact that gonadotropin levels are known to increase with age, being particularly high in early menopause, which may explain the increased incidence of ovarian cancer in post-menopausal women. Additionally, infertile women who use fertility drugs have been reported to

have an increased risk of EOC [20]. Cramer and Welch [22] proposed that after inclusion cysts form, differentiation, proliferation, and malignant transformation occur as a result of either direct stimulation by gonadotropins or by indirect stimulation by steroids stimulated by gonadotropins. They argued that the fact that pregnancy decreases the risk of ovarian cancer supports the gonadotropin stimulation hypothesis because pregnancy may cause permanent changes in the pituitary gland that affect the secretion of trophic hormones. Similarly, they believe that the fact that oral contraceptive use lowers gonadotropin secretion and decreases a woman's risk of ovarian cancer supports their theory [22]. A recent study using a genetically modified mouse model lacking ovulation with high gonadotropin levels found increased incidence of ovarian tumors, which provides additional support for the gonadotropin stimulation hypothesis [23].

Other hypotheses that were developed to explain the development of ovarian cancer that may be considered not as widely accepted include the inflammation hypothesis, the hormonal stimulation hypothesis, and the pelvic contamination theory. The inflammation hypothesis suggests that lymphocytes, growth factors, and prostaglandins associated with inflammation may contribute to cancer growth and to immunosuppression associated with cancer advancement [24, 25]. The inflammation-induced transcription factor, nuclear factor κB, is believed to play a key role in cancer through the inhibition of apoptosis [26]. With respect to ovarian cancer, inflammation is induced in damaged OSE with each ovulation and aspects of the ovulatory process have been described as resembling an acute inflammatory process [27]. Evidence supporting this theory includes the fact that use of non-steroidal anti-inflammatory drugs (NSAIDs) may reduce the

risk of ovarian cancer [28-31], exposure to inflammation-inducing agents such as talc or asbestos increases risk [reviewed in [20]], and conditions such as endometriosis or pelvic inflammatory disease are associated with ovarian cancer development [25].

Theories involving the effect of hormones on ovarian cancer risk suggest that androgens and possibly estrogens promote carcinogenesis, while progestins may have protective effects. Evidence in support of the hormonal stimulation hypothesis include the fact that levels of androgens in growing follicles <10mm in diameter are more than 10 times greater than that of estradiol, which may mean that epithelial cells within inclusion cysts would be exposed to high androgen levels [reviewed in [21]]. The presence of androgen receptors on human OSE cells and the ability of these cells to increase proliferation and decrease apoptosis with androgen stimulation have been shown [32]. Additionally, an analysis of the expression of hormone receptors in 94 samples of untreated ovarian tumor samples showed that androgen receptors were present in 90% of samples, while estrogen and progesterone receptors were present in 55% and 52%, respectively [33]. Furthermore, PCOS is associated with increased androgens and increased risk of ovarian cancer, and oral contraceptive use decreases ovarian testosterone production [reviewed in [21]]. Progesterone, on the other hand, decreases risk of EOC [reviewed in [20]] and induces apoptosis in human ovarian cancer cell lines [34]. It has also been suggested that estrogens may play a role in ovarian cancer development. Studies have indicated an increase in the ratio of the two estrogen receptor (ER) subtypes, ERα to ERβ, in ovarian tumors and cells isolated from ovarian tumors [35, 36]. It is hypothesized that ERβ may stimulate anti-proliferative effects that protect against ERα- induced

hyperproliferation [37], which may explain why ERβ is down-regulated in neoplastic ovarian tissue [38, 39]. In addition, epidemiological data suggest that long-term use of hormone replacement therapy increases risk of EOC [reviewed in [40]]. Indirect evidence that supports a role for estrogens in ovarian carcinogenesis includes the fact that breast-feeding and oral contraceptive use, which both decrease risk of EOC also decrease estradiol production [reviewed in [21]]. Pregnancy, however, increases serum estradiol as much as 100-fold and has a protective effect [reviewed in [21]].

The pelvic contamination theory suggests that ovarian cancer is initiated after carcinogens ascend the reproductive tract and act at the ovary, while others believe that systemic carcinogens such as tobacco smoke may be the impetus behind ovarian cancer initiation [reviewed in [22]]. It remains to be seen whether one of these theories or perhaps a combination of two or more of these theories accurately define the cause of ovarian cancer. Until one is proven doctrine, all theories should be considered sources of intriguing opportunities for research potential.

Several prognostic factors are used to determine the relevant treatment for ovarian carcinoma patients including surgical stage, volume of residual tumor after primary surgery and histological grade [4]. Surgery is most commonly the first treatment followed by chemotherapy, although the reverse of this order is used in some cases. A combination of paclitaxel and carboplatin or carboplatin alone is the standard first-line chemotherapy for advanced ovarian cancer [41]. Paclitaxel is a mitotic inhibitor that binds the β-subunit of tubulin, which prevents microtubule breakdown [42]. As described in a recent review [43], microtubule stabilization can lead to the induction of apoptosis through a p53-dependent pathway or through the MAPK pathway,

which activates pro-apoptotic proteins such as BAD. Additionally, the fact that the pro-apoptotic protein, BIM, is bound to a microtubule-bound ATPase indicates that paclitaxel-induced microtubule stabilization could directly activate an apoptotic pathway [43]. Carboplatin is a DNA alkylating agent that induces the formation of platinum-DNA adducts, crosslinks, and strand breaks which inhibit DNA replication [42]. The interstrand and intrastrand crosslinks formed by platinum-DNA adducts also activate the mismatch repair pathway, which triggers apoptosis [reviewed in [43]]. The standard second line for ovarian cancer treatment depends on a number of factors including the type of initial treatment, time elapsed before recurrence, tolerability, the patient's performance status, and cost-effectiveness [41].

Because the aim of tumor-debulking surgery is to remove as much of the tumor as possible and most ovarian carcinomas are not diagnosed until the cancer has spread beyond the ovaries, considerable morbidity is associated with surgery as treatment for ovarian cancer [44]. Despite this, surgery appears to be necessary for ovarian cancer treatment because the recurrence of cancer for patients receiving only systemic chemotherapy is around 80% and recurrence is a predictor of poor outcome [41, 45]. Additionally, most patients with advanced EOC exhibit poor initial response to chemotherapy or develop resistance to chemotherapy [46, 47]. Chemoresistance in ovarian tumors can occur due to genetic or nongenetic alterations that allow the tumor to increase drug inactivation, enhance efflux of the drug, increase DNA damage repair, decrease apoptotic pathways, or increase survival signals [43]. In addition, quiescent cells within the tumor may be unaffected by chemotherapy because most chemotherapeutic drugs require the cell to be active in the cell cycle [42]. There is evidence that resistance to chemotherapeutic agents varies among

histologic subtypes of EOC due to differences in tumor cell biology among the subtypes, reflected in differences in biomarker expression [10]. The poor efficacy of treatments of advanced ovarian cancer make studies aimed at enhancing prevention or early detection of the disease pertinent. As a means to this end, many studies are focused on determining relevant pathways involved in the development or progression of ovarian cancer.

The Cyclooxygenase Enzymes in Ovarian Cancer

The COX enzymes are the key regulatory enzymes in the prostaglandin synthesis pathway (Fig. 1-1). Abnormal production of prostaglandins due to aberrant expression of the COX enzymes has been associated with multiple malignancies, including ovarian cancer. Prostaglandins are thought to contribute to carcinogenesis through the enhancement of cell growth, inhibition of apoptosis, and modulation of the immune system, and have also been associated with increased cell proliferation, enhanced tumor invasiveness, and metastatic potential [49-51]. Additionally, the production of stable oxy radicals and the formation of malondialdehyde are catalyzed by prostaglandins and both may lead to the formation of mutations that may eventually trigger tumor formation [49]. Few studies actually document changes in the levels of prostanoids in cancer and data regarding the expression of the COX enzymes and downstream isomerases are used to deduce the involvement of prostanoids in tumor progression [52].

The five major prostanoids produced from the COX reactions are prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin I_2 (PGI_2), and thromboxane A_2 (TxA_2) [52]. As explained by

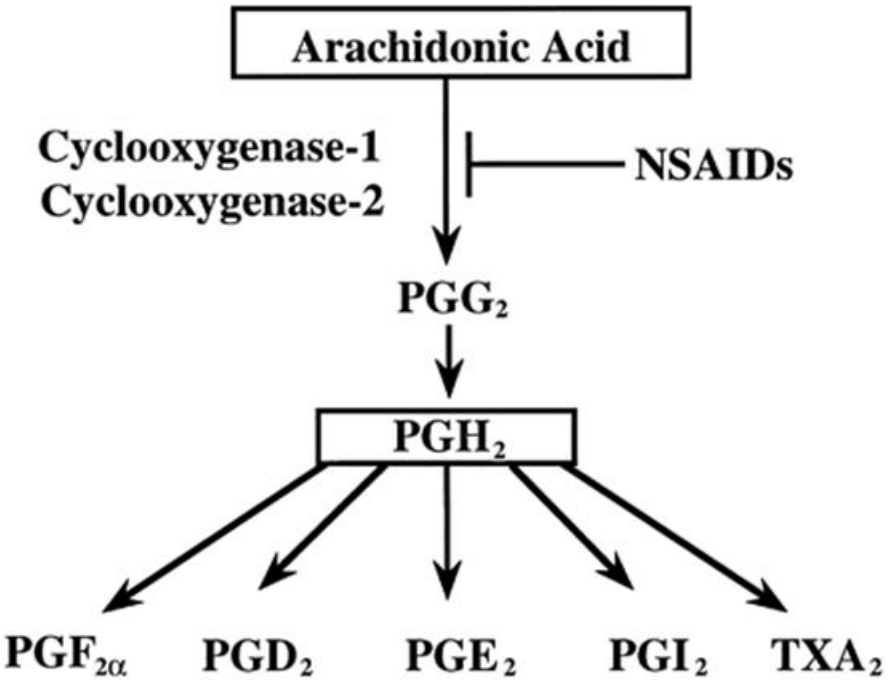

Figure 1-2. Production of prostglandins from arachidonic acid.

Adapted from Dubois *et al.* [48].

Narumiya *et al*., prostaglandins are named according to modifications to their cyclopentane ring, while thromboxanes have an oxane ring in the place of the cyclopentane ring. PGI and TxA are chemically unstable with a half-life of 30 seconds to a few minutes before they are degraded into inactive products. Other prostaglandins are chemically stable but are metabolized quickly, in a single passage through the lung [reviewed in [53]]. Prostaglandins are therefore believed to act locally and participate in a wide range of biological functions including but not limited to platelet aggregation, immune function, kidney development, renal homeostasis, uterine function, pregnancy, parturition, sleep wake cycle, body temperature, and inflammation [reviewed in [52]].

Two COX isoforms currently being studied with respect to cancer risk are COX-1 and COX-2. COX-1 and COX-2 share around 60% sequence identity and are ~600 amino acids in chickens and mammals. Additionally, both isoforms catalyze identical reactions in the conversion of arachidonic acid to prostaglandins. COXs have bifunctional enzymatic activity that includes a cyclooxygenase reaction, that produces an endoperoxide-containing prostaglandin G_2 (PGG_2), and a peroxidase reaction, in which a hydroperoxyl in PGG_2 is reduced to a hydroxyl to form PGH_2 [Figure 1-2; reviewed in [54]]. Specific synthases then transform PGH_2 into different prostaglandins. The peroxidase activity of the COX enzymes can function independent of the cyclooxygenase activity, but the reverse is not true [55]. Despite the similarities, COX-1 and COX-2 are located on different genes that are separately regulated and have distinct expression patterns.

Habenicht *et al.* [56] noted that treatment of Swiss 3T3 cells (an

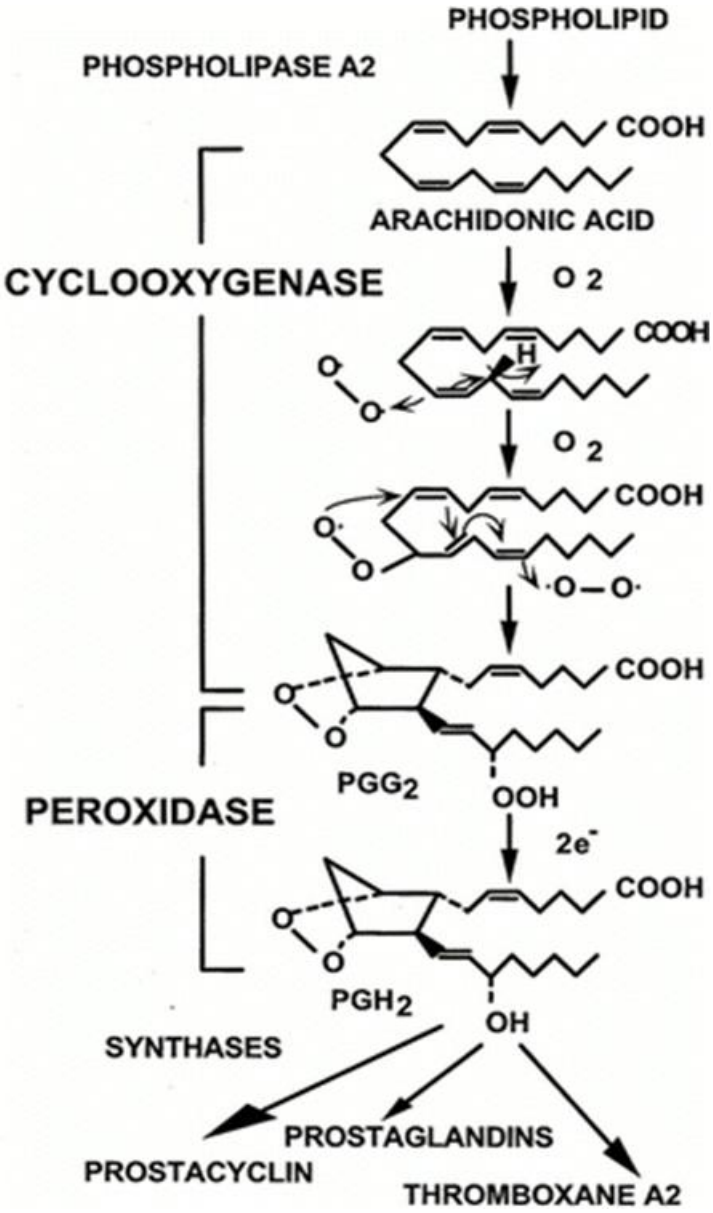

Figure 1-3. The formation of prostaglandin from arachidonic acid, catalyzed by the cyclooxygenase and peroxidase activities of the COX enzymes.

Adapted from Smith *et al.* [58].

immortal line of fibroblast-like cells derived from whole trypsinized embryos of Swiss mice) with platelet-derived growth factor (PDGF), induced prostaglandin synthesis in two separate peaks: an early peak at 10 min and a late peak 2-4 hours after treatment. This led to the conclusion that PDGF-stimulated prostaglandin synthesis must occur through two direct effects on the COX system, one involving a protein-synthesis independent mechanism and another that requires rapid translation of COX [56]. In 1991 the combined work of three independent groups led to the identification of COX-2 [reviewed in [54]]. The origin of multiple cyclooxygenase enzymes is believed to be the result of the duplication of the *COX* gene independently in invertebrates and vertebrates, although the reason for separate isozymes is unknown [57]. Despite the probable common origin, the two isoforms are differentially regulated in terms of mRNA splicing, stability, and translational efficiency [48]. In addition, COX-1 and COX-2 appear to be able to utilize different substrate pools. For example, in both fibroblasts and immune cells, endogenous arachidonic acid is utilized by COX-2, while COX-1 requires exogenous substrate [48]. This suggests that the availability of endogenous or exogenous arachidonic acid is a regulatory element that may differentially control prostaglandin production by the two COX isoforms in certain cell types.

COX-1 had long been known as the constitutively expressed COX isozyme and is involved in producing prostaglandins that perform "housekeeping" functions including smooth muscle contraction, the regulation of renal water and sodium metabolism, stomach acid secretion, platelet aggregation, renin release, and parturition [reviewed in [59], 60, 61]. Recent data indicate that COX-1 may be inducible under certain conditions

[62] and, as will be discussed below, COX-1 may not only be involved in "housekeeping" functions. COX-1 expression can be found in most mammalian tissues [48, 63]. COX-2 had been known as the inducible isoform associated with inflammatory states [64]. COX-2 expression is inducible in endothelial cells, macrophages, fibroblasts, and ovarian follicles by various stimuli including mitogens, cytokines, growth factors, and hormones [reviewed in [48], 65, 66, 67]. Despite the fact that COX-2 is considered inducible, it is now known to be constitutively expressed in some tissues including the brain and kidney of rodents and in human and rabbit gastric mucosa [68, 69, 70].

The generation of COX knockout (KO) mice resulted in surprising phenotypes. Because COX-1 was considered the isoform responsible for homeostasis, it was expected that COX-1 deficiency would be detrimental. Lagenbach *et al.* [71] used homologous recombination to disrupt COX-1 in the mouse and surprisingly found that the only abnormality observed was a decreased inflammatory response to arachidonic acid. Neither male nor female fertility was affected, development was normal, and no significant pathology was observed in the liver, spleen, kidney, heart, lungs, GI or reproductive tracts. Breedings between homozygous mutant females and heterozygous males (and vice versa) resulted in litter sizes and surviving pups close to normal. Only breedings between homozygous mutant females and homozygous mutant males resulted in death of all resulting pups despite normal litter sizes. The death of pups was hypothesized to be due to impaired onset of labor [71]. Results from COX-2 KO mice, reported by Dinchuk *et al.* [72], included renal dysplasia (100% penetrance) and cardiac fibrosis (50% penetrance). Although COX-2 deficiency was not lethal *in utero*, neonatal

death was common and average lifespan of null animals was 3.5 months. COX-2 deficient females were largely infertile and had ovaries that were virtually void of corpora lutea but had apparently normal follicle development. These data were interpreted as showing that inhibition of COX-2 inhibits ovulation [72]. The results from COX-1 and COX-2 KO mice indicate that the functional roles of these two isozymes are not as defined or distinct as was once believed. For example, if COX-1 is only responsible for homeostasis and if COX-2 is only responsible for inflammatory responses, then the lack of significant pathology and decreased inflammatory response seen in mice lacking COX-1 activity is contradictory. Furthermore, the normal inflammatory response observed in COX-2 KO mice indicates that COX-1 contributes to this process.

In addition to the data gathered from COX KO mice, further evidence shows that the COX enzymes serve multiple functional roles. As mentioned above, the inducible isoform, COX-2, now is known to be constitutively expressed in some tissues. Most notably, constitutive expression of COX-2 in human gastric mucosa [70] adds to evidence that suggests that COX-2 may catalyze the production of prostaglandins with a protective function [reviewed in [73]]. Data show that gastric and intestinal damage in rats is only seen after both COX-1 and COX-2 are inhibited [74, 75] and specific inhibition of COX-2 results in decreased gastric ulcer healing in both mice and rats [76, 77]. COX-2 is also involved in normal renal development and contributes to myocardial protection [63]. A study by Gilroy *et al.* in 1999 [78] showed that COX-2 activity is associated with anti-inflammatory actions, in addition to its known pro-inflammatory function. In their rat model of inflammation, two peaks of COX-2 activity were observed: one at 2 hours and a second at 48

hours. The activity observed at the second peak was 350% greater than that at the first peak and coincided with a decrease in the inflammatory reaction. Inhibition of COX-2 using indomethacin or a specific COX-2 inhibitor decreased inflammation at 2 hours but resulted in increased inflammation at 48 hours [78]. These combined data suggest that interactions and functions of COX-1 and COX-2 are more complex than was originally believed and further research is warranted to accurately determine effects of inhibiting these enzymes.

In the last 2 decades, many studies have focused on the possibility that the COX enzymes may play a role in cancer formation or progression. COX-2 expression has been associated with cancers of the colon, breast, head, neck, pancreas, lung, and stomach, among others [79-85]. Additionally, studies in transgenic mice have shown that overexpression of COX-2 is sufficient to induce mammary gland tumorigenesis, possibly by altering levels of anti- and pro-apoptotic proteins [86]. Most early studies regarding the COX enzymes and ovarian cancer focused on COX-2, possibly because of the association of COX-2 with carcinomas of various origins (described above) and due to the fact that COX-2 was known as the inducible isoform. Many studies that examined COX-2 expression in ovarian cancer indicate that COX-2 is expressed in ovarian tumors or some human ovarian cancer cell lines [87-100]. COX-2 is believed to contribute to tumorigenesis by producing prostaglandins that increase cell growth, prevent apoptosis, increase cell motility and adhesion, and increase angiogenesis [101].

The expression of COX-2 in ovarian cancer may not be surprising, however, because COX-2 is involved in ovulation and both COX-1 and COX-2 are normally expressed in the ovary [102]. Some studies, however, found no

COX-2 expression in ovarian cancer [103, 104] and others have shown that most ovarian cancer cell lines do not express COX-2 [90, 105-107]. One study indicated that COX-2 expression is induced by many of the cytotoxic drugs used to treat ovarian cancer. They used tumor samples from individuals that had not undergone previous chemotherapeutic treatment and found no COX-2 expression [105]. This indicates that COX-2 expression may be induced by chemotherapy and therefore may not be involved in ovarian tumor formation. Furthermore, studies using ovarian cancer cell lines show that the few lines that express COX-2 do not respond to cytokines and lose the ability to stimulate a normal inflammatory response that is observed in normal OSE cells [108, 109]. It is hypothesized that ovarian cancer cells may inactivate COX-2 in order to decrease inflammation in an effort to avoid immune surveillance [108].

COX-1 was largely overlooked in earlier studies of ovarian cancer. A study by Dore *et al.* in 1998 [103] was the first to show COX-1 but not COX-2 expression in human ovarian adenocarcinomas. In 2003, a second group verified these findings and also showed COX-1 expression in a human ovarian cancer cell line [105]. Since then, COX-1 expression has been further verified in human ovarian tumor samples and cell lines [90, 91, 93, 94, 96, 107, 110-113]. Additionally, a rat model of epithelial ovarian cancer correlated COX-1 with ovarian tumors [114] and mouse models of ovarian cancer also correlate COX-1 with ovarian cancer rather than COX-2 [115, 116]. Although the exact contribution of COX-1 to ovarian cancer remains undefined, a recent paper suggests that COX-1 may be acting through the PPARδ-ERK signaling pathway to contribute to epithelial ovarian cancer through the stimulation of cell proliferation [116]. COX-1 derived prostaglandins may also contribute to

ovarian cancer and studies have specifically correlated COX-1 and PGE_2 in ovarian cancer [107, 110]. PGE_2 in particular is believed to be involved in ovarian cancer progression due to its involvement with processes such as angiogenesis, cell proliferation and apoptosis [50, 51, 96, 105], and the ability of PGE_2 to stimulate proliferation and reduce apoptosis in EOC cells has been confirmed [117].

The similarity of sequences for COX-1 and COX-2 and the fact that COX-1 expression in ovarian cancer was not considered until recently raises the question of whether antibodies used for studies that only considered COX-2 cross-reacted with COX-1 to give false-positive results. This, in addition to the fact that COX-2 is induced by many chemotherapeutic agents [105], suggests that COX-1 may play a significant role in ovarian cancer. Conflicting results in different studies could also be due to differences in detection methods used or differences in tissue processing [105]. KO animal models have suggested that the normal biological functions of COX-1 and COX-2 are not as distinct as was once believed. Therefore, it should not be surprising that both COX enzymes may be involved in cancer development and progression. The possibility that one isoform may be able to compensate for the other [108] additionally warrants examination of both isoforms in studies of tumorigenesis. Future studies in this area will hopefully elucidate the exact role of the COX enzymes in ovarian cancer initiation and/or progression.

The Role of Vascular Endothelial Growth Factor in Ovarian Cancer

The hypothesis that tumor cells secrete a factor that increases the permeability of blood vessels was presented in 1979 [118] and vascular

permeability factor (VPF) was identified in 1983 in the ascites of rodent tumors [119]. A few years later a protein with selective mitogenic activity for endothelial cells, called vascular endothelial growth factor (VEGF), was isolated and it was established that VEGF and VPF were the same protein [reviewed in [119]]. VEGF plays a key role in normal ovarian processes including follicle development and selection, ovulation, and corpus luteum formation [121]. In 1993, a study identified the presence of VEGF in human ovarian adenocarcinoma effusions [122] and one year later the constitutive expression of VEGF in normal and neoplastic human ovaries was characterized [123].

It has been shown that 97% of ovarian cancers express VEGF and there is a significant increase in VEGF expression in stage III and stage IV tumors as compared to ovaries without ovarian cancer [124, 125]. Furthermore, a significant correlation between ovarian tumor VEGF expression and disease stage, histological grade, and patient survival has been found [126-128]. In addition to ovarian tumor expression, VEGF expression has been correlated with ascites formation associated with advanced ovarian cancer. As described by Nagy *et al*. [129], the mechanism that links VEGF to ascites formation involves the ability of VEGF to stimulate increased permeability of vessels lining the peritoneal cavity. Hyperpermeability of these vessels leads to the release of a plasma exudate that nourishes the malignant cells within the ascites [129]. VEGF released by malignant cells within the peritoneum therefore serves a parallel purpose to VEGF released by solid tumors, to increase angiogenesis for nourishment of (and waste removal from) the solid tumor. The concentration of VEGF in ascites has been shown to correlate to tumor stage and ascites volume, and negatively correlate to patient survival

[128].

Studies aimed at determining the prognostic significance of the concentration of VEGF in the serum of ovarian cancer patients have produced mixed results. It has been reported that serum levels of VEGF were significantly increased in patients with ovarian cancer as compared to unaffected patients, and patients with metastatic ovarian cancer have increased serum VEGF levels as compared to patients with localized tumors [130]. Preoperative serum VEGF levels in women with invasive epithelial ovarian cancer were also shown to be increased as compared to women with low malignant potential ovarian cancer or benign ovarian tumors [131]. Other studies, however, found no difference in serum levels of VEGF between patients with ovarian carcinoma and those with benign ovarian dysplasia or lacking gynecological disease [128, 132]. The contradiction in results regarding the usefulness of serum VEGF as a prognostic tool leaves the question of whether VEGF can be used to screen women for ovarian cancer unanswered.

The prognostic significance of VEGF expression in ovarian tumors and ascites of patients with ovarian cancer, and the possible significance of VEGF in the serum of ovarian cancer patients have sparked investigations into antiangiogenic treatments targeting VEGF. The types of anti-angiogenic treatments available include bevacizumab (a monoclonal antibody that blocks the interaction between VEGF and its receptor), receptor decoys that intercept VEGF such as VEGF-Trap, small molecule inhibitors that inhibit receptor tyrosine kinases for VEGF, and ribozyme (angiozyme), which targets pre-mRNA for VEGF receptors [133]. Studies show that inhibition of VEGF reduces both ascites formation and ovarian tumor burden [134-138]. These

results raise interest in determining the pathways associated with VEGF expression in ovarian cancer.

Interestingly, a link between VEGF and the COX pathways has been found in ovarian cancer studies. A correlation between COX-2 and VEGF expression in ovarian cancer has been reported by a number of studies [93, 98, 100, 139, 140], while less data are available for COX-1. Lack of data associating COX-1 with VEGF may be because COX-1 was overlooked in early ovarian cancer studies, as was described above. One study that considered both COX isozymes showed that both COX-1 and COX-2 selective inhibitors could decrease endothelin-1 induced VEGF production in human ovarian cancer cells [94]. Additionally, VEGF mRNA in human ovarian cancer tissue was localized to regions expressing high levels of COX-1 mRNA and a COX-1 specific inhibitor decreased VEGF production by an ovarian cancer cell line, while a COX-2 specific inhibitor did not [105]. These combined data suggest that COX-derived prostaglandins may contribute to ovarian cancer progression through stimulation of VEGF, which in turn stimulates neovascularization and angiogenesis. Targeting the COX pathways in ovarian cancer treatments may therefore decrease ascites burden and tumor growth by decreasing VEGF production.

NSAIDs as Chemopreventative/Chemotherapeutic Agents

Ovarian cancer is the most fatal gynecologic malignancy due in part to lack of effective screening techniques to detect early cases and inadequate treatment options for patients with advanced stages of the disease. Factors such as these make research of chemoprevention and chemotherapy options

for women with ovarian cancer particularly important. Because of the association of the COX enzymes and cancer, described above, it is of interest to determine whether NSAIDs may aid in the prevention or treatment of ovarian cancer.

Evidence suggests that frequent use of common NSAIDs may aid in the prevention of carcinogenesis in tissues such as colon, breast, and pancreas among others [141-144]. Additionally, epidemiological studies have indicated that the use of aspirin or other nonselective NSAIDs may be associated with a risk reduction for ovarian cancer [28-31]. Because of the fact that many of the common NSAIDs that are classified as nonselective are generally better inhibitors of COX-1 than COX-2 [145], these data support the idea that COX-1 may play an important role in the development of ovarian cancer.

Most NSAIDs inhibit the COX enzymes through reversible or irreversible binding to the active site of COX enzymes, thus blocking the entry and binding of arachidonic acid. Aspirin is an exception. Aspirin acetylates a serine residue (Ser^{530} in COX-1 and Ser^{516} in COX-2) in the channel that leads to the active sites of COX-1 and COX-2 [reviewed in [146]], which causes irreversible inhibition of the cyclooxygenase but not the peroxidase activity of the enzymes [147]. Aspirin is therefore considered a nonselective NSAID because it inhibits both COX-1 and COX-2, although it is a more potent inhibitor of COX-1 than COX-2 [148]. COX-1 specific NSAIDs block the entry of arachidonic acid into the COX-1 active site but do not effectively block the entry of arachidonic acid into the larger active site of COX-2. COX-2 specific NSAIDs take advantage of the fact that the active site of COX-2 has a hydrophobic pocket not present in the active site of COX-1 and is larger than the active site of COX-1, due to a residue difference within the active site as

well as other residue differences that line the active site [149].

The development of COX-specific NSAIDs permitted studies focused on the independent contributions of the COX isoforms to ovarian cancer. One study showed that the COX-1 selective inhibitor, SC-560, significantly inhibited PGE_2 formation and VEGF secretion in human ovarian cancer cell lines while the COX-2 selective inhibitor, celecoxib, had no effect [105]. It should be noted, however, that the cell lines used do not express COX-2 [105]. Another study indicated that the use of celecoxib, a COX-2 selective inhibitor, had little effect on ovarian tumor growth, while the COX-1 selective inhibitor, SC-560, reduced the growth of ovarian tumors [104]. A number of studies have reported the effectiveness of selective COX-2 inhibitors in reducing growth of human ovarian cancer cell lines [111, 150, 151]. These results again may be surprising in light of the fact that the cell lines used in these studies, namely OVCAR-3, SCOV-3 and Caov-3, have been shown to lack COX-2 protein expression [90, 105, 106]. Denkert *et al.* [113] showed that even though COX-2 expression was inducible in OVCAR-3 cells after stimulation with interleukin-1β, the same was not true for SKOV-3 cells. They also found that a COX-2 specific inhibitor, NS-398, significantly decreased cell proliferation in both OVCAR-3 and SKOV-3 cell lines. Using siRNA for both COX enzymes, they further verified that NS-398 decreased cell proliferation through a COX-independent mechanism [113]. Another study by Bijman *et al.* [106] verified that the antitumor effects of a selective COX-2 inhibitor, celecoxib, on ovarian cancer cell lines occurs in the absence of COX-2 expression. They also studied the intriguing effect of celecoxib catalyzing cisplatin resistance and noted that this effect is also independent of COX-2 expression [106]. Results showing that celecoxib is more effective at inhibiting cell growth and inducing

apoptosis in SKOV-3 cells with wild-type p53 as compared to SKOV-3 cells with null type p53 suggest that the COX-independent effects of celecoxib may involve the p53 pathway [152]. It is not clear from these studies how COX-2 selective inhibitors are causing effects in ovarian cancer cells. Importantly, the fact that celecoxib may also decrease the efficacy of chemotherapeutic drugs [106] warrants more research before these drugs are used in ovarian cancer treatments.

Explanations for the effectiveness of NSAIDs in the absence of COX-1 or COX-2 have been proposed. The presence of COX-1 variants, including COX-3, that are affected by NSAIDs is one possibility for alternate targets [153]. Other known mechanisms of action of NSAIDs include the ability to inhibit calcium movement, enhance intracellular levels of cyclic AMP, inhibit NF-κB, and inhibit phospholipase C [reviewed in [154]]. With respect to ovarian cancer, studies have shown non-COX actions of NSAIDs that include the depletion of cellular thiols, up-regulation of the antitumorigenic protein NSAID activated gene -1 (NAG-1), inhibition of macrophage migration inhibitory factor, and suppression of NF-κB activity [155-157]. Any of these mechanisms working alone or in combination with decreased prostaglandin synthesis could account for the anti-cancer effects of NSAIDs [154].

The Hen as a Model for Ovarian Cancer

One of the main impediments to the advancement of ovarian cancer research has been the lack of a suitable animal model. Mice do not spontaneously develop ovarian cancer at a rate high enough to study and spontaneous tumors in mice differ histopathologically from most tumors in

women [158]. Several genetically engineered mouse models of human ovarian cancer have been developed, however. Orsulic *et al.* [159] found that when any two of the oncogenes, *c-myc*, *K-ras*, or *akt,* were introduced in transgenic mice deficient in p53, the mice developed ovarian cancer with similar origin, progression, and intraperitoneal metastatic spread as human ovarian cancer. Another rodent EOC model was developed through the introduction of an engineered DNA fragment that placed the oncogene SV40 T antigen downstream of the mullerian inhibiting substance type II receptor promoter, which restricted expression of the oncogene to the ovary [160]. Mice in this model developed ovarian tumors in ~50% of cases. Inactivation of *p53* and *Rb1* by intrabursal administration of recombinant adenovirus expressing Cre was also found to effectively induce EOC in mice [161]. Wu *et al.* [162] developed a more recent mouse model by deregulating the PI3K/Pten and Wnt/beta-catenin signaling pathways. Greater than 75% of mice in this model developed adenocarcinomas morphologically similar to human ovarian endometroid adenocarcinoma, with similar biological behavior and gene expression patterns [162].

The induction of genetic alterations to initiate ovarian cancer in mice may be introducing changes that do not accurately portray the spontaneous etiology of ovarian cancer. A model that spontaneously develops EOC may more accurately depict the pathogenesis of the disease in women. Most animals, however, do not spontaneously develop ovarian cancer [158, 163], possibly due to the fact that the common natural reproductive state of most animals is pregnancy or lactation. It has also been suggested that human OSE cells are incompletely committed and arrested in a progenitor state, unlike the OSE of laboratory animals, which may make these cells in humans more prone

to neoplastic transformation [8, 164]. The interaction between the OSE and the extracellular matrix in women may contribute to the phenotypic plasticity of OSE cells, which enables these cells to perform the diverse functions for the maintenance and remodeling of the dynamic ovarian cortex [165]. Another major difference between human OSE cells and those of laboratory animals is the connection between the cells and the underlying tissues. OSE cells in women are loosely attached to the underlying tunica albuginea, which separates the OSE from the ovarian stroma and partially inhibits the transfer of bioactive materials between the stroma and the OSE [8]. The OSE of rats more firmly adheres to the ovarian stroma, which may expose these cells to a different microenvironment [164]. Exposure of the OSE in women to the ovarian stroma in the form of inclusion cysts, therefore, may be a more drastic environmental change that permits neoplastic progression as compared to exposure of rodent OSE cells to the ovarian stroma. While studying the differences between humans and animals that do not frequently spontaneously develop EOC (including nonhuman primates [reviewed in [166]] may provide insight into protective effects for the disease, a spontaneous model of ovarian cancer may prove invaluable to investigating the underlying events preceding ovarian carcinogenesis.

The laying hen (*Gallus domesticus*) is a persistent ovulator and spontaneously develops ovarian adenocarcinomas [167-170]. In an analysis of the hen as a model of ovarian cancer in 1987, Fredrickson found an ovarian tumor incidence of 32% in 466 hens studied for 3.5 years ranging in starting age from 2-7 years. He also determined that ovarian tumors are uncommon in hens less than 2 years of age [167]. As explained by Murdoch *et al*. [17], the surface epithelial cells located near a follicular rupture are exposed to

oxyradicals that are liberated during ovulation. They confirmed in the hen that there are elevated levels of 8-oxo-guanine adducts in OSE cells collected from the apical surfaces of preovulatory follicles and perimeters of postovulatory follicles. A surface epithelial cell with these adducts that escapes repair or apoptosis could then give rise to a transformed phenotype. These adducts in the hen therefore may be indicative that genomic damage to the OSE caused by frequent ovulation is a main contributor to ovarian cancer initiation [17].

The hen reproductive tract normally consists of a single oviduct and ovary on the left side of the animal. The single ovary (Fig. 1-3, A) consists of an ovarian cortex in which primordial, primary, and secondary follicles are located. Cortical follicles (<1mm in diameter) are located throughout the ovarian stroma. Protruding from the cortex are several thousand growing, non-hierarchical follicles ranging from small white follicles (~1mm in diameter) to small yellow follicles (~6-10mm in diameter). The largest protruding follicles are organized into a hierarchy identified as F_6 through F_1. The F_1 is the next follicle to ovulate, which is initiated at the stigma, and the F_2 follicle ovulates roughly 24 hours later. Tumorigenesis in the hen ovary (Fig. 1-3, B) is characterized by disruption or complete loss of the ovarian hierarchy. In these cases, the non-cortical follicles are replaced by cauliflower-like nodules, fluid-filled cysts and/or hemorrhagic follicles.

Ovarian cancer in the hen morphologically and histopathologically resembles the human disease [167, 171-174]. As mentioned previously, cauliflower-like nodules are commonly found within or extending from the cortex of the ovary and glandular-like areas are present in histological sections of ovarian tumors. Similar to humans, metastases to other organs within the

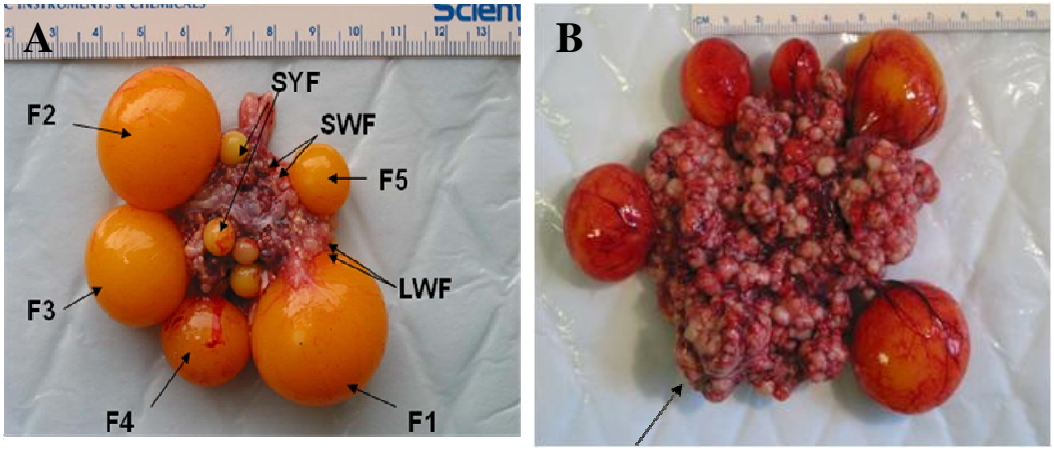

Figure 1-4. Example of a normal hen ovary (A) and an ovarian tumor (B).

A normal hen ovary (A) consists of follicles ranging in size from small white follicles (SWF), large white follicles (LWF), small yellow follicles (SYF), and a hierarchy of large yellow follicles (labeled F5 to F1). Disruption of the ovarian hierarchy and cauliflower-like nodules (B, arrow) are typical of ovarian tumors in the hen (B).

peritoneal cavity are often associated with ovarian cancer progression [175], with metastasis to the oviduct, intestinal tract, and liver being most common. Additionally, metastasis is rarely found outside the peritoneal cavity in women [176, 177] as well as in hens [178]. Also similar to humans, the development of ascites within the peritoneal cavity of hens is associated with advanced cases of ovarian cancer [179].

Although several studies from 1941-1969 reported the occurrence of ovarian adenocarcinoma in hens [168-170, 180, 181], it was not until 1987 that Fredrickson published his landmark study of ovarian cancer incidence in hens. Following this, it was not until 2001 that studies began to evaluate the validity of the hen as a model for a mammalian carcinoma. Rodriguez-Burford *et al.* [171] identified expression of antigens in hen ovarian tumors using antibodies made against human antigens. These included cytokeratin AE1/AE3 and pan cytokeratin (epithelial markers); EGFR and erB-2 (epidermal growth factor receptors); and Lewis Y, CEA, and Tag 72 (oncofetal tumor markers). Additionally, antibodies that stain surrogate endpoints for human ovarian cancer that cross-reacted in the chicken include PCNA (marker of proliferation), p27 (cell cycle inhibitor), and TGF-$\propto$ (growth factor receptor). This study suggested that the hen may be a useful model to study the efficacy of chemopreventative agents [171].

The first study to isolate and culture chicken OSE cells was complete by Giles *et al.* [172], which further developed the hen as a model for ovarian adenocarcinoma. This study also verified positive expression of cytokeratin and PCNA in hen ovarian tumors and cultured OSE cells, and determined the expression of progesterone receptor (PR) within these samples. PR immunohistochemical staining in OSE cells was similar in the chicken to what

is observed in humans. Expression of PR in the tumors of hens may also suggest that tumors in hens are hormone-responsive [172].

The origin of ovarian adenocarcinoma in hens was addressed in a study that determined the expression of an oviductal protein, ovalbumin, in ovarian samples collected from 12 hens with adenocarcinoma because of the observation that many advanced peritoneal cancers in hens involved both the ovary and the oviduct. The presence of ovalbumin in the ovary of all 12 hens was interpreted as indicating that most adenocarcinomas in hens are oviductal in origin [182]. It should be noted that oviductal lesions were also observed in all 12 hens used in the study. Interestingly, it has also been suggested that a higher percentage of cases of ovarian cancer in women may actually have fallopian origin than was originally believed [183]. Twenty years after Haritani *et al.* [182], Giles *et al*. [184] demonstrated that ovalbumin was expressed in ovarian tumors of the hen that lacked oviductal involvement. This result was interpreted as suggesting that ovarian tumor cells dedifferentiate during tumorigenesis and therefore resemble serous (fallopian tube-like) tumors in humans [184]. This finding was particularly useful in validating the hen as a model for human ovarian cancer in light of the fact that serous tumors are believed to be the most common type of ovarian tumors in women.

Fredrickson's original study in 1987 showed a difference in susceptibility to ovarian cancer among different flocks of hens [167]. This suggested that there is a level of genetic susceptibility to ovarian cancer in hens. Johnson *et al.* [185] confirmed a difference between two genetic strains of hens and ovarian cancer incidence. They found a greater incidence of ovarian neoplasms in the Cornell C strain of White Leghorn hens than the

Cornell K strain hens. In addition, they found elevated levels of plasma estradiol and lower levels of plasma immunoreactive inhibin in the C strain as compared to the K strain [185]. This study furthered the understanding of differences between strains of hens that may predispose one strain to developing ovarian cancer.

Jackson *et al*. [186] reported that chicken ovarian tumors express cancer antigen 125 (CA125), which is a tumor marker that is found to be elevated in blood in many patients with ovarian cancer and is used as a screening agent for the disease [187]. They analyzed CA125 expression in dispersed tumor cells and ovarian tumors of the hen using immunohistochemical and Western blot analyses [186]. Although they did publish the first protocol on tumor dispersion in the hen, the data regarding CA125 expression may be questionable. Necessary controls were omitted from immunocytochemical analysis and Western blot results indicate the CA125 band at a size that is smaller from the expected size. Furthermore, it was previously determined that CA125 is not expressed in ovarian tumors of the hen [171] and no study to date (including unpublished reports from our lab) has confirmed the findings of Jackson *et al*. [186].

Recently, an increase in the expression of COX-1 mRNA in ovarian tumors as compared to normal hen ovaries was shown [188], which confirmed an earlier study from our lab [189]. Both studies showed no difference in COX-2 expression between normal ovaries and ovarian tumors. The more recent study of COX expression [188] also found a significantly increased expression of COX-1 mRNA in the first post-ovulatory follicle (POF-1) as compared to POF-2 or POF-3, and no difference in COX-2 expression among POFs. The expression of COX-1 in POFs is interesting in light of the

hypothesis that the POF could serve as a potential site for the formation of clefts or inclusion cysts and subsequent incorporation of OSE into the ovarian stroma [188]. No difference in COX-1 or COX-2 mRNA in the OSE isolated from individual follicles in the hierarchy was found [188}. This study therefore confirmed and expanded the previous characterization of the COX enzymes in the hen ovary. Finally, in 2008, the expression pattern of selenium-binding protein 1, an important mediator of the anti-cancer effects of selenium, in the hen was shown to parallel that found in women with ovarian cancer [174].

Most studies using the hen model of ovarian cancer have focused on validation of the model by determining expression patterns of genes and proteins associated with the etiology of the disease in women, as described above. Only two studies to date have moved beyond validation of the hen model and have begun to utilize hens to study early detection or prevention methods. One study that evaluated the effectiveness of medroxyprogesterone acetate (Depo-Provera) as a chemopreventative agent in the hen model of ovarian cancer found evidence of reduced incidence of adenocarcinoma in the Depo-Provera group as compared to control [190]. This evidence may be considered comparable to the reduced incidence of ovarian cancer observed with oral contraceptive use in women. It should be noted that only a partial reduction in ovulation was achieved, the hens were only treated for one year, and the data indicate that up to 10% of the hens may have had ovarian cancer prior to initiation of the study [190]. A study aimed at utilizing ultrasound as a method for early detection of ovarian cancer was reported by Barua *et al* [173]. Their results show that ovarian tumors in the hen can be detected using *in vivo* transvaginal sonographic techniques. They additionally showed that

blood flow velocity increased in ovaries of hens with malignant tumors [173]. The ability to non-invasively identify hens with tumors permits hens to be utilized to test the efficacy of chemopreventative or chemotherapeutic reagents.

The main objective of the work presented in this dissertation was to study the potential role of the COX enzymes in ovarian cancer in the hen as a model for the human disease. The mRNA and protein expression of the COX enzymes and VEGF was determined in normal ovaries and ovarian tumors of the hen, as well as ascites cells collected from hens with ovarian tumors. The efficacy of several NSAIDs to decrease VEGF expression and to affect the viability of ascites cells was also examined in subsequent *in vitro* studies. Finally, the *in vivo* effects of feeding hens a diet containing aspirin, a common NSAID, on ovarian cancer incidence and progression were determined.

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CHAPTER 2

CYCLOOXYGENASE 1 AND 2 mRNA AND PROTEIN EXPRESSION IN THE *GALLUS DOMESTICUS* MODEL OF OVARIAN CANCER*

Abstract

Objective. Our purpose was to determine the mRNA and protein expression of cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) in ovarian tumors and normal ovaries of the hen, which is an excellent model for human ovarian cancer. Tissue concentrations of prostaglandin E_2 (PGE_2) and PGE_2 metabolites were also determined.

Methods. Tissue was obtained from ovarian tumor (n=18) and normal ovary (n=29) of 2-4-year old Single-comb White Leghorn hens. Quantitative real-time PCR with Sybr Green was used to quantify the mRNA expression of COX-1 and COX-2, using 18S expression as an internal control for COX normalization. Immunohistochemistry using antibodies for COX-1 and COX-2 was used to localize protein expression of each isoform in a subset of tumor (n=5) and normal samples (n=6). For determination of tissue prostaglandin concentration, tissue was obtained from ovarian tumor (n=8) and normal ovary (n=8). PGE_2 and PGE_2 metabolites were measured using competitive enzyme immunoassays (EIAs).

Results. Our results indicate that COX-1 mRNA expression is

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significantly higher ($p < 0.05$) in ovarian tumor samples compared to normal ovaries while there is no significant difference in expression of COX-2 between the samples. Immunohistochemistry results support this finding and show COX-1 expression only in tumor samples and COX-2 expression unchanged between normal ovary and tumor samples. PGE_2 levels are significantly higher ($p<0.05$) in tumor samples compared to normal ovaries and there is no significant difference in PGE_2 metabolite levels between the samples.

Conclusion. These findings may implicate COX-1 as a suitable target for the prevention or treatment of ovarian cancer.

Introduction

Ovarian cancer continued to be one of the leading causes of death due to cancer in women in 2005 [1]. More than 85% of human ovarian cancers are believed to arise from the ovarian surface epithelial layer [2]. These epithelial ovarian cancers are characteristically heterogenous in nature and insights into the underlying molecular and genetic events leading to ovarian adenocarcinoma are still largely unknown. One of the main impediments to the advancement in ovarian cancer research has been the lack of a suitable animal model for spontaneous ovarian cancer. Mouse models may be considered inefficient due to lack of spontaneous cancer occurrence [3] and more recent models in which genetic alterations are induced to cause ovarian cancer [4-6] may be introducing changes that do not accurately portray spontaneous development of ovarian cancer. The laying hen (*Gallus domesticus*) has proved to spontaneously develop epithelial ovarian adenocarcinomas similar to the human female with a fairly high incidence [7,

8, 9]. The hen has thus provided a spontaneous model to study the etiology and/or treatment of ovarian cancer.

The cyclooxygenase (COX) enzymes are key rate-limiting enzymes responsible for catalyzing the synthesis of prostaglandins from arachidonic acid. Two isoforms currently being studied with respect to cancer risk are COX-1 and COX-2. Separate genes encode COX-1 and COX-2 and the two isoforms have distinct expression patterns. Evidence suggests that the COX enzymes may be involved in both the establishment of tumors [13] and maintenance of existing tumors [14]. The role of the COX enzymes in ovarian cancer, however, remains unknown and conflicting evidence has been published regarding the expression of both isoforms in human ovarian tumors. Past studies have associated COX-2 expression with carcinogenesis including that of the lung, colon, breast, head and neck [15, 16, 17]. For this reason, most studies of ovarian tumor formation focused on COX-2 expression and COX-1 has been largely overlooked in studies of ovarian cancer [14]. Although a number of studies have shown increased COX-2 expression in ovarian tumors or a subset of tumors, other studies indicate that COX-2 is overexpressed in preneoplastic lesions rather than established ovarian tumors [10,18]. Conflicting results may be due to case selection or differences in the detection method, tissue processing, or specificity of the antibody used. Recent studies also indicate that ovarian cancer exhibits increased expression of COX-1, rather than COX-2 [14, 19, 20] and other studies that considered COX-1 [10, 18, 21, 22], found positive expression in human ovarian cancer. These data combined with the fact that some studies found no COX-2 expression in ovarian cancer [14, 19, 20] suggest that COX-1 may be more highly correlated with ovarian adenocarcinoma than COX-2.

The reactions catalyzed by COX-1 and COX-2 produce a number of lipids known as prostaglandins. Increased prostaglandin levels have been associated with many of the major types of cancer, although the role of prostaglandins in the pathology and progression of these cancers is not certain [23]. Prostaglandins are thought to contribute to carcinogenesis through the enhancement of cell growth, inhibition of apoptosis, and modulation of the immune system, and have also been associated with increased cell proliferation, enhanced tumor invasiveness, and metastatic potential [23, 24, 25]. The production of stable oxy radicals and the formation of malondialdehyde are both catalyzed by prostaglandins and both may lead to the formation of mutations that may eventually trigger tumor formation [23]. A recent paper reported that COX-1 was the prominent pathway responsible for prostaglandin production in a genetically modified mouse model of epithelial ovarian cancer [14]. Studies have indicated that prostaglandin E_2 (PGE_2), in particular, is elevated in ovarian cancer [25, 26]. For this reason, we focused on PGE_2. The goal of the following experiment was to assess the abundance of COX-1 and COX-2 mRNA in normal ovarian tissue as well as in ovarian tumor samples of the hen and to localize COX-1 and COX-2 protein using immunohistochemistry. Concentrations of PGE_2 and PGE_2 metabolites were also determined using competitive enzyme immunoassays (EIAs).

Materials and Methods

Tissue Samples. Ovarian tumor (n=18) and normal ovarian (n=29) tissue samples were collected from 2-4-year old Single-comb White Leghorn hen ovaries for PCR analysis and a subset of these samples was used for

immunohistochemistry (ovarian tumor n= 5; normal ovary n= 6). Tissue was also collected from ovarian tumors (n=8) and from normal ovaries (n=8) of 2-5-year old Single-comb White Leghorn hen ovaries for PGE_2 and PGE_2 metabolite EIA analysis. Diagnosis was made by histopathologic analysis of hematoxylin and eosin sections.

RNA Extraction. RNA was extracted from hen ovarian tissue using the RNeasy Mini Kit (Qiagen) and integrity was verified through use of 2100 Expert Bioanalyzer (Agilent Technologies). Extracted RNA was subjected to reverse transcription to cDNA.

PCR Analysis. Quantitative real-time PCR with Sybr Green was used to quantify the expression of each COX isoform, using 18S expression as an internal control for COX normalization. Chicken-specific primers designed to span intron regions were used and predicted a product of 74 bp for COX-1 (forward: 5'TGGCGGAGTCCTTTTCCATG3'; reverse: 5'TTAATGCCAACGTACTGGGA3') and 88 bp for COX-2 (forward: 5'CTCGTGCAAGTGGAACTCGT3'; reverse: 5'GAGGTGTATGCATGACAACA3'). Control reagent was Ambion Quantum RNA Universal 18S primers (# 1718) and controls were run for each sample. A standard curve was established from a pool of the RNA of 5 normal hen ovaries, which was reverse transcribed to cDNA. Control reactions lacking reverse transcriptase and template were also run on each plate. Reactions were 50 μl in total volume consisting of a final concentration of 1x Quantitect SYBR Green PCR Master Mix (Qiagen), 0.3 μM of COX primer or 0.2 μM of 18S primer solution, 4 μM Rnase-free water, and 0.4 μM cDNA. All sample amplifications were run in duplicate using an ABI Prism 7000 Sequence Detection System and the mean value was calculated relative to 18S reactions.

Sequence Detection System Software (Applied Biosystems) was used to determine mRNA concentration.

Immunohistochemistry. Polyclonal rabbit anti-mammalian COX-1 antibody was purchased from Oxford Biomedical Research Inc. (# PG 20) and rabbit polyclonal anti-chicken COX-2 antibody was generously donated by Oxford Biomedical Research Inc. Paraffin sections were deparaffinized and rehydrated through a series of xylene and ethanol treatments. Antigen was retrieved through heating in citrate buffer (COX-1) or incubation at 37 °C with pronase (COX-2). Sections were blocked with 10% goat serum in PBS for 30 minutes at 37 °C and treated with primary and secondary antibodies for 1 hour at 37 °C. Control slides were treated with normal rabbit IgG (Upstate Self Signaling Solutions #12-370). All primary antibodies were diluted 1:25. The secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular Probes #A-11034) diluted to 1.3 μg/mL. Slides were viewed using a Nikon eclipse E600 microscope with fluorescence capability and images were captured using a SPOT RT Slider camera with a standardized exposure of 6 sec.

PGE_2 Competitive Enzyme Immunoassay (EIA). PGE_2 and PGE_2 metabolites were extracted according to manufacturer's protocol (Cayman Chemical) and measured using the PGE_2 EIA Kit (# 514010) and PGE metabolite EIA kit (# 514531), respectively. Concentrations (pg/mL) were estimated from the absorbance of the calculated standard curve. Results were calculated using the Cayman Chemical computer spreadsheet.

Statistics. All data were analyzed with SAS using Proc GLM and the significance level was $P < 0.05$. Duncan's multiple range test was used to separate means.

Results

Quantitative Real-Time PCR. Figures 2-1 and 2-2 show results of quantitative real-time PCR for COX-1 and COX-2 mRNA expression, respectively. After normalization to 18S expression, results indicate that COX-1 expression was significantly increased in ovarian tumor samples as compared to normal ovarian samples ($p< 0.05$). No significant difference in COX-2 expression was observed between ovaries with tumors and normal ovaries. Figure 2-3 indicates that the ratio of COX-1 expression to COX-2 expression was significantly higher in ovarian tumor samples as compared to normal ovarian samples ($p< 0.05$).

Immunohistochemistry. Figure 2-4 shows representative photomicrographs of immunohistochemistry results of normal ovary and ovarian tumor samples. Panels A and B show representative hematoxylin and eosin stains. Normal ovary sections indicate the presence of follicles (panel A), while ovaries with tumors typically have fewer follicles than normal ovaries and have glandular-like structures within the ovary (panel B). Normal ovary showed no specific COX-1 staining (panel C) as compared to the control adjacent section (panel E). COX-1 specific staining was observed within the glandular structures of ovarian tumor (panel D) as compared to the IgG control (panel F). Widespread COX-2 specific staining was observed in normal ovary (panel G) as compared to adjacent section control (panel I) and ovarian tumor (panel H) as compared to adjacent section control (panel J). All tumor samples (n=5) and normal ovary samples (n=6) showed the same expression pattern.

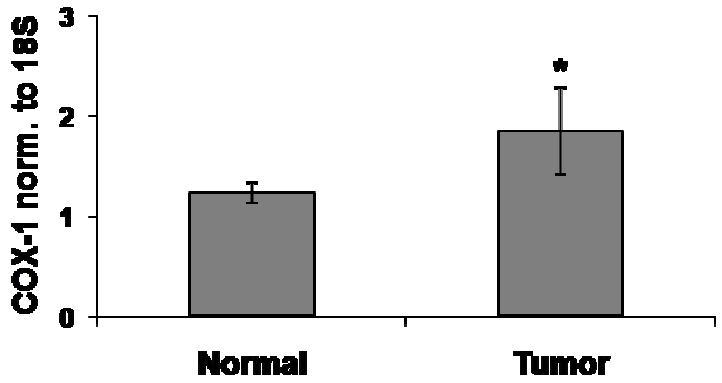

Figure 2-1. COX-1 mRNA expression in ovaries with tumors and normal ovaries.

Asterisks indicate a significant increase in ovaries with tumors as compared to normal ovaries ($p<0.05$). Bars indicate standard error.

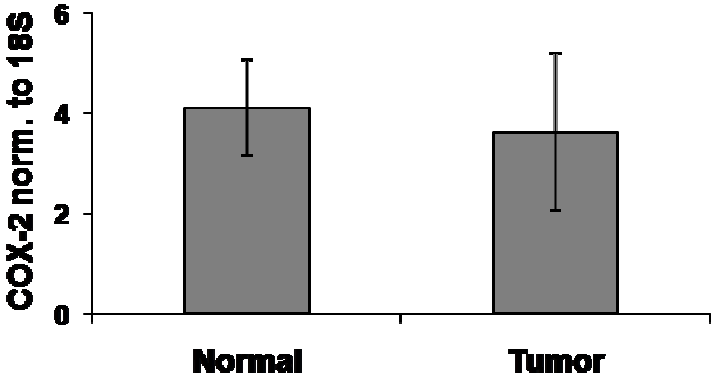

Figure 2-2. COX-2 mRNA expression in normal ovaries and ovaries with tumors.

No significant differences were found. Bars indicate standard error.

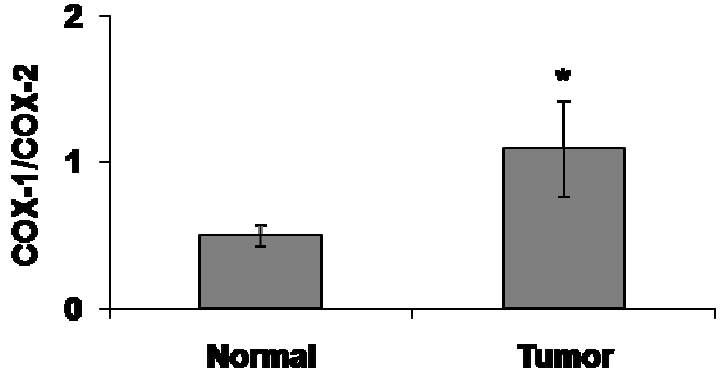

Figure 2-3. Ratio of COX-1/COX-2 mRNA expression in normal ovaries and ovaries with tumors.

Asterisks indicate a significant increase in ovaries with tumors as compared to normal ovaries ($p<0.05$). Bars indicate standard error.

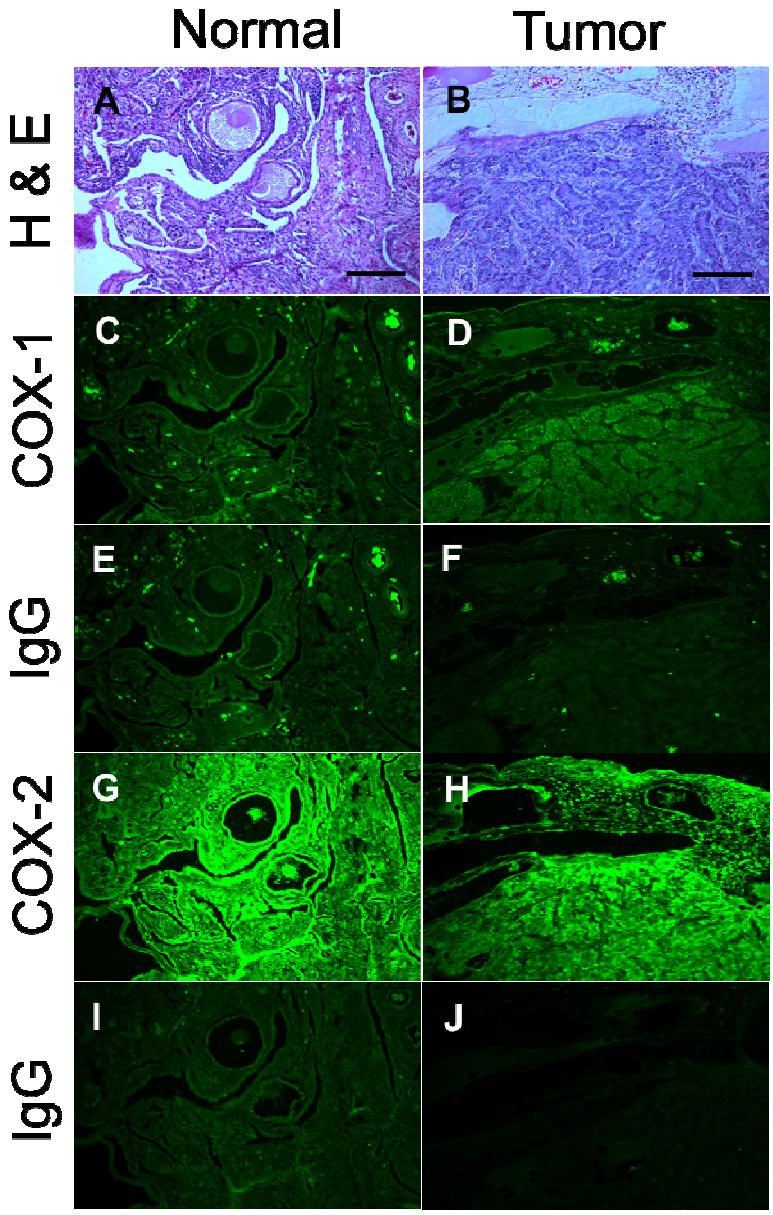

Figure 2-4. **COX immunohistochemistry results.**

Normal ovary and ovarian tumor stained with Hematoxylin & Eosin (A, B), COX-1 antibody (C, D), normal rabbit IgG (E, F), COX-2 antibody (G, H), and normal rabbit IgG (I, J). COX-1 staining is observed only in ovarian tumor. Positive COX-2 staining is observed in both normal ovary and ovarian tumor.

PGE_2 Competitive Enzyme Immunoassay (EIA). Results in Figure 2-5 show that PGE_2 levels are significantly increased in ovarian tumor as compared to normal ovarian samples ($p<0.05$). No significant difference in PGE_2 metabolite levels was observed between the samples (Figure 2-6).

Discussion

Our quantitative real-time PCR analysis indicates that COX-1 mRNA is elevated in hen ovarian tumor samples as compared to normal hen ovarian samples. These results were supported by our immunohistochemistry results, which showed specific COX-1 staining in glandular areas within the ovaries with tumors. The localization of COX-1 only to the glandular areas of ovaries with tumors could explain the large standard error associated with COX-1 mRNA expression (Fig. 2-1). Samples for quantitative PCR were taken at random from the main body of the ovaries with tumors, which could have resulted in variability in the amount of glandular areas included in the PCR analysis for each sample. COX-1 expression was undetectable by immunohistochemistry in normal ovary, presumably due to low abundance. These results suggest that a correlation exists between COX-1 expression and ovarian tumors of the hen.

We found no correlation between COX-2 expression and ovarian tumors. No difference in COX-2 mRNA expression was observed between normal ovaries and ovaries with tumors and widespread COX-2 protein staining was observed in both normal ovaries and ovaries with tumors. Enhanced COX-2 expression in the ovary may have been due to the relatively

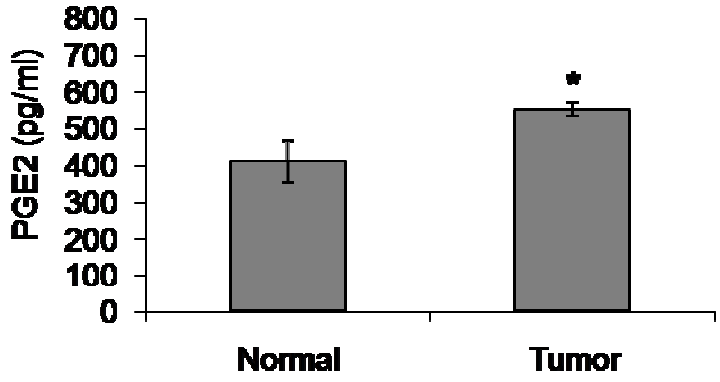

Figure 2-5. **Prostaglandin E_2 production in ovaries with tumors and normal ovaries.**

Asterisks indicate a significant increase in ovaries with tumors as compared to normal ovaries ($p<0.05$). Bars indicate standard error.

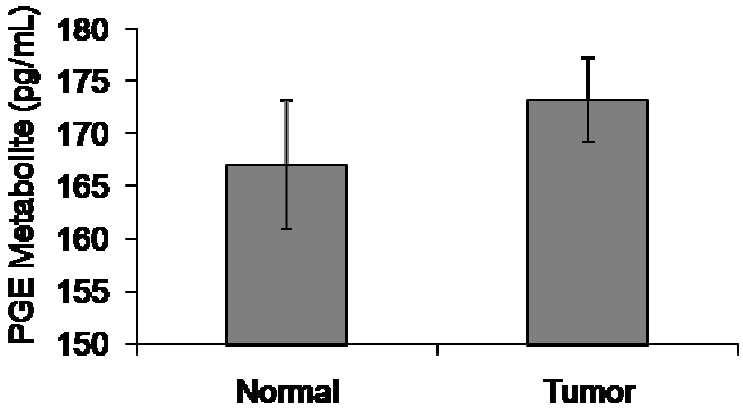

Figure 2-6. **Prostaglandin E_2 metabolite production in normal ovaries and ovaries with tumors.**

No significant differences were found. Bars indicate standard error.

old age of the hens used. Studies show that COX-2 expression may increase with age [29]. We selected older birds, because hens display increasing susceptibility to tumor formation after 2 years of age [8]. It is possible that the COX-2 expression levels in this group of hens may be higher than that in younger hens. We have not yet examined COX expression in hens younger than 2 years of age. Studies have shown that in human ovaries, COX-2 expression is found in follicles prior to ovulation and in interstitial tissue following ovulation [31]. The wide-spread COX-2 expression found in this experiment in both tumors and normal ovaries could therefore, be associated with the frequent ovulation which occurs in the hen.

Increased expression of COX enzymes generally results in increased levels of prostaglandins, which have been correlated to a number of cancers [23]. Prostaglandin E_2 (PGE_2) in particular has been correlated with ovarian cancer [25, 26]. The present study indicates that PGE_2 concentration is greater in hen ovarian tumor samples compared to normal ovary samples. Because of the labile nature of PGE_2 and prostaglandins in general, the metabolite levels of PGE_2 were measured to be sure that there were no differences in the metabolism of PGE_2 between normal samples and samples with ovarian tumors. No difference in PGE_2 metabolite levels was found. Previous studies found that PGE_2 induced angiogenesis in chicken embryos [27] and stimulated proliferation and inhibited apoptosis in two epithelial ovarian cancer cell lines [28]. PGE_2 may also suppress immune function, thus leading to increased susceptibility to tumor formation and impaired defense against previously formed tumors [23]. One recent study indicates that PGE_2 production in ovarian cancer cell lines is regulated by COX-1 [30]. Specifically, the study

showed a correlation between PGE_2 production and COX-1 mRNA and protein. Inhibition of PGE_2 production was achieved through a selective COX-1 inhibitor, while COX-2 inhibitors had no effect.

Our data suggest that the increase in PGE_2 found in hen ovaries with tumors may be due to COX-1. To examine the relative expression of the two COX isoforms in these tissues to be sure that COX-2 under-expression was not a compensation for COX-1 over-expression, we compared the ratio of COX-1 to COX-2 in ovaries with tumors to this ratio in normal ovaries. We found that this ratio is higher in ovaries with tumors as compared to normal ovarian samples. Although these data are not relevant to the biologic activity of either isozyme, the data do show that minor differences in COX-2 expression do not alter the main result of increased COX-1 expression in ovarian tumor samples as compared to normal ovaries. It is therefore plausible that the increase in PGE_2 in tumor samples is due to the COX-1 pathway.

Future studies utilizing non-steroidal anti-inflammatory drugs (NSAIDs) in the hen may provide the insight needed to determine whether COX-1 is responsible for the increase in PGE_2 expression found in ovarian tumor samples. It is not clear from this study whether COX-1 is involved in the establishment or in the maintenance of ovarian tumors of the hen. This could also be determined through studies using NSAIDs. NSAIDs prevent prostaglandin biosynthesis through inhibition of the COX enzymes. Recently, NSAIDs have been considered for use in cancer prevention. One study indicates that the use of celecoxib, a COX-2 selective inhibitor, had little effect on ovarian tumor growth, while the COX-1 selective inhibitor, SC-560, reduced the growth of ovarian tumors [14]. Additionally, a recent population-based, case-control study showed that the use of NSAIDs, including aspirin,

was associated with a reduction in the risk of ovarian cancer [32]. Because of the fact that many of the common NSAIDs that are classified as nonselective are generally better inhibitors of COX-1 than COX-2 [33], these data suggest that COX-1 NSAIDs may be effective at reducing the risk of ovarian cancer.

Although COX-2 has been implicated in a range of other cancers, our results indicate that in the hen, COX-1 mRNA and protein are increased in tumors. Our data also indicate an increase in PGE_2 and suggest that this increase may be due to COX-1. Interestingly, supporting evidence for this finding was also obtained in a genetically modified mouse model of epithelial ovarian cancer [14]. These combined results suggest that COX-1 could be a suitable target for the prevention and treatment of epithelial ovarian cancer.

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adenocarcinoma. Gynecol Oncol 2004;95:530-533.

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CHAPTER 3

VEGF EXPRESSION AND THE EFFECT OF NSAIDS ON ASCITES CELL PROLIFERATION IN THE HEN MODEL OF OVARIAN CANCER*

Abstract

Objectives. We aimed to determine the expression of vascular endothelial growth factor (VEGF) and the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on the proliferation of cells isolated from ascites in the hen model of ovarian cancer.

Methods. Ovarian tumor and normal ovary were collected from hens and ascites cells were isolated from hens with ovarian cancer. Quantitative real-time PCR was used to quantify mRNA expression. Immunohistochemical and/or Western blot analyses were used to localize protein expression in ovarian tumors, normal ovaries, and ascites cells. Cells were treated with a nonspecific, COX-1 specific, or COX-2 specific NSAID and proliferation was determined.

Results. VEGF mRNA was increased in ascites cells and there was a trend for a correlation between VEGF mRNA in ascites cells and ascites volume. VEGF protein was localized to theca cells of normal ovaries, in glandular areas of tumors, and to the cytoplasm of ascites cells. Aspirin and a COX-1 specific inhibitor decreased the proliferation of ascites cells, whereas a COX-2 specific inhibitor did not.

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Conclusions. VEGF may play a role in ovarian cancer progression in the hen and the proliferation of ascites cells can be decreased by targeting the COX-1 but not COX-2 pathway.

Introduction

At diagnosis, nearly 70% of women with ovarian cancer have an advanced stage of the disease which has spread beyond the ovaries [1]. Advanced ovarian carcinoma is characterized by the accumulation of malignant ascites, which is inversely associated with survival [reviewed in [2]]. Vascular endothelial growth factor (VEGF) has been correlated to the metastasis of ovarian cancer cells as well as to peritoneal ascites accumulation [3], and studies have shown that inhibition of VEGF reduces ascites formation [4, 5] and ovarian tumor burden [6].

Evidence suggests that frequent use of nonsteroidal anti-inflammatory drugs (NSAIDs) may aid in the prevention of carcinogenesis in tissues such as colon, breast, and pancreas [7-9]. In addition, epidemiological studies have indicated that use of NSAIDs is correlated with decreased ovarian cancer risk [10-13]. An explanation for the chemopreventative effect of these drugs is inhibition of the cyclooxygenase (COX) enzymes. Interestingly, COX-1 expression has been correlated with VEGF expression in human ovarian cancer. VEGF mRNA in human ovarian cancer tissue was localized to regions expressing high levels of COX-1 mRNA and a COX-1 specific NSAID inhibited VEGF production by an ovarian cancer cell line, while a COX-2 specific NSAID did not [14].

The domestic hen spontaneously develops ovarian cancer with many similarities to the human disease, including the accumulation of ascites. We determined the expression of VEGF in the hen model of ovarian cancer and examined the effects of a nonspecific, COX-1 specific, and COX-2 specific NSAID on the proliferation of cells isolated from ascites collected from hens with ovarian cancer. Finally, we aimed to determine the effects of these NSAIDs on VEGF expression in ascites cells.

Materials and Methods

Animals. White Leghorn hens were individually caged, with free access to water and feed and were maintained on a 15 hour light and 9 hour dark schedule. Hens with ovarian cancer were selected from a flock of approximately 918 2-7 year-old hens by observation of ascites accumulation and palpation and verified by histological examination. Animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Sample Collection. Ascites fluid was aspirated from hens after euthanasia (n=15) and cells were isolated using centrifugation. Results from ascites of individual hens were averaged for each experiment. Ascites volume (ml) was determined by subtracting hen body weight (g) after removal of total ascites volume from hen weight (g) prior to ascites removal. Ovarian tissue was stored in RNAlater (Qiagen) for RNA extraction or fixed in buffered formalin for immunohistochemical analysis.

Cell Culture. Cells were isolated from ascites fluid (n=13) and resuspended in M199. Ascites cells were plated in 96-well plates at a density of

2-4 x 10^3 cells/well and in 6-well plates or on coverslips at a density of 50 x 10^3 cells/well. Cells were maintained in M199 containing 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

RNA Extraction. RNA was extracted using the RNeasy Mini Kit (Qiagen), including DNase digestion. Cell cultures were terminated by removal of medium and addition of Buffer RLT (supplied in RNeasy Mini Kit). Cells were collected by gentle scraping and homogenized.

Quantitative PCR. Quantitative real-time PCR with SYBR Green was used to quantify the expression of VEGF, COX-1, and COX-2 mRNA, using 18S rRNA as an internal control (n=6-7). VEGF primers (designed to span introns) were described by Makanya *et al.* [15] and COX-1 and COX-2 primers were described previously [16]. Control reagent was Quantum RNA Universal 18S primers (Ambion #1718), and controls were run for each sample. A standard curve was established from a pool of RNA from 5 normal hen ovaries. Control reactions lacking reverse transcriptase or template were run on each plate. Reactions were 50 µl in total volume with a final concentration of 1× Power SYBR Green PCR Master Mix (Qiagen), 0.3 µM of VEGF primer or 0.2 µM of 18S primers, and 0.4 µM cDNA. Sample amplifications were run in duplicate using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the comparative threshold cycle (C_T) method was used to determine mRNA concentration relative to 18S.

Immunohistochemistry. Pieces of ovary from normal hens (n=5) and from hens with ovarian cancer (n=5) were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4-5 µm by the Cornell University Veterinary School. Antigen was retrieved by incubation at 37°C with pronase. Sections were blocked with 10% goat serum in PBS for 30 min at 37°C and

treated with polyclonal rabbit anti-human VEGF antibody (Santa Cruz, diluted 1:25). Control slides were treated with normal rabbit IgG (Upstate Self Signaling Solutions). Secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular Probes, diluted to 1.3 µg/ml). Sections were treated with secondary antibody for 1 h at 37°C. To identify nuclei, sections were incubated with propidium iodide (Sigma, diluted to 1µg/ml) in PBS for 15 min at room temperature following secondary antibody. Hematoxylin and eosin staining was performed by the Cornell University Veterinary School. Slides were viewed using a Nikon eclipse E600 microscope and pictures were taken using a SPOT RT Slider camera.

Western Blot Analysis. Ascites cell homogenates (n=4) and media collected from cell cultures (n=5 preparations) were concentrated using Microcon 30 devices (Millipore). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce) and 30 g of total protein were loaded onto 10% Precise Protein Gels (Pierce) along with a Biotinylated protein ladder (Cell Signaling). Electrophoresis was performed under reducing conditions and gels were blotted to nitrocellulose membranes (Thermo Scientific). Blots were blocked in 1X Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% milk for 30 min, washed and incubated overnight at 4°C with VEGF antibody (diluted 1:70) in TBST containing 1% milk. Blots were washed and incubated with a peroxidase labeled goat anti-rabbit IgG (KPL, diluted 1:1000) and an anti-biotin antibody (Cell Signaling, diluted 1:1000) in TBST containing 5% milk for 1 hour at room temperature. Detection was performed using a Lumiglo Chemiluminescent Substrate Kit (KPL). Western blot was performed with untreated cell homogenates and media at the lowest, medium, and highest dose of each NSAID tested in proliferation assays.

Immunocytochemistry. Ascites cells (n=6 preparations) were fixed using methanol or 80% acetone. Cells were incubated with VEGF antibody (diluted 1:10), COX-1 antibody ([16], diluted 1:10), COX-2 antibody ([16], diluted 1:25), or rabbit IgG at equivalent concentrations, followed by secondary antibody.

Treatments. Aspirin (Sigma), SC-560 (Cayman Chemical), and NS-398 (Cayman Chemical) were solubilized in dimethyl sulfoxide (DMSO, Sigma). Cells incubated in medium without treatment were included as controls. For vehicle controls, cells were treated with the maximum concentration of DMSO used.

Prostaglandin Analysis. Concentrations of PGE_2 (pg/ml) were determined in media using a PGE_2 EIA Kit (Cayman Chemical).

Cell Proliferation Analysis. Number of viable cells was determined using the CellTiter 96 Aqueous One Solution Assay (Promega).

Statistics. Data were analyzed using PROC GLM of SAS and means were compared using Duncan's multiple range test.

Contribution of Authors. ME Urick developed and completed the experiments presented in the manuscript. Drs. JR Giles and PA Johnson contributed technical assistance, intellectual insight, and funding.

Results

VEGF mRNA expression is higher ($p< 0.02$) in cells isolated from ascites (n=6) as compared to normal ovarian samples (n=7, Fig. 3-1). VEGF mRNA expression is intermediate in samples collected from hens with ovarian tumors (n=7). There is a trend for a correlation between VEGF mRNA

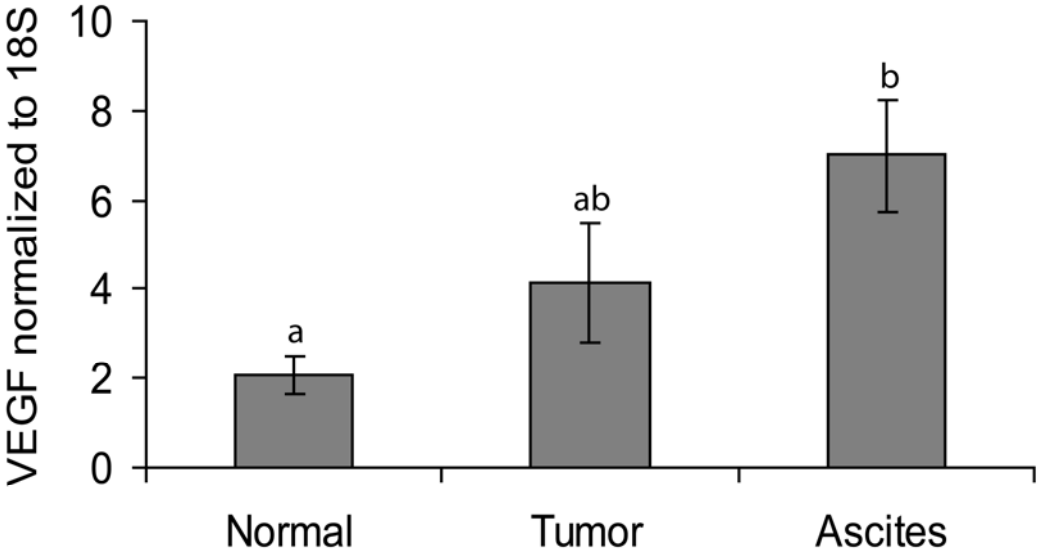


Figure 3-1. VEGF mRNA expression in normal ovaries, ovarian tumors, and ascites from hens with ovarian cancer.

Letters indicate significant differences ($p<0.02$). Bars indicate standard error (n=6-7).

expression in ascites cells and ascites volume (Fig. 3-2, p=0.087).

Cytoplasmic VEGF is present in both normal ovaries (n=5) and ovaries of hens with ovarian cancer (n=5, Fig. 3-3). Concentrated expression is observed in theca cells of normal ovaries (panel C arrow) and in glandular areas and the stroma surrounding glandular areas of ovarian tumors (panel D arrowheads). IgG controls show no specific staining (panels G, H, I, & J). VEGF protein in ascites cells (n= 6 preparations) was detected by Western blot and immunocytochemistry (Fig. 3-4). The VEGF band was observed at the expected size, approximately 24kDa (panel A). Two cell phenotypes were observed within the primary cultures: round epithelial-like cells and cells that appeared dendritic or fibroblast-like. VEGF protein (panel B) is localized to the cytoplasm of some epithelial and dendritic-like cells. No staining is evident in IgG controls (panel C). COX-1 and COX-2 mRNA and protein are expressed in ascites cells from tumor-bearing hens (Fig. 3-5). No staining is evident in IgG controls (data not shown).

PGE_2 concentrations (pg/ml) were decreased in medium after treatment of cultures for two days with a nonspecific (aspirin), COX-1 specific (SC-560), or COX-2 specific (NS-398) NSAID (Fig. 3-6, n=2-3 preparations per dose of each treatment, $p<0.05$). Proliferation assay results show a significant decrease in proliferation ($p< 0.05$) in cells treated with 200 and 300μm of SC-560 (COX-1 inhibitor) but not in cells treated with equivalent doses of NS-398 (COX-2 inhibitor) (Fig. 3-7, n=3-7 preparations per dose of each treatment). Proliferation was also decreased in cells treated with 5 and 10mM doses of aspirin ($p< 0.05$).

VEGF mRNA in untreated ascites cells and cells treated with aspirin, SC-560, or NS-398 showed no decrease with any NSAID treatment (data not

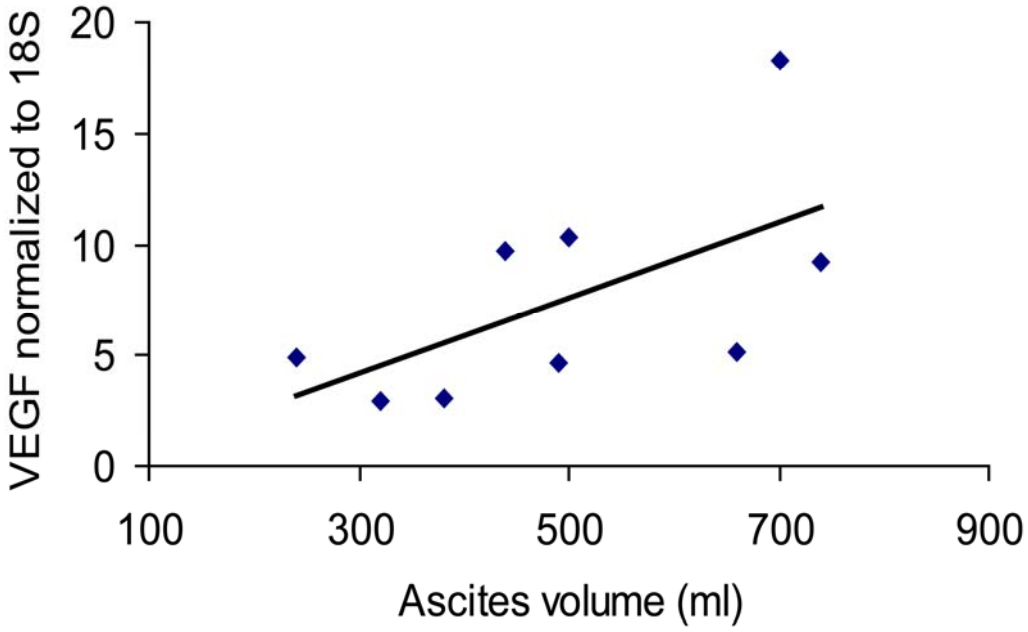
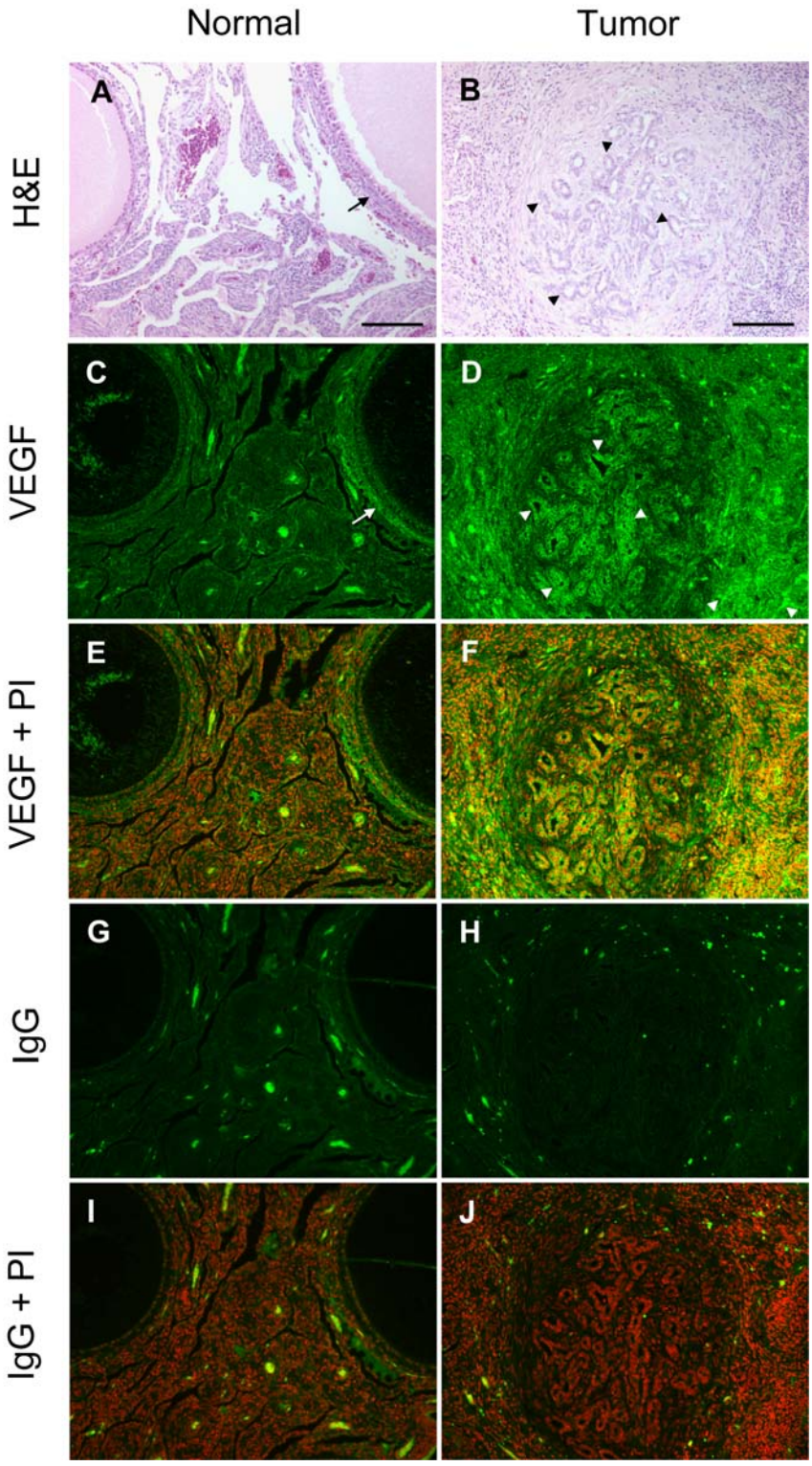


Figure 3-2. VEGF mRNA expression in ascites cells graphed against the volume of ascites (ml).

There is a trend for a significant correlation between VEGF expression in ascites cells and ascites volume (p= 0.087).

Figure 3-3. **(Following Page) VEGF immunohistochemistry results.**

Representative photomicrographs of normal ovary and ovarian tumor stained with hematoxylin and eosin (A, arrow indicates theca layer; B, arrowheads indicate glandular areas), VEGF antibody (C, arrow indicates theca staining; D, arrowheads indicate glandular and stromal staining), VEGF antibody and propidium iodide (PI; E, F), rabbit IgG (G, H), and rabbit IgG with PI (I, J). Scale bars represent 100 μm.


Normal
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VEGF + PI
IgG
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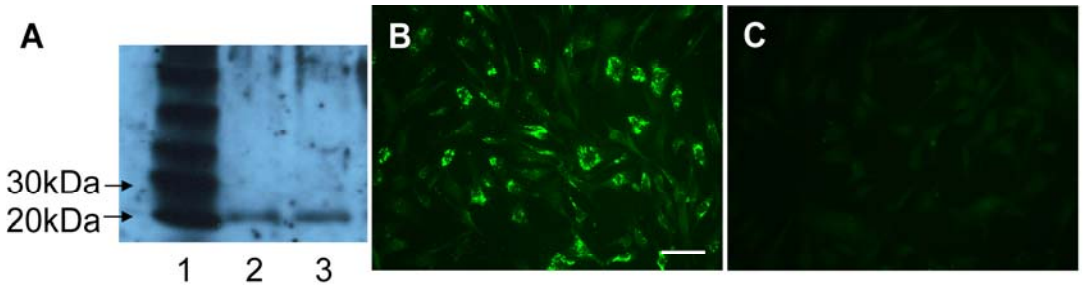

Figure 3-4. VEGF protein expression in ascites cells.

VEGF protein in two representative ascites cell homogenates (lanes 2 and 3) detected by Western blot (A; ladder lane 1). Representative photomicrograph of ascites cells stained with VEGF antibody (B), and normal rabbit IgG (C). Scale bar represents 100 μm.

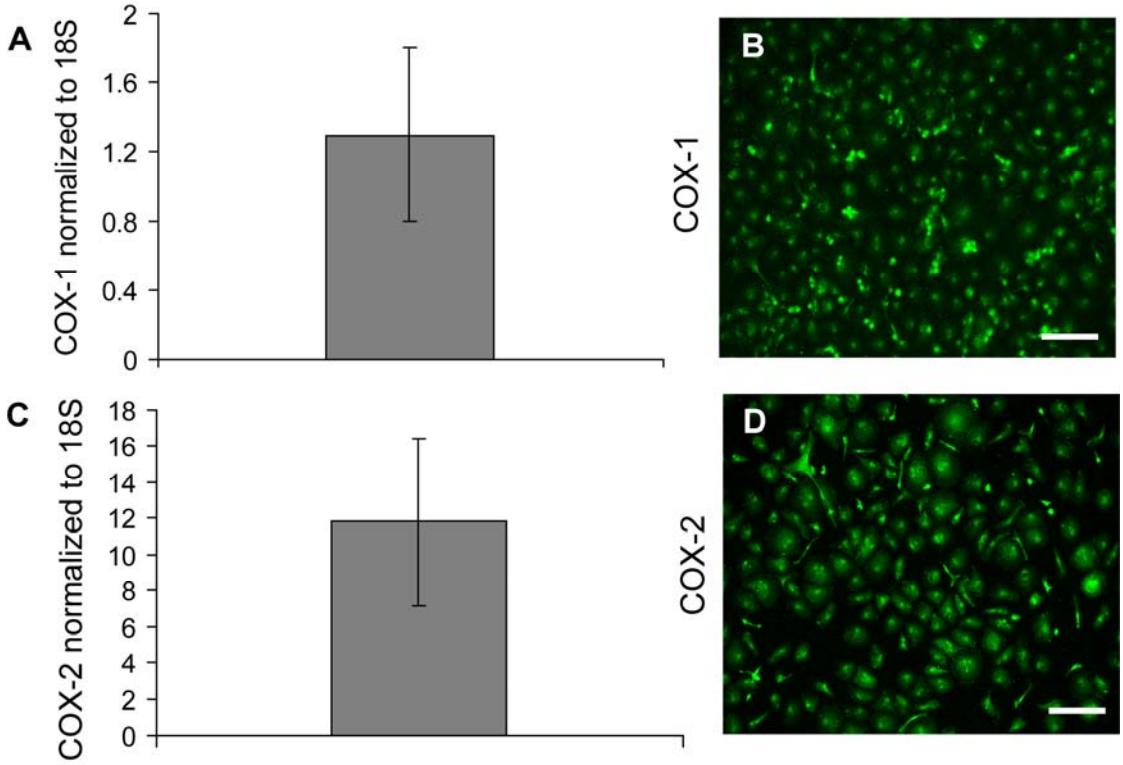

Figure 3-5. COX expression in ascites cells.

COX-1 (A) and COX-2 (C) mRNA, and COX-1 (B) and COX-2 (D) protein in cells isolated from ascites of hens with ovarian cancer. Bars indicate standard error (n=6). Scale bars represent 100 μm.

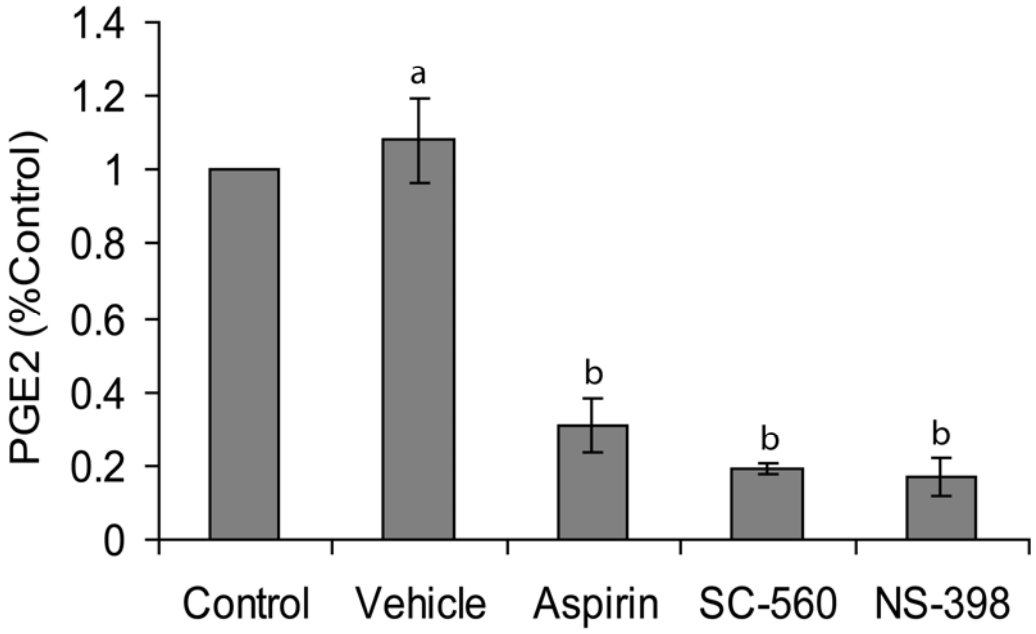

Figure 3-6. Effects of NSAIDs on PGE_2 production in ascites cells.

Prostaglandin E_2 in media collected from ascites cells cultured for 2 days with a nonspecific (aspirin; doses = 5 μm - 10 mM), COX-1 specific (SC-560; doses = 5 μm - 20 μm), or COX-2 specific (NS-398; doses = 5 μm - 20 μm) NSAID. Asterisks indicate significant decreases in PGE_2 ($p < 0.05$). Bars indicate standard error (n = 2-3).

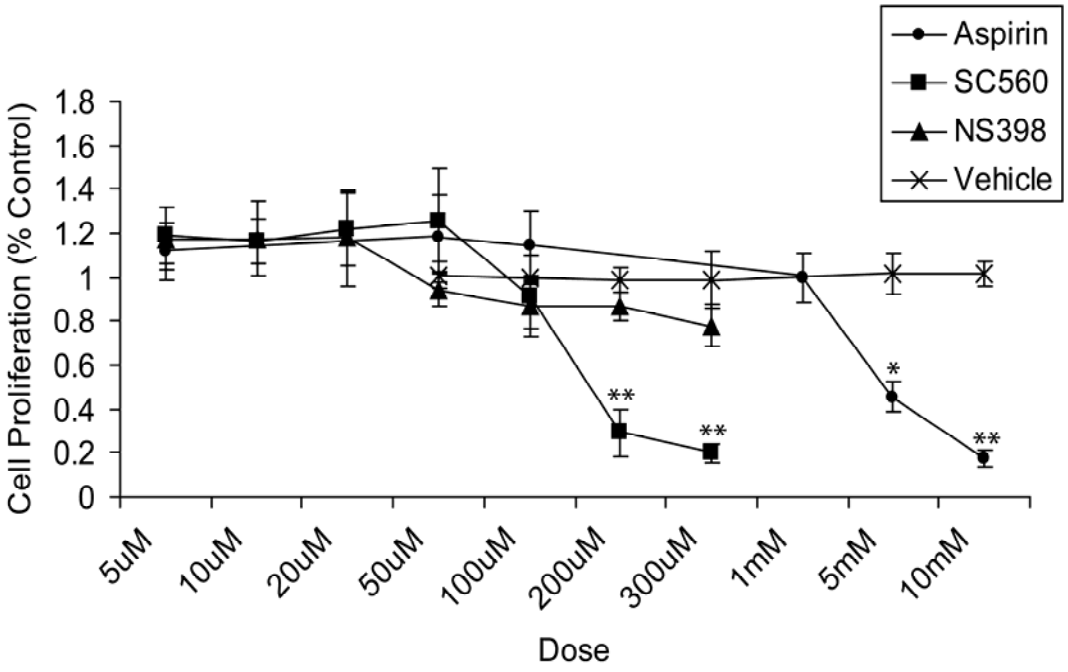

Figure 3-7. Effect of NSAIDs on ascites cell proliferation.

Proliferation of ascites cells treated for 4 days with DMSO (vehicle) or a nonspecific (aspirin), COX-1 specific (SC-560), or COX-2 specific (NS-398) NSAID. Asterisks indicate significant decreases in proliferation ($p < 0.05$). Bars indicate standard error (n=3-7).

shown). Similarly, no effect of any NSAID was found on VEGF protein (data not shown).

Discussion

To assess whether VEGF may be associated with ovarian cancer in the hen, we determined VEGF expression in ovarian tumors and ascites cells. Ovaries with tumors are often heterogeneous, with normal tissue intermixed with cancerous areas. This might explain why we observed an intermediate amount of VEGF mRNA in ovarian tumor samples. We found a trend for a correlation between VEGF mRNA expression of ascites cells and volume of ascites. Data indicate that ascitic VEGF expression may be a useful prognostic factor for ovarian cancer patients [17] and our results suggest the same could be true for the hen.

VEGF protein is evident in glandular regions and stroma surrounding glandular regions within ovarian tumors (Fig. 3-3 D&F). We have previously shown localization of COX-1 protein to glandular areas of tumor sections [16]. Expression of COX-1 and VEGF protein in glandular areas of ovarian tumors is similar to the findings of Gupta *et al.*, which show that COX-1 mRNA expression is associated with VEGF mRNA expression in human ovarian cancer [14]. For this reason, we investigated the effects of a nonspecific, COX-1 specific, and COX-2 specific NSAID on the proliferation of, and VEGF expression in, ascites cells.

Aspirin, which is a more potent inhibitor of COX-1 than COX-2 [18], and the COX-1 specific inhibitor, SC-560, decreased the proliferation of ascites cells, whereas the COX-2 inhibitor did not. These results are consistent with results from a mouse model of ovarian cancer which showed that SC-560

reduced the growth of tumors while a COX-2 selective inhibitor did not [19]. Additionally, our previous report shows an increase in COX-1, but not COX-2, mRNA in ovarian tumors of the hen [16] and others have reported that COX-1 is increased in human ovarian cancer rather than COX-2 [14, 20]. Avian clearance of NSAIDs was shown to be more rapid than human clearance and metabolic products of NSAIDs in birds have been shown to differ from humans [21-23]. These differences explain why higher doses of NSAIDs have been used in previous studies with avian tissue [24, 25] and were used in these experiments with chicken cells.

PGE_2 has been associated with ovarian cancer [16] and all three NSAIDs used in this study significantly reduced PGE_2 levels at concentrations that did not affect proliferation. It was shown that the antiproliferative effects of NSAIDs may not be explained solely by decreased prostaglandin synthesis [26] and other known mechanisms of action of NSAIDs may explain their antiproliferative effects [reviewed in [27]].

A COX-1 specific NSAID inhibited VEGF production by the OVCAR-3 ovarian cancer cell line in a previous study, while a COX-2 specific NSAID did not [14]. The OVCAR-3 cell line is a human ovarian cancer cell line derived from peritoneal ascites of ovarian cancer patients. Difference in results obtained from the OVCAR-3 cell line and our data may be due to species differences or the fact that OVCAR-3 cells are from a homogenous cell line, while our experiments used primary cultures. We chose to utilize primary cultures to ensure that our results were as close to *in vivo* conditions as possible. *In vivo*, interactions of malignant cells with other cell types may affect the viability of the malignant cells [28, 29] and may help explain why chemotherapeutic drugs do not have the same effects *in vivo* as in isolated

tumor cells [29]. Our results indicate that *in vivo*, treatments with NSAIDs may not affect VEGF production by cells found within ascites fluid.

This study is the first to report VEGF mRNA and protein expression in ovaries and ascites cells of tumor-bearing hens as well as normal hen ovaries. These data suggest that VEGF may play a role in ovarian cancer progression in the hen, including the accumulation of ascites. Since ascites accumulation is inversely associated with survival (reviewed in 2), the hen might be a model to study therapies that target VEGF expression. We have shown that the proliferation of ascites cells can be decreased using aspirin or a COX-1 specific, but not COX-2 specific, NSAID. Our findings in the hen may or may not apply to epidemiological data that implicate NSAIDs as preventative agents in ovarian cancer initiation.

Acknowledgments

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CHAPTER 4

DIETARY ASPIRIN DECREASES THE STAGE OF OVARIAN CANCER IN THE HEN[*]

Abstract

Objective. We aimed to determine the effects of dietary aspirin treatment on ovarian cancer incidence and progression in the hen as a model for the human disease.

Methods. Hens were fed a standard layer diet (control) or the same diet containing 0.1% aspirin for 1 year. Liver prostaglandin E_2 (PGE_2) was measured using an enzyme immunoassay. Incidence and stage of ovarian cancer were determined through necropsy and immunohistochemical analysis of ovarian sections for each hen.

Results. Aspirin treatment decreased liver PGE_2 in treated hens as compared to control hens. Treatment with aspirin did not decrease ovarian cancer incidence. Significantly more control hens developed late stage ovarian cancer than early stage, while the same was not true for aspirin-treated hens. Hens that developed ovarian cancer, even early ovarian cancer, produced significantly fewer eggs in the year prior to diagnosis than hens without ovarian cancer.

Conclusions. Aspirin treatment may inhibit the progression of ovarian cancer in the hen and egg production may be used to identify hens with early stages of the disease.

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Introduction

Evidence suggests that frequent use of nonsteroidal anti-inflammatory drugs (NSAIDs) may aid in the prevention of carcinogenesis in tissues including the colon, breast, and pancreas [1-4]. In addition, epidemiological studies indicate that the use of aspirin or other NSAIDs may be associated with a risk reduction for ovarian cancer [5-8]. Several observational studies, however, demonstrate no association between ovarian cancer risk and NSAID use [reviewed in 9]. Wernli *et al*. recently hypothesized that the contradictions in results may be due to differences in factors such as parity and oral contraceptive use across study populations. They found a reduction in ovarian cancer risk associated with the use of NSAIDs in women who never used oral contraceptives or were nulliparous. They did not find a risk reduction in women who used oral contraceptives or parous women, possibly because these groups of women already had reduced risks of ovarian cancer [9]. The inconsistency of results in epidemiological studies illuminates the need for laboratory and clinical studies to test the efficacy of NSAIDs to decrease ovarian cancer risk and/or progression.

The ability of NSAIDs to negatively affect the viability of ovarian cancer cells *in vitro* has been shown by a number of studies [10-14]. Results using nude mice xenografted with tumors derived from human ovarian cancer cell lines show that NSAIDs have anti-proliferative effects *in vivo* [13, 15]. No study to date, however, has investigated the effect of NSAIDs in a spontaneous *in vivo* model of ovarian cancer. The hen is the only animal that spontaneously develops ovarian cancer at a rate high enough to study [16, 17]. We have previously shown that aspirin (a common nonspecific NSAID) or a COX-1 specific NSAID can decrease the viability of ascitic cells collected from

hens with ovarian cancer [18]. The purpose of the following experiment was to examine the effects of dietary aspirin treatment on ovarian cancer incidence and progression in the hen as a model for the human disease.

Materials and Methods

Experimental Design. Three age groups (Table 4-1) of the Babcock B300 strain of single-comb White Leghorn hens were individually caged, with free access to water and feed. Hens were maintained on a 15 hour light and 9 hour dark schedule. Half of the hens in each group were fed a standard layer diet (control, n=99), while the other half were fed the same diet which contained 0.1% aspirin (aspirin-treated, n=99) for 1 year. Eggs were collected daily and birds were weighed monthly. Percent egg production was calculated by dividing the number of days an egg was produced by the total number of days per month. Necropsy was performed on all hens, including those that died prior to the end of the experiment (n=30 control; n=28 aspirin-treated), as well as hens euthanized (CO_2) at the end of the experiment (n=69 control; n=71 aspirin-treated). Animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Sample Collection. Samples of ovary from all hens were fixed in buffered formalin for immunohistochemical analysis. Samples of liver were collected from hens euthanized at the end of the experiment and snap frozen in liquid nitrogen for prostaglandin analysis.

PGE_2 Immunoassay. PGE_2 was extracted from liver samples according to the manufacturer's protocol (Cayman Chemical) and measured using a PGE_2

Table 4-1. **Three age groups used for the *in vivo* study.**

Ending age shows the age of the hens in each group that survived until the end of the experiment (n=57 from the 2006 hatch, 47 from the 2005 hatch, 36 from the 2004 hatch).

	Starting Age (years)	**Ending Age (years)**	**Starting Number of Hens**
2006 Hatch	0.5	1.5	60
2005 Hatch	1.5	2.5	60
2004 Hatch	2.5	3.5	78

EIA Kit (#514010). Concentrations (pg/mL) were estimated from absorbance compared to the standard curve using the data analysis tool provided by Cayman Chemical and normalized to the weight of the liver sample (g) used for extraction.

Immunohistochemical Analysis. Paraffin sections were cut and hematoxylin and eosin staining of ovarian sections was performed by the Cornell Histology Laboratory. Two investigators blinded to the experimental treatment analyzed at least two sections from each hen. Sections were identified as containing cancerous cells (cancer) or no significant neoplastic lesions (NSL). Diagnosis of cancer was based on the classifications in Table 4-2, which were adapted from previous staging systems used in hens [17, 19].

Statistics. Data were analyzed using SAS. PGE_2 and egg production data were analyzed using the PROC GLM function and means were compared using Duncan's multiple range test. Overall cancer incidence was analyzed using the PROC LOGISTIC function. Analyses of cancer incidence with respect to age and tumor stage data were performed using the PROC FREQ function followed by a chi-square test of independence. Survival data were analyzed using the PROC LIFETEST function. Completion of a power analysis to establish initial sample size and determination of relevant statistical analyses were made after consultation with a statistician at the Cornell Statistical Counseling Unit.

Results

When hens with ovarian cancer or other pathology noted at necropsy were excluded, aspirin caused a significant decrease in PGE_2 in treated hens as compared to control hens ($p<0.05$, Fig. 4-1). The incidence of ovarian cancer

Table 4-2. Stages of ovarian cancer in the hen.

Adapted from previous staging systems used in the hen [17, 19].

Stage	Description
1	Tumor restricted to ovary and detectable by histology
2	Tumor restricted to ovary and observable at necropsy
3	Extensive ovarian tumor with abdominal seeding
4	Extensive ovarian tumor with abdominal seeding and ascites

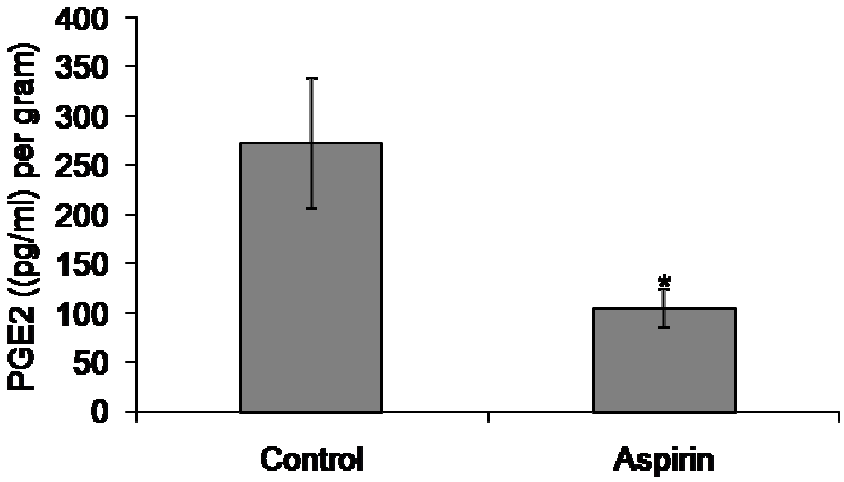

Figure 4-1. Effect of dietary aspirin on liver PGE_2 production.

Prostaglandin E_2 (PGE_2) in liver samples collected from a random subset of hens that lacked noticeable pathology at necropsy and were fed standard layer diet (Control, n=11) or standard layer diet containing 0.1% aspirin (Aspirin, n=12) for 1 year. The asterisk indicates a significant decrease in PGE_2 ($p<0.05$). Bars indicate SEM.

significantly increased with age in both control and aspirin treated hens ($p<0.05$, Fig. 4-2). Treatment of hens for 1 year with 0.1% aspirin did not significantly decrease ovarian cancer incidence when all three age groups were analyzed together (Table 4-3, All Hens). We found, however, that there were significantly more control hens with late stage ovarian cancer (stages 3 or 4) than early stage ovarian cancer (stage 1 or 2), while the same was not true for aspirin-treated hens ($p<0.05$, Table 4-4). Additionally, aspirin treatment may increase the survival of hens with ovarian cancer as compared to control hens (Fig. 4-3, $p=0.26$). Data from the 2005 hatch hens also show the potential for aspirin to decrease ovarian cancer incidence (Table 4-3, 2005 Hatch).

No differences in egg production (Fig. 4-4) or body weight (data not shown) were observed in aspirin-treated hens as compared to control hens. In addition, no obvious gastric or intestinal pathologies were noticeable at necropsy, but thorough examination of tissues was not performed. We found a significant decrease in egg production in hens that developed ovarian cancer as compared to hens without ovarian cancer ($p<0.05$, Fig. 4-4). Additionally, we found that hens with stage 1 ovarian cancer have significantly decreased egg production in the year preceding diagnosis as compared to hens that do not have ovarian cancer ($p<0.05$, Fig. 4-5).

Discussion

The dose of 0.1% aspirin in feed was chosen based on results from a pilot study, which showed decreased COX activity in liver samples of hens fed this dose of aspirin. Moreover, previous work in the hen showed that 0.1% dietary aspirin decreased the production of shell-less eggs through the inhibition of prostaglandins in the reproductive tract [20], while increased

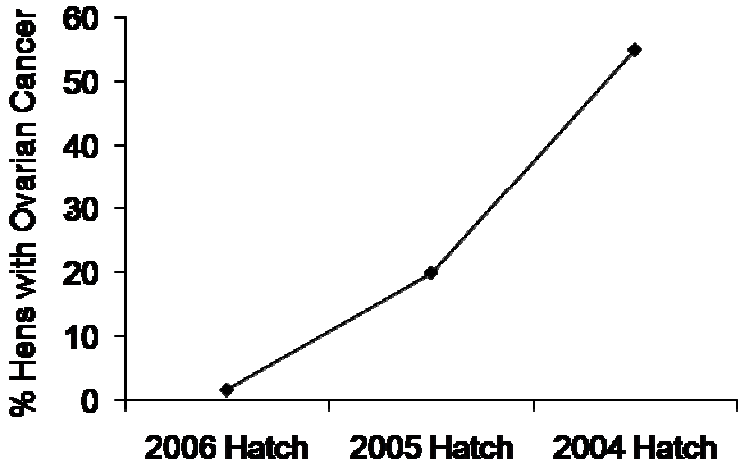

Figure 4-2. Percent of hens with ovarian cancer.

A significant increase in ovarian cancer incidence with age was found ($p<0.05$; control and aspirin-treated combined).

Table 4-3. **Percent ovarian cancer in control and aspirin-treated hens.**

Results for all hens at the end of the experiment.

	2006 Hatch	2005 Hatch	2004 Hatch	All Hens
Control	0.0 (0/30)	23.3 (7/30)	51.3 (20/39)	27.3 (27/99)
Aspirin	3.3 (1/30)	16.7 (5/30)	59.0 (23/39)	29.3 (29/99)

Table 4-4. Stage of ovarian cancer in control and aspirin-treated hens. Number of hens (3 age groups combined) with early stage (stages 1 and 2) and late stage (stages 3 and 4) ovarian cancer in hens fed standard layer diet (Control) and hens fed standard layer diet containing 0.1% aspirin (Aspirin). There is a significantly higher incidence of late stage ovarian cancer than early stage ovarian cancer in the control hens ($p<0.05$).

	Early Stage	**Late Stage**	**% Late Stage**
Control	4	23	85.2*
Aspirin	11	18	62.1

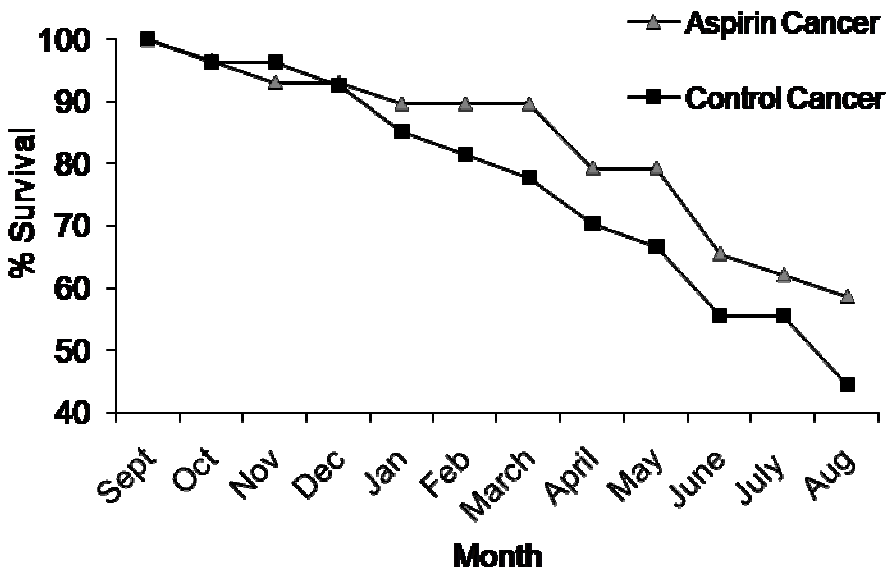

Figure 4-3. Percent survival for each month of the experiment.

Although not significant (p=0.26), aspirin-treated hens with ovarian cancer (Aspirin Cancer) survived longer than control hens with ovarian cancer (Control Cancer).

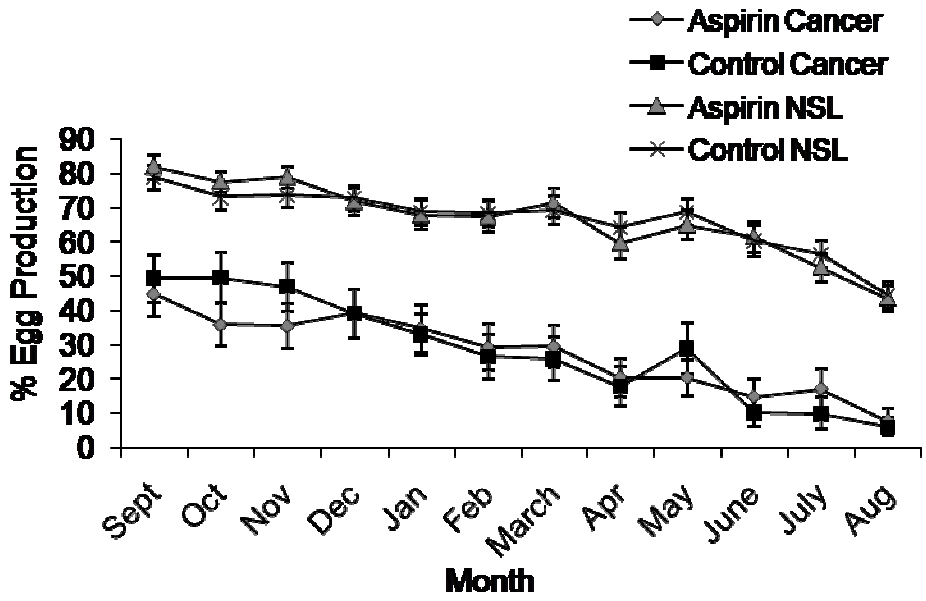

Figure 4-4. Egg production in control and aspirin-treated hens with ovarian cancer.

Percent egg production in hens fed control diet that developed ovarian cancer (Control Cancer, n=27), hens fed control diet that did not develop ovarian cancer (Control NSL, n=71), hens treated with aspirin that developed ovarian cancer (Aspirin Cancer, n=29), and hens treated with aspirin that did not develop ovarian cancer (Aspirin NSL, n=69). Hens that developed ovarian cancer produced significantly fewer eggs than hens without ovarian cancer ($p<0.05$). Bars indicate SEM.

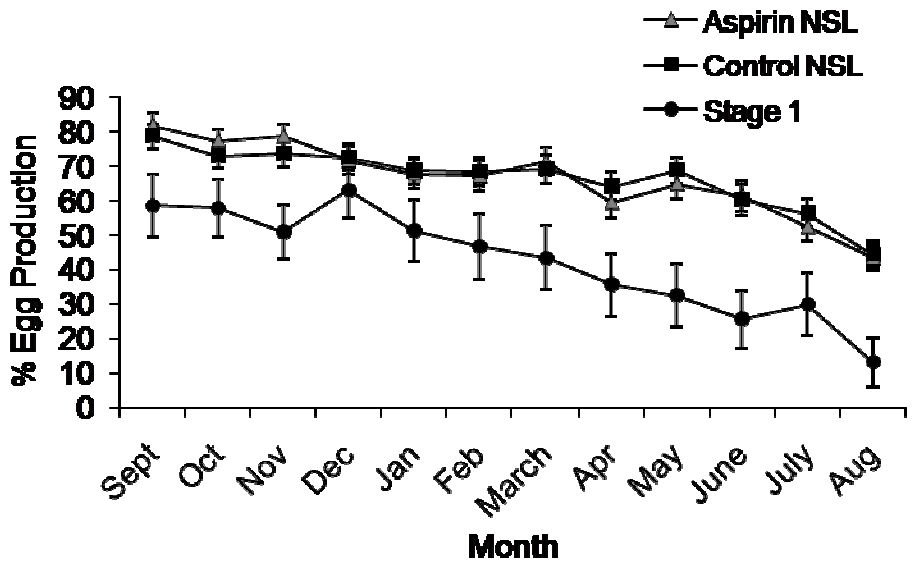

Figure 4-5. Egg production in hens with stage 1 ovarian cancer.

Percent egg production in hens fed control diet that did not develop ovarian cancer (Control NSL, n=71), hens treated with aspirin that did not develop ovarian cancer (Aspirin NSL, n=69), and hens with stage 1 ovarian cancer (Stage 1, n=12; 3 control hens, 9 aspirin-treated hens). Hens with stage 1 ovarian cancer produced significantly fewer eggs than hens without ovarian cancer ($p<0.05$). Bars indicate SEM.

Mortality was observed with a higher dose (0.2%) of aspirin [21]. In order to verify that 0.1% aspirin was a sufficient dose to affect systemic COX activity, we measured PGE_2 levels in liver samples from control and aspirin-treated hens. We previously found increased PGE_2 in ovarian tumors of the hen as compared to normal ovaries [22] and PGE_2 is elevated in human ovarian cancer [23, 24]. We therefore chose to evaluate liver PGE_2 as a measure of the systemic effects of aspirin treatment. There was an overall trend for a decrease in PGE_2 in the liver of aspirin-treated hens as compared to control hens ($p=0.0897$) but the various pathologies associated with the failing health of the 2004 hatch hens, including ovarian cancer, may have affected liver PGE_2 levels. When the hens with failing health were removed, there was a significant decrease in PGE_2 levels in the liver of aspirin-treated hens as compared to control hens ($p<0.05$, Fig. 4-1).

Fredrickson showed an increase in ovarian cancer incidence in White Leghorn hens after 2 years of age [17]. The incidence of ovarian cancer in the Babcock B300 commercial strain of White Leghorn hens had not been characterized prior to this study and, therefore, three different ages of hens were used (Table 4-1). Our findings verify the increase in ovarian cancer incidence in hens after 2 years of age ($p<0.05$, Fig. 4-2), and additionally show a higher incidence (55.1% at 3.5 years of age) in Babcock B300 hens than has been previously reported for other strains [17, 25-27]. These data indicate that this modern commercial strain may be particularly useful for ovarian cancer studies in the hen.

Although we did not find a significant effect on cancer incidence, we observed a significant decrease in the stage of ovarian cancer in aspirin-treated hens as compared to control hens ($p<0.05$, Table 4-4). This could indicate that

aspirin treatment may have decreased the progression of ovarian cancer. Aspirin also tended to increase the survival of hens with ovarian cancer (Fig. 4-3).

Only one hen in the 2006 hatch hens was identified with ovarian cancer and therefore, it might have been advantageous to treat these hens for longer than 1 year. This may have permitted a determination of the efficacy of aspirin to prevent the initiation of ovarian tumors. The 2004 hatch hens were 2.5 years old at the start of the experiment and showed a high incidence (85.0% of control; 69.6% of aspirin-treated) of late stage ovarian cancer. This suggests that ovarian carcinogenesis had initiated in many of these hens prior to the onset of the experiment. The decrease in the percentage of late stage ovarian cancer in aspirin-treated hens of this hatch suggests that aspirin treatment may decrease the progression of ovarian cancer even if treatment begins after carcinogenesis is initiated.

Based on previous data documenting an increase in ovarian cancer incidence in hens after 2 years of age [17] and the fact that only one hen was identified with ovarian cancer in the 2006 hatch hens (ending age of 1.5 years), it is likely that most of the hens in the 2005 hatch (starting age 1.5 years) did not have ovarian cancer at the onset of this experiment. In addition, at the end of the experiment, these hens were 2.5 years of age (Table 4-1). The data from these hens, therefore, may provide the most insightful information regarding the effect of aspirin intake on ovarian cancer incidence. We found a significant decrease in stage of ovarian cancer with aspirin treatment in hens of this age ($p<0.05$; 2005 Hatch data not shown separately) and a potential for aspirin to decrease ovarian cancer incidence (Table 4-3, 2005 Hatch). Additionally, 50% (3/6) of 2005 Hatch control hens that died prior to the end

of the experiment were diagnosed with ovarian cancer, while only 14.3% (1/7) of aspirin-treated hens of this age died with ovarian cancer. A more pronounced effect of aspirin treatment on ovarian cancer incidence and/or the survivability of hens with ovarian cancer may have been observed if the hens were allowed to survive until natural death.

There was a significant decrease in egg production in hens with ovarian cancer ($p<0.05$, Fig. 4-4). This may suggest that egg production can be used to identify hens with the disease. Sonography is currently the only method of detection in the hen that does not require euthanasia of the animal [19] and monitoring egg production may be a more convenient method to distinguish hens with ovarian cancer. The failing health of hens with advanced stages of ovarian cancer may arguably have caused the decrease in egg production shown in Figure 4-4. Our data show, however, that hens with stage 1 ovarian cancer produce significantly fewer eggs in the year prior to diagnosis than hens that do not have ovarian cancer ($p<0.05$, Fig. 4-5). Stage 1 ovarian cancer in the hen can only be detected histologically (Table 4-2), which suggests that egg production decreases before the disease has significantly affected the overall health of the hen. A decrease in egg production may therefore identify hens with an earlier stage than can be identified with physical inspection or sonography. Studies in our laboratory are currently underway to confirm and assess this method.

To our knowledge, this is the first study to evaluate the effectiveness of aspirin in decreasing ovarian cancer incidence in the hen. The fact that the hen spontaneously develops ovarian cancer permits the *in vivo* testing of chemotherapeutic or chemopreventative agents. This study provides vital information and encouraging data for future studies evaluating these agents

using hens. Dietary aspirin decreased liver prostaglandin production and resulted in a significantly reduced stage of ovarian cancer. This suggests that aspirin may inhibit the progression of the disease. Our data also indicate that a decrease in egg production may be used to distinguish hens with early stages of ovarian cancer. Finally, we have identified a commercial strain of White Leghorn hens as having a higher incidence of ovarian cancer at 3.5 years than previously noted.

Acknowledgements

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CHAPTER 5

SUMMARY AND INTERPRETATION OF RESULTS

Our data indicate that COX-1 mRNA is significantly increased in ovarian tumors of the hen as compared to normal hen ovaries, while there is no difference in COX-2 mRNA between normal ovaries and ovarian tumors (chapter 2). Prostaglandin E_2 (PGE_2) levels were also significantly increased in ovarian tumors as compared to normal ovaries, which indicates that the increase in COX-1 mRNA was a functional increase. In addition, COX-1 protein localized to glandular areas of ovarian tumors. These data are similar to findings in women, which indicate that ovarian cancer exhibits increased expression of COX-1 rather than COX-2 [1-3]. Expression data from women and our data from the hen, therefore, both suggest that COX-1 is more highly correlated with ovarian adenocarcinoma than COX-2.

The accumulation of large volumes of malignant ascites is often associated with advanced stages of ovarian cancer in women [reviewed in [4]], as well as in the hen. In order to further elucidate the roles of both COX enzymes in ovarian cancer progression, we determined the expression of both isoforms in ascites cells collected from hens with ovarian cancer and studied the effects of treatment of the cells with different nonsteroidal anti-inflammatory drugs (NSAIDs) (chapter 3). We found mRNA and protein expression of both COX isoforms in cells isolated from ascites collected from hens with ovarian cancer. Treatment with aspirin (a nonspecific NSAID that is a more potent inhibitor of COX-1 than COX-2 [5]) or SC-560 (a COX-1 specific NSAID) significantly decreased the proliferation of ascites cells, while equivalent concentrations of NS-398 (a COX-2 specific NSAID) did not

significantly affect proliferation. These results support data from a mouse model of ovarian cancer, which showed that SC-560 reduced the growth of tumors while a COX-2 selective inhibitor did not [1]. These data in combination with our characterization data further suggest that COX-1 may play a more prominent role in ovarian carcinogenesis than COX-2.

Vascular endothelial growth factor (VEGF) expression has been positively correlated to the metastasis of ovarian cancer cells as well as to peritoneal ascites accumulation in women [6]. Interestingly, VEGF expression has also been correlated to COX-1 expression in ovarian cancer in women. Colocalization of COX-1 and VEGF mRNA were reported and a COX-1 specific NSAID inhibited VEGF production by an ovarian cancer cell line, while a COX-2 selective inhibitor did not [3]. Therefore, we wanted to determine whether treatment with NSAIDs affected the expression of VEGF in ascites cells collected from hens with ovarian cancer (chapter 3). We found VEGF mRNA and protein expression in ascites collected from hens with ovarian cancer as well as in ovarian tumors. Although we did not observe a decrease in VEGF expression with any NSAID treatment, we did find that VEGF mRNA expression was significantly increased in ascites cells as compared to normal hen ovaries. Additionally, we found a positive correlation between VEGF mRNA expression in ascites cells and ascites volume. These data suggest that VEGF may be a useful prognostic factor for ovarian cancer in the hen, as it also may be in women [7].

The agreement between the results from our first two studies (chapters 2 & 3) and those found in studies with ovarian cancer patients provide strong evidence that the hen is a valid model to use in studies testing the efficacy of treatments targeting the COX enzymes. Epidemiological studies indicate that

the use of aspirin or other NSAIDs may be associated with a risk reduction for ovarian cancer [8-11]. At least five observational studies, however, demonstrate no association between ovarian cancer risk and NSAID use [reviewed in [12]]. No clinical trials have been reported examining the effectiveness of NSAID treatments in decreasing ovarian cancer risk, decreasing the progression of the disease, or enhancing the efficacy of current treatments. The purpose of the final study presented in this dissertation (chapter 4), therefore, was to complete the first *in vivo* study examining the effects of aspirin on ovarian cancer incidence and progression in the hen, the only animal that spontaneously develops the disease at a rate high enough to study.

Although we did not find a significant effect on cancer incidence, we did observe a significant effect of aspirin treatment on the stage of ovarian cancer, which indicates that aspirin may slow the progression of the disease. Our data also indicate that treatment with aspirin may potentially decrease ovarian cancer incidence or increase the survivability of hens with ovarian cancer as compared to untreated hens if the hens are allowed to survive until natural death. This experiment not only provided vital information and encouraging data for future studies using the hen for *in vivo* studies, it also added to the growing amount of evidence suggesting that NSAIDs may be useful as chemotherapeutic or chemopreventative agents for ovarian cancer [8-12].

In addition to providing novel data regarding the potential role of the COX enzymes in ovarian carcinogenesis, the experiments presented in this dissertation provided additional information regarding the hen model. We identified the commercial Babcock B300 strain of single-comb White Leghorn

hens as having a higher incidence of ovarian cancer than has been previously reported for other strains [13-16] and have verified the age-dependency of ovarian cancer incidence that was observed by Fredrickson in 1987 [13] (chapter 4). In addition, we found that hens with ovarian cancer produce significantly fewer eggs in the year prior to diagnosis (chapter 4). This finding is in contrast to data reported by Fredrickson [13], which showed no difference in egg lay between hens that developed genital tumors and those that did not. Continuous laying records were not maintained for individual birds in Fredrickson's study, however. The number of eggs for individual hens was recorded by trapping hens as they entered nests for 14 nonconsecutive months over the 3.5-year observational period [13]. The hens in our experiment were individually caged, which permitted the recording of continuous laying records for each hen and may explain the difference in results. According to our results, monitoring egg production may provide a new method to detect ovarian cancer in the hen.

Sonography and palpation are the only current methods of detection that do not require anesthesia [17]. Our data show that hens with stage 1 ovarian cancer, which can only be detected histologically, lay significantly fewer eggs in the year prior to diagnosis than hens that do not have ovarian cancer (chapter 4). This indicates that monitoring egg production may be a more sensitive method of detection than sonography. The mechanism behind the decrease in egg lay in hens with stage 1 ovarian cancer is unknown. Examination of histological sections in our lab has led to the observation that early lesions in hen ovaries may frequently occur near postovulatory follicles (unpublished observation). The hen postovulatory follicle (POF) serves as a source of prostaglandins that play a role in stimulating oviposition, which is

demonstrated by a delay in oviposition observed after removal of the POF [18, 19]. It is possible that a neoplastic lesion near the POF may disrupt prostaglandin production and result in an initial delay in oviposition. A subsequent decrease in egg lay may follow as the neoplasm affects the normal hormonal milieu of the ovary. The ability of hen ovarian tumors to produce steroid hormones is indicated by the presence of 3-beta-hydroxysteroid dehydrogenase (3-β-HSD) in glandular areas of ovarian tumors, which suggests that these tumors can synthesize progesterone and androgens [20]. Progesterone produced from the F_1 follicle stimulates the release of GnRH from the hypothalamus, which in turn triggers the LH surge prior to ovulation in the hen. Continuous production of progesterone from the ovarian tumor could therefore negatively feedback to the hypothalamic-pituitary axis and decrease gonadotropin production, which would ultimately inhibit ovulation. Progesterone produced by neoplastic cells could also inhibit ovulation through the disruption of follicular development. Studies have shown that administration of large doses of progesterone causes follicular atresia and inhibition of ovulation [21]. Testosterone has also been shown to stimulate ovulation [22] and a testosterone antagonist was shown to inhibit ovulation and preovulatory surges of progesterone, LH, and estradiol in laying hens [23]. Production of testosterone by neoplastic cells, therefore, could also act through a negative feedback mechanism to disrupt ovulation. Alternatively, the decrease in egg lay in hens with stage 1 ovarian cancer may reflect a prior hormonal imbalance that affected egg lay and facilitated the formation of the neoplasm.

In summary, we have presented novel data showing the expression of the COX enzymes and VEGF in the normal hen ovary and over-expression of

COX-1 and VEGF mRNA in ovarian adenocarcinomas of the hen. We have published the first work using ascites cells collected from hens with ovarian cancer and have shown that the proliferation of these cells can be decreased using aspirin or a COX-1 specific NSAID. Finally, we have completed the first *in vivo* study testing the efficacy of NSAIDs in decreasing the risk or progression of ovarian cancer in the only spontaneous model of the disease and have shown a significant effect on the stage of ovarian cancer with aspirin treatment. Taken together, the studies presented in this dissertation provide a significant contribution of literature that validates utilizing the hen as a model for ovarian cancer as well as provides experimental evidence that the COX enzymes are involved in ovarian carcinogenesis.

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