UVB Exposure and Topical Estrogen Effects on the Development of Skin Cancer in a Pre- and Post-Menopausal Mouse Model

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Michelle Creamer, DVM

Graduate in Comparative and Veterinary Medicine

The Ohio State University

2012

Master’s Examination Committee:

Tatiana Oberyszyn, PhD, Advisor
Judy Hickman-Davis, DVM, PhD
Krista LaPerle, DVM, PhD
Abstract

Ultraviolet light (UV) is the most common complete carcinogen which we are exposed to on a daily basis that initiates and promotes tumor development and growth in the skin. Thus, multiple exposures to UV radiation from the sun can increase the incidence of skin cancer. Estrogen based hormone replacement therapy is now prescribed as a topical ointment. Estrogen, a known carcinogen, has been linked to increased risks of various reproductive systems cancers. Prior to this study, the effects of topical estrogen applied to sun exposed areas on the development of skin cancer had not been extensively studied. The specific aims of this study are twofold: (1) to determine the acute effects of topical estrogen on UVB induced inflammation, DNA damage, and repair and (2) to investigate the potential carcinogenic effect of topical estrogen on chronically UVB exposed skin in pre-menopausal and post-menopausal mouse models of human disease. We hypothesized that previously UVB irradiated skin treated with topical estrogen (10nmol) would result in increased inflammation and DNA damage acutely and ultimately result in increased tumor burden and severity. For the acute study, SKH1 mice were irradiated with UVB light once, treated topically with vehicle, Surgilube®, or estrogen and sacrificed at 24, 48, 72, or 96 hours (n=48). In the chronic study, mice were irradiated three times a week for 10 weeks (n=55). They were then randomized into two groups and underwent either surgical removal of their ovaries or a sham surgical procedure simulating pre- and post-menopausal conditions. These two groups were
further randomized into 4 groups and treated topically with estrogen or Surgilube® three times a week for 15 weeks. Inflammatory mediators, myeloperoxidase, hydrogen peroxide, and prostaglandin E$_2$ (PGE$_2$) as well as markers of DNA damage, cyclobutane pyrimidine dimers and p53 were measured in the acute and chronic studies. Tumor growth was measured weekly in the chronically irradiated mice. The acute study data shows that topical treatment with estrogen did not alter UVB induced increases in hydrogen peroxide and PGE$_2$. However topical estrogen treatment did appear to affect the infiltration of neutrophils into the skin following UVB exposure. This suggests that estrogen may play a role in modulating select inflammatory mediators when applied after UVB radiation. In the chronic study, there were no significant differences in the effects of estrogen in control or ovariectomized mice. However, estrogen treated mice developed more tumors of a higher grade compared to mice treated with Surgilube®. The results of this study suggest that estrogen may be increasing the severity of tumors through the inhibition of DNA repair as shown by the decreased levels of p53 in the skin surrounding tumors with increased levels of p53 in the actual tumors. Although the results of this study did not reach statistical significance, the clinical relevancy of developing more malignant versus benign neoplasms with topical estrogen treatment is important. Future studies are needed to investigate biological causes for the increased severity of neoplasms in SKH1 mice treated with topical estrogen.
Dedication

To my parents, siblings, and my Matthew whose love and support have made everything in my life meaningful and possible; and to my sweet dogs Penny, Mannie, and Pinky whose unconditional love and companionship constantly remind me why I became a veterinarian.
Acknowledgments

Research cannot be done without the help of others. I cannot express how thankful I am to have been welcomed into Dr. Oberyszyn’s lab and mentored without any hesitation. The mentorship of Dr. Tatiana Oberyszyn and Dr. Kathy Tober was beyond my expectations and I will forever consider them both colleagues and friends. Members of the Oberyszyn lab, Judy Riggenbach, Erin Burns, Keith Lamping, and Paul Cipriani, were crucial players in helping me complete my masters. Without them I truly don’t know how I would have done it! What I enjoyed most about being a part of the Oberyszyn lab was the team attitude. Everyone was incredibly helpful, friendly, and sincerely cared about their fellow lab members. Dr. Donna Kusewitt also deserves many thanks for analyzing hundreds of tumors in such a short amount of time.

I would have never gotten the opportunity to pursue my research interests if it had not been for Dr. Valerie Bergdall and Dr. Judy Hickman-Davis who directed me towards the Oberyszyn lab. I inquired about research involving surgery and oncology and without hesitation Drs. Bergdall and Hickman-Davis knew just who to call and for that I am so thankful. Their support in allowing time away from my clinical obligations as a laboratory animal medicine resident was vital to completing the masters successfully. I can’t thank the entire ULAR veterinary and technician staff enough for their support.
during this time. Whether it was covering clinical obligations or simply asking how things were going in the lab, everyone was so kind and encouraging.

I would also like to thank Dr. Krista LaPerle whose expertise is immeasurable. She offered much guidance and suggestions for troubleshooting our immunohistochemical procedures. She has also been a phenomenal committee mentor and has never doubted my abilities in the somewhat unfamiliar land of research.
Vita

June 2002 .......................................................Park View Senior High School

May 2006 .......................................................B.S. Animal and Poultry Science, Virginia Polytechnic Institute and State University

May 2010 .......................................................D.V.M. Virginia-Maryland Regional College of Veterinary Medicine

June 2010 to present ..................................Resident, Laboratory Animal Medicine, The Ohio State University

June 2010 to Present ...............................Graduate Research Associate, Department of Veterinary Preventative Medicine, The Ohio State University

Fields of Study

Major Field: Comparative and Veterinary Medicine
List of Figures

Figure 1. Myeloperoxidase levels in SKH1 mice over time after acute UV radiation ........15

Figure 2. Prostaglandin E₂ in SKH1 mice over time after acute UV radiation. ...............16

Figure 3. Representative sample of histochemical staining for hydrogen peroxide in the skin of mice treated with estrogen .................................................................17

Figure 4. Hydrogen peroxide in SKH1 mice over time after acute UV radiation. ............18

Figure 5. Representative sample of immunohistochemical staining for p53 in the skin of mice treated with estrogen .................................................................19

Figure 6. p53 levels in SKH1 mice over time after acute UV radiation .........................20

Figure 7. Dot Blot and template of CPD levels in SKH1 mice over time. ......................21

Figure 8. Graph of CPD levels in SKH1 mice over time..................................................22

Figure 9. Representative sample of immunohistochemical staining for Ki67 in the skin of mice treated with estrogen.................................................................23

Figure 10. Ki67 levels in SKH1 mice over time after acute UV radiation......................24

Figure 11. Scatter plot of tumor burden for all treatment groups and estrogen versus Surgilube® ....................................................................................................34
Figure 12. Tumor grades in estrogen or Surgilube® treated SKH1 mice after chronic UV radiation. ................................................................................................................................35

Figure 13. Representative photomicrographs of benign cutaneous neoplasms classified as a papillomas (pap) grades 1-3. ........................................................................................................36

Figure 14. Representative photomicrographs of malignant cutaneous neoplasms classified as microinvasive squamous cell carcinoma (MiSCC) grades 1-3, squamous cell carcinoma (SCC), and spindle/anaplastic cell sarcoma. .........................................................37

Figure 15. Myeloperoxidase in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation..............................................................................................................38

Figure 16. Prostaglandin E2 levels in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation ........................................................................................................39

Figure 17. Hydrogen peroxide levels in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation .........................................................................................................40

Figure 18. p53 levels in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation...........................................................................................................................41

Figure 19. Ki67 levels in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation .........................................................................................................................................42
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-oxo-dG</td>
<td>8-oxo-deoxyguanosine</td>
</tr>
<tr>
<td>8-oxo-G</td>
<td>8-oxo-guanine</td>
</tr>
<tr>
<td>AAALAC</td>
<td>Association for the Assessment and Accreditation of Laboratory Animal Care International</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CC</td>
<td>Cytosine-Cytosine</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CPDs</td>
<td>Cyclobutane pyrimidine dimers</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzine</td>
</tr>
<tr>
<td>E₂</td>
<td>Estrogen</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immuno-Assay</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen Receptor Alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>Estrogen Receptor Beta</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone Replacement Therapy</td>
</tr>
<tr>
<td>HTAB</td>
<td>Hexadecyltrimethylammonium Bromide</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NMSC</td>
<td>Nonmelanoma Skin Cancer</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomized</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline with Tween 20</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Sham</td>
<td>Sham surgical procedure</td>
</tr>
<tr>
<td>TC</td>
<td>Thymine-Cytosine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Veh</td>
<td>Vehicle Control, Surgilube®</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

Cutaneous neoplasms, both nonmelanoma skin cancer (NMSC) and melanoma, resulting from ultraviolet light (UV) exposure, account for nearly one half of all cancers in the United States with increasing incidence in females less than 40.\textsuperscript{1,5} Sunlight is composed of three types of radiation classified by wavelength into three bands: UVA (320–400 nm), UVB (290–320 nm) and UVC (< 290 nm). The UV component of the sunlight reaching the earth surface (daylight UV) consists of UVA and UVB (290–400 nm). UVA radiation penetrates to the dermis and is responsible for chronic skin damage due to upregulation of metalloproteinases which deteriorate collagen and elastin.\textsuperscript{11} UVB penetrates to the level of the epidermis and is responsible for erythema, or redness, associated with sunburn.\textsuperscript{11, 38, 51, 60} The UVB portion shows a strong carcinogenic effect on the skin and some involvement of UVA radiation in carcinogenesis of the skin has also been implicated.\textsuperscript{24}

The phototoxic effect of UVA radiation is much lower than that of UVB radiation because unlike UVA light, UVB light uses DNA as a chromophore. Incident photons from UVB light are directly absorbed by DNA bases leading to cytotoxic and mutagenic changes.\textsuperscript{23} Although both UVA and UVB radiation cause DNA damage within the skin, UVB radiation causes direct mutations in DNA replication. These mutations occur through the creation of cyclobutane pyrimidine dimers (CPDs) and 6,4-photoproducts at sites where two pyrimidine bases are adjacent. Among CPDs, thymine-cytosine (TC) and cytosine-cytosine (CC) dimers are shown to be the most mutagenic, and these mutations are frequently found in the p53 gene of UV induced cancer cells.\textsuperscript{23} These UV induced damages, CPDs and 6,4-photoproducts, are assumed to cause UV-specific mutations which, if unrepaired contribute to the formation of skin tumors.\textsuperscript{23,24 25,34,50}
UVB radiation also causes indirect DNA damage by inducing oxidative stress in cells through the production of reactive oxygen species (ROS) by neutrophils and other phagocytic cells.\textsuperscript{33,38} ROS attack DNA and can produce oxidative base damage such as 8-oxo-guanine (8-oxo-G). They also attack cellular nucleotide pools, producing oxidized nucleotides such as 8-oxo-deoxyguanosine (8-oxo-dG).\textsuperscript{19,29,45,61} Damaged, unrepaired DNA leads to mutated DNA still available for use in DNA replication. Thus, UV radiation can induce oxidative stress-mediated mutations in the cellular genome through an indirect mechanism.\textsuperscript{51} In response to UV radiation, infiltrating inflammatory cells including neutrophils and macrophages release myeloperoxidase during cutaneous inflammation which generates ROS.\textsuperscript{51} In addition, keratinocytes act as a source of reactive oxygen species by transferring hydrogen peroxide (H$_2$O$_2$) to melanocytes. In epidermal cells, H$_2$O$_2$ levels increase in response to oxidative stress from environmental trauma, such as UVB radiation causing damage to biomolecules. There is also a basal level of oxidants in keratinocytes cells that is a by-product of normal endogenous processes, which are normally kept inactive through a system of enzymatic and non-enzymatic antioxidants. During times of oxidative stress the antioxidant defenses can be overwhelmed and lead to a disruption of cellular function.\textsuperscript{39} These ROS induce indirect DNA damage via oxidative stress through generation of oxidized nucleotides, mainly 8-oxo-dG.\textsuperscript{33,38,51}

Inflammatory cells have significant effects on tumor development. Early in tumorigenesis neutrophils, which are the first cells to infiltrate to sites of inflammation, induce DNA damage in proliferating cells through the production of reactive oxidative and nitrogen species. These oxidizing components, particularly myeloperoxidase and hydrogen peroxide, are normally produced by neutrophils to fight infection. Myeloperoxidase, released by activated neutrophils during inflammatory events, is an enzyme that converts hydrogen peroxide to hypochlorous acid.\textsuperscript{10} Additionally these cells mediate damage through the generation of arachidonic acid derivatives including prostaglandins which are capable of producing an intense inflammatory response. The production of prostaglandin E$_2$ (PGE$_2$) through the cyclooxygenase (COX) pathway specifically contributes to reactive oxygen species production as it forms oxygen radicals.
during conversion of prostaglandins. As a byproduct of prostaglandin synthesis, reactive oxygen species that can induce the formation of oxidative adducts such as 8-oxo-dG are formed. An increase in PGE$_2$ production and function appears to be critical to the observed damaging effects of UVB light on the skin.$^{10,53}$ Studies suggest that reactive intermediates such as those following UVB exposure may also contribute to the mutation of tumor suppressor genes such as p53, allowing for an increased rate of accumulation of genetic damage in the cell.$^{10}$

Mutations in p53, a tumor suppressor gene, are important in the development of UVB induced squamous cell carcinomas.$^{30,60}$ p53 acts as a checkpoint in the cell cycle, either preventing or initiating apoptosis. p53 becomes activated in response to various stressors which include, but are not limited to, DNA damage (induced by UV or chemical agents such as hydrogen peroxide) and oxidative stress.$^{20}$ Breaks in DNA strands and UV radiation initiate a series of phosphorylation events that disturb p53 and its regulatory protein, MDM2, causing p53 degradation. Ultimately UV radiation is a complete carcinogen as it initiates cancer through DNA mutations and promotes cancer growth through the inflammatory process inherent in cumulative UV exposure.

Through epidemiologic studies, the American Cancer Society has reported that the incidence of NMSC development in men is twice that of women which is generally attributed to occupational differences between the genders.$^{38}$ However there is also a suggestion that estrogen in women is protective against certain diseases, namely cardiovascular disease and colorectal cancer.$^{46,55}$ Estrogen (E$_2$) applied topically has been well known to improve skin firmness, elasticity, and decrease wrinkle depth and pore size.$^{43}$ The effects of estrogens are mediated by the estrogen receptors alpha and beta which bind 17-β estradiol. Estrogen receptor alpha (ER$\alpha$) is found in the mammary gland, reproductive tissues, cardiovascular system, bone and regions of the brain.$^{7,52}$ Estrogen receptor beta (ER$\beta$) appears to be more widely expressed and has shown to be present in reproductive tissues, lungs, bladder, heart, adrenals, thymus, kidneys, pituitary, hypothalamus and skin.$^{4,7,9,4249,52}$ The skin locally synthesizes significant amounts of this sex hormone making topical application for cosmetic or therapeutic use an effective route
of administration through the presence of local estrogen receptors. Topical estrogen is now the number one prescribed form of estrogen for hormone replacement therapy (HRT) and numerous studies advocate the use of topical estrogen for its cosmetic benefits to the skin. However, estrogen based HRT has been linked as a significant risk factor for uterine, ovarian and breast cancer. The effects of topical estrogen applied to sun exposed areas for a period of time on the development of skin cancer has not been studied extensively. Preliminary unpublished data using intact female SKH1 mice found a significant increase in the number of skin tumors in mice treated topically with estrogen following UVB exposure compared to mice treated with a vehicle control. This suggests that increased levels of estrogen in the skin combined with UV exposure may act to enhance initiation and promotion of UV-induced skin cancers.

The current studies are based on the theory that many women are voluntarily exposed to potentially harmful amounts of UVB light in their late teens and twenties through indoor and outdoor tanning. As women age, they typically become more cognizant of the harmful effects of excessive UVB radiation and begin to take protective measures such as applying sunscreen or limiting sun exposure. Women may begin applying estrogen based products for their anti-aging cosmetic benefits in efforts to reduce the effects of chronic skin aging due to UV radiation. Due to the abundance of estrogen based topical products and the advent of the Internet, a prescription is no longer necessary to obtain the drug. This study is designed to mimic such a scenario.

The specific aims of this study are twofold: (1) to determine the acute effects of topical estrogen on UVB induced inflammation, DNA damage, and repair and (2) to investigate the potential carcinogenic effect of topical estrogen on chronically UVB exposed skin in pre-menopausal and post-menopausal mouse models of human disease. The two endogenous estrogen statuses in our mouse models mimic the major populations of women using estrogen based products. Pre-menopausal women may be using topical estrogen for its anti-aging benefits on the skin and post-menopausal women are either using estrogen for HRT as prescribed by their physicians or for its anti-aging benefits. We hypothesize that ovariectomized and intact hairless mice previously irradiated with
UVB light and then treated with topical estrogen will have a higher incidence and greater severity of cutaneous neoplasms compared to valid controls.
Chapter 2: Acute Inflammation and Topical Estrogen Effects Over Time

2.1 Acute Inflammation

Short exposure to UVB radiation can result in an acute inflammatory response, which is characterized by erythema and edema due to increased vascular flow and permeability. Increases in vascular flow and permeability, in turn, aid in the recruitment and infiltration of inflammatory cells, including neutrophils, into the skin. These cells and their released contents lead to indirect DNA damage which can be evaluated using the various markers mentioned below. DNA is also instantly damaged through the production of CPDs because DNA directly absorbs UVB radiation. A study evaluating the acute effects of UVB damage in the skin treated with topical estrogen was performed to determine the level of DNA repair over time and the length of time the various markers of inflammation are present in UVB damaged skin. This information will be used to help analyze data in estrogen treated skin chronically exposed to UVB radiation.

2.2 Materials and Methods

Forty-eight female, 8 week old SKH1 outbred mice purchased from Charles River Laboratories (Wilmington, MA) were housed in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. All procedures involving animal use were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Mice were group housed in solid bottom polycarbonate shoebox cages with autoclaved corncob bedding on an individually ventilated cage rack system. The room was kept on a 12:12h light:dark cycle, relative humidity range of 30-70%, and 20°C +/- 4°C temperature. Mice were provided ad libitum access to chlorinated, reverse osmosis
water and irradiated feed. Upon arrival mice were anesthetized with isoflurane (1-3% by inhalation) for identification tattoos at the base of the tail and removal of <5mm of the distal tail tip for genotyping for future studies.

Mice (n=4) were grouped based on topical treatment, estrogen or Surgilube® (sterile surgical lubricant), and the time of sacrifice post UVB exposure. One group of mice from each topical treatment group was not exposed to UVB light and only treated with estrogen or Surgilube® at time point zero. The remaining groups of mice were exposed to 2240J/m² of UVB light (290-320nm) equivalent to 1 minimal erythemic dose, using Phillips FS40 UVB bulbs (American Ultraviolet Company, Murray Hill, NJ). Mice received one topical treatment of 100µl Surgilube® or 100µl of 10nmol 17-β estradiol dissolved in Surgilube® immediately post UVB exposure on their back. Mice were euthanized at 24, 48, 72, and 96hr post UVB exposure and topical treatment. The control (non-UVB) mice were euthanized 48hr post topical treatment.

Mice were euthanized by carbon dioxide (CO₂) asphyxiation. Dorsal skin and serum were harvested for analysis. Skin tissue was fixed in 4% paraformaldehyde and placed in optimal cutting temperature (OCT) medium. The remaining tissues were frozen at -80°C. Various bench top procedures were completed to analyze components within the tissues and are described below.

2.21 Myeloperoxidase

Myeloperoxidase (MPO), an enzyme that converts hydrogen peroxide to hypochlorous acid, is released by activated neutrophils during inflammatory events. The levels of MPO in the cutaneous tissue were determined biochemically and used as a measure of neutrophil infiltration, as described. Briefly, 10mm punch biopsies of frozen dorsal skin were homogenized in 1.25mL of 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50mM potassium phosphate buffer, pH 6.0 at 4°C, then subjected to three rounds of sonication, freezing in liquid nitrogen, and thawing. The samples were then centrifuged for 10 min at 13,000rpm at 4°C and the supernatants were transferred to new tubes for analysis. Ten µL of sample and 290µL of substrate (0.167mg/mL o-
dianisidinedihydrochloride and 0.0005% H_2O_2 in 50mM potassium phosphate buffer, pH 6.0) were assayed in a 96-well plate for MPO activity with a spectrophotometer (Molecular Devices, Menlo Park, CA) over a 5 min period at 450nm. The data are expressed as mean units of MPO activity. The amount of MPO required to degrade 1µmol of peroxidase per minute at 25°C is equal to 1U of MPO activity. The content of MPO activity in each sample was calculated based on a standard curve.

2.22 Prostaglandin E_2

Prostaglandin E_2 (PGE_2) an inflammatory signaling product of cyclooxygenase-2 (COX-2) has been shown to be a critical factor mediating the contribution of the COX-2 pathway to cancer development. PGE_2 levels in the skin were determined using the monoclonal PGE_2 Enzyme Immunoassay (EIA) kit from Cayman Chemical (Ann Arbor, MI) according to the manufacturer’s instructions. Briefly, a section of frozen dorsal skin was crushed and sonicated in PGE_2 Extraction Buffer (0.1M Phosphate Buffer, pH 7.4, containing 1mM EDTA). Samples were incubated and vortexed over ice every 5 minutes for 50 min before being centrifuged twice at 10,000g for 10 min. The supernatant was removed and a 1:10 dilution of sample (10µl) and PGE_2 Extraction Buffer (90µl) was prepared for a BCA assay to determine protein concentrations. Following the BCA assay, 50µl of sample in triplicate, PGE_2 EIA standard, PGE_2 Acetylcholinesterase Tracer, PGE_2 Monoclonal Antibody, and 100µl of EIA buffer were added to their respective wells on a 96 well plate and incubated for 18 hours at 4°C. The plate was washed prior to the addition of Ellman’s Reagent and Tracer to their respective wells and read at 415nm.

2.23 Hydrogen Peroxide

The presence of hydrogen peroxide, an inflammatory product that induces oxidative DNA damage, was determined via histochemical staining as described. Briefly, fixed frozen skin was cut using a cryostat into 10µm sections, placed on slides and allowed to incubate at room temperature overnight. Control slides were pre-incubated for 15 minutes with catalase, a well known enzyme that destroys hydrogen peroxide. Sample and control slides were incubated for 6 hours with 150mL of 0.1M Tris-HCl
containing 0.15g glucose and 0.15g diaminobenzine (DAB) at a pH of 7.5. Catalase was added to the control slides during the 6 hour incubation. The reaction was terminated with a wash in deionized water. The slides were dehydrated and mounted with coverslips. Photomicrographs were taken using a Nikon Eclipse E400 microscope with a DXM1200 digital camera at 20X from five random sections of each slide for image analysis with Image Processing and Analysis in Java, ImageJ. The percent area positive for H₂O₂ in each field of view was determined and then averaged for each sample.

2.24 p53

Fixed frozen skin was cut using a cryostat into 10µm sections, placed on slides and allowed to incubate at room temperature overnight. Tissue sections were baked at 60°C for 30 minutes before being rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H₂O₂) in water for 10 minutes and washed in phosphate buffered saline with Tween 20 (PBST). Heat induced antigen retrieval was then accomplished using antigen unmasking solution (Vector Labs Burlingame, CA) and a microwave. Slides were blocked with 1X casein for 10 min at room temperature (RT) prior to incubation with p53 antibody (1:500 in 1X casein, NCL-p53-CM5p; Novacastra Laboratories Ltd, Newcastle, UK) for 1 hr at RT. The sections were then rinsed with PBST, incubated with Rabbit Link Solution (Biogenex, San Ramon, CA) for 30 min at RT, then rinsed again. Rabbit Label Solution (Biogenex) was applied to the slides for 30 min at RT before the slides were rinsed with PBST again. Immunohistochemical detection was visualized using DAB (Vector). The slides were finally counterstained with hematoxylin, dehydrated and mounted. Photographs of slides were taken using a Nikon Eclipse E400 microscope with a DXM1200 digital camera. Data are represented as the percentage of p53 positive nuclei counted within all cells of the epidermis in ten 60X fields of view per tissue section.

2.25 Cyclobutane Pyrimidine Dimers

Cyclobutane pyrimidine dimers are formed within DNA as a result of UVB radiation. The number of dimers within tissues can serve as a marker of direct DNA
damage with greater amounts of CPDs suggesting more extensive DNA damage. Epidermal tissue was separated from dermal tissue by heating a section of skin in a 60°C water bath for 10 seconds, transferring it to an ice bath for 10 seconds and peeling the epidermal tissue from the dermis using forceps. DNA was isolated using Invitrogen’s Purelink Genomic DNA Mini Kit (Life Technologies Corporation, Carlsbad, CA) protocol for mammalian tissue. The concentration of DNA was determined fluorometrically using the Qubit double stranded DNA Broad Range assay kit (Invitrogen). DNA was denatured in 0.8M NaOH 20mM EDTA for 10 minutes at 95°C followed by neutralization in 2M cold ammonium acetate. 100ng of denatured and neutralized DNA was transferred to a 0.45µm HyBond ECL nitrocellulose using a dot blot apparatus and vacuum. The membrane was dried and blocked with 1X TBS-0.1% Tween with 5%BSA before being incubated overnight with 1:500 mouse anti-thymine dimer antibody (Kamiya Biomedical) in 1X TBS-0.1% Tween with 5%BSA. After washing in TBST, a 1:10,000 dilution of cell signaling HRP-linked anti-mouse antibody in 5%BSA/TBST was added for 45min. The membrane was then incubated with Cell signaling LumiGlo and photographed using Quantity One 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA). The membrane was then counterstained with 1:5000 Sybr Gold in 1X PBS-0.1%Tween for 1hr and photographed with transillumination.

2.26 Ki67

The Ki67 protein is associated with cellular proliferation and is used as a proliferation marker in tissue.\textsuperscript{44} Fixed frozen skin was cut using a cryostat into 10µm sections, placed on slides and allowed to incubate at room temperature overnight. Tissue sections were baked at 60°C for 30 minutes before being rehydrated. Endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes and washed in PBST. Heat induced antigen retrieval was then accomplished using antigen unmasking solution (Vector Labs Burlingame, CA) and a steamer. Tissues were then blocked with 1X casein for 10 min at RT prior to incubation with rat anti-mouse Ki67 primary antibody (1:200 in 1X casein) overnight at 4°C. The sections were then rinsed with PBST and incubated with biotinylated rabbit-anti-rat IgG at a 1:200 dilution in 1X casein
for 30 minutes at room temperature. Following another PBST wash, ABC Elite was added for 30 minutes at room temperature. Immunohistochemical detection was visualized using DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA). The slides were finally counterstained with hematoxylin, dehydrated and mounted. Photographs of slides were taken using a Nikon Eclipse E400 microscope with a DXM1200 digital camera. Data are represented as the percentage of Ki67 positive nuclei counted within all cells of the epidermis in five 20X fields of view per tissue section.

2.3 Statistics

Results from immunohistochemical and EIA procedures were normally distributed and evaluated using an analysis of variance (ANOVA). Posthoc comparisons were performed using Bonferroni and Tukey to identify differences among and within groups when differences were detected. All data was reported as mean ± standard error of the mean (SEM) and considered significant at p<0.05 and analyzed using computerized software (SPSS Statistics, version 19, IBM, Armonk, NY).

2.4 Results

2.41 Myeloperoxidase (MPO) levels in skin of mice following acute exposure to UVB radiation.

The ability of topical estrogen to alter MPO activity, a marker for neutrophil infiltration and activation was evaluated. At baseline, Surgilube® and estrogen treated mice not exposed to UV light had low levels of MPO measuring 0.0013 ± 5.87E-05 and 0.0012 ± 1.78E-05 mean units of MPO respectively (Figure 1). Levels of MPO were significantly (p<0.01) increased at 24 hours and peaked at 48 hours in mice exposed to UV radiation and treated with topical Surgilube® or estrogen. At 72 hours, MPO levels were still significantly (p<0.01) higher in mice treated estrogen and exposed to UV radiation compared to mice not exposed to UV radiation suggesting that estrogen slows the removal of MPO following UV exposure. In addition, at 96 hours, levels of MPO did not return to baseline in either the UV/Surgilube®, 0.0032 ± 0.00078 mean units MPO or UV/Estrogen, 0.0028 ± 0.00047 mean units MPO skin remaining three to four times
greater than the amount of MPO in mice not exposed to UV radiation. Topical treatment with either estrogen or Surgilube® did not result in any statistically significant differences in MPO at any time point examined (p=0.71) however estrogen may slow the recovery of MPO levels to baseline as evidenced at the 72 hour time point.

2.42 Prostaglandin E₂ levels in skin of mice following acute exposure to UVB radiation.

The effects of topically applied estrogen compared to Surgilube® on PGE₂ production were determined using an enzyme immunoassay. PGE₂ is a COX-2 principle metabolic product and is synthesized after an inflammatory event. The COX-2/PGE₂ pathway can affect multiple aspects of cell physiology required for tumor development and maintenance. Levels of PGE₂ were not significantly different among treatment groups (p=0.77) (Figure 2). Throughout the time course, mice exposed to UV radiation and treated with estrogen had greater levels of PGE₂ averaging 48.83 ± 3.983 pg/µg of protein compared to mice not exposed to UV radiation and treated with estrogen, 40.77 ± 3.63 pg/µg. At 72 hours, levels of PGE₂ exhibited near significant (p=0.052) increases in UV/Estrogen mice compared to mice not exposed to UV radiation and treated with estrogen. Regardless of exposure to UV radiation, mice treated with the vehicle control, Surgilube®, had relatively constant levels of PGE₂ in the skin averaging 41.49 ± 6.17 pg/µg for the No UV group and 41.77 ± 6.49 pg/µg for the UV group.

2.43 Hydrogen Peroxide levels in skin of mice following acute exposure to UVB radiation.

Hydrogen peroxide produced during an inflammatory response can result in oxidative damage. Quantitative analysis using histochemical procedures and the image analysis program, ImageJ revealed that at 24 hours levels of hydrogen peroxide in mice exposed to UV radiation and treated with topical estrogen were significantly (p<0.01) elevated compared to mice not exposed to UV radiation (Figures 3,4). In addition, at 24 hours, the levels of hydrogen peroxide were significantly (p=0.02) higher in the skin of UV exposed mice treated topically with estrogen compared to Surgilube®, 0.46% ± 0.070 vs. 0.26% ± 0.044, respectively. While hydrogen peroxide levels significantly
(p<0.01) peaked at 48 hours in the UV/Surgilube® treated skin and began to decline, hydrogen peroxide levels remained significantly (p<0.05) elevated in UV/Estrogen skin at 72 hours compared to mice not exposed to UV radiation further suggesting that estrogen slows the recovery of inflammatory mediators to levels in mice not exposed to UV radiation.

2.44 p53 levels in skin of mice following acute exposure to UVB radiation

DNA damage resulting from UVB radiation induces an increase in p53 expression. To determine levels of overall DNA damage, p53, a tumor suppressor gene was evaluated in the skin using immunohistochemical analysis. The percentage of p53 positive cells in the epidermis of mice not exposed to UV radiation was essentially 0% (0.11% ± 0.063 Surgilube®, 0.18% ± 0.14 estrogen) (Figures 5,6). Regardless of treatment group, estrogen or Surgilube®, levels of p53 significantly (p<0.01) peaked at 24 hours in mice exposed to UV radiation. At 24 hours, those treated with estrogen had an average of 17.6% ± 0.09 positive cells within the skin compared to mice treated with Surgilube® which had an average of 14.3% ±2.39. At 48 hours, levels of p53 remained significantly (p<0.01) elevated in mice exposed to UV radiation and treated with estrogen while levels declined in mice treated with Surgilube® suggesting that estrogen may slow the recovery of p53 to pre-UV levels. Although levels of p53 were consistently increased in the skin of mice treated with estrogen, there were no statistically significant differences in levels of p53 between treatment groups throughout the time course.

2.45 Cyclobutane Pyrimidine Dimer levels in skin of mice following acute exposure to UVB radiation

UVB radiation directly damages DNA through the formation of CPD. To detect CPDs a southwestern blot was performed using dorsal skin collected from each mouse. Levels of CPDs in UVB irradiated mice were significantly (p<0.001) higher at 24 and 48 hours when compared to mice not exposed to UVB radiation (Figures 7,8). These levels then decreased over time and by 96 hours CPD levels were not significantly different
than those found in mice not exposed to UV radiation. In addition, there were no significant differences between topical treatment groups.

2.46 Ki67 levels in skin of mice following acute exposure to UVB radiation

Ki67 is a common protein found in proliferating and dividing cells. Because of its absence in quiescent cells and its universal expression in proliferating tissues Ki67 is considered a marker of cell proliferation. Immunohistochemistry using dorsal skin was used to determine the level of Ki67 among treatment groups over time. The percentage of Ki67 positive cells in the epidermis of mice exposed to UV radiation peaked at 48 hours in both treatment groups (Surgilube® 44.74% ± 3.26; estrogen, 45.39% ± 2.68) compared to their No/UV counterparts (Surgilube® 14.93 ±1.03; estrogen 14.14 ± 3.12) (Figures 9,10). Ki67 levels decreased overtime to levels observed in the skin of mice not exposed to UV radiation. Again, no significant differences (p=0.14) in the induction of Ki67 were seen between the UV/Surgilube® and UV/Estrogen treatment groups.
Figure 1. Myeloperoxidase levels in SKH1 mice over time after acute UV radiation. Levels of MPO were significantly (*, p<0.01) increased at 24 hours and peaked at 48 hours in mice exposed to UV radiation and treated with topical Surgilube® or estrogen. At 72 hours, MPO levels were still significantly (*, p<0.01) higher in mice treated with estrogen and exposed to UV radiation compared to mice not exposed to UV radiation suggesting that estrogen slows the removal of MPO following UV exposure.
Figure 2. Prostaglandin E₂ in SKH1 mice over time after acute UV radiation. Levels of PGE₂ were not significantly different among treatment groups (p=0.77). However, throughout the time course, mice exposed to UV radiation and treated with estrogen had greater levels of PGE₂ averaging $48.83 \pm 3.983$ pg/µg of protein compared to mice not exposed to UV radiation and treated with estrogen, $40.77 \pm 3.63$ pg/µg. At 72 hours, levels of PGE₂ exhibited near significant (*, p=0.052) increases in UV/Estrogen mice compared to mice not exposed to UV radiation and treated with estrogen.
Figure 3. Representative sample of histochemical staining for hydrogen peroxide in the skin of mice treated with estrogen. 20X magnification.
Figure 4. Hydrogen peroxide in SKH1 mice over time after acute UV radiation. At 24 levels of hydrogen peroxide in mice exposed to UV radiation and treated with topical estrogen were significantly (*, p<0.01) elevated compared to mice not exposed to UV radiation and significantly (#, p=0.02) higher in the skin of compared to Surgilube®, 0.46% ± 0.070 vs. 0.26% ±0.044, respectively. While hydrogen peroxide levels significantly (*, p<0.01) peaked at 48 hours in the UV/Surgilube® treated skin and began to decline, hydrogen peroxide levels remained significantly (*, p<0.05) elevated in UV/Estrogen skin at 72 hours compared to mice not exposed to UV radiation.
Figure 5. Representative sample of immunohistochemical staining for p53 in the skin of mice treated with estrogen. 60X magnification.
Figure 6. p53 levels in SKH1 mice over time after acute UV radiation. Levels of p53 significantly (*, p<0.01) peaked at 24 hours in mice exposed to UV radiation. At 48 hours, levels of p53 remained significantly (*, p<0.01) elevated in mice exposed to UV radiation and treated with estrogen while levels declined in mice treated with Surgilube® suggesting that estrogen may slow the recovery of p53 to pre-UV levels.
Figure 7. Dot Blot and template of CPD levels in SKH1 mice over time.
Figure 8. Graph of CPD levels in SKH1 mice over time. Levels of CPDs in UVB irradiated mice were significantly (*, p<0.001) higher at 24 and 48 hours when compared to mice not exposed to UVB radiation. These levels then decreased over time and by 96 hours CPD levels were not significantly different than those found in mice not exposed to UV radiation. There were no significant differences between topical treatment groups.
Figure 9. Representative sample of immunohistochemical staining for Ki67 in the skin of mice treated with estrogen. 20X magnification.
Figure 10. Ki67 levels in SKH1 mice over time after acute UV radiation. The percentage of Ki67 positive cells in the epidermis of mice exposed to UV radiation peaked at 48 hours in both treatment groups (Surgilube® 44.74% ± 3.26; estrogen, 45.39% ± 2.68) compared to their No/UV counterparts (Surgilube® 14.93 ±1.03; estrogen 14.14 ± 3.12). Ki67 Levels decreased overtime to levels observed in the skin of mice not exposed to UV radiation. Again, no significant differences (p=0.14) in the induction of Ki67 were seen between the UV/Surgilube® and UV/Estrogen treatment groups.
Chapter 3: Chronic Inflammation and Topical Estrogen Effects Over Time

3.1 Chronic Inflammation

Chronic exposure to UVB radiation resulting in repeated inflammatory responses has been linked to the development of skin cancer.\textsuperscript{12,13,40,51,56,57,59} Chronic exposure to UV radiation also augments the functional decline in skin often associated with aging including thickened, wrinkled, and leathery appearing skin.\textsuperscript{14} Women, in particular, spend a fair amount of time exposing themselves to UVB radiation while sunbathing outside or in tanning beds during their teens and twenties. As premature signs of aging develop in their skin, women may become more vigilant about applying sunscreen and may also begin applying cosmetic creams or lotions to slow the signs of aging in their skin. In addition, as women age and estrogen levels decline, similar signs of skin aging are evident such as increased wrinkle depth, elasticity and pore size. Perimenopausal women are encouraged by some physicians to use estrogen based topical compounds to reverse or slow these signs of aging skin as well as use topical estrogen for hormone replacement therapy.\textsuperscript{43} The purpose of this study of chronic exposure to UVB radiation was to mimic a compliant patient, either pre-menopausal or post-menopausal, whom after years of sun exposure takes measures to decrease their exposure to UVB radiation while using a topical estrogen product for cosmetic or therapeutic purposes. The application of topical estrogen may be prescribed for hormone replacement therapy or used by a woman based upon anecdotal evidence, without a prescription solely for cosmetic benefits. Thus the study modeled pre-menopausal women with normal levels of circulating estrogen and post-menopausal women with decreased estrogen levels who would be applying an estrogen based topical product for hormone replacement or cosmetic purposes. This study sought to evaluate the use of a known carcinogen, estrogen, on previously chronically UVB irradiated skin and its effects on cutaneous tumor development and progression.
3.2 Materials and Methods

Seventy female SKH1 mice, 6-8 weeks old, purchased from Charles River Laboratories were housed in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. All procedures involving animal use were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Mice were group housed in solid bottom polycarbonate shoebox cages with autoclaved corncob bedding on an individually ventilated cage rack system. The room was kept on a 12:12h light:dark cycle, relative humidity range of 30-70%, and 20°C +/- 4°C temperature. Mice were provided *ad libitum* access to chlorinated, reverse osmosis water and irradiated feed. Upon arrival mice were anesthetized with isoflurane (1-3% by inhalation) for identification tattoos at the base of the tail and removal of <5mm of the distal tail tip for genotyping for future studies.

Mice were divided into two initial groups: UVB radiation exposure (n=50) and no UVB radiation exposure (n=20). Mice were exposed dorsally to UVB light thrice weekly on nonconsecutive days for 10 weeks. Individual exposures to UVB light (290-320nm) were 2240J/m², equivalent to 1 minimal erythemic dose, using Phillips FS40 UVB bulbs (American Ultraviolet Company). Exposure times were approximately 5-8min depending on the output of the UVB lights. Time of exposure was determined prior to each exposure using the following formula: mJ = (mW/cm²) x (Area in cm²) x (Time in sec).

Following radiation exposure, the original 70 mice were further grouped into ovariectomy (OVX) or sham surgery with topical treatments of estrogen or vehicle (Veh) control. The final groupings included: (1) No UVB, Sham, Veh; (2) No UVB, Sham, E₂; (3) No UVB, OVX, Veh; (4) No UVB, OVX, E₂; (5) UVB, Sham, Veh; (6) UVB, Sham, E₂; (7) UVB, OVX, Veh; (8) UVB, OVX, E₂.

Mice were anesthetized with 1-3% isoflurane by inhalation for their respective surgical procedures. Left and right lateral abdominal skin was prepped with 2% chlorhexidine beginning at the site where the incision was made moving in a circular
pattern away from the incision site. The area was then swabbed with 70% ethanol in the same motion. The chlorhexidine/ethanol scrub procedure was repeated 2 additional times. A 5mm incision was made through the skin of the flank centered between the bottom of the rib cage and the front of the hind limb. A second incision was made through the muscle layers and held open with forceps. The fat pad surrounding the ovary was pulled through the incision to exteriorize the ovary. For mice receiving an ovariectomy, a mosquito hemostat was placed at the boundary between the oviduct and uterus and the ovary was removed with scissors. The remaining tissue was returned to the abdominal cavity and the skin incision closed with wound clips. This procedure was repeated to remove the other ovary. The procedure was identical for mice in the sham surgery groups except the exteriorized ovary was returned to the abdominal cavity prior to wound closure. Mice were given 0.025mg/kg subcutaneous buprenorphine prior to removal of anesthesia and placed in a recovery cage for monitoring. Once the mice were able to move around the recovery cage, they were transferred to their original cage. Wound clips were removed 14 days post-surgery and topical treatments with estrogen or vehicle were started.

Topical treatment was applied to the dorsal skin of mice every Monday, Wednesday and Friday; either 10nmol 17-β estradiol mixed with the vehicle, Surgilube®, or the vehicle alone for 15 weeks. During the topical treatment period, mice were weighed weekly in conjunction with weekly dorsal tumor counts and measurements. Digital calipers were used for recording the length and width of all tumors greater than 1mm.

Mice were euthanized by CO₂ asphyxiation. Dorsal skin with complete punch biopsies of any tumors >2mm in length or width and serum were collected for analysis. Skin tissue was fixed in 4% paraformaldehyde and placed in OCT. The remaining tissues were frozen at -80°C. Various bench top procedures were completed to analyze components within the tissues and are described below.

3.21 Myeloperoxidase
Myeloperoxidase (MPO), an enzyme that converts hydrogen peroxide to hypochlorous acid, is released by activated neutrophils during inflammatory events. The levels of MPO in the cutaneous tissue were determined biochemically and used as a measure of neutrophil infiltration, as described. Briefly, 10mm punch biopsies of frozen dorsal skin were homogenized in 1.25mL of 0.5% HTAB in 50mM potassium phosphate buffer, pH 6.0 at 4°C, then subjected to three rounds of sonication, freezing in liquid nitrogen, and thawing. The samples were then centrifuged for 10 min at 13,000rpm at 4°C and the supernatants were transferred to new tubes for analysis. Ten μL of sample and 290μL of substrate (0.167mg/mL o-dianisidinedihydrochloride and 0.0005% H₂O₂ in 50mMpotassium phosphate buffer, pH 6.0) were assayed in a 96-well plate for MPO activity with a spectrophotometer (Molecular Devices) over a 5 min period at 450nm. The data are expressed as mean units of MPO activity. The amount of MPO required to degrade 1μmol of peroxidase per minute at 25°C is equal to 1U of MPO activity. The content of MPO activity in each sample was calculated based on a standard curve.

3.22 Prostaglandin E₂

PGE₂, an inflammatory signaling product of COX-2, has been shown to be a critical factor mediating the contribution of the COX-2 pathway to cancer development. PGE₂ levels in the skin were determined using the monoclonal PGE₂ EIA kit from Cayman Chemical according to the manufacturer’s instructions. Briefly, a section of frozen dorsal skin was crushed and sonicated in PGE₂ Extraction Buffer (0.1M Phosphate Buffer, pH 7.4, containing 1mM EDTA). Samples were incubated and vortexed over ice every 5 minutes for 50 min before being centrifuged twice at 10,000g for 10 min. The supernatant was removed and a 1:10 dilution of sample (10μl) and PGE₂ Extraction Buffer (90μl) was prepared for a BCA assay to determine protein concentrations. Following the BCA assay, 50μl of sample in triplicate, PGE₂ EIA standard, PGE₂ Acetylcholinesterase Tracer, PGE₂ Monoclonal Antibody, and 100μl of EIA buffer were added to their respective wells on a 96 well plate and incubated for 18 hours at 4°C. The
plate was washed prior to the addition of Ellman’s Reagent and Tracer to their respective wells and read at 415nm.

3.23 Hydrogen Peroxide Staining

The presence of hydrogen peroxide, an inflammatory product that induces oxidative DNA damage, was determined via histochemical staining as described.\textsuperscript{8,48} Briefly, fixed frozen skin was cut using a cryostat into 10µm sections, placed on slides and allowed to incubate at room temperature overnight. Control slides were pre-incubated for 15 minutes with catalase, the classic enzyme that destroys hydrogen peroxide. Sample and control slides were incubated for 6 hours with 150mL of 0.1M Tris-HCl containing 0.15g glucose and 0.15g DAB at a pH of 7.5. Catalase was added to the control slides during the 6 hour incubation. The reaction was terminated with a wash in deionized water. The slides were dehydrated and mounted with coverslips. Photomicrographs were taken using a Nikon Eclipse E400 microscope with a DXM1200 digital camera at 20X from five random sections of each slide for image analysis with Image Processing and Analysis in Java, ImageJ. The percent area positive for H\textsubscript{2}O\textsubscript{2} in each field of view was determined and then averaged for each sample.

3.24 p53

Fixed frozen skin was cut using a cryostat into 10µm sections, placed on slides and allowed to incubate at room temperature overnight. Tissue sections were baked at 60°C for 30 minutes before being rehydrated. Endogenous peroxidase activity was blocked with 3% (H\textsubscript{2}O\textsubscript{2}) in water for 10 minutes and washed in phosphate buffered saline with Tween 20 (PBST). Heat induced antigen retrieval was then accomplished using antigen unmasking solution (Vector Labs) and a microwave. Slides were blocked with 1X casein for 10 min at (RT) prior to incubation with p53 antibody (1:500 in 1X casein, NCL-p53-CM5p; Novacastra Laboratories Ltd) for 1 hr at RT. The sections were then rinsed with PBST, incubated with Rabbit Link Solution (Biogenex) for 30 min at RT, then rinsed again. Rabbit Label Solution (Biogenex) was applied to the slides for 30 min at RT before the slides were rinsed with PBST again. Immunohistochemical detection
was visualized using DAB (Vector). The slides were finally counterstained with hematoxylin, dehydrated and mounted. Photographs of slides were taken using a Nikon Eclipse E400 microscope with a DXM1200 digital camera. Data are represented as the percentage of p53 positive nuclei counted within all cells of the epidermis in ten 60X fields of view per tissue section.

3.25 Ki67

The Ki67 protein is associated with cellular proliferation and is used as a proliferation marker in tissue. Fixed frozen skin was cut using a cryostat into 10µm sections, placed on slides and allowed to incubate at room temperature overnight. Tissue sections were baked at 60°C for 30 minutes before being rehydrated. Endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes and washed in PBST. Heat induced antigen retrieval was then accomplished using antigen unmasking solution (Vector Labs) and a vegetable steamer. Tissues were then blocked with 1X casein for 10 min at RT prior to incubation with rat anti-mouse Ki67 primary antibody (1:200 in 1X casein) overnight at 4°C. The sections were then rinsed with PBST and incubated with biotinylated rabbit-anti-rat IgG at a 1:200 dilution in 1X casein for 30 minutes at room temperature. Following another PBST wash, ABC Elite was added for 30 minutes at room temperature. Immunohistochemical detection was visualized using DAB Peroxidase Substrate Kit (Vector Laboratories). The slides were finally counterstained with hematoxylin, dehydrated and mounted. Photographs of slides were taken using a Nikon Eclipse E400 microscope with a DXM1200 digital camera. Data are represented as the percentage of Ki67 positive nuclei counted within all cells of the epidermis in five 20X fields of view per tissue section.

3.26 Tumor Grade

Fixed, frozen tumors embedded in optimal cutting temperature (OCT) medium were cut into 10µm sections for H&E staining and grading. The tumors were graded by a veterinary pathologist, Donna Kusewitt, DVM, PhD, Diplomate American College of Veterinary Pathologists, masked to the various treatment groups. Tumors were considered benign if classified as hyperplasia or papilloma and were considered malignant if classified as microinvasive squamous cell carcinoma, squamous cell
carcinoma, or spindle cell sarcoma (Figures 13,14). The grading scheme for UV induced skin tumors in SKH1 mice detailed by Kusewitt et al describes papillomas as tumors that show no evidence of stromal invasion composed primarily of epithelium without a pronounced papillary pattern (grade 1), as a well differentiated papillary mass (grade 2), or a well differentiated papillary mass with a few finger like projections of atypical cells as the base (grade 3). Microinvasive squamous cell carcinomas are characterized by penetration into the dermis with breaching of the basement membrane and, frequently, development of an inflammatory stromal response. Only tumors that invade into the panniculus carnosus are classified as fully invasive squamous cell carcinomas.³

3.27 Tumor Burden

For each mouse, the sum of the area of all tumors was calculated based on the weekly length and width tumor measurements. The number of tumors per mouse as well as the size of the tumors makes up the tumor burden.

3.3 Statistics

A two-way ANOVA was used to evaluate tumor burden at 24 weeks. One factor was sham surgery vs. ovariectomy and the other was topical vehicle vs. topical estrogen. Due to the large variability in the measurements, the data was log10 transformed in order to meet the assumptions of the model. Adjustment for multiple comparisons, comparing each group to the surgery status and topical treatment was achieved using Dunnett’s adjustment. No significant differences were found between mice with intact or removed ovaries. For further analyses, treatment groups were combined regardless of surgery status and the effects of estrogen and Surgilube® were compared. Results from the immunohistochemical and EIA procedures were normally distributed and evaluated using an ANOVA. All data was reported as mean ± SEM and considered significant at p<0.05 and analyzed using computerized software (SPSS Statistics, version 19, IBM, Armonk, NY).

3.4 Results
All mice that were not exposed to UVB radiation prior to topical treatment remained free of tumors. Mice exposed to UVB radiation for ten weeks prior to the initiation of topical treatment developed tumors beginning at weeks 10 or 11. Tumor burden between mice with intact ovaries (Sham) and mice that were ovariectomized (OVX) did not significantly differ (p=0.26) between topical treatment groups throughout the experiment. This suggests that endogenous estrogen levels may not play a role in the outcome of tumor development and progression. For further statistical analyses mice were re-grouped based on UVB status and topical treatments regardless of endogenous estrogen status. Therefore, all mice treated topically with Surgilube® (Sham and OVX) and all mice treated topically with estrogen (Sham and OVX) were grouped for further analysis. Topical treatment with estrogen for 15 weeks following the end of UVB exposure resulted in an average tumor burden of 29.39 ±7.54mm² compared to 15.13 ±3.83mm² tumors per mouse in the Surgilube® treated groups (Figure 11). Due to variability in tumor burden resulting from the outbred nature of the SKH1 strain, tumor burden in mice treated with estrogen or surgilube did not achieve statistical significance (p=0.19). However, interestingly, the percentage of tumors graded malignant was significantly (p=0.03) higher in mice receiving topical estrogen at 31.43 ± 3.55 compared to 17.07 ± 2.39 for mice receiving topical Surgilube®. Representative photomicrographs of tumors graded as benign papillomas 1-3 and malignant tumors including microinvasive squamous cell carcinomas 1-3, squamous cell carcinoma, and spindle cell sarcomas are shown in Figures 13 and 14, respectively.

To examine the effect of topical estrogen treatment following the cessation of UVB exposure on cutaneous inflammation several inflammatory markers were examined. Measures of the inflammatory mediator PGE₂ as well as hydrogen peroxide, an indicator of oxidative stress, did not significantly differ between either treatment group (Figures 15,16). Levels of PGE₂ and hydrogen peroxide did remain elevated in both treatment groups exposed to UVB radiation compared to mice not exposed to UVB radiation. Mice that were treated with topical estrogen and exposed to UVB radiation had significantly (p<0.01) increased amounts of PGE₂ compared to mice that were not exposed to UV radiation and treated topically with estrogen. When evaluating another inflammatory
mediator, MPO, we found that the mean units of MPO were decreased in UVB irradiated mice treated with estrogen, $0.00118 \pm 5.1E-05$, compared to mice treated with Surgilube®, $0.00194 \pm 0.00012$ (p<0.001) (Figure 17). Myeloperoxidase was also significantly decreased in mice exposed to UVB radiation, compared to mice not exposed and treated with Surgilube®, $0.0026 \pm 0.00028$ (p=0.019) or estrogen, $0.0017\pm 4.05E-05$ (p<0.001).

To evaluate direct DNA damage, the percentage of p53 positive foci determined as 3 or more adjacent positively stained cells was measured using immunohistochemistry. The percentage of p53 foci in the skin surrounding tumors was significantly (*, p<0.01) elevated in mice exposed to UV radiation averaging $0.0057\% \pm 0.0043$ compared to 0% p53 foci in mice not exposed to UV radiation. However, the percentage of p53 foci was not different (p=0.6) between treatment groups and mice treated with estrogen $0.053 \pm 0.0047$ or Surgilube® $0.063 \pm 0.0040$ (Figure 18). There also was no significant difference (p=0.7) between the percentage of Ki67 positive cells in the skin of mice treated with estrogen $16.81 \pm 1.36$ or surgilube $17.48 \pm 0.977$ (Figure 19).
Figure 11. Scatter Plot of tumor burden for all treatment groups and estrogen versus Surgilube®. Tumor burden between mice with intact ovaries (Sham) and mice that were ovariectomized (OVX) did not significantly differ ($p=0.26$) between topical treatment groups throughout the experiment. For further statistical analyses mice were re-grouped based on UVB status and topical treatments regardless of endogenous estrogen status.
Figure 12. Tumor grades in estrogen or Surgilube® treated SKH1 mice after chronic UV radiation. The percentage of tumors graded malignant was significantly (#, p=0.03) higher in mice receiving topical estrogen at 31.43 ± 3.55 compared to 17.07 ± 2.39 for mice receiving topical Surgilube®.
Figure 13. Representative photomicrographs of benign cutaneous neoplasms classified as papillomas (pap) grades 1-3. Hematoxylin and eosin 4X and 10X.
Figure 14. Representative photomicrographs of malignant cutaneous neoplasms classified as microinvasive squamous cell carcinoma (MiSCC) grades 1-3, squamous cell carcinoma (SCC), and spindle/anaplastic cell sarcoma. Hematoxylin and eosin 4X and 10X.
Figure 15. Prostaglandin E₂ levels in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation. Although not statistically significant, levels of PGE₂ in mice treated with topical estrogen were higher than mice treated with topical Surgilube®. In addition, mice that were treated with topical estrogen and exposed to UVB radiation had significantly (p<0.01) increased amounts of PGE₂ compared to mice that were not exposed to UV radiation and treated topically with estrogen.
Figure 16. Hydrogen peroxide levels in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation. There were no differences in levels of hydrogen peroxide between treatment groups.
Figure 17. Myeloperoxidase in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation. Levels of MPO were significantly decreased (*, p<0.001) in UVB irradiated mice treated with estrogen compared to mice treated with Surgilube®. Myeloperoxidase was also significantly decreased in mice exposed to UVB radiation compared to mice not exposed and treated with Surgilube® (#, p=0.019) or estrogen (p<0.001, #)
Figure 18. p53 levels in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation. The mean number of foci of p53 in the skin surrounding tumors was significantly (*, p<0.01) elevated in mice exposed to UV radiation averaging 0.0057% ± 0.0043 compared to 0% p53 foci in mice not exposed to UV radiation. The percentage of p53 foci was not different (p=0.6) between treatment groups and mice treated with estrogen 0.053% ± 0.0047 or Surgilube® 0.063% ± 0.0040.
Figure 19. Ki67 levels in SKH1 mice treated with estrogen or Surgilube® after chronic UV radiation. There was no significant difference (p=0.7) between the percentage of Ki67 positive cells in the skin of mice treated with estrogen 16.81% ± 1.36 or surgilube 17.48% ± 0.977
Chapter 4: Discussion

The incidence of skin cancer has been increasing at an alarming rate in the United States with an estimated one million new cases each year.\textsuperscript{18} When acutely damaged by UV light, the skin cells can respond to the damage by repairing DNA or inducing apoptosis to remove potential mutagenic cells from the skin if the damage is too great.\textsuperscript{31} Failure to control the damage associated with UV radiation can result in the loss of control over proliferation and lead to tumor development through the activation of oncogenes or inactivation of tumor suppressor genes.\textsuperscript{34} UV radiation also causes immunosuppression in the skin which creates a favorable environment for the development and progression of tumors. Direct and indirect DNA damage and the resulting inflammation due to chronic UV irradiation causes skin aging, mutations in oncogenes and tumor suppressor genes, and skin carcinogenesis.\textsuperscript{34}

High levels of endogenous estrogen have been linked to various cancers including breast,\textsuperscript{2,26,28,35} uterine,\textsuperscript{17} and ovarian cancer.\textsuperscript{15} With the long term use of estrogen based hormone replacement therapy, the relative risk of breast, uterine, or ovarian cancer is increased.\textsuperscript{52} Estrogen based HRT is now being supplied as a topical cream or gel and this formulation is the number one prescribed estrogen HRT in Europe and Canada. However the use of estrogen, a known carcinogen, on skin exposed to a complete carcinogen, UV light, has not been previously studied. The purpose of this study was to evaluate topical estrogen applied to skin previously exposed to UVB light. Package inserts for topical estrogen based HRT do not mention protecting the area of estrogen application from direct sunlight. However the experimental design was modeled after a compliant patient who would take precautionary measures to limit their current exposure to UV radiation but did previously have chronic UV exposure as a young adult.

The results of the acute study show the progression of inflammatory mediators and markers of direct DNA damage over time after exposure to UV radiation. The peak
inflammatory response to UV radiation occurs 24-48hr post exposure and is visibly evident with edema, erythema, and increased sensitivity in the skin. Increased vascular dilation and permeability following UV radiation causes erythema and tissue leukocytosis. An increase in tissue white blood cells, particularly neutrophils account for the increase in inflammatory products within the tissue following UV radiation. Neutrophils release myeloperoxidase which in turn catalyzes the production of hypochlorous acid and tyrosyl radicals using hydrogen peroxide. These products are cytotoxic and can contribute to the inflammatory damage. In response to UV damage to the skin, PGE₂ is generated through a COX-2 controlled enzymatic cascade resulting in increased vascular permeability and hyperalgesia. In general, our data shows that increases in inflammatory mediators are directly related to exposure to UV radiation. While most inflammatory mediators measured do not significantly vary with topical treatments of estrogen or Surgilube®, levels remained significantly elevated for a longer period of time in mice treated with topical estrogen compared to mice not exposed to UV radiation. This suggests that estrogen may be slowing the recovery of these inflammatory products to their No/UV levels. In addition, hydrogen peroxide was significantly elevated at 24 hours post UVB exposure and topical estrogen treatment compared to mice treated with surgilube. Overall, this suggests that estrogen may play a role in increasing the onset of select inflammatory mediators and indicators of oxidative stress after UV radiation and delaying recovery. Increased proliferation in keratinocytes, assessed by the presence of Ki67, is maximal at 48hr after UV radiation of the skin and parallels hyperplasia. The hyperplastic effect results in an overall reduction in the barrier function of the skin.⁶ UVB radiation causes deleterious effects on permeability barrier function as evidenced by increased transepidermal water loss. The decreases in barrier function appear to be dependent on epidermal hyperplasia and thymocyte mediated responses.²¹

DNA acts as a chromatophore and directly absorbs UV radiation the instant it reaches the skin. Thus the formation of CPDs is immediate. As seen in the acute study, peak levels of CPDs were found at 24 and 48hrs and decreased over time as the body repaired the damaged DNA. These premutagenic lesions alter the structure of DNA, which inhibits DNA replication. The lesions can be repaired by nucleotide excision repair
but if left uncorrected the dimers are considered mutagenic.\textsuperscript{16} The p53 gene is the most frequently mutated gene in human cancers and it functions as a sensor for the integrity of the genome. p53 is activated by different forms of cellular stress including DNA damage and its actions are associated with cell cycle arrest, rendering time for damage repair, or with induction of apoptosis to prevent division of the damaged cells.\textsuperscript{31} The observed increased levels of p53 24 hours post acute UV radiation were present in both topical treatment groups, Surgilube\textsuperscript{®} and estrogen. However levels of p53 remained significantly elevated in mice with topical estrogen treatment for 48 hours post UVB exposure compared to only 24 hours with Surgilube\textsuperscript{®} treatment.

The results of the chronic study show that although the use of topical estrogen applied to mouse skin previously exposed to UVB radiation does not significantly increase or decrease the incidence of cutaneous neoplasms, there was an overall increase in tumor burden as well as a significant increase in the histologic grade of tumors in mice treated with estrogen. SKH1 mice treated with topical estrogen following UVB exposure developed more malignant cutaneous neoplasms than the mice treated with the vehicle. The endogenous estrogen status of the mice in this study did not have an effect with the topical treatments suggesting that at least in the murine model a pre-menopausal or post-menopausal status does not result in an increased risk of developing skin cancer after the application of topical estrogen. However, the outbred nature of the mouse model, with its inherent increased biological variability and the limited study numbers, may account for the lack of statistical significance of tumor burden. The significantly lower levels of myeloperoxidase in mice chronically exposed to UVB radiation and treated with estrogen do support a current theory that estrogen can act as an anti-inflammatory agent by disrupting neutrophil endothelial adhesion.\textsuperscript{37} This disruption decreases the number of neutrophils migrating to areas of inflammation through diapedesis and ultimately lessens the effects of oxidative stress due to inflammation. Furthermore, levels of PGE\textsubscript{2} were significantly elevated in mice chronically exposed to UVB radiation and treated topically with estrogen compared to mice not exposed to UVB radiation and treated with estrogen. This suggests that estrogen may play a role in modulating select inflammatory mediators when applied after UVB radiation. In addition, mice were exposed to UVB radiation for
10 weeks prior to the application of topical therapy. During the 15 week topical treatment mice were no longer exposed to UVB radiation. All inflammatory damage was done prior to the start of topical treatments.

However, this study suggests that estrogen may be increasing the severity of tumors through inhibition of DNA repair as shown by the increased levels of p53 in the skin surrounding tumors and increased levels of p53 in the actual tumors. Functional inactivation of p53 through relocalization to the cytoplasm has been observed with estrogen treatment. By acting as a proliferating agent, estrogen may drive cell proliferation to diminish cell cycle checkpoint function and accompanying DNA repair activities resulting in increased tumor grade.

Although the results of this study did not reach statistical significance as a result of the low sample size because of mortality and the outbred nature of the mouse model, the clinical relevancy of developing more malignant versus benign neoplasms is important. Many of the results in this study were trending towards significance and if repeated with a larger sample size, statistically significant results may be achieved. This study also uses an outbred stock of mouse, which models the variability observed in humans and contributes to the inherent biological variability that decreases the power of this study. Future studies are needed to investigate biological causes for the increased severity of neoplasms in SKH1 mice treated with topical estrogen. It is possible that a particular biomarker might correlate with tumor severity. Additionally, women with increased levels of such a biomarker might have an increased risk of developing malignant neoplasms should they apply topical based estrogen products. This biomarker could then potentially be used in humans to develop individualized treatment plans.
REFERENCES


carcinogenesis is associated with decreased CpG island methylation and increased mRNA and protein expression of the colonic vitamin D receptor. Oncology Research, 11(6), 255-264.


