THE EFFECTS OF BLACK RASPBERRY EXTRACT ON UVB-INDUCED INFLAMMATION AND CARCINOGENESIS

DISSERTATION

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By

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ABSTRACT

Non-melanoma skin cancers (NMSCs), especially squamous cell carcinoma (SCC), are a significant problem in the general population, and a deadly one among immunosuppressed populations. Nearly a third of transplant patients die from aggressive SCCs, and the risk for development increases as the length of time under immunosuppression increases. It is not yet fully understood how immunosuppression impacts UVB-induced inflammation and carcinogenesis. In Chapter 2, we determine the impact of different immunosuppressive drugs on UVB-induced inflammation and carcinogenesis. We found that cyclosporine (CsA) exacerbates all markers of inflammation and carcinogenesis, including angiogenesis. Mycophenolate mofetil (MMF), when co-administered with CsA reduced the markers of inflammation and carcinogenesis to near vehicle levels. Choice of immunosuppression, therefore, plays a role in the development of SCC in transplant patients.

The changes wrought in keratinocytes by the change from normal cell to malignant cell are poorly understood. Understanding the changes in cytokine and chemokine expression, as well as the changes in programmed cell death pathways would give new avenues for treatment and prevention. To expand the knowledge of these changes, we used an in vitro system of normal (JB6) and pre-malignant (308) keratinocytes in the
presence and absence of UVB (Chapter 3). We found that 308 cells produce more inflammatory cytokines than JB6 cells, and 308 cells have an altered expression of autophagy proteins. These changes may illustrate the steps necessary for malignant transformation.

Preventing the transformation of normal cells would be a life saver to the general population. The use of a treatment that would not interfere with immunosuppressive medications would be a boon to transplant patients. To address both of these we developed a post-UVB-exposure treatment derived from black raspberries (BRE). In Chapter 4, we explore the efficacy of BRE in the prevention of UVB-induced inflammation and carcinogenesis. BRE is able to reduce all markers of inflammation, with the exception of neutrophil infiltration. BRE is also able to reduce carcinogenesis, possibly through a reduction in tumor-infiltrating regulatory T cells.

In Chapter 5, we further explored the mechanisms behind the extract’s ability to reduce the effects of UVB exposure. We again used the in vitro system of JB6 and 308 cells in the absence or presence of UVB. BRE was able to alter the cytokine and chemokine production of both cells lines whether UVB was used or not, but the results did not always match in vivo results. BRE also was able to return the expression of autophagy proteins in 308 cells more to a pattern resembling JB6 cells. We also used acutely exposed dorsal skin and UVB-induced tumors in attempt to confirm the in vitro findings. The in vitro and in vivo data correlated well.
DEDICATION

This work is dedicated to Erin, my wife. You are the source of all that is good in my life. I would not have been able to do this without your love and support. I can never thank you enough. I love you.
I would like to acknowledge all of the members of the Oberyszyn lab, past and present for their help in the completion of the work contained in this document. Their help in the carrying out of the experiments, and the humor offered when the experiments didn’t work got me through the hard times. Having someone to share the good times with only made them better.

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Immunosuppressants Influence UVB-Induced Tumor Size Through Effects on


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LIST OF ABBREVIATIONS

8−oxo-dG  8-hydroxydeoxyguanosine
α  alpha
AIDS  acquired immuno-deficiency syndrome
APC  antigen-presenting cell
Atg  autophagy related protein
β  beta
BCC  basal cell carcinoma
BRE  black raspberry extract
°C  degrees Celsius
CD  cluster of differentiation
CPD  cyclobutane pyrimidine dimer
CsA  cyclosporine
DAPI  4′,6-Diamidino-2-phenylindole dihydrochloride
EDTA  ethylenediaminetetraacetic acid
ELR  glutamic acid leucine arginine motif
EtOH  ethanol
γ  gamma
g  gram(s)
H₂O₂  hydrogen peroxide
HIV  human immuno-deficiency virus
HNSCC  head and neck squamous cell carcinoma
HRP  horseradish peroxidase
HTAB  hexadecyltrimethylammonium bromide
IL  interleukin
i.p.  intra-peritoneal
J  Joule
kg  kilogram
KC  keratinocyte-derived chemokine
L  liter(s)
LC  Langerhans cell
LC3B  light chain 3 B
m  meter
mL  milliliter
mm  millimeter
μg  microgram
M  moles per liter
MCP  monocyte chemotactic protein
MED  minimal erythemal dose
miSCC  micro-invasive squamous cell carcinoma
MMF  mycophenolate mofetil
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<td>mycophenolic acid</td>
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<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>NFκB</td>
<td>nuclear factor kappa B</td>
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<td>nm</td>
<td>nanometer</td>
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<td>NMSC</td>
<td>non-melanoma skin cancer</td>
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<td>PCD</td>
<td>programmed cell death</td>
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<tr>
<td>PGE$_2$</td>
<td>prostaglandin E 2</td>
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<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<td>RNS</td>
<td>reactive nitrogen species</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>reverse transcriptase polymerase chain reaction</td>
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<td>SCC</td>
<td>squamous cell carcinoma</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>SE</td>
<td>standard error</td>
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<td>SRL</td>
<td>sirolimus</td>
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<td>TAM</td>
<td>tumor-associated macrophage</td>
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<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>UVRAG</td>
<td>ultraviolet radiation associated gene</td>
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CHAPTER 1

Introduction

Skin

The skin is the largest organ in the body, and serves a critical role as a barrier to infectious agents, toxins and carcinogens. The first layer of the skin, the epidermis, is comprised mainly of keratinocytes and Langerhans cells. In humans, the epidermis is comprised of keratinocytes of various stages of differentiation. As keratinocytes progress outward from the basal layer, they lose their organelles and become cornified as they move to the outermost layer.

As keratinocytes act as the main barrier between the outside world and the interior of the body, they have to be a multi-functional cell. Keratinocytes can produce a broad range of cytokines and chemokines in response to extracellular pathogens, during the wound healing process and in response to UVB exposure\textsuperscript{1-4}. Keratinocytes are known to produce chemotactic factors for neutrophils and cytokines to activate the neutrophils once they reach the dermis\textsuperscript{5}. They also produce cytokines to enhance the inflammatory response. The complete array of changes that occur in the keratinocyte to transform it into a malignant cell are as yet unknown.

Beneath the epidermis is the dermis, comprised of many cell types, extracellular matrix, blood and lymphatic vessels. The dermis gives support to the epidermis, both
Figure 1.1: Anatomy of the skin. (A) resting state and (B) after UVB exposure.
physical and in the form of nutrients from blood vessels. Blood vessels also serve as routes of infiltration for trafficking cells during the inflammatory response. The majority of trafficking cells leaves the blood vessels of the dermis and do not travel far from the blood vessels.

The function of neutrophils during an inflammatory response is to remove invading pathogens. To do this, neutrophils generate large quantities of reactive oxygen and reactive nitrogen species (ROS and RNS)\(^6\). The ROS go, in part, to fuel the production of hypochlorous acid. Neutrophils express an enzyme, myeloperoxidase (MPO) that converts ROS into hypochlorous acid\(^7\). Experimentally, MPO is used as a marker for neutrophil activation. Neutrophils also carry secretory granules and lysosomes that contain substances (proteinases, collagenases, cytokines, chemokines and various receptors) to aid in the inflammatory response\(^8\). These granules are released after the neutrophil is fully activated.

**UV Light**

Ultraviolet light is composed of three distinct wavelengths: UVA (400-320 nm), UVB (320-280 nm) and UVC (208-100 nm). UVC is blocked by the ozone layer, but is often used as a germicidal agent in research laboratories. UVA is the majority (~98%) of the sunlight that reaches the surface of the earth. UVA can penetrate through the epidermis down to the dermis, and over time cause wrinkling of the skin. UVA damages collagen by inducing the production of reactive oxygen species and initiating the production of matrix metalloproteins, many of which are collagenases\(^9\). ROS species activate AP-1, which impairs collagen synthesis\(^10\). Collagen damaged by UVA alters the
structural integrity of the skin and inhibits the production of fresh collagen to replace the UVA damaged collagen\textsuperscript{11}. UVB makes up the remaining 2\% of sunlight that reaches the earth, but it is the primary cause of squamous cell carcinoma, and may play a role in other diseases (i.e. triggering cutaneous lupus)\textsuperscript{12}. UVB initiates a strong inflammatory response in the skin, resulting in edema, neutrophil infiltration and activation, and DNA damage (direct and indirect)\textsuperscript{5}. The danger in UVB is that this response is initiated by every exposure. Many groups have shown that chronic inflammatory states are associated with or enhance the development of cancer in a number of organ systems, and this has been shown to be true in the development of skin cancer as well\textsuperscript{13,14}.

### Acute and Chronic Effect of UV on the Skin

There are two broad categories of skin cancer: melanoma and non-melanoma skin cancers (NMSCs). NMSCs can be further broken down into more categories, the two most prevalent of which are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). In the US alone, there are over one million new cases of NMSCs diagnosed each year. In the general population, BCC is the more frequently diagnosed cancer, but SCC is more deadly. In transplant patients, SCC is more prevalent than BCC.

Although UVB only makes up about 2\% of the light that reaches the surface of the earth, it is the predominant cause of SCC. It is more deadly than BCC because BCC rarely metastasizes, while SCC may metastasize, especially in certain patient subsets. In transplant patients, it is thought that the immunosuppressants used to prevent allograft rejection contribute to the more aggressive nature of SCC in transplant patients. Part of
our work is aimed at elucidating the role of different immunosuppressants in UVB-induced inflammation and carcinogenesis.

Chronic inflammation has been linked to the development of cancers, including NMSCs. There are many reasons why this is thought to be. Inflammation in the skin produces ROS, which damage DNA\textsuperscript{15}. This damage results in the formation of 8-oxodeoxyguanosine adducts. UVB can also directly damage cutaneous DNA.
**Figure 1.2:** DNA damage initiated by UVB. (A) 8-oxo-dG, initiated by ROS. 6-4 photoproduct (B) and cyclobutane pyrimidine dimer (C), formed by DNA exposure to UVB. Taken from Wu LL et al, Clinica Chimica Acta 339 (1-2): 1-9 and Cadet J et al Mutation Research 571(1-2): 347.
Direct DNA damage is caused by the absorption of UVB by DNA, which results in the formation of 6-4 photoproducts and cyclobutane pyrimidine dimmers (CPD) (Figure 1.2B and C). While there are cellular mechanisms that repair DNA damage, over the time course of chronic UVB exposure the imprecise repair of UVB-induced adducts results in mutations and ultimately tumor development.

The inflammatory response initiated by UVB in the skin initiates the release of a number of cytokines and chemokines. It has been shown in a number of organ systems that the cytokines released during an inflammatory response can promote the formation and growth of tumors. Interleukin 1-alpha (IL-1α), IL-6, IL-8 (the human equivalent of murine keratinocyte-derived chemokine or KC), monocyte chemotactic protein 1 (MCP-1), IL-23 and IL-17 are all released during inflammatory responses (including those caused by UVB exposure), and all have been shown to promote carcinogenesis\textsuperscript{16-18}.

One of the ways that these factors promote carcinogenesis is through the induction of angiogenesis, the growth of new blood vessels. To grow past a certain size, a tumor needs to have a ready supply of nutrients, which can only be supplied by a blood vessel\textsuperscript{19}. The larger the tumor grows, the larger and more numerous the supporting blood vessels need to be. Angiogenesis can occur during the acute inflammatory response, but for it to be sustained there must be the involvement of other factors, such as the ones mentioned above\textsuperscript{20,21}. Without sufficient nutrient supply, the tumors will die off.
Apoptosis and Autophagy

The majority of DNA damage is caused by the direct effects of UVB\textsuperscript{5,22}. The cells that are damaged beyond repair have two ways to undergo cell death: apoptosis and autophagy. Both are programmed cell deaths (PCDs), but they are very different pathways. Autophagy can even be a pro-survival mechanism, as described below.

Apoptosis is a well-known cell death pathway controlled by the activation (cleavage) of caspases. Cells undergoing apoptosis shrink as the cytoskeleton is dismantled, their DNA is cleaved into small pieces and the cell surface expresses markers for phagocytosis by macrophages and other APCs.

Autophagy, on the other hand, is caspase independent and is initiated by nutrient deprivation\textsuperscript{23}. It is primarily a mechanism for cells to recover cellular building blocks, and can be used to remove damaged organelles and proteins to prevent cellular damage. It is tightly regulated by the mammalian target of rapamycin (mTOR), a protein downstream of Akt in the PI3K/Akt signaling pathway\textsuperscript{24}. Sirolimus, a potent immunosuppressive agent explored in this thesis, and other derivatives of Rapamycin are therefore able to initiate autophagy.

Beclin-1 is the main regulatory protein in the autophagy pathway\textsuperscript{25}. Once Beclin-1 is activated, other proteins in the pathway are activated and regulate each other. The end result of autophagy is the formation of the autophagolysosome, where organelles and proteins are degraded\textsuperscript{26}. Figure 1.3 shows a comparison between apoptosis and autophagy.
Transplantation

Transplantation, or at least the idea of transplantation, is a very old one. Many cultures have stories regarding the transfer of body parts from one person to another.
Figure 1.3: Autophagy and Apoptosis.
Perhaps the most well-known story is from Catholic tradition. Saints Damian and Cosmas successfully transplanted the leg of an Ethiopian cadaver onto Justinian, the deacon of Rome. Justinian’s leg had been removed after it had turned gangrenous. Not all mythological transplants were for medical reasons. A Chinese myth has a surgeon exchanging the hearts of a strong-willed and a weak-willed man in attempt to alter their strength of character.

Successful transplantation of an allograft was not achieved until the 20th century. There are two early reports of allografts to immunologically privileged sites (eye, testes), but it was not until the advent of the immunosuppressant cyclosporine that long-term survival of an allograft was possible. Since the early ground-breaking transplants, the number of transplants has increased dramatically. The Scientific Registry of Transplant Recipients estimates that at the end of 2007 there were roughly 200,000 people in the United States living with solid organ transplants (OPN/SRTR Annual Report).

There have been other immunosuppressants discovered (or in the case of mycophenolate mofetil (MMF), made) since cyclosporine. Cyclosporine, tacrolimus and sirolimus were all originally isolated from soil microbes. Sirolimus is an inhibitor of the mammalian target of rapamycin (mTOR). Sirolimus (and other rapamycin derivatives) block the T cells from responding to IL-2. MMF was not discovered; it is the first designed immunosuppressant. MMF is metabolized in the body to its active form, mycophenolic acid (MPA). MPA blocks the proliferation of T and B cells, and induces apoptosis in activated T cells. These immunosuppressants, and others, have been combined in various ways with each other and with steroids to combat the many deleterious side effects of the immunosuppressants. Cyclosporine and tacrolimus are
calcineurin inhibitors; their main effect is to prevent the production of IL-2, a cytokine critical for the activation of T cells\textsuperscript{31}. Cyclosporine has been shown to be nephrotoxic, cause hyperlipidemia, hypertension, hirsutism, and increase the risk for malignancies, such as skin cancer\textsuperscript{32-34}. In fact, skin cancer is the number one cancer these patients develop post-transplant\textsuperscript{35}. Transplant (and other immunosuppressed) patients are 60-250 fold more likely to develop NMSCs than the general population\textsuperscript{36-39}. However the reasons for cutaneous involvement are currently not well identified.

**Black Raspberry Extract**

To address the need for a treatment to reduce UVB-induced inflammation and carcinogenesis without affecting immunosuppressive medications, we developed a topical, post-exposure treatment derived from black raspberries. As the extract is derived from a natural product known to not affect drug metabolism pathways, the likelihood of interference with immunosuppressants is low.

Black raspberries are high in antioxidants known as anthocyanins (Figure 1.4), which are found in dark-pigmented fruits\textsuperscript{40}. Anthocyanins are powerful antioxidants, and black raspberries are among the fruits containing the highest levels of anthocyanins. Antioxidants are being explored as treatments or prophylactics in many diseases, including cancer\textsuperscript{41,42}.

Black raspberries have been shown to have a number of beneficial properties. Not only are they powerful antioxidants, but they also reduce inflammation by blocking signaling pathways, including NF\textsubscript{κ}B, which has been shown to be a key pathway linking chronic inflammation and cancer\textsuperscript{43,44}. Black raspberries also act directly on cancer cells,
inducing them to undergo apoptosis while having no effect on normal cells\textsuperscript{45}. An extract of black raspberries has been used in early clinical trials to treat oral intraepithelial neoplasias (IENs), with a therapeutically beneficial outcome\textsuperscript{46}. 
Figure 1.4: Anthocyanins. These are the three most potent anthocyanins present in black raspberries, with a general anthocyanin structure at the top. Taken from Hecht SS et al, Carcinogenesis, 27(8): 1617-1626.
SKH-1 Mouse

SKH-1 mice are commonly used in skin research models. The SKH-1 mouse is a hairless, outbred strain with a fully competent immune system (Figure 1.4). The hairless phenotype of the SKH-1 mouse results from a stably integrated retrovirus in intron 6 of the hairless gene\textsuperscript{47}. This results in impaired splicing of the transcript and hairloss. The mice are born with hair, but lose the hair with a few weeks after birth. The hairless mouse has been used extensively to explore skin diseases, including wound healing and photoaging\textsuperscript{48,49}. The bulk of research performed using SKH-1 mice, however, is in skin cancer, both photocarcinogenesis and chemically-induced carcinogenesis.
Figure 1.5: The SKH mouse.
The following chapters will describe our studies examining UVB-induced inflammation and carcinogenesis. Studies exploring the role of immunosuppressive drugs in UVB-induced inflammation and carcinogenesis to determine if the choice of immunosuppressive agent impacts either are described in Chapter 2. *In vitro* models were used to explore the changes in keratinocytes during the transformation to a papilloma cell (Chapter 3). In Chapter 4, the efficacy of BRE as an anti-inflammatory and chemopreventive agent was determined. In the final chapter, the effects of BRE on keratinocytes and papilloma cells exposed to UVB were explored.
Abstract

Immunosuppressive therapies allow long-term patient and transplant survival, but are associated with increased development of UV-induced skin cancers, particularly squamous cell carcinomas. The mechanisms by which CsA, MMF, tacrolimus (TAC) or sirolimus (SRL), alone or in dual combinations, influence tumor development and progression are not completely understood. In the current study, chronically UV-exposed mice treated with SRL alone or in combination with CsA or TAC developed more tumors than mice treated with vehicle or other immunosuppressants, but the tumors were significantly smaller and less advanced. Mice treated with CsA or TAC developed significantly larger tumors than vehicle-treated mice, and a larger percentage in the CsA group were malignant. The addition of MMF to CsA, but not to TAC, significantly reduced tumor size. Immunosuppressant effects on UVB-induced inflammation and tumor angiogenesis may explain these findings. CsA enhanced both UVB induced inflammation and tumor blood vessel density, while MMF reduced inflammation. Addition of MMF to CsA reduced tumor size and vascularity. SRL did not affect inflammation, but significantly reduced tumor vascularity. Thus the choice of immunosuppressants has important implications for tumor number, size and progression,
likely due to the influence of immunosuppressants on UVB-induced inflammation and angiogenesis.

**Introduction**

Transplant recipients have a 60–250 fold increased risk for developing non-melanoma skin cancer, and tend to develop multiple aggressive skin tumors that can be life threatening. Indeed, one Australian study estimated that 27% of heart transplant recipients who had survived greater than 4 years post-transplantation died of metastatic squamous cell carcinoma (SCC). Sun exposure is the main risk factor for non-melanoma skin cancer development in immunocompetent individuals. In addition to sun exposure, the level and duration of immunosuppression contribute to increased skin cancers in transplant patients. The mechanisms underlying this increased risk are not completely understood, nor is it known whether all immunosuppressive regimens increase skin cancer to the same extent.

Although less than 1–2% of the UV light from the sun is UVB radiation (290–320 nm), UVB is primarily responsible for the skin damage from acute and chronic sun exposure. One immediate physiologic consequence of UVB exposure is skin inflammation, characterized by edema and dermal neutrophil infiltration. Neutrophils contribute to skin inflammation by producing myeloperoxidase (MPO), reactive oxygen intermediates and pro-inflammatory cytokines (reviewed in), which can contribute to tumor growth by enhancing angiogenesis. Reducing inflammation
in these early stages reduces both angiogenesis (reviewed in 79) and tumor growth 14,71. However, once tumors are established, increasing the inflammatory response through the use of immune modulators has been shown to be an effective skin cancer treatment 55,80-82.

To determine how clinically relevant immunosuppressant treatments influence UVB-induced tumor development and progression, we assessed the effects of CsA, MMF, tacrolimus (TAC) and sirolimus (SRL) alone or in combination on tumor number, size, progression, angiogenesis and on UVB-induced inflammation. We observed that SRL-based regimens resulted in more tumors than vehicle (VEH) controls, but these tumors were significantly smaller. In contrast, CsA did not increase the number of tumors compared to VEH, but the tumors were larger and a greater percentage was malignant. Furthermore, CsA exacerbated UVB-induced inflammation and resulted in more vascular tumors compared to VEH, while the addition of MMF to CsA reduced both inflammation and tumor vascularity. Thus, our data indicate that specific immunosuppressant regimens influence tumor size and progression through reduced inflammation and tumor angiogenesis.

**Materials and Methods**

**Mice**

Adult female SKH-1 hairless mice (Charles River Laboratories, Wilmington, MA), 8–12/group, were maintained in an accredited Ohio State University vivarium. All
procedures were approved by the Institutional Laboratory Animal Use and Care Committee.

**UV Irradiation:** Mice were exposed to 2240 J/m² of UVB, generated by FS40UVB lamps (American Ultraviolet Company, Lebanon, IN) covered by Kodacel filters (Eastman Kodak, Rochester, NY). UVB levels were measured using a UVX radiometer (UVP Inc, Upland, CA).

**Immunosuppressants:** CsA (Sandimmune®, Novartis, Basel, Switzerland), MMF (CellCept®, Roche Pharmaceuticals, Nutley, NJ) and tacrolimus (TAC–Prograf®, Astellas, Deerfield, IL) were all purchased through The Ohio State University pharmacy; Sirolimus (rapamycin) (SRL) was obtained from LC Laboratories (Woburn, MA). CsA, MMF or vehicle (VEH, PBS) was injected i.p. once daily at a dose of 20 mg/kg/day, while TAC and SRL were given at a dose of 2 mg/kg/day. The immunosuppressants were either given alone or in the following combinations: CsA+ MMF, TAC + MMF, CsA + SRL, TAC + SRL. Drug doses were based on published effective immunosuppressive doses in rodents. Drug levels were not changed in combination therapies.

**Study design (Figure 2.1):** For acute studies, mice received immunosuppressants or VEH i.p. daily for 1 week prior to receiving 0 or 2240 J/m² UVB. Mice were euthanized, skin fold thickness determined and tissues recovered for analysis 48 h after UVB exposure. For carcinogenesis studies, mice received 2240 J/m² UVB thrice weekly for
23 weeks. Beginning in week 10, mice were randomly assigned to receive VEH, CsA, MMF, TAC, SRL, CsA + MMF, CsA + SRL, TAC + MMF or TAC + SRL i.p. once a day for the remaining 13 weeks. Beginning at week 11, tumors ≥ 1mm diameter were counted in a blinded fashion. Measurements were performed using digital calipers, and tumor area calculated multiplying length X width. Mice were euthanized and tissues collected for analysis at the end of week 23.

**Tumor staging:** Tumor stage was determined histologically in a blinded fashion by a board-certified veterinary pathologist (DFK). Grading of tumor progression is based on invasion. Exophytic papillomas stages 1–3 are considered premalignant. Micro-invasive SCC (miSCC) and SCC, which invades the panniculus carnosus are considered malignant.

**Myeloperoxidase (MPO) assay:** MPO activity was assessed as previously described. Briefly, MPO activity in a 10-mm$^2$ dorsal skin punch was measured over a 5-min period at 450 nm with a programmable microplate reader. Results are reported as the fold-increase over activity in matched, non-UVB-exposed controls.

**Fluorescent staining for CD31:** Frozen sections of tumors were fixed in acetone and blocked, then incubated with anti-CD31 antibody (BD Biosciences, San Diego, CA).
Figure 2.1: Schematic of experimental design.
Staining was visualized using goat anti-rat IgG Alexa Fluor 594 (Invitrogen, Carlsbad, CA). Vascularity was determined similarly to the method of Bolontrade et al.\textsuperscript{86} Images were captured at 200X (20X objective, 10X eyepiece) on a digital fluorescent microscope and loaded into Image J v1.38b (http://rsb.info.nih.gov/ij/), which was used to adjust the threshold so that only CD31\textsuperscript{+} staining was measured. A 640 X 640 pixel box was drawn and the percent of the pixels above threshold were measured. This was repeated four times for a total of five measurements/slide, with 8–12 slides/group. Images of a micrometer were captured under identical conditions and used to determine pixels/mm. The area in pixels was then converted to area in mm\textsuperscript{2}. Data are shown as the average area in mm\textsuperscript{2} X 10\textsuperscript{5} ± SE.

**Statistical analyses:** To determine if there were significant differences in the numbers of tumors per group, MPO activity or CD31 staining, normally distributed data were analyzed using Student’s \textit{t}-test, while non-normally distributed data were analyzed using the Wilcoxon rank-sum test. Individual p-values <0.025 were considered significant in the pair-wise comparisons. To determine if there were significant differences between groups with respect to the size of the tumors, a repeated measures analysis of variance model was fit to the data using the MIXED procedure in SAS (Institute, Cary, NC), version 9.1. The model assumptions of normality and equal variances of the residuals were checked and the data transformed using a natural log transformation. Outliers in the data still remained after transformation. The residuals from the model were still not normally distributed and this could have an effect on the p-values presented. The exact Wilcoxon rank-sum test was performed to compare diagnosis level between the groups.
and between the sizes of tumors. For each of these analyses, a Bonferroni adjustment was made to each set of comparisons to assure that the overall significance level remained at 0.05. Thus, to be significant, a p-value from a specific test had to be 0.025 or lower for the two-pair comparisons. For the comparisons with VEH, the p-value had to be 0.0063 or lower.

Results

Effects of immunosuppression on UVB-induced skin cancer

Tumor number and size: To assess the effects of different immunosuppressive treatments on tumor development in a well-established animal model that mimicked a common clinical situation, SKH hairless mice were exposed to UVB for 23 weeks, and immunosuppressed for the final 13 weeks. VEH-treated animals developed $12.7 \pm 3.5$ tumors per mouse (Figure 2.2). Neither CsA, TAC nor MMF significantly altered the number of tumors per animal, yielding $10.4 \pm 3.8$, $14.3 \pm 2.5$ and $14.9 \pm 5$ tumors per mouse, respectively. In contrast, SRL treatment, either alone or in combination with CsA or TAC, resulted in increased tumor numbers compared to VEH ($20.6 \pm 5.1$, $p = 0.002$; $21.0 \pm 7.6$, $p = 0.02$; $21.0 \pm 5.1$, $p = 0.002$, respectively). We also assessed tumor size in these treatment groups (Table 2.1). Tumors from CsA or TAC-treated animals were significantly larger than those from VEH-treated mice ($p < 0.001$ and $p = 0.0057$, respectively). In contrast, tumors from MMF-treated animals were not significantly different in size from those of VEH-treated animals. The addition of MMF to CsA significantly reduced the tumor size compared to CsA treatment ($p = 0.007$), such that
Figure 2.2: Average number of tumors/mouse at 23 weeks. SKH-1 hairless mice (8-12/group) were exposed to UVB and immunosuppressed as described in Materials and Methods. At the end of 23 weeks, tumor numbers were counted. Data are shown as the average number of tumors per mouse ± SEM. Data are combined from two independent experiments, and each group contains at least eight mice. * indicates significant difference compared to vehicle, p < 0.012.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of tumors</th>
<th>Median size, mm$^2$</th>
<th>Size range, mm$^2$</th>
<th>p-Value, compared to vehicle$^a$</th>
<th>p-Value of combination to calcineurin inhibitor alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH</td>
<td>89</td>
<td>3.97</td>
<td>1.0–72.52</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CsA</td>
<td>115</td>
<td>5.55</td>
<td>1.3–279.84</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>MMF</td>
<td>164</td>
<td>4.4</td>
<td>1.0–67.94</td>
<td>0.64, N.S.</td>
<td></td>
</tr>
<tr>
<td>TAC</td>
<td>129</td>
<td>5.09</td>
<td>1.2–34.98</td>
<td>0.0057</td>
<td></td>
</tr>
<tr>
<td>SRL</td>
<td>247</td>
<td>2.50</td>
<td>1.0–56.09</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>CsA + MMF</td>
<td>103</td>
<td>5.05</td>
<td>1.1–77.6</td>
<td>0.1216, N.S.</td>
<td>0.007</td>
</tr>
<tr>
<td>CsA + SRL</td>
<td>168</td>
<td>3.06</td>
<td>1.0–35.91</td>
<td>0.0028</td>
<td>0.001</td>
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<tr>
<td>TAC + MMF</td>
<td>141</td>
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<td>1.1–252.17</td>
<td>0.0022</td>
<td>0.7252, N.S.</td>
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<td>2.21</td>
<td>1.0–91.4</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$^a$Statistical analyses were performed on the log-transformed size data, as the sizes were not normally distributed. For comparisons with VEH, p-values less than 0.0003 were considered significant.
these tumors were not significantly different in size compared to VEH-treated mice. Addition of MMF to TAC did not significantly decrease tumor size compared to TAC alone. In contrast, all of the SRL treatment groups (SRL, CsA + SRL and TAC + SRL) had significantly smaller tumors than VEH (p < 0.001, p = 0.0028, p < 0.001, respectively).

Tumor progression: To determine if immunosuppression influenced tumor progression, three randomly selected tumors (one each small, medium, large) from each animal were staged on the basis of invasion $^8$. Figure 2.3A shows the stages of the benign and malignant tumors in each treatment group. Tumors from VEH-treated animals showed a range of benign and malignant phenotypes, with benign tumors being most prevalent and malignant tumors comprising 26.9% of the tumors examined. With the exception of CsA, similar patterns were observed with other immunosuppressants. Malignant tumors were the most prevalent tumors in CSA-treated animals, comprising 46.4% of the tumors. Addition of MMF or SRL to CSA reduced the percentage of malignant tumors from 46.4% to 26.9% and 22.2%, respectively. The percentage of malignant tumors in animals treated with SRL alone was only slightly decreased, but the percentage of stage 1 papillomas increased from 11.5% to 32%. Thus, it appears that CsA treatment may enhance tumor progression, but the addition of MMF or SRL reduces that enhancement. Further, SRL may slightly slow tumor progression, as stage 1 papillomas were increased in this group. Increasing tumor size is thought to correlate with increased malignancy. However, only pathological analysis can adequately determine tumor stage. Figure 2.3B demonstrates the association of tumor size with increasing malignancy. Overall,
Figure 2.3: Distribution of tumor stages. (A) Diagnosis of all tumors per treatment group. H&E-stained sections from formalin-fixed, randomly selected tumors (1 each small, 1-5 mm²; medium, 5-12 mm²; and large >12 mm²; if available) from each animal were staged by a board-certified veterinary pathologist as described in Materials and Methods. The percentage of each tumor stage was determined for each treatment group. White section = papilloma 1, light gray = papilloma 2, dark gray = papilloma 3, black = malignant (micro-invasive SCC and SCC combined). Minimaly, 24 tumors per group were analyzed. (B) Schematic representation of benign versus malignant diagnoses in small and large tumors. Small and large tumors from each animal were staged as described in Figure 3A, and the size relative to malignancy analyzed. Since papilloma stages 1-3 are considered benign, these stages are combined for ease of viewing and are shown as medium gray. Malignant tumors are shown as black bars. Significant differences between small and large tumors were observed overall (p < 0.001) and in the VEH (p = 0.0012), CsA (p < 0.001) and the MMF (p = 0.0029) groups, which are denoted by an asterisk.
malignant tumors were significantly increased in large tumors compared to small tumors (p < 0.001) or medium tumors (p < 0.001, not shown). As expected, there was a significant increase in malignancy going from small to large tumors in the control group VEH (p=0.0012). Further, both CsA (p<0.001) and MMF(p=0.0029) groups also showed a similar association of size with malignancy. Interestingly, there was no significant increase in malignancy comparing small versus large tumors in the TAC or SRL groups, or the CsA + MMF group. These data therefore suggest that TAC, SRL or CsA + MMF may slow conversion compared to VEH.

Effects of immunosuppression on the acute inflammatory response to UVB

MPO activity: UVB-induced inflammation, characterized by an increase in skin MPO activity, is linked to the development and progression of skin tumors. Since CsA-treated mice had larger tumors, we hypothesized that CsA might exacerbate UVB-induced inflammation (Figure 2.4). Compared to VEH, CsA significantly increased (p = 0.013), while MMF significantly reduced skin MPO activity (p <0.003). Neither TAC nor SRL altered MPO activity relative to VEH. However, the addition of either MMF or SRL to CsA reduced MPO activity to VEH levels. This reduction was statistically significant compared to CsA alone (p <0.02).

Blood vessel density: Chronic inflammation is associated with increased angiogenesis [76-78] that is essential for tumor growth and progression [87-89]. We hypothesized that the increased inflammation in CsA-treated animals would result in more vascular tumors than VEH. We assessed CD31 immunofluorescence in tumors from VEH, SRL, CsA,
Figure 2.4: Effect of immunosuppressant on skin MPO activity. SKH-1 mice were immunosuppressed and exposed to 2240 J/m² UVB as described in Materials and Methods. MPO activity in a 10 m² punch biopsy was determined as described in Materials and Methods. Data are shown as the average fold increase ± S.D. of UVB-exposed samples, compared to MPO activity in matched non-UVB exposed controls. Baseline, no UVD MPO activity is shown with a dashed line. Data are combined from at least three independent experiments. None of the treatments altered MPO activity in the non-UVB-exposed controls. SKH-1 mice were injected with VEH or immunosuppressants for 7 days prior to UVB exposure. **MIF significantly reduced MPO compared to VEH (p < 0.002). TAC, SRL, CsA + MMF, TAC + MMF and CsA + SRL had MPO activity similar to VEH, and significantly lower than CsA-treated skin (all p < 0.021).
MMF and CsA + MMF groups and calculated the percentage of each section staining positively for CD31 (Figure 2.5A). SRL treatment significantly reduced (p = 0.009) CD31 staining relative to VEH treatment. MMF treatment alone did not significantly alter CD31 staining, although there was a trend toward less staining (p = 0.032) compared to VEH. The addition of MMF to CsA resulted in significantly less CD31 staining compared to CsA and to VEH (p=0.002, p=0.017, respectively). Tumors from SRL-treated mice had less CD31 staining compared to VEH-treated mice (Figure 2.5B, panels a, b), indicating reduced angiogenesis in these tumors. In contrast, tumors from CSA-treated mice had increased CD31 staining compared to tumors from VEH-treated animals (Figure 2.5B, panel c). MMF did not significantly alter CD31 staining compared to VEH (panel d). However, the addition of MMF to CsA greatly reduced CD31 staining (panel e). Thus, our data indicate that CsA allows increased tumor growth and progression, likely due in part to an exacerbation of UVB-induced inflammation and angiogenesis, as shown by increases in MPO and CD31 staining, respectively. MMF does not appear to directly inhibit angiogenesis significantly, but can reduce the enhanced levels of inflammation and angiogenesis caused by CsA, and may thereby slow tumor growth. In contrast, SRL reduces tumor size in this de novo tumor model through an inhibition of angiogenesis likely separate from effects on inflammation.
Non-melanoma skin cancers, especially SCC, are dramatically increased in immunosuppressed populations, such as transplant recipients\textsuperscript{36,39,54,90}, HIV/AIDS patients\textsuperscript{51,91,92}, cancer patients\textsuperscript{50,93-96} and patients with autoimmune diseases\textsuperscript{97-100}. Organ transplant recipients have the highest risk for developing non-melanoma skin cancers, and skin cancers in these patients demonstrate more aggressive characteristics than skin cancers in non-transplant recipients, including early dermal invasion, an infiltrative growth pattern and greater depth of invasion at diagnosis\textsuperscript{101}. This is thought to be due to a combination of sun exposure and the continued administration of immunosuppressive drugs to prevent or treat organ rejection\textsuperscript{39,60}.

Using data from human transplant patients, it has been difficult to determine which specific immunosuppressive drugs are responsible for the increased skin cancer risk\textsuperscript{61} or to determine the general mechanisms underlying the increased risk\textsuperscript{61,102,103}. Most transplant recipients are adults when they receive their new organs, and have accumulated years of sunlight-related skin damage. Although some patients modify their behavior to use sunscreen and stay out of the sun, most do not\textsuperscript{104-106}. We therefore used the hairless mouse model of post-transplantation skin cancer to study the effects of specific immunosuppression regimes on UVB-induced inflammation and to mimic both previous and ongoing sun exposure of adult transplant patients. It is important to note that this is a \textit{de novo} model of tumor development, and the animals, other than being treated with immunosuppressants, are immunocompetent.
Figure 2.5: Effect of immunosuppressant type on angiogenesis. SKH-1 mice were immunosuppressed and exposed to 2240 J/m² UVB for 23 weeks as described in Materials and Methods. CD31 staining in frozen sections from small tumors (1-5 mm³) was determined as described. (A) Graphical representation of the area staining positive for CD31. Data are shown as average staining +/- SE from five fields per group. (B) Representative staining of a) VEH, b) CsA, c) SRL, d) MMF and e) CsA + MMF. 5 μm sections were stained as described. Dotted line represents the epidermis/dermis border. Blue = DAPI staining of nuclei, Red = CD31.
CsA is reported to promote tumorigenesi s through increased transforming growth factor β (TGF-β) production and decreased DNA repair and increased angiogenesis.

We expected that CsA-treated animals would develop more tumors than VEH-treated mice in response to chronic UVB exposure. Although CsA and VEH-treated mice developed similar numbers of tumors, the tumors were significantly larger in the CsA group (median sizes of 5.55 mm$^2$ compared to 3.97 mm$^2$) and overall, more of the tumors had progressed to malignancy (46.4% in CsA), compared to VEH-treated mice. TAC treatment also resulted in larger tumors compared to VEH treatment. MMF treatment alone had no effect on tumor number or size. Combining MMF with CsA significantly reduced the median tumor size from 5.55 mm$^2$ to 5.05 mm$^2$ (p=0.007), and MMF blocked the development of extremely large tumors. In contrast, combining MMF with TAC did not reduce tumor size or the variability in sizes. Some of the size differences, although statistically significant, were subtle, and may not be biologically important. CsA + MMF reduced the median size to 5.05 mm$^2$, which was not different from VEH. However, 5.09 mm$^2$ for TAC was significantly larger. There was a wide range of tumor sizes, with some tumors in the CsA and TAC + MMF groups becoming extremely large. These very large tumors were invariably malignant. Thus, the ability of MMF to prevent the growth of very large tumors may be important. The outbred nature of the hairless mice may partially explain the wide range of tumor sizes. Definitive comparisons of the different treatment groups for their effects on tumor progression are not possible, as we only had 26–30 tumors per group available for analysis and the p-values to achieve significance needed to be less than 0.0063. Nonetheless, there was a trend toward increased
malignancy with CsA and reduced malignancy with SRL, compared to VEH. Likewise, the addition of MMF to CsA appeared to ameliorate the increased malignancy observed with CsA alone, but this did not achieve statistical significance. We did observe a statistically significant association of increasing tumor size with increasing malignancy in the VEH, CsA and MMF groups, and among the different tumor sizes overall. Based on these data, we suggest that CsA specifically promotes tumor growth and progression, but that this effect can be ameliorated by the addition of MMF to the treatment regimen. Although we used established dosing regimens for all drug combinations, a general limitation of our studies is that we were unable to obtain systemic levels for all drug combinations. Therefore, we cannot rule out that blood levels of immunosuppressants may have influenced our results.

Interestingly, SRL treatment resulted in increased tumor numbers, but the tumors were significantly smaller than tumors in VEH-treated mice and more of these were stage 1 papillomas (32% vs. 11.5% for VEH). In contrast to VEH, the SRL treatment did not show a statistically significant association between increasing size and increasing malignancy, suggesting that SRL may slow or inhibit tumor progression. The increase in tumor number with SRL was unexpected based on previous reports indicating an anti-tumor effect of SRL. Many reports showing anti-tumor effects of mTOR inhibitors have assessed the growth of transformed cell lines injected into mice, including the landmark paper by Guba et al.\(^{108}\), which concluded that SRL limited tumor growth by inhibiting angiogenesis. Two reports have investigated mTOR inhibitors in \textit{de novo} tumor formation in genetically engineered mice. Koehl et al. showed an anti-tumor effect of SRL compared to MMF in p53\(^{-/-}\) mice; in these studies, mice treated with SRL had
reduced tumor burden compared to controls or to animals treated with CsA or MMF. Likewise, Mabuchi et al. showed that another mTOR inhibitor, everolimus, reduced tumor formation and progression in a transgenic model of spontaneous ovarian adenocarcinoma. These two reports did not directly assess the mechanism underlying this effect, but the findings are consistent with effects of mTOR inhibitors on angiogenesis. There are two main differences between our study and the previous reports. First, our source of mTOR inhibitor and mode of administration is different: Koehl et al. obtained SRL from Wyeth and Mabuchi obtained their mTOR inhibitor from Novartis, while we used a generic version of SRL obtained through LC laboratories. Secondly, Koehl et al. fed the SRL to the mice, while we administered SRL i.p. We cannot rule out the possibility that the fluctuation of SRL levels with i.p. injection influenced tumor development. Perhaps most importantly, both of the previous reports examined the spontaneous development of tumors in genetically engineered animal models, while our data were obtained from a non-genetically engineered model of UVB carcinogenesis closely paralleling the clinical situation. Mice were exposed to UVB for 10 weeks prior to being immunosuppressed, so much of the mutational damage had already occurred, similar to an adult transplant recipient who has already had a lifetime of sun exposure. Our data indicate that SRL does not prevent tumor appearance or, by inference, the mutational damage preceding tumorigenesis. However, the markedly reduced size of the tumors and the slightly delayed tumor progression suggested that SRL inhibition of angiogenesis may account for our observations. In addition, spread of the large tumors in the other treatment groups could mask several smaller tumors, potentially leading to a misleadingly low tumor number.
To investigate the mechanisms by which the different immunosuppressive treatments affected tumor size, we assessed the effects of immunosuppression on UVB-induced inflammation. The link between chronic inflammation from sun exposure and skin cancer development has been established in both humans\(^{111-113}\) and animal models\(^{71,114}\). Chronic inflammation is believed to promote genetic changes, tumor angiogenesis, tumor progression and metastasis (reviewed in \(^{76,77,115}\)). In animal models, administration of anti-inflammatory compounds, orally or topically, reduces UVB-induced inflammation in the skin and also reduces the number of subsequent skin tumors by 35–50\%\(^{14,116}\).

Inflammatory responses to UVB were measured in immunosuppressed hairless mice 48 hours after exposure. We analyzed edema and MPO activity, which are routinely used as indicators of the degree of the cutaneous inflammatory response\(^{84,85}\). Edema results from enhanced vascular permeability, while MPO is produced by neutrophils to catalyze the conversion of hydrogen peroxide to hypochlorous acid and is generally used as an indicator of neutrophil activation\(^{117}\). Only CsA increased both skin edema (not shown) and MPO activity. Calcineurin inhibitors like CsA are used to treat atopic dermatitis and other skin inflammatory conditions\(^{118}\); however, these are applied topically, not used systemically as was done in our study. Moreover, the two FDA-approved topical calcineurin inhibitor formulations are pimecrolimus and TAC, not CsA. We tested systemic TAC in our system and found that it had no effect on UVB-induction of MPO activity. Furthermore, CsA is reported to decrease DNA repair in the skin, and persistent DNA damage may independently lead to increased inflammation\(^{107}\). Interestingly, while SRL alone had no effect on MPO activity, the addition of SRL to CsA significantly reduced MPO activity compared to CsA alone.
MMF significantly reduced MPO activity, alone or when combined with CsA. Although MMF is used widely to prevent transplant rejection and was designed to inhibit inosine monophosphate dehydrogenase, it has multiple effects on many different immune cells (reviewed in 119). Farivar et al. reported that MMF treatment reduced MPO activity and leukocytic infiltration in a model of lung reperfusion injury, clearly demonstrating that MMF has anti-inflammatory properties 120. MMF is used in dermatology to reduce inflammation in dermatitis and vasculitis, and its efficacy is thought to be related to effects on endothelial cells that reduce neutrophil infiltration 121-123.

We used CD31 staining to assess tumor blood vessel density as a marker for angiogenesis in tumors from VEH, CsA, MMF, CsA + MMF and SRL groups. Tumors from SRL-treated mice had less, while tumors from CsA treated mice had more CD31 staining compared to VEH. These data confirm the findings of Guba et al. 108, in a physiologic, de novo tumor model. There was a trend toward reduced CD31 staining with MMF treatment, although it did not reach significance. Interestingly, when combined with CsA, MMF significantly reduced CD31 staining. Recently, Koehl et al. 124 observed that MMF had powerful, consistent anti-angiogenic effects on vascular endothelial cells in vitro, but had less consistent effects in an in vivo model using injected, transformed cell lines. These authors presented data suggesting that the different effects may relate to the bioavailability of MMF in vivo 124. We used significantly lower doses of MMF (20 mg/kg/day vs. 40 mg/kg/day and 80 mg/kg), so we cannot rule out that MMF bioavailability was limited in our studies. However, additional explanations are that MMF may act preferentially to reduce neutrophil activity rather than acting on endothelial cells to directly reduce angiogenesis. Thus, MMF may have variable direct
effects on angiogenesis *in vivo*. However, when CsA is present and neutrophil activity is dramatically increased, MMF could reduce the enhanced neutrophil infiltration and/or activity and therefore indirectly reduce angiogenesis. Alternatively, MMF may act on UVB-exposed and/or neoplastic epithelium, which elaborates several angiogenic factors.

Our experiment mimicked an adult transplant recipient who received regular UVB exposure throughout their life and did not alter their sun behavior post-transplantation. Thus, inflammation and DNA damage were repeatedly induced throughout the study. Although our experimental design may best reflect the current clinical situation, understanding the precise effects of immunosuppression on skin tumor formation and progression requires additional experimental designs. For example, it will be necessary to assess the effects of immunosuppression when UVB exposure precedes immunosuppression, as in transplant recipients with a lifetime of sun exposure who alter their sun behavior post-transplantation. Data indicate that when immunosuppressive treatments are administered in the absence of continued UVB exposure, CsA and SRL treatments both result in fewer tumors than VEH, but CsA tumors are larger, and SRL tumors smaller, than VEH (Wulff et al., in review). Likewise, immunosuppression may have different effects when administered concomitantly with UVB exposure from the beginning of the experiment, mimicking the experience of a pediatric transplant recipient. Preliminary data indicate that in this instance, SRL treatment results in fewer and smaller tumors than VEH (DeGruijl and Geissler, in review).

In conclusion, our data indicate that the choice of immunosuppressant administered after chronic UVB exposure can have significant effects on tumor number.
and size. It is likely that the influence of immunosuppressants on UVB-induced inflammation and angiogenesis play important roles in mediating these effects.

Acknowledgments

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CHAPTER 3

Alterations in Cytokine and Autophagy Profiles in Normal and Pre-Malignant Keratinocytes

Abstract

Keratinocytes are our first line of defense against UVB exposure, and they are consequently the cells most damaged by UVB. The alterations in the inflammatory profile that occurs during the transformation from normal keratinocyte to pre-malignant lesion are not completely understood. In an attempt to understand this process, we have explored the cytokine profiles and survival pathways in normal immortalized murine keratinocytes (JB6 cells) and a cell line established from a murine epidermal papilloma (308 cells), in the absence of UVB and after UVB exposure. In the current chapter, we show that at a resting state, 308 cells produce more transcript for Interleukin-6 (IL-6) and Interleukin-23 (IL-23) compared to JB6 cells. 308 cells produce more keratinocyte-derived chemokine (KC) KC than JB6 cells, and only 308 cells produce Interleukin 1-alpha (IL-1α). All cytokine production was increased after exposure to UVB, except IL-1α at 72 and 96 hours after UVB exposure. Compared to JB6 cells, pre-malignant 308 cells up-regulated autophagic proteins after UVB exposure. We confirmed these in vitro results by performing in vivo experiments. Dorsal skin from mice exposed to UVB showed a significant increase in KC. Tumors from mice exposed to UVB for 25 weeks showed differential expression of autophagic proteins compared to non-exposed skin.
Together, these data give some insight into the changes keratinocytes undergo after acute and chronic exposure to UVB, and how they change from normal keratinocytes to pre-malignant lesions.

**Introduction**

Keratinocytes are exposed daily to a number of environmental carcinogens, including light in the ultraviolet B (UVB) spectrum. Each encounter with UV light leaves its fingerprint upon the cells, in the form of mutations in the DNA. Exposure of skin to UVB results in two forms of damage. Direct DNA damage, in the form of 6,4-photoproducts and cyclobutane pyrimidine dimers (CPD) is a result of DNA absorbing light in the UVB spectrum. Indirect damage is caused by reactive oxygen species (ROS) initiated by UVB exposure, and results in the formation of 8-oxo-deoxyguanosine (8-oxo-dG) adducts. This is the initiation of the carcinogenic process.

UVB is a complete carcinogen. Not only does it initiate the carcinogenesis process by causing DNA mutations, it also promotes the process. This is due, in part, to the inflammatory cytokines and chemokines released from damaged keratinocytes following exposure to UVB. The damage to keratinocytes stimulates the production of chemotactic factors which call neutrophils into the dermis and fully activate them. In mice, important inflammatory chemokines are KC (CXCL1) and monocyte chemotactic protein-1 (MCP-1; CCL2), among others. The neutrophils release hypochlorous acid, which causes further tissue damage, and the furthering of the inflammatory cascade.
Inflammatory cytokines and chemokines have been shown by a number of groups to support the growth and progression of cancer cells. Pro-inflammatory cytokines such as IL-1$\alpha$ and IL-6 have been shown to promote angiogenesis and produce growth factors (such as PGE$_2$) that promote the growth and survival of transformed cells$^{71,130,131}$. A new subset of T cells, Th17, has also been shown to play an important role in the promotion of tumors$^{132-135}$. Th17 cells can induce IL-6 and IL-8 (KC in mice) in many types of cells, and they can be pro-angiogenic, and can increase tumor invasiveness$^{16,136,137}$. IL-6 and IL-23 are important in the differentiation and survival of Th17 cells$^{138,139}$, while IL-2 is critical for the survival of all T cells. The role of these cells in skin inflammation or cancer has not been explored.

Cytokines and cellular damage initiated by UVB exposure also play a role in the fate of damaged cells. Damage can be repaired, or the genetic changes can be incorporated as mutations. Cells that have been damaged beyond repair can die in one of three ways: necrosis, apoptosis or autophagy$^{140}$. Necrosis is not initiated by the cell; it occurs in response to damage caused by external factors (ie hypochlorous acid from neutrophils). Apoptosis and autophagy are forms of programmed cell death; they each follow set pathways in preparing the cell for death. Although it is associated with cell death, autophagy is also a pro-survival pathway, allowing the cell to survive nutrient-deprived conditions.

While apoptosis has been shown to occur in UVB irradiated skin, the process of autophagy is still an emerging concept in the skin. Autophagy is controlled by Beclin-1$^{141}$. After initiation by Beclin-1, two pathways act sequentially in the formation of the autophagosome, Atg12 and LC3B$^{142}$. These two pathways can regulate each other. After
the formation of the autophagosome, it eventually combines with a lysosome to form an autophagolysosome and the proteins and/or organelles are degraded.

Our lab and others have shown that chronic inflammation leads to the transformation of keratinocytes to pre-malignant and, eventually, malignant phenotypes. As yet, it is not fully known how the changes between the two cellular states gives the transformed keratinocytes a survival advantage over normal cells, although changes in tumor suppressor and oncogene expression certainly plays a major role. Understanding the steps that result in a change from a normal to a transformed keratinocyte is important in understanding how non-melanoma skin cancers form and progress. In the current work, we have explored the role of cytokines and survival pathways in JB6 (an immortalized keratinocyte cell line) and 308 (a pre-malignant papilloma cell line) cells in the absence of UVB and after exposure to UVB light.

Materials and Methods

Cells

The 308 cells were a kind gift from Dr. Stuart Yuspa, NIH/NCI, Bethesda, MD. They were developed from pooled papillomas initiated in BALB/c mice with DMBA and promoted by a phorbol ester. JB6 cells were procured from ATCC (Manassas, VA), and are also on a BALB/c background. JB6 cells were grown in EMEM (BioWhittaker, Walkersville, MD) supplemented with sodium pyruvate (Invitrogen, Carlsbad, CA), non-essential amino acids (Invitrogen), sodium bicarbonate (Invitrogen), penn/strep (Invitrogen), L-glutamine (Invitrogen) and FCS (Invitrogen). 308 cells were grown in
EMEM (BioWhittaker, Walkersville, MD) supplemented with calcium-chelated FCS (Invitrogen), antibiotic/antimycotic (Invitrogen), calcium chloride (Sigma-Aldrich, St. Louis, MO) and L-glutamine (Invitrogen). All cells were allowed to grow to near confluency before experimental protocol was initiated. Cells were exposed to 600J of UVB.

Skin

Six to eight week old female SKH-1 hairless mice (Charles River Laboratories, Wilmington, MA) were housed in a vivarium at The Ohio State University according to protocols established by the American Association for Accreditation of Laboratory Animal Care. Mice were housed at constant temperature and humidity levels. Food and water containing the antibiotic Baytril (Bayer HealthCare, LLC, Shawnee Mission, KS) was provided *ad libitum*. All procedures performed were approved by the Institutional Laboratory Animal Care and Use Committee.

*Carcinogenesis model*: Mice (n=10 per group) were exposed to one minimal erythemal dose (1 MED) of UVB, which was previously established in our lab as 2240 J/m². UVB levels were measured using a UVX radiometer (UVP Inc, Upland, CA). UVB light was generated by a bank of Philips FS40UVB lamps (American Ultraviolet Company, Lebanon, IN) covered by Kodacel filters (Eastman Kodak, Rochester, NY) to block UVC wavelengths. Groups of mice were exposed to UVB in large rat cages. The positions of these cages were rotated on a weekly basis to standardize exposure conditions. Mice received 1 MED of UVB thrice weekly on non-consecutive days for 25 weeks. Mice were sacrificed at 48 hours following the final UVB exposure, which is the
peak UVB-induced inflammatory time point. Non-irradiated age-matched mice were used as controls. Dorsal skin and tumors were snap frozen in liquid nitrogen for protein isolation.

**Acute inflammation model**: Mice (n=6 per group) were exposed to one MED of UVB (2240 J/m²). Mice were sacrificed 48 hours following UVB exposure. This time point was chosen because it represents the height of the acute UVB-induced cutaneous inflammatory response. Non-irradiated age-matched mice were used as controls. Dorsal skin was snap frozen in liquid nitrogen for protein isolation.

**RT-PCR**

The media was removed and either JB6 or 308 cells were washed with PBS before Trizol (Sigma Aldrich, St. Louis, MO) was added directly to the cells. The suspensions were pipetted repeatedly and collected into DNA/RNAsse free tubes (Fisher Scientific, Pittsburgh, PA). The tubes were placed into -80 for at least 24 hrs prior to extraction. The purity and quality of the RNA was assayed by the Microarray Core at OSU. The RNA then underwent reverse transcription, and the resulting cDNAs were used in PCR reactions using the following primer sets:

- IL-2 (forward: GCAGGATGGAGAATTACA; reverse: GTGTTGTCAGAGGCTTTTAGT)
- IL-2R (CD25) (forward: GCACCAGCAACTCCCATGACAAAT; reverse: TGGCCACTGCTACCTTAT)
- IL-4 (forward: GCAAGCAGAAACCCACCACAGAG; reverse: GCAAGACCTTCCAYCCAGTGGC)
- IL-6 (forward: GCAAGAGACTCCAYCCAGTGGC; reverse: TTACCTCTTGGTGAAGATATGA)
- IL-10 (forward:
GGACTTTAAGGGTTACTTGGGTTG; reverse: CCTTGGTCTTGAGCTTATT) and IL-23A (forward: CCAGCGGGACATATGAATCTAC; reverse: AGCTGTTGGCAGGAAGCCCTCAG). All results were normalized against HPRT (forward: AGTCCCAGCGTCGTGATTAGCGATGAT; reverse: TGGTTAAGGGTTGCAAGCTTGCTGG) and expression was quantified using the Kodak 1D image analysis software program.

**ELISA**

Supernatants from cell cultures were collected, filtered and stored at -20º C. ELISAs for IL-1α and KC were performed using matched antibody pair kits from R&D Systems (Minneapolis, MN). The PGE2 ELISA was obtained from Cayman Chemical (Ann Arbor, MI).

**Western Blots**

Culture media was removed and cells were washed twice in PBS. Adherence was broken using 0.5% Trypsin/0.25% EDTA solution and incubation at 37 ºC for 30 minutes. Cells were collected in 50 mL conical tubes by washing dishes with culture media. The tubes were spun at 1500 rpm for 7 minutes. Supernatant was removed and the cells were washed before a second spin. After the second spin, the supernatant was removed and cells were resuspended in NP40 buffer. The cell suspension was sonicated and placed on ice. After all samples were sonicated, the suspensions were placed into microcentrifuge tubes and spun at 13,500 rpm for 30 minutes. The resulting supernatant was then transferred to a new microcentrifuge tube and placed at -20 ºC.
Skin samples (10mm punch biopsies) were collected at the time of sacrifice and snap frozen in liquid nitrogen. Samples were placed into a 50 mL conical tube with NP40 buffer and homogenized. The resulting suspension then underwent the same sonication and spin protocol as above.

All samples underwent Bradford assays to determine protein concentrations. 40 μg of protein was loaded onto precast denaturing gels (Bio-Rad, Hercules, CA) with appropriate controls. Activated caspase 3 and LC3B assays were performed using 15% gels, while Beclin-1, Atg-5 and Atg-7 assays were performed using 10% gels. All antibodies were purchased from Cell Signal (Danvers, MA). Primary antibodies were used at 1:1000 in 5% BSA in TBST and were incubated overnight at 4 °C while rocking gently. Anti-rabbit IgG, HRP-linked antibody (Cell Signal) was used for detection at 1:2,000 in 5% milk in TBST. Blots were developed using Lumi-Glo (Cell Signal).

**Results**

*Pre-malignant keratinocytes transcribe pro-tumorigenic cytokines*

After forty-eight hours in culture after the addition of fresh media, 308 cells expressed more pro-inflammatory cytokines than JB6 cells (Figure 3.1). Figure 3.1A shows that 308 cells express roughly three times more IL-6 than JB6 cells, while Figure 3.1B shows that 308 cells transcribe twice the IL-23 of JB6 cells. Interestingly, at 72 hours, 308 cells begin to show transcript for IL-2 (3.1C). The expression of all cytokines was normalized to HPRT.
Figure 3.1: 308 cells express more message for inflammatory cytokines than JB6 cells. JB6 and 308 cells were grown to 90% confluency in 100 mm petri dishes as described. Fresh media was added and cells were harvested at 24 hour intervals. Harvested cells then underwent DNA isolation and RT-PCR as described in Materials and Methods. 308 cells produced more IL-6 (A) than JB6 cells at 48 and 72 hours, but not 24 hours. 308 cells produced more IL-23 (B) and IL-2 (C) message than JB6 cells at all time points. All levels were normalized to HPRT expression.
Pre-malignant keratinocytes express cytokines and chemokines that encourage tumor cell growth and recruit tumor stroma

308 cells produce more KC than MCP-1, while JB6 cells produce more MCP-1 than KC (Figures 3.2A-D). Only 308 cells secreted IL-1α (Figure 3.2E; JB6 not shown). While both cell lines produced PGE$_2$, 308 cells produced more than JB6s. All cytokine production increased after UVB.
Figure 3.2: Cytokine production is increased in both cell lines after UVB exposure. JB6 and 308 cells were grown to 90% confluence before half were exposed to UVB; the other half were used as No UV controls. Data resulting from ELISAs show that cytokine production increased in both cell lines after UVB exposure. B,D: JB6 cells. A, C, E, and F: 308 cells. ** = p<0.01, compared to No UV matched controls, as determined by Student’s two-tailed t test.
Pre-malignant keratinocytes down-regulate key proteins in the autophagic pathway in the absence of UVB, but up-regulate autophagic proteins after UVB

308 cells at 48 hours after the addition of fresh media had lower expression of Beclin-1, compared to the JB6 cells (Figure 3.3A). 308 cells produced less LC3B at both 24 and 48 hours (Figure 3.3B). After UVB exposure, 308 cells produced more Beclin-1 than the JB6 cells at all time points (Figure 3.3C). 308 cells produced more LC3B at 24 hours, but the expression was similar at 48 hours (Figure 3.3D). Similar to Beclin-1, Atg7 was higher in the 308 cells at all time points (figure 3.3E). Atg7 was not detectable in cells that had not been exposed to UVB.
Figure 3.3: JB6 and 308 cells express different levels of autophagy proteins. JB6 and 308 cells were grown in culture as described. Cells were collected at 24 hour time points and underwent protein isolation. Isolated proteins were used in Western blots to determine the expression of autophagy proteins. Densitometry data was generated using Image J. As 308 cells grow in culture, they express more Beclin-1 (A) and less LC3B (B) proteins over time, while JB6 cells express less of both proteins. After both cell lines were exposed to 600 J of UVB, 308 cells increased Beclin-1, LC3B and Atg7 (C-E) over time, while JB6 cells only increase LC3B levels (D).
There is no difference in apoptosis between normal and pre-malignant keratinocytes

Pre-malignant 308 cells did not show any difference in the levels of activated caspase 3 compared to normal JB6 cells via Western blot, either at a resting state or after UVB exposure at 24, 28, 72 or 96 hours (data not shown).

Correlation between in vitro and in vivo observations

In dorsal skin isolated from mice acutely exposed to UVB, levels of LC3B and Beclin-1 decreased, compared to mice unexposed to UVB (Figures 3.4A and 3.4B). In tumors isolated from mice exposed to UVB for 35 weeks, Beclin-1 and Atg7 were reduced, compared to unexposed mice (Figure 3.4C and 3.4D). Only LC3B showed an increase in mice chronically exposed to UVB (Figure 4E).

Whole dorsal skin from mice exposed to a single MED of UVB showed a decrease in IL-1α levels (3.4F), and an increase in KC levels (3.4G).
**Figure 3.4**: Correlation between *in vitro* and *in vivo* data. SKH-1 mice (n=5) were exposed to 2240 J/m² of UVB. 48 hours later, the mice were sacrificed and dorsal skin was harvested for protein isolation. Autophagy protein expression was determined via Western blot. UVB decreases autophagy protein expression in the dorsal skin of mice exposed to UVB (A and B). SKH-1 mice were exposed to one MUD of UVB thrice weekly for 26 weeks, and protein was isolated from the resulting tumors. Beclin-1 and Atg7 expression is decreased in tumors (C and E), while LC3B expression is increased (D). IL-1α expression is decreased in the dorsal skin of mice acutely exposed to one MED of UVB (F), while KC expression is increased (G).
IL-6 has garnered a great deal of research in the last few years. Chung et al showed that IL-6 is produced by keratinocytes and dermal fibroblasts in response to UVB \textsuperscript{2}. IL-6 has since been shown to be associated with carcinogenesis following chronic inflammation \textsuperscript{13,144} and IL-6 levels have been shown to be elevated in head and neck squamous cell carcinomas (HNSCCs)\textsuperscript{145}. Tumor-associated macrophages (TAMs) have been shown to be at least one of the major producers of IL-6 in the HNSCCs\textsuperscript{146}. TAMs are macrophages that have been conditioned by transformed cells to produce growth factors that promote the growth and survival of tumor cells, and to produce cytokines that dampen the host immune response to the tumor\textsuperscript{147}. We have shown in our current study that papilloma cells themselves can produce IL-6 transcript, showing that papilloma cells can secrete cytokines that promote the growth and survival of cancer cells.

In the current study, we show that papilloma cells (308 cells) produce roughly twice the transcript for IL-23 than keratinocytes. It is interesting that keratinocytes (JB6 cells) produce IL-23 transcript, as this suggests the IL-23/IL-17 pathway may be important in normal skin homeostasis. One report has shown that the receptor for IL-17, IL-17RC, is present in many normal human tissues, including the skin\textsuperscript{148}. The increase in IL-6 and IL-23 message in 308 cells may suggest an increased role for Th17 cells in skin cancer. A larger sample size and repeated experiments are needed to confirm the trends we have reported here. Also, message does not always equal protein. It is therefore important that any future studies undertaken to look more closely at the role of Th17 cells in normal skin and skin tumors look at the expression of these proteins.
directly. There is a lack of information on whether these novel cells play a role in UVB-induced inflammation and carcinogenesis, so further work is necessary.

KC (keratinocyte-derived chemokine) is one of the primary neutrophil chemoattractants in mice. Our lab and others have shown that it may also be important for the full activation of neutrophils once they reach the site of inflammation (see Chapter 4). Mice exposed to UVB and treated with a natural anti-inflammatory compound (black raspberry extract, BRE) had significantly reduced levels of KC in dorsal skin compared to mice treated with vehicle. This reduction in KC did not lead to a reduction in the number of neutrophils infiltrating the dermis of mice treated with BRE; in fact, there was a slight (but not significant) increase in dermal-infiltrating neutrophils in BRE-treated mice. However, there was a decrease in the level of MPO produced by the infiltrating neutrophils in mice treated with BRE, compared to vehicle. It therefore seems KC may play more of a role in inflammation than just neutrophil homing, namely neutrophil activation.

Other groups have shown that KC alters the tumor microenvironment, enhancing tumor growth, including the promotion of angiogenesis\cite{149-151}. The human KC equivalent, IL-8, has been shown to be elevated in patients with SCC and in SCC cell lines\cite{151,152}. IL-1\alpha has been shown to increase KC production, and KC production correlates with metastatic potential\cite{153}.

In our work, we have shown that 308 cells secrete more KC than JB6 cells irrespective of UVB exposure. This, when taken into account with the cytokine data, begins to give an insight into the tumor microenvironment in SCC. An environment rich in IL-1\alpha and KC leads to more aggressive tumors, while an environment rich in IL-23
and KC can recruit neutrophils that can secrete growth factors promoting tumor cell
growth, such as TNF-α and IL-6\textsuperscript{154-156}. The secretion of IL-6 can then promote Th17 cell
differentiation, furthering tumor promotion.

Monocyte chemoattractant protein 1 (MCP-1, CCL2) is an ELR\textsuperscript{+} (glutamic acid
leucine arginine motif) CC chemokine that induces the migration of T cells, basophils,
mast cells, dendritic cells and Langerhans cells\textsuperscript{157}, and can be secreted by fibroblasts,
endothelial cells, monocytes, macrophages and tumor cells\textsuperscript{158}. ELR\textsuperscript{+} chemokines
promote angiogenesis\textsuperscript{151}. MCP-1 is a cellular protein whose role in cancer is difficult to
determine. Zachariae et al found that \textit{in vitro} the addition of MCP-1 to macrophages
enhanced their anti-tumor properties\textsuperscript{159}, and Rollins et al found that MCP-1 blocked
tumor growth\textsuperscript{160}. More recent studies have shown that MCP-1 may be promote tumor
growth. MCP-1 has been show to recruit TAMs in SCC and in breast cancer\textsuperscript{161,162}. MCP-1 increases the secretion of Tumor Necrosis Factor alpha (TNFα) by monocytes\textsuperscript{163},
which in turn increases the secretion of MCP-1 and IL-8 by breast tumor cells\textsuperscript{18}. Further,
Vitello et al showed that a reduction in MCP-1 led to an increase in Interferon gamma (IFN-γ), a cytokine important in tumor rejection\textsuperscript{164}.

In our studies, JB6 cells produced more MCP-1 than the 308 cells. MCP-1
initially decreased at 24 hours after UVB exposure in both cell types. In the JB6 cells,
MCP-1 secretion increased at all time points after UVB exposure. In the 308 cells MCP-
1 secretion dramatically increased at 48 and 72 hours, and decreased at 96 hours. Taken
together, these data show that UVB exposure initiates the secretion of these cytokines
that may persist for many days. Chemokine levels at the at later time points may be due
to accumulation of the proteins, so more detailed studies are warranted to understand the kinetics of chemokine production by keratinocytes and papillomas.

Smith et al have shown that both primary and metastatic squamous cell carcinomas produce elevated levels of IL-1α, IL-6, KC and GM-CSF\textsuperscript{17}. IL-1α has been shown to have different impacts upon carcinogenesis, depending on whether it is secreted or not. Secreted IL-1α leads to a more aggressive and invasive phenotype, while intracellular or membrane-bound IL-1α is anti-carcinogenic\textsuperscript{20,165-167}. Our data shows that a papilloma cell line (308) secretes IL-1α constitutively, and the secretion increases after exposure to UVB at 48 and 96 hours.

Our current data show that there is a shift in the cytokines and chemokines released between keratinocytes (JB6 cells) and papillomas (308 cells). There is a shift towards cytokines known to support the growth of transformed cells. Perhaps most interesting is the shift away from chemokines known to increase the homing of cells of a myeloid lineage (MCP-1) to one known to attract (and possibly activate) neutrophils to inflammatory sites (KC). Not only is there a shift in which chemokines are produced, but there is a dramatic up regulation in the amount of KC produced by the 308 cells.

Autophagy is a new and intriguing process. Until recently, it was believed there were two cell death pathways: necrosis and apoptosis. Necrosis was thought to be a response to over-whelming cell damage. Now there is evidence that necrosis is a programmed cell death pathway initiated by the depletion of cellular ATP to a level where the cell can no longer survive\textsuperscript{140}. Apoptosis is a programmed cell death that follows a well delineated program. Apoptosis is dependent upon caspase activation and
results in DNA cleavage and the formation of apoptotic bodies\textsuperscript{140}. Recently, apoptosis has been termed programmed cell death type I (PCD1).

Autophagy (programmed cell death type II, PCD II) is a novel programmed cell death pathway that is caspase independent and does not fragment DNA. Autophagy is also the process by which cells are able to recycle organelles and proteins, and can, in times of nutrient starvation, be a pro-survival pathway. Autophagy is blocked by mTOR activation, a key regulator of the cellular response to nutrient starvation\textsuperscript{24}.

One of the earliest proteins involved in autophagy is Beclin-1. Beclin-1 directly interacts with Bcl-2 and has been shown to be a haploinsufficient tumor suppressor in mice\textsuperscript{141}. The formation of the double-walled phagophore is regulated by a number of Atg (autophagy-related proteins), including Atg7. Other Atg proteins interact with LC3B to form the autophagosome, in which the organelles or proteins undergoing degradation are sequestered\textsuperscript{26}. The autophagosome then merges with a lysosome to form an autophagolysosome.

UV exposure is known to induce autophagy in cells via a Beclin-1 dependent mechanism involving the UV irradiation resistance-associated gene (UVRAG)\textsuperscript{168}. Cao et al showed the importance of Beclin-1 in the induction of autophagy and in the prevention of cancer, and that Atg7 is a tumor suppressor as well\textsuperscript{25}. Interestingly, three reports showed that cancer cells can use autophagy to survive nutrient starvation\textsuperscript{23,169,170}.

Our \textit{in vitro} data show that the 308 cells express more early autophagic proteins after UVB exposure than JB6 cells. We hypothesize that the papilloma cells are increasing autophagy to escape cell death, while the keratinocytes are undergoing autophagic cell death. In the skin, autophagy proteins are decreased. This may reflect
decreased cell death, or the fact that autophagy is a slow process. In tumors, autophagy proteins are decreased, except LC3B. This may show that there is a loss of the two tumor-suppressive proteins, Beclin-1 and Atg7 in the tumors. Even though those proteins are decreased, the increase in LC3B may mean that there is still autophagy taking place in the tumors, possibly a cell survival mechanism. There were no differences in apoptosis, as measured by activated caspase 3.

The autophagy data presented in this chapter only characterizes the expression of proteins in the autophagy pathway. We did not perform functional assays to determine if the changes in protein expression result in altered autophagy. This is mainly due to the fact that there are, as yet, no reliable ways to determine autophagy levels. As assays become available, it would be necessary to repeat these experiments to fully understand the role of autophagy in these cell lines.

There is some disconnect between the in vitro data and that obtained from whole dorsal skin. This may be explained from the fact that we used whole dorsal skin, while the in vitro results only show what happens in keratinocytes (either normal or transformed). We cannot say with certainty that the results obtained from the JB6 cells are invalidated by the in vivo data, nor can we argue that it is not. Further studies using epidermal scrapes are warranted to confirm or refute our findings.

In conclusion, our data shows that there is a change in cytokine profiles and autophagy between normal keratinocytes and papilloma cells that benefits the growth of transformed cells. These changes are confirmed in tumors from mice exposed chronically to UVB. These data will help understand how normal skin cells undergo carcinogenesis.
CHAPTER 4

Topical Treatment with Black Raspberry Extract Reduces Cutaneous UVB-Induced Carcinogenesis and Inflammation

Abstract

Light in the UVB spectrum (280-320 nm) induces a number of changes in the epidermis and dermis of mice and humans, resulting in a robust inflammatory response. Black raspberry extracts (BRE) have been effective in reducing signaling pathways commonly initiated by inflammatory stimuli. In this study, we determined whether these extracts could reduce cutaneous UVB-induced inflammation and carcinogenesis. In our carcinogenesis model, female SKH-1 hairless mice were exposed to one minimal erythemal dose of UVB thrice weekly on non-consecutive days for 25 weeks. Immediately after each exposure, the mice were treated topically with either BRE dissolved in vehicle or with vehicle only. Beginning in week 19, mice treated with BRE had a significant reduction in tumor number and in average tumor size. This reduction correlated with a significant reduction in tumor-infiltrating CD3\(^+\)foxp3\(^+\) regulatory T cells. In the acute model, mice were exposed to a single minimal erythemal dose of UVB and treated topically with BRE or with vehicle. At 48 hours post UVB exposure topical BRE treatment significantly reduced edema, p53 protein levels, oxidative DNA damage
and neutrophil activation. The ability of topical BRE to reduce acute UVB induced inflammation and to decrease tumor development in a long term model provides compelling evidence to explore the clinical efficacy of BRE in the prevention of human skin cancers.

**Introduction**

Exposure to UV light, particularly UVB (280-320 nm), initiates a robust inflammatory response \(^{14}\) characterized by the influx and activation of innate immune cells, predominantly neutrophils and macrophages. Infiltration and activation of neutrophils is mediated via the release of chemokines and cytokines from the epidermis \(^{70,130}\). Macrophages and neutrophils are thought to be the main sources of the reactive oxygen species (ROS) that amplify the inflammatory response and thereby cause secondary DNA damage in keratinocytes \(^{6,15}\), though resident skin cells (keratinocytes, macrophages, Langerhans cells) have also been shown to be a source of ROS production \(^{172}\).

The activation and infiltration of innate immune cells leads to the influx of cells of the adaptive immune system \(^{155}\). We have previously shown that reducing CD4\(^+\) T cells in mice exacerbates the UVB-induced acute cutaneous inflammatory responses and increases tumor development \(^{85}\). Others have shown that CD8\(^+\) T cells are critical in the inhibition of carcinogenesis by natural products \(^{173}\). It is also known that regulatory T cells (CD3\(^+\)CD4\(^+\)foxp3\(^+\)) have functional skin homing markers \(^{174}\). Understanding the interplay of these populations in UVB induced carcinogenesis is crucial to understanding how tumors evade immune surveillance.
The most serious effect of chronic UVB exposure is the development of non-melanoma skin cancer (NMSC), the most frequently diagnosed cancer in the United States \textsuperscript{175}. Recent work has shown that there is a strong link between chronic inflammation and carcinogenesis in a number of organ systems \textsuperscript{44,176}. This association holds true in the development of UVB-induced skin cancer as well \textsuperscript{14,177}.

As we have shown previously (see Chapter 2), the choice of immunosuppressant after solid organ transplantation has a significant impact upon UVB-induced inflammation and carcinogenesis. Of the immunosuppressants we tested, cyclosporine (CsA) significantly worsened both UVB-induced inflammation and carcinogenesis. There have been reports that CsA can inhibit the repair of DNA damage caused by UVB exposure\textsuperscript{107,178}. There is research from a number of countries showing that transplant patients suffer from aggressive SCC. It is therefore extremely important to have an effective treatment for UVB-induced inflammation that does not interfere with immunosuppressive medications.

The UVB-induced generation of ROS can produce oxidative DNA damage, resulting in the formation of 8-oxo-deoxyguanosine (8-oxo-dG) adducts \textsuperscript{15}. There has been increased interest in the last few years in the ability of natural compounds to combat inflammatory responses. One of the main classes of compounds investigated has been plant products that are high in antioxidants including anthocyanins and carotenoids. Studies from a number of laboratories have focused on testing a variety of extracts made from black raspberries \textsuperscript{42,179}. The ethanol/water extract of black raspberries (BRE) contains a number of powerful anti-oxidants, including cyanidin-3-O-glucoside, cyanidin 3-O-(2(G)-xylosylrutinoside) and cyanidin 3-O-rutinoside \textsuperscript{40,180}. These molecules are not
only effective antioxidants but also are able to affect signaling pathways activated in inflammatory responses \textsuperscript{41}. The promising findings regarding the ability of natural products to inhibit the inflammatory process have carried over to cancer studies in a variety of organs including the oral cavity and the skin. Natural compounds, such as tea polyphenols, fruit extracts and spices, have shown promise \textit{in vivo} in reducing carcinogenesis in the skin (reviewed in \textsuperscript{181}). In \textit{in vitro} models, BRE has been found to induce apoptosis in a number of transformed cell lines, but had no effect upon the growth of normal cells \textsuperscript{45}.

The present study was designed to determine if topical BRE treatment is effective at reducing carcinogenesis and the inflammatory response in murine skin following UVB exposure. We have shown that topical treatment with BRE immediately following UVB exposure significantly reduced UVB-induced tumor formation and progression. We also have shown that treatment with BRE reduced the edema response, neutrophil activation, hydrogen peroxide production, and, oxidative DNA damage, indicating that BRE was a strong inhibitor of the inflammatory response initiated by UVB exposure. These data suggest that topical application of BRE following UVB exposure may be an effective treatment for the prevention of UVB-mediated inflammation as well as tumor development.
Materials & Methods

Standardized BRE Preparation

Standardized BRE was prepared as previously reported\(^{182}\). Briefly, Jewel varietal black raspberries were grown at the Stokes Raspberry Farm (Wilmington, Ohio), harvested, washed and frozen according to previously determined protocols\(^{183}\). The berries were assayed by Covance Laboratories to determine levels of pesticides/herbicides/fungicides, which were found to be negligible. Berries were then freeze-dried and shipped frozen to the laboratory of Dr. S. Hecht at the University of Minnesota where the extract was prepared as previously reported\(^{40}\). The most prevalent peaks in a high performance liquid chromatograph of the ethanol/water (80/20) extract of black raspberries are anthocyanins, and make up 5-10% of the dry berry weight\(^{184}\). Anthocyanins, particularly cyanidin-3-\(O\)-glucoside, cyanidin-3-\(O\)-rutinoside and cyanidin-3-\(O\)-xylosylrutinoside have been show to be effective anti-cancer agents in a number of model systems, both \textit{in vitro} and \textit{in vivo}\(^{40,180,185-195}\). These extracts have been shown to be rapidly up taken and pass rapidly through tissues, which, coupled with the post-exposure application and low dosage, makes the likelihood of accumulation in the skin and the production of a sun blocking effect very low\(^{196}\).

Animal Treatment

Six to eight week old female SKH-1 hairless mice (Charles River Laboratories, Wilmington, MA) were housed in a vivarium at The Ohio State University according to protocols established by the American Association for Accreditation of Laboratory
Animal Care. Mice were housed at constant temperature and humidity levels. Food and water containing the antibiotic Baytril (Bayer HealthCare, LLC, Shawnee Mission, KS) was provided ad libitum. All procedures performed were approved by the Institutional Laboratory Animal Care and Use Committee. All mice treated with extract received 500 μg of BRE dissolved in 100 μl vehicle (KY Jelly; McNeil Consumer & Specialty Pharmaceuticals, Fort Washington, PA). This dosage was decided upon after preliminary dose-response experiments testing the efficacy of BRE concentrations ranging from 100 μg to 1 mg showed that the 500 μg dose was optimal for the inhibition of acute UVB induced inflammatory responses in the skin.

Carcinogenesis model: Mice (n=10 per group) were exposed to one minimal erythemal dose (1 MED) of UVB, which was previously established in our lab as 2240 J/m². UVB levels were measured using a UVX radiometer (UVP Inc, Upland, CA). UVB light was generated by a bank of Philips FS40UVB lamps (American Ultraviolet Company, Lebanon, IN) covered by Kodacel filters (Eastman Kodak, Rochester, NY) to block UVC wavelengths. Groups of mice were exposed to UVB in large rat cages. The positions of these cages were rotated on a weekly basis to standardize exposure conditions. Mice received 1 MED of UVB followed immediately by topical application of 500 μg of BRE dissolved in 100 μl vehicle (KY Jelly) or 100 μl vehicle alone thrice weekly on non-consecutive days for 25 weeks. Several laboratories have shown that anthocyanins are readily absorbed by epithelial cells and excreted by the body, with peak levels of anthocyanin excretion occurring at 4-8 hours. This rapid absorption and processing and the gap between treatments reduced the risk of sunscreen effects. BRE was placed in the middle of the dorsum of the mouse and manually gently rubbed into the
dorsal skin by glove-wearing personnel immediately after UVB exposure. All mice were treated within two minutes of the cessation of UVB exposure, which is the peak UVB-induced inflammatory time point, and topical treatment. Non-irradiated age-matched control mice were treated topically with vehicle or BRE. Beginning in week 11, the length and width of each tumor greater than 1mm in each direction were measured using digital calipers; these measurements were used to calculate tumor area. At harvest tumor samples were fixed in 10% neutral buffered formalin or placed in OCT (Tissue-Tek, Sakura Finetek, Torrance, CA) for further processing and histological analysis. The remaining dorsal skin was snap frozen in liquid nitrogen for protein isolation.

Acute inflammation model: Mice (n=6 per group) were exposed to one MED of UVB (2240 J/m²) followed immediately by topical application of 500 μg of BRE dissolved in 100μl vehicle or 100μl vehicle alone as described above. Mice were sacrificed 48 hours following UVB exposure. This time point was chosen because it represents the height of the acute UVB-induced cutaneous inflammatory response. Non-irradiated age-matched control mice were treated topically with vehicle or BRE. At harvest, edema, as determined by skin-fold thickness measurements, was recorded and dorsal skin samples were fixed in 10% neutral buffered formalin or placed in OCT (Tissue-Tek, Sakura Finetek, Torrance, CA) for further processing and histological analysis. Skin punches were snap-frozen in liquid nitrogen for subsequent determination of myeloperoxidase (MPO) activity. The remaining dorsal skin was snap frozen in liquid nitrogen for protein isolation.

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Immunofluoresence Staining of Tumors

At the time of sacrifice tumors were removed and placed into OCT before being frozen on dry ice. To determine the presence of specific sub-populations of T cells, 5 μm sections were cut and stained with antibodies recognizing CD3, CD4, (Pharmingen, San Diego, CA) or foxp3 (eBioscience, San Diego, CA). A total of 15 tumors per group were analyzed. Sections were incubated with primary antibody (CD3) overnight at 4º C before washing to remove the primary antibody. The sections were then incubated with the appropriate Alexa Fluor conjugated secondary antibody (Invitrogen, Carlsbad, CA). Sections were then incubated with the final primary (CD4 or foxp3) and secondary antibodies (Alexa Fluor) at room temp (1 hour) before being counterstained with DAPI (Sigma Aldrich, St. Louis, MO) and cover slipped using Prolong Gold (Invitrogen). For T cell measurements, the number of positive cells within tumor margins was counted for each animal normalized to tumor area and the mean was determined for each group.

Edema Measurement

Dorsal skin edema induced by acute UVB exposure was measured by determining skin fold thickness at harvest using digital calipers. Data shown are the mean ± standard deviation of each group.

Myeloperoxidase Assay

The level of myeloperoxidase (MPO) was measured as previously described 7. Briefly, a 10-mm skin biopsy was snap frozen at harvest and stored at –80º. Biopsies were incubated on ice in phosphate buffer containing hexadecyltrimethylammonium
bromide (HTAB). Tissue samples were homogenized and then underwent three cycles of sonication and freeze-thawing. Cellular debris was removed by centrifugation. The MPO levels of the supernatants were then analyzed spectrophotometrically. Results are reported as fold-increase compared to matched control samples from mice not exposed to UVB. The rate of $\text{H}_2\text{O}_2$ consumption was measured spectrophotometrically over a 5 minute period.

**Immunohistochemical Staining**

Skin samples from the inflammation model were removed and fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 $\mu$m. Sections were stained for Ly6G (a neutrophil marker) to identify infiltrating neutrophils as reported previously $^7$. Briefly, sections were rehydrated and blocked using 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 hour. The slides were then incubated overnight at 4° C with the primary antibody (monoclonal rat anti-mouse Ly6G, Pharmingen). The next day the slides were washed and incubated with the biotinylated anti-rat secondary antibody (Vector Laboratories) for 30 minutes at room temperature. Slides were then incubated with an avidin/horseradish peroxidase complex (ABC Elite, Vector Laboratories) for 30 minutes. The slides were developed using a DAB kit (Vector Laboratories) and counterstained with hematoxylin before being dehydrated in a series of ethanol washes ending in Clear-Rite. Slides were then cover slipped. To determine the number of neutrophils infiltrating into the dermis, the mean number of neutrophils per ten fields per 5$\mu$m section per mouse at 600x magnification was calculated.
p53 levels in keratinocytes were measured using a similar method. Dorsal skin was harvested 48 hours after UVB exposure and treatment with vehicle or 500 μg BRE. Paraffin embedded sections were cut and treated with the Mouse-on-Mouse (M.O.M) kit (Vector Laboratories), then stained with anti-rabbit p53 (Leica Microsystems, Bannockburn, IL). Link-Label (Biogenex, San Ramon, CA) was used to amplify signal and the slides were developed using the DAB system (Vector Laboratories). The number of p53 positive cells per ten fields per 5μm section per mouse at 600x magnification per mouse in the epidermis was counted and averaged.

8-oxo-deoxyguanosine (8-oxo-dG) Adduct Detection

To identify differences in ROS-induced DNA damage, flash-frozen dorsal epidermis was scraped from whole skin and used to isolate DNA. Briefly, frozen scraped epidermis was incubated for four hours at 45°C in solubilization buffer (10% SDS, 50mM EDTA, 100mM Tris, 100mM NaCl, 200 μg/mL proteinase K). After cooling to room temperature, 200 μL of a 4.21 M NaCl, 0.63 M KCl, 10mM Tris solution was added and incubated at 4°C for 30 minutes to precipitate proteins. Samples were centrifuged at 13,000 X g for 10 minutes and supernatant removed for DNA precipitation. DNA was precipitated with 100% EtOH at 4°C overnight. DNA was pelleted by centrifugation at 2000 X g for 7 minutes and washed with 80% EtOH 3 times. Washed DNA was resuspended in 0.4M NaOH/10mM EDTA and the concentration was determined spectrophotometrically. Equal amounts of DNA from each animal in the different treatment groups were pooled for 8-oxo-dG south-western blots.
South-western immunoblots were performed as previously reported\textsuperscript{15}. Briefly, 5 μg of epidermal DNA was loaded onto a nitrocellulose membrane and dried for 1 hr before being blocked with 5% BSA in TBST (TBS + 0.1% v/v Tween-20 [Fisher Scientific, Pittsburgh, PA]). The blot was then incubated overnight with the 8-oxo-deoxyguanosine primary antibody (Genox Corporation, Baltimore, MD). After overnight incubation, the blot was washed and incubated with a goat anti-mouse HRP-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) for 45 minutes at room temperature. The blot was washed and incubated with West Femto Supersignal chemiluminescent reagent (Bio-Rad, Hercules, CA) and exposed to Kodak Biomax MR Film (Kodak, Rochester, NY). It was then incubated in DAPI and scanned with a Pharos FX scanner (Bio-Rad) to normalize for DNA adhesion. The ratio of 8-oxo-dG dot intensity to DAPI dot intensity was determined using ImageJ version 1.28b (http://rsb.info.nih.gov.ij).

**Statistical Analysis**

We performed statistical analyses using Excel (Microsoft Corporation, Redmond, WA). After consultation with a biostatistician, the Student’s two-tailed t Test was used to determine statistical significance, as the data was normally distributed. p<0.05 was considered significant and p<0.01 was considered highly significant.
Results

*BRE treatment significantly reduced UVB-induced carcinogenesis*

Tumor measurements were collected beginning in week 11. As can be seen in Figure 4.1A, by twenty-five weeks, vehicle-treated mice developed, on average, 30 tumors/mouse (± 3.1). In contrast, treatment with BRE significantly reduced the total number of tumors on the dorsum of UVB-exposed mice to 17 tumors/mouse (± 1.9, p=0.003; Student’s two-tailed t Test). Thus topical treatment with BRE following UVB exposure reduced the number of tumors that developed in response to chronic UVB exposure.
Figure 4.1: BRE significantly reduced UVB induced carcinogenesis. Female SKH-1 mice (n=10/group) were treated with vehicle or BRE after exposure to 2240 J/m² of UVB thrice weekly for 25 weeks. Tumor measurements (number, length and width) were taken beginning in week 11. BRE was able to significantly reduce tumor number (A) and tumor size (B) (p= 0.003 for both, at 25 weeks). Data are shown as mean ±SE. (C) BRE also significantly reduced the number of large tumors at 25 weeks (p=0.004) ±SE. ** p<0.01.
BRE treatment decreased tumor size and the number of large tumors

BRE treatment also significantly reduced the size of the tumors per mouse, from a mean of 30.2 mm² in vehicle-treated mice to 10.1 mm² in BRE-treated mice (Figure 4.1B, p=0.003). The number of tumors measuring greater than 10 mm² in area were counted and averaged for each group. BRE treatment reduced the average number of large tumors from 6.9 to 1.6 (Figure 4.1C, p=0.004). Thus BRE treatment significantly retarded the growth of UVB-induced tumors.

Tumors from mice treated with BRE contain significantly fewer CD3⁺foxp3⁺ T cells

To determine how BRE was able to alter tumor progression, we explored how BRE affected T cell infiltration into tumors. BRE reduced the number of tumor-infiltrating CD4⁺ T cells within tumors from a mean of 206 in mice treated with vehicle to 77 in mice treated with BRE (p<<0.001; Figure 4.2C). Representative micrographs of tumors from vehicle (A) and BRE (B) treated mice are shown in Figure 4.2. Analysis of the number of CD3⁺foxp3⁺ T cells within the tumors demonstrated a significant reduction, down from 10 in vehicle-treated mice to 2.2 in BRE-treated mice (p=0.01; Figure 4.3C). Representative micrographs of tumors from vehicle (A) and BRE (B) treated mice are shown in Figure 4.3. There was no significant difference in the number of infiltrating CD8⁺ T cells in any tumor (data not shown).
Figure 4.2: BRE altered tumor-infiltrating CD4+ T cell numbers. Tumors (n=15) from mice exposed to 2240 J/m² of UVB for 25 weeks were stained for cells expressing CD3 and CD4. The number of double positive cells infiltrating tumors of vehicle-treated mice (A) was significantly higher than BRE-treated mice (B). Magnification for all images is 600X. Graphical representation of the data in (C; * p =0.04). Data are shown as mean ±SE.
Figure 4.3: BRE altered tumor-infiltrating foxp3+ T cell numbers. Tumors (n=15) from mice exposed to 2240 J/m² of UVB for 25 weeks were stained for cells expressing CD3 and foxp3. The number of double positive cells infiltrating tumors of vehicle-treated mice (A) was significantly higher than BRE-treated mice (B). Magnification for all images is 600X. Graphical representation of the data in (C; * p =0.05). Data are shown as mean ±SE.
BRE treatment reduced acute UVB-induced edema

To determine if the observed anti-tumor effects of BRE treatment were being mediated at least in part by a decreased inflammatory response we examined the effects of BRE treatment on the acute UVB-induced inflammation. The most visible responses to acute UVB exposure are erythema and edema, which can be used as an indicator of the severity of the inflammatory response. All mice were sacrificed 48 hours post UVB exposure, at the peak of the inflammatory response in the skin. Post-exposure treatment with 500 μg of BRE significantly reduced UVB-induced edema (determined via single fold dorsal skin thickness) at 48 hours following UVB exposure (Figure 4.4A; p=0.001, compared to UVB-exposed vehicle-treated controls).
Figure 4.4: BRE significantly reduced UVB-induced edema and neutrophil activation. Mice (n=10) were exposed to 2240 J/m² UVB and immediately treated with either vehicle (KY Jelly) or 500 µg BRE. 48 hours later, mice were sacrificed and dorsal skin was harvested. UVB induced edema as measured via single fold dorsal skin thickness was significantly decreased (**p=0.001, compared to UVB-exposed vehicle-treated controls) (A). BRE significantly inhibited the UVB-induced increase in dorsal skin MPO activity compared to vehicle (*p=0.03) (B). Data are shown as mean fold increase of MPO activity over matched no UV controls ± SD. BRE did not reduce dermal neutrophil infiltration, as determined by immunohistochemical staining for Ly6G, a neutrophil marker (C). Data are shown as mean Ly6G⁺ cells per 600X field ± SE.
BRE treatment reduced neutrophil activation, but did not reduce neutrophil infiltration

While topical BRE treatment decreased edema, topical BRE treatment did not reduce dermal neutrophil numbers compared to vehicle (Figure 4.4C). Groups are as follows: mice unexposed to UVB and treated with vehicle (No UV Vehicle), mice unexposed to UVB and treated with BRE (No UV BRE), mice exposed to UVB and treated with vehicle (UV Vehicle) and mice exposed to UVB and treated with BRE (UV BRE). BRE treatment did significantly reduce neutrophil activation as measured by myeloperoxidase (MPO) levels (Figure 4.4B, p=0.03, compared to vehicle). The ability of BRE to decrease MPO without affecting neutrophil homing suggests BRE may selectively inhibit neutrophil activation.

BRE treatment decreased p53 expression

To estimate the overall DNA damage response in BRE- and vehicle-treated skin, dorsal skin was stained for p53 and the mean number of keratinocytes with positive nuclei per 600X magnification field was determined. Treatment with BRE significantly reduced the number of UVB-induced p53-positive cells (Figure 4.5, p=0.002, compared to vehicle), suggesting less DNA damage.

BRE treatment significantly reduced the levels of indirect DNA adducts but not direct adducts

To assess the level of indirect DNA damage induced by ROS formed during the inflammatory response to UVB, we performed a south-western immunoblot for 8-oxo-dG.
Figure 4.5: BRE reduced UVB-induced p53 expression. p53 was immunohistochemically localized and the number of positive cells was enumerated. The average number of p53+ cells per X600 field ±SD is shown (** p=0.002).
on DNA isolated from the epidermis (Figure 4.6A). The densitometry data show that BRE treatment significantly (Figure 4.6B; p=0.04, compared to vehicle controls) reduced the levels of 8-oxo-dG adducts in the epidermis of mice exposed to UVB down to those seen in mice that had not been exposed to UVB. Immunohistochemical staining of dorsal skin sections for the presence of cyclobutane pyrimidine dimers (CPD), a measure of direct UV-induced DNA damage, (Figure 4.6C) show that treatment with BRE did not significantly reduce the formation of direct DNA adducts.
Figure 4.6: BRE reduced ROS-mediated DNA damage, but not direct DNA damage. After flash freezing, DNA was isolated from epidermal scrapes and used in a south-western immunoblot (A). Densitometry was performed using Image J. Data is reported as 8-oxo-dG levels, error bars are ratio of DNA to 8-oxo-dG ± SD (* p=0.04) (B). Paraffin sections were cut and stained for cyclobutane pyrimidine dimers; keratinocytes staining positive were enumerated. Data is shown as mean number of CPD positive cells per X600 ± SD(C).
Many groups have explored the use of natural compounds in the prevention of several types of cancer, including SCC of the skin. For example, myricetin, a phytochemical found in nuts and dark pigmented fruits, was shown to significantly reduce tumor formation in mice chronically exposed to UVB light \(^{200}\). Other dietary botanicals including apigenin, curcumin, resveratrol, and green tea have been shown to reduce the deleterious effects of UVB exposure \(^{181}\). Anthocyanins, found in red, blue, and purple fruits and vegetables, have also been studied for their chemopreventive properties. Ohio-grown black raspberries and extracts made from these berries, which have high levels of anthocyanins and other compounds, have been shown in an animal model to be efficacious in preventing esophageal cancer \(^{201}\). Ongoing studies are determining their efficacy in both colon and oral cancers \(^{46,184}\). To date, however, no *in vivo* studies have examined the efficacy of BRE in the prevention of cutaneous UVB-induced damage or tumor development.

Many groups have shown that inhibition of inflammation is associated with a reduction in carcinogenesis \(^{7,14,176,177}\). We have previously demonstrated that topical application of the anti-inflammatory drug celecoxib reduced both acute UVB-mediated inflammation and chronic UVB-mediated tumor formation. We have also found that agents increasing UVB-induced inflammation lead to an increase in tumors with an area greater than 10mm\(^2\), which were of a high malignant grade \(^{14,177}\). These studies supported the link between UVB-induced inflammation and skin cancer development. Natural products, such as \((-\)-Epigallocatechin-3-gallate, a green tea polyphenol, have also been
shown to reduce acute inflammation and inhibit UVB-induced carcinogenesis \(^{202}\). Our present data demonstrates the ability of BRE, a natural product, to inhibit several hallmarks of UVB-mediated inflammation including the production of DNA damaging reactive oxygen species (ROS) in the skin.

Exposure to light in the UVB wavelength (280-320 nm) produces both direct and indirect DNA damage. Direct DNA damage from UVB absorption results in the formation of cyclobutane pyrimidine dimers (CPD) \(^{203-205}\). Indirect DNA damage, including signature DNA adducts such as 8-oxo-deoxyguanosine (8-oxo-dG), results from the activity of ROS generated by keratinocytes and infiltrating inflammatory cells, especially neutrophils, during the inflammatory response to UVB \(^{15,206}\). These ROS can damage keratinocytes, which induces the production of more pro-inflammatory cytokines and chemokines, thus amplifying the inflammatory response \(^5\). We have previously shown that the inhibition of UVB-induced neutrophil infiltration and especially neutrophil activation can lead to decreased inflammation and tumor development \(^{14}\). Our current data demonstrated that while BRE treatment had no effect on neutrophil infiltration, it significantly reduced MPO (Figures 4.4B), thus decreasing ROS levels in the skin and ultimately oxidative DNA damage.

Epidermal keratinocytes have mechanisms to repair both direct and indirect damage initiated by UVB exposure. One of the ways they accomplish this is by p53 production and activation, which halts the cell cycle and allows DNA repair mechanisms to correct the CPD and 8-oxo-dG adducts, or induce apoptosis if the damage is beyond repair \(^{207}\). p53 stabilization, indicated by nuclear immunoreactivity, has previously been used as an indicator of overall levels of DNA damage \(^{85,208}\). The present study found that
topical application of BRE following UVB exposure was able to reduce the number of p53 positive epidermal cells, indicating less overall damage in the epidermis. Our studies determined that topical BRE application following exposure to UVB did not decrease the formation of the direct DNA damage adducts CPDs, demonstrating that BRE was not acting as a sunscreen. However, similar to other topical inhibitors of inflammation \textsuperscript{14,209}, BRE did significantly inhibit the formation of the ROS induced 8-oxo-dG adducts. We cannot discern from our data whether the reduction in 8-oxo-dG adducts was attributable to inhibition of initial adduct formation or enhancement of DNA repair. Ongoing studies are further examining this question. Regardless of the mechanism of reduction of 8-oxo-dG formation, the formation of fewer oxidative DNA adducts would ultimately result in decreased carcinogenesis.

In addition to decreasing inflammation and oxidative DNA damage another possible mechanism by which BRE may be altering UVB-mediated tumor development and progression is by altering the infiltration of the adaptive immune cells. It is now known that the influx of regulatory T cells is one mechanism by which transformed cells can evade immune surveillance \textsuperscript{210}. Yang et al was one of the first to report that treatment with resveratrol, a natural product derived from grape skins, reduced the number of regulatory T cells in the spleens of mice who had been injected with syngeneic lymphoma or colon cancer cells \textsuperscript{211}. It is also been shown in mice that chronic UVB exposure leads to an increase in regulatory T cells \textsuperscript{212} and that this cell type expresses functional skin homing receptors \textsuperscript{174}. While there have been no previous reports of ananthocyanins having an effect on the adaptive immune response, our data demonstrates that T cells with a regulatory phenotype are reduced in the tumors isolated from BRE
treated mice (Figure 4.3C). The reduction in putative regulatory T cells, even after accounting for differences in tumor size between the groups, suggests a possible mechanism for the observed reduction in the numbers of large tumors in BRE skin (Figure 4.1C). It is important to note that while we did see a decrease in CD4$^+$ cells within tumors isolated from BRE treated mice, we did see did not see a reduction in CD8$^+$ T cells in any of the tumors (data not shown). The observed reduction in the number of cells with an immunosuppressive regulatory phenotype may then allow CD4$^+$ and CD8$^+$ effector cells to aid the innate and adaptive immune system to reject tumor cells. Although our data is suggestive, we did not isolate the cells with a regulatory T cell phenotype and test their ability to suppress immune responses. Neither did we remove these cells from the mice to determine what effect that would have on tumor development. These types of future studies will provide further confirmation of the importance of immune cell regulation in the skin by BRE treatment. Completion of these preclinical studies will provide valuable information leading to more effective skin cancer prevention strategies.

Acknowledgements

We would like to thank Dr. Stephen Hecht of the University of Minnesota Cancer Center for providing the black raspberry extract.
CHAPTER 5

The Effects of Black Raspberry Extract on Cytokine Production and Survival Pathways in JB6 and 308 Cell Lines as well as Normal Skin and UVB-induced Tumors

Abstract

UVB-induced carcinogenesis is a multi-step process that commences with an acute inflammatory response. The acute response to UVB results in neutrophil infiltration and activation, cytokine and chemokine production, generation of reactive oxygen species and activation of immune cells already present in the skin (macrophages, Langerhans cells). The acute inflammatory response results in DNA damage and the generation of a microenvironment conducive to the growth and survival of transformed cells. Black raspberry extract (BRE) has been shown to be effective at reducing both acute inflammation and carcinogenesis resulting from chronic UVB exposure. In the current chapter, we set out to determine the mechanism(s) behind BRE’s efficacy. We used two cells lines, a keratinocyte cell line (JB6) and a cell line made from murine papilloma cells (308) to determine the effects of BRE treatment on cytokine and chemokine production as well as the effects of BRE treatment upon autophagy. We then confirmed our findings in vivo by determining if the effects seen in vitro were still present in acutely irradiated dorsal skin and in tumors taken from mice chronically exposed to UVB. We were able to show that BRE does impact the secretion of inflammatory cytokines and chemokines in
vitro and in vivo, and BRE was able to impact the ability of tumors cells to use autophagy as a cell survival mechanism.

**Introduction**

Damage to keratinocytes from UVB exposure is a prominent cause of non-melanoma skin cancers (NMSCs)\textsuperscript{14,213}. UVB can damage keratinocytes in two ways, directly and indirectly. Direct damage results from light in the UVB range striking DNA, which results in cyclobutane pyrimidine dimers (CPD)\textsuperscript{125,203}. Indirect damage results from the production of reactive oxygen species (ROS) resulting from the initiation of an inflammatory response. ROS damage to DNA results in the formation of 8-oxodeoxyguanosine adducts\textsuperscript{15}. The inflammatory response is often exacerbated by the release of cytokines and chemokines from the damaged keratinocytes\textsuperscript{2,214-216}.

The damage to keratinocytes stimulates the production of chemotactic factors which call neutrophils into the dermis and fully activate them. In mice, the main neutrophil chemokines are keratinocyte-derived chemokine (KC; CXCL1) and monocyte chemotactic protein-1 (MCP-1; CCL2)\textsuperscript{6,129}. The neutrophils release hypochlorous acid, which causes further tissue damage, and the furthering of the inflammatory cascade\textsuperscript{6}.

Activated neutrophils and keratinocytes exposed to UVB secrete a number of pro-inflammatory cytokines\textsuperscript{2,8,136,217-219}. Pro-inflammatory cytokines such as IL-1\textalpha and IL-6 have been shown to promote angiogenesis and produce growth factors (such as PGE\textsubscript{2}) that are beneficial for transformed cells\textsuperscript{71,130,131}. Th17, a recently discovered lineage of T cells, may play an important role in the tumor promotion\textsuperscript{132-135,220}. These T cells can
induce the secretion of factors that are pro-angiogenic and can support the metastasis of tumors$^{132,134,221}$.

Cells damaged by UVB, either directly or indirectly, have two ways to resolve the damage. They can either repair the damage, which usually necessitates a pause in the cell cycle initiated by p53$^{222}$. Or, if the damage is too extensive, they can undergo programmed cell death via either apoptosis or autophagy.

Autophagy is a programmed cell death pathway conserved in all eukaryotes$^{223}$. The induction of autophagy is controlled by Beclin-1, which is also a known tumor suppressor$^{141}$. After initiation by Beclin-1, two pathways that can regulate each other act sequentially in the formation of the autophagosome, Atg12 and LC3B$^{142}$. After the formation of the autophagosome, it eventually combines with a lysosome to form an autophagolysosome and the proteins and/or organelles are degraded.

ROS are known to influence autophagy, and an active inflammatory response significantly up regulates the concentration of ROS$^{7,71}$. ROS are also increased in cancer cells, which is due in part to an increase in cellular metabolism in cancer cells$^{224}$. It has been shown that Beclin-1 expression is induced in cancer cells via ROS$^{224}$.

ROS have also been shown to interact with another tumor suppressor, phosphatase and tensin homolog deleted on chromosome 10 (PTEN)$^{225,226}$. PTEN is a well-characterized tumor suppressor that is deleted in a large number of human cancers$^{227}$, and is lost in squamous cell carcinoma of the skin$^{228}$. It is also known that PTEN increases autophagy, through its blockade of the mammalian target of rapamycin (mTOR)$^{224}$.  

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Inhibiting ROS production and the production of pro-inflammatory cytokines after exposure to UVB would perhaps reduce DNA damage and reduce carcinogenesis after chronic inflammation. We have an extract from black raspberries (BRE) that reduces inflammation and carcinogenesis in a hairless mouse model of UVB-induced inflammation and carcinogenesis. The extract is known to contain a number of bio-active compounds, including anthocyanins. Anthocyanins are powerful antioxidants that give pigmented fruits their dark colors. Also present in the extract are ellagitannins, vitamins A, C, E and folic acid, and minerals, such as calcium, selenium and zinc \(^ {184}\).

In the current study, we explored the role of BRE in affecting the cytokine response and autophagy in a number of models. In *in vitro* studies we treated both immortalized murine keratinocytes (JB6 cell line) and pre-malignant murine epidermal papillomas (308 cell line) with BRE, while *in vivo* we examined skin, T cells and tumors from mice treated topically with BRE. Understanding how BRE affects the inflammatory response in keratinocytes, whole skin and tumors will allow us to begin to determine the mechanism(s) behind BRE’s efficacy in preventing UVB-induced inflammation and carcinogenesis.

**Materials and Methods**

**Cells**

The 308 cells were a kind gift from Dr. Stuart Yuspa, NIH/NCI, Bethesda, MD. They were developed from pooled papillomas initiated in BALB/c mice with DMBA and promoted by a phorbol ester\(^ {143} \). JB6 cells were procured from ATCC (Manassas, VA), and are also on a BALB/c background. JB6 cells were grown in EMEM (BioWhittaker,
Walkersville, MD) supplemented with sodium pyruvate (Invitrogen, Carlsbad, CA), non-
essential amino acids (Invitrogen), sodium bicarbonate (Invitrogen), penn/strep (Invitrogen), L-glutamine (Invitrogen) and FCS (Invitrogen). 308 cells were grown in
EMEM (BioWhittaker, Walkersville, MD) supplemented with calcium-chelated FCS (Invitrogen), antibiotics/antimycotic (Invitrogen), calcium chloride (Sigma-Aldrich, St. Louis, MO) and L-glutamine (Invitrogen). All cells were allowed to grow to near
confluence before experimental protocol was initiated. Cells were exposed to 600J of UVB. Either media containing $64 \mu g$ BRE or fresh control media was added at this time.

For cell number determination, cells were harvested and viable cells were counted on a hematocytometer.

**Skin**

Six to eight week old female SKH-1 hairless mice (Charles River Laboratories, Wilmington, MA) were housed in a vivarium at The Ohio State University according to
protocols established by the American Association for Accreditation of Laboratory
Animal Care. Mice were housed at constant temperature and humidity levels. Food and
water containing the antibiotic Baytril (Bayer HealthCare, LLC, Shawnee Mission, KS)
was provided *ad libitum*. All procedures performed were approved by the Institutional
Laboratory Animal Care and Use Committee. All mice treated with extract received $500$
$\mu g$ of BRE dissolved in 100 $\mu l$ vehicle (KY Jelly; McNeil Consumer & Specialty
Pharmaceuticals, Fort Washington, PA). This dosage was decided upon after preliminary
dose-response experiments testing the efficacy of BRE concentrations ranging from $100$
$\mu g$ to 1 mg showed that the $500 \mu g$ dose was optimal for the inhibition of acute UVB
induced inflammatory responses in the skin.
Carcinogenesis model: Mice (n=10 per group) were exposed to one minimal erythemal dose (1 MED) of UVB, which was previously established in our lab as 2240 J/m². UVB levels were measured using a UVX radiometer (UVP Inc, Upland, CA). UVB light was generated by a bank of Philips FS40UVB lamps (American Ultraviolet Company, Lebanon, IN) covered by Kodacel filters (Eastman Kodak, Rochester, NY) to block UVC wavelengths. Groups of mice were exposed to UVB in large rat cages. The positions of these cages were rotated on a weekly basis to standardize exposure conditions. Mice received 1 MED of UVB followed immediately by topical application of 500 μg of BRE dissolved in 100 μl vehicle or 100 μl vehicle alone thrice weekly on non-consecutive days for 25 weeks. Several laboratories have shown that anthocyanins are readily absorbed and excreted by the body, with peak levels of anthocyanin excretion occurring at 4-8 hours. This rapid absorption and processing and the gap between treatments reduced the risk of sunscreen effects. BRE was placed in the middle of the dorsum of the mouse and manually gently rubbed into the dorsal skin by glove-wearing personnel immediately after UVB exposure. All mice were treated within two minutes of the cessation of UVB exposure. Mice were sacrificed at 48 hours following the final UVB exposure, which is the peak UVB-induced inflammatory time point, and topical treatment. Non-irradiated age-matched control mice were treated topically with vehicle or BRE. At harvest tumor samples were snap frozen in liquid nitrogen for protein isolation.

Acute inflammation model: Mice (n=6 per group) were exposed to one MED of UVB (2240 J/m²) followed immediately by topical application of 500 μg of BRE dissolved in 100μl vehicle or 100μl vehicle alone as described above. Mice were sacrificed 48 hours following UVB exposure. This time point was chosen because it
represents the height of the acute UVB-induced cutaneous inflammatory response. Non-irradiated age-matched control mice were treated topically with vehicle or BRE. At harvest dorsal skin was snap frozen in liquid nitrogen for protein isolation.

**T cell isolation**

T cells were isolated from mice exposed to a one MED of UVB thrice on non-consecutive days. Dorsal skin was removed and placed dermis side down in a solution of dispase before being incubated at 37°C for 30 minutes. The skin was then placed epidermis side down on a clean petri dish. The dermis was then carefully peeled away from the epidermis. The epidermis was then placed in a trypsin solution and incubated at 37°C for 30 minutes. The epidermis then underwent mechanical disruption via a cell strainer (BD Pharmingen). The resulting cell suspension was placed on a nylon wool column and incubated for 1.5 hour at 37 °C. The T cell enriched suspension was then spun and washed twice before being placed into Trizol (Sigma Aldrich, St. Louis, MO). The suspension was then frozen prior to DNA isolation.

**RT-PCR**

The media was removed and either JB6 or 308 cells were washed with PBS before Trizol (Sigma Aldrich, St. Louis, MO) was added directly to the cells. The suspensions were pipetted repeatedly and collected into DNA/RNAse free tubes (Fisher Scientific, Pittsburgh, PA). The tubes were placed into -80 for at least 24 hrs prior to extraction. The purity and quality of the RNA was assayed by the Microarray Core at OSU. The RNA then underwent reverse transcription, and the resulting cDNAs were
used in PCR reactions using the following primer sets: IL-2 (forward: GCAGGATGGAGAATTACA; reverse: GTGTTGTCAAGAGGCCTTTAGT), IL-2R (CD25) (forward: GCACCAGCAACTCCCCCATGACAAAT; reverse: TGGCCACTGCTACCTTAT), IL-4 (forward: GCAACGAAGAACACCACAGAGA; reverse: TGCAGCTTATCGATGAATCCAGGC), IL-6 (forward: GCAAGAGACTTCCAYCCAGTTG; reverse: TTAGCTCTTGGTTGAAGATATGA), IL-10 (forward: GGACTTTAAGGCTTGTGACAG; reverse: CCTTGGTCTTGAGGCTTATT) and IL-23A (forward: CCAGCAGGGACATATGATC; reverse: AGCTGTTGGCATAAAGGGCTCAG). All results were normalized against HPRT (forward: AGTCCCAGCGTCGTGATTAGCGATG; reverse: TGGTTAAGGCTTGTGACAG) and expression was quantified using the Kodak 1D image analysis software program.

**ELISA**

Supernatants from cell cultures were collected, filtered and stored at -20º C. ELISAs for IL-1α and KC were performed using matched antibody pair kits from R&D Systems (Minneapolis, MN). The PGE2 ELISA was obtained from Cayman Chemical (Ann Arbor, MI).

**Western Blots**

Culture media was removed and cells were washed twice in PBS. Adherence was broken using 0.5% Trypsin/0.25% EDTA solution and incubation at 37 ºC for 30
minutes. Cells were collected in 50 mL conical tubes by washing dishes with culture media. The tubes were spun at 1500 rpm for 7 minutes. Supernatant was removed and the cells were washed before a second spin. After the second spin, the supernatant was removed and cells were resuspended in NP40 buffer. The cell suspension was sonicated and placed on ice. After all samples were sonicated, the suspensions were placed into microcentrifuge tubes and spun at 13,500 rpm for 30 minutes. The resulting supernatant was then transferred to a new microcentrifuge tube and placed at -20 ºC.

Skin samples (10mm punch biopsies) were collected at the time of sacrifice and snap frozen in liquid nitrogen. Samples were placed into a 50 mL conical tube with NP40 buffer and homogenized. The resulting suspension then underwent the same sonication and spin protocol as above.

All samples underwent Bradford assays to determine protein concentrations. 40 mg of protein was loaded onto precast denaturing gels (Bio-Rad, Hercules, CA) with appropriate controls. Activated caspase 3 and LC3B assays were performed using 15% gels, while Beclin-1, Atg-5 and Atg-7 assays were performed using 10% gels. All antibodies were purchased from Cell Signal (Danvers, MA). Primary antibodies were used at 1:1000 in 5% BSA in TBST and were incubated overnight at 4 ºC while rocking gently. Anti-rabbit IgG, HRP-linked antibody (Cell Signal) was used for detection at 1:2,000 in 5% milk in TBST. Blots were developed using Lumi-Glo (Cell Signal).
Results

*BRE reduces inflammatory cytokine message*

Figure 5.1 shows the effect of BRE on inflammatory cytokine message levels in JB6, 308 and T cells isolated from the dorsal epidermis of mice exposed to UVB. There was no measureable IL-2 or IL-2Rα (CD25) message in either JB6 or 308
Figure 5.1: BRE affects cytokine message in JB6 and 308 cells, as well as freshly isolated T cells from dorsal epidermis. JB6 and 308 cells were grown to 90% confluency in 100 mm petri dishes as described. Fresh media or fresh media containing 64 µg of BRE was added and cells were harvested at 24 hour intervals. Harvested cells then underwent DNA isolation and RT-PCR as described in Materials and Methods. T cells were isolated from the dorsal skin of mice exposed to one MED of UVB thrice as described in Materials and Methods. BRE reduced message for IL-2, IL-2Rα, and IL-6 in T cells (A, B and C). BRE reduced message for IL-6 and IL-23 in JB6 cells (C and D), and increased IL-23 message in T cells (D). BRE had a differential effect upon IL-6 and IL-23 message (C and D). All levels were normalized to HPRT expression.
cells (5.1A and 5.1B). BRE decreased message for both cytokines in epidermal T cells.

Both cell lines showed a reduction in IL-6 message early, but this effect was lost as time went on (5.1C). Indeed, at 48 hours after the addition of fresh media, the 308 cells showed an increase in IL-6. In the T cells isolated from the epidermis, BRE was able to reduce IL-6 message.

308 cells produced more message for IL-23 than the JB6 cells (5.1D). Both cell lines showed a reduction at 24 hours in cells treated with BRE. Both showed an increase at 48 hours, while JB6 cells showed a decrease at 72 hours. IL-23 message increased in BRE-treated cells at 72 hours. IL-23 message was highest in T cells isolated from mice treated with BRE.

**BRE treatment alters the production of inflammatory cytokines in epidermal cell lines**

At 48 and 96 hours BRE was able to significantly affect the production of KC in JB6 cells, exposed to UV or not (Figure 5.2A). Maximal production in the papilloma cells was 13,666 pg/mL/10^5 cells at 96 hours in UVB-exposed, BRE-treated cells. BRE was able to significantly affect the production of KC in 308 cells as well (Figure 5.2B). Maximal production in the papilloma cells was 299 pg/mL/10^5 cells at 72 hours in UVB-exposed, BRE-treated cells.
Figure 5.2: BRE decreased UVB-induced secretion of inflammatory cytokines and chemokines in vitro. JB6 and 308 cells were grown to 90% confluence before half were exposed to UVB, the other half were used as No UV controls. Half of each received fresh media, the other half received fresh media containing 64 μg of BRE. Supernatants were collected at 24 hour time points and used in ELISAs. (A and B) BRE increased KC production in both keratinocyte (JB6) and papilloma (308) cells. (C and D) BRE increased MCP-1 production in both JB6 and 308 cells. There was also a shift in which chemokine was produced in greater quantity between cell types. JB6 cells produced more MCP-1, while 308 cells produced more KC. (E) BRE increased IL-1α secretion by 308 cells. JB6 cells did not secrete IL-1α. *p < 0.05, **p < 0.01, compared to matched No UV samples.
JB6 cells treated with BRE showed a significant changes in MCP-1 at all time points (Figure 5.2 C). Maximal production was 105 pg/mL/10⁵ cells at 48 hours in cells exposed to UVB. 308 cells at all experimental conditions showed significant alterations in MCP-1 production (figure 5.2D). Maximal production was 4.2 pg/mL/10⁵ cells at 96 hours in non-UVB-exposed, non-BRE-treated cells.

IL-1α production was only seen in 308 cells. BRE treatment significantly altered IL-1α production in from 48 on (Figure 5.2D). Maximal IL-1α production (16 pg/mL/10⁵ cells) was in UVB-expose, BRE-treated cells at 96 hours.

BRE alters the autophagic response in epidermal cell lines

**Un-irradiated cells:**

In JB6 cells, Beclin-1 was down-regulated at 24 hours and up-regulated at 48 hours (Figure 5.3A). LC3B was down-regulated at both time points (3B). In 308 cells, Beclin-1 was up-regulated at 24 hours and down-regulated at 48 hours (3C). LC3B was down-regulated at both time points in 308 cells (5.3D). Atg7 was not observed in either cell line.
Figure 5.3: BRE decreases early and increases late autophagy proteins in the absence of UVB. JB6 and 308 cells were grown in culture as described in Materials and Methods. Cells were collected at 24 hour time points and underwent protein isolation. Isolated proteins were used in Western blots to determine the expression of autophagy proteins. Densitometry data was generated using Image J. BRE increased Beclin-1 in 308 cells at 48 hours (C), while it decreased Beclin-1 (A) in JB6 cells. BRE decreased LC3B in JB6 cells at 48 hours (B), while it increased LC3B in 308 cells at the same time point (D).
Irradiated cells:

Beclin-1, LC3B and Atg7 were all up-regulated in JB6 cells treated with BRE (Figure 5.4A-C). Both Beclin-1 and Atg7 were down-regulated at 24 hours and up-regulated at 48 hours in 308 cells (5.4D and 5.4E). LC3B was up-regulated at 24 hours and down-regulated at 48 (5.4F).
Figure 5.4: BRE affects autophagy in keratinocytes (JB6) and papilloma (308) cells after UVB exposure. JB6 and 308 cells were grown in culture as described in Materials and Methods. Cells were collected at 24 hour time points and underwent protein isolation. Isolated proteins were used in Western Blots to determine the expression of autophagy proteins. Densitometry data was generated using Image J. BRE alters the expression of Beclin-1 (D and A) and Atg7 (E and C) in 308 cells to resemble that of JB6 cells. The alteration in LC3B is less dramatic (F and B).
BRE alters 308 cell numbers but not JB6 cell numbers, irrespective of UVB exposure

In cultures of cells grown in the presence or absence of UVB, 308 cells grown in media containing 64 μg of BRE (Figure 5.5C) showed a reduction in cell number, compared to cells grown in control media (Figure D). JB6 cell numbers did not decrease after addition of BRE (Figure 5.5B). In fact, BRE seems to have enhanced cellular proliferation at 72 and 96 hours.

Correlation between cell lines, dorsal skin and tumors

Skin

In the dorsal skin of mice, IL-1α production was decreased after UVB exposure, and there was no difference between mice treated with vehicle and those treated with BRE (Figure 5.6A). There was a significant decrease in the production of KC in mice treated after UVB with BRE (Figure 5.6B). Beclin-1 and LC3B decreased in the dorsal skin of mice exposed to UVB, (Figure 5.6C and 5.6D). There was no difference between mice treated with vehicle and those treated with BRE.

Tumors

In tumors isolated from mice treated with BRE and exposed to UVB, Beclin-1 and Atg7 expression was significantly increased compared to mice treated with vehicle (Figure 5.6A and 5.6B). There was no change in LC3B expression (data not shown). Phosphorylated phosphate and tensin homologue (p-PTEN) was also significantly increased in mice treated with BRE (Figure 5.6C).
Figure 5.5: Effect of BRE on cell number. JB6 and 308 cells were grown in culture as described in Materials and Methods. Cells were collected at 24 hour time points and viable cells were counted. JB6 cell numbers were similar in cultures without BRE (A) or with 64 μg of BRE (B). In 308 cultures, cell numbers were decreased in cells grown with 64 μg of BRE (D), compared to 308 cells grown in control media (C).
Figure 5.6: The effects of BRE on acutely-exposed dorsal skin. SKH-1 (n=10) were acutely exposed to 2240 J/m² UVB and treated topically as described in Materials and Methods. 48 hours after UVB exposure, mice were sacrificed and dorsal skin was harvested. Dorsal skin was homogenized for protein isolation used in ELISAs (A and B) and Western blots (C and D). BRE slightly increased IL-1α (A), decreased KC (B), and had little effect on autophagic proteins Beclin-1 (C) and LC3B (D).
Figure 5.7: Effect of BRE on tumor suppressor protein expression. Mice were exposed to one MED of UVB thrice weekly for 25 weeks, as described in Materials and Methods. After 25 weeks, mice were sacrificed and tumors harvested for protein isolation. Protein isolates were used for Western blots, and densitometry was performed using Image J. BRE increased the levels of Beclin-1 (A), Atg7 (B) and p-PTEN (C) in tumors taken from mice treated with BRE, compared to vehicle-treated controls.
Discussion

Numerous groups have shown that there is a connection between cytokine production and carcinogenesis. We have previously shown that there is a shift in cytokine and chemokine profiles between normal keratinocytes and epidermal papilloma cells (Chapter 3). As it has been shown that BRE can affect signaling pathways, we set out to determine if BRE affects the production of immune mediators in JB6 and 308 cell lines, and in T cells isolated from the dorsal skin of mice.

IL-2 is the critical cytokine in the activation and survival of T cells. The high-affinity receptor for IL-2, IL-2Rα (CD25) is often used as a marker for T cell activation in vitro and in vivo. In our model of acute UVB-induced inflammation, BRE was able to reduce message for both IL-2 and IL-2Rα in T cells isolated from female murine dorsal skin. This may indicate BRE has an impact upon T cell activation, which may be due to an inhibition of AP-1, a protein important in the activation of T cells. Sharma et al showed that resveratrol, a component of BRE, is able to inhibit T cell proliferation. As we have shown in Chapter 4, BRE is able to reduce UVB-induced inflammation, which may combine with AP-1 inhibition to reduce T cell activation. Future studies on the direct effects of BRE on T cell function will be performed. Although both cell lines showed slight expression of IL-2, it is unknown whether this is biologically significant. It has been reported that a variable percentage of keratinocytes express IL-2Rα, so IL-2 may play some role in normal keratinocyte biology.

IL-6 is known to be an important cytokine regulating carcinogenesis in a number of models. Egler et al showed that IL-6 is an important indicator of prognosis in
childhood patients with neuroblastoma. Malinowska et al found that IL-6 is critical in the growth of prostate cancer cells. IL-6 has also been shown to regulate the migration of ovarian cancer cells. BRE was able to reduce IL-6 message levels in the cell lines, except at the 48 hour time point in the 308 cells. BRE was able to reduce IL-6 message in T cells isolated from the dorsal skin of mice exposed to UVB as well. Berberine, a compound isolated from traditional Chinese medicine, was found to inhibit IL-6 production after TPA treatment. Resveratrol was able to inhibit the production of IL-6 by stimulated macrophages. Taken together, these data suggest that one way BRE may be inhibiting UVB-induced carcinogenesis is the reduction of UVB-induced IL-6 produced by T cells, keratinocytes and papilloma cells.

IL-23 is a key cytokine in the pathway of a new class of T cells, Th17. While these cells were first described in models of auto-immunity, they are now being implicated as having a role in carcinogenesis as well. IL-23 levels have been shown to be elevated in gastric cancer, and in chemically-induced skin cancer. In the JB6 cell line, BRE reduced the transcript for IL-23. In 308 cells, BRE decreased message at 24 hours, but increased it at 48 and 72 hours. BRE also increased IL-23 message in T cells isolated from mice exposed to UVB.

In examining the results from the IL-6 and IL-23 experiment, it is important to note that these cytokines may have over-lapping functions in the generation of Th17 cells. Recent work has also suggested that IL-23 may not be necessary for the generation of Th17 cells, but it may be a survival factor, prolonging the life of Th17 cells. As mentioned by a number of reviews, the function of IL-23, beyond its importance in some way to Th17 cells, is poorly understood.
KC (CXCL1) is the murine equivalent of IL-8 in humans, the key neutrophil chemokine. While KC is important in inflammation, it also may play a role in carcinogenesis\(^{149-151}\). KC is elevated both in patients with SCC and in SCC cell lines\(^{151,152}\), and may predict whether a tumor will metastasize\(^{153}\). The same study showed that KC production is at least partially controlled by IL-1\(\alpha\). These data illustrate how many of these proteins work in concert to promote tumor growth. JB6 cells produce significantly less KC than 308 cells, even after exposure to UVB. BRE treatment significantly increased KC production in both cell types at multiple time points. KC was significantly reduced in the dorsal skin of mice exposed to UVB and treated with BRE.

A reduction in KC by BRE \textit{in vivo} would be anti-carcinogenic in a number of ways. A reduction in angiogenesis would starve the tumors and restrict their growth, which could be reflected in the tumor size data from Chapter 4. A reduction in neutrophil numbers during carcinogenesis could also reduce the number of Th17 cells, which would in turn reduce the levels of a number of pro-carcinogenic growth factors, such as IL-6, KC and MCP-1\(^{242,243}\). BRE may also restrict the ability of metastases to grow into tumors. Smith et al found that metastatic tumors of SCCs produced more KC, IL-1\(\alpha\), IL-6 and GM-CSF than primary tumors\(^{17}\). Reducing these growth factors may impair the ability of metastases to grow beyond single cells.

MCP-1 is a chemotactic factor for monocytes and cells of a monocyte lineage, including macrophages\(^{161}\). In response to acute inflammation, macrophages can secrete myeloperoxidase, although to a lesser extent than neutrophils\(^{213}\). Macrophages are also a source of pro-inflammatory cytokines. Inhibition of MCP-1 during the acute inflammatory response would act to reduce the UVB-induced inflammation in the skin.
As discussed earlier, TAMs produce a number of factors that support the growth of tumors. Reducing the levels of MCP-1 during tumor formation would reduce the number of TAMs infiltrating the tumor stroma, and would impair the growth of the tumor.

We have previously shown that there is a shift between KC and MCP-1 production in JB6 and 308 cells (Chapter 3). JB6 cells produce more MCP-1, while 308 cells produce more KC. BRE significantly increased MCP-1 production in both cell types at multiple time points.

IL-1α is an important inflammatory cytokine that is up regulated after UVB exposure\textsuperscript{2,130}. The location of IL-1α (secreted versus membrane-bound or intracellular) determines whether it is beneficial to host or tumor cell\textsuperscript{20,165-167}. IL-1α was only secreted by 308 cells, and was increased in cells exposed to UVB. BRE was able to augment this increase even more at multiple time points. In the \textit{in vivo} murine acute exposure model, there was no difference in IL-1α levels or in autophagic protein expression after BRE treatment. It is not clear why we were unable to detect differences in IL-1α levels as have been reported in human skin, where there is a significant increase in IL-1α after UVB exposure\textsuperscript{244}. It is possible that the levels of IL-1α are increased early in the UVB response, and then are inhibited by the secretion of anti-inflammatory cytokines, perhaps even to levels below that of un-exposed skin.

Although we attempted to explore more closely the results obtained \textit{in vivo}, there are obvious differences in the results obtained \textit{in vivo} and those obtained \textit{in vitro}. \textit{In vivo}, KC levels were decreased after BRE treatment, while the opposite occurred \textit{in vitro}. The cause for this difference is unknown, although we do plan to explore this more fully in the future. One possible explanation is that a regulatory loop exists between
keratinocytes (or papilloma cells) and infiltrating cells that causes the keratinocytes to halt production of the chemokines. This is an untested hypothesis at this point.

The damage to cells from UVB exposure and the resulting inflammatory response can be overwhelming, forcing the cell to undergo programmed cell death. Programmed cell death (PCD) follows one of two pathways. Programmed cell death type one (PCD1) is apoptosis. Apoptosis is a programmed cell death that follows a well delineated program. Apoptosis is dependent upon caspase activation (namely caspase 3) and results in DNA cleavage and the formation of apoptotic bodies\textsuperscript{140}.

Apoptosis is markedly different from PCD2, also known as autophagy. The induction of autophagy is controlled by Beclin-1, which is also a tumor suppressor\textsuperscript{25,141,245}. After Beclin 1 induces the autophagy pathway, two proteins (Atg12 and LC3B) act in concert to form the autophagosome\textsuperscript{142}. Of the two proteins, LC3B is the most important in the formation of the autophagosome\textsuperscript{26}. After forming the autophagosome around the organelles of proteins to be recycled, the autophagosome merges with a lysosome to form an autophagolysosome, and the targets are degraded\textsuperscript{142}.

Autophagy is caspase-independent, although there likely is cross-talk between autophagy and apoptosis\textsuperscript{246}. Autophagy recycles unused and damaged organelles and proteins, especially in times of nutrient deprivation. As such, it is regulated by the PI3K pathway, in particular by the mammalian target of rapamycin (mTOR)\textsuperscript{247}. mTOR inhibitors (such as rapamycin and its derivatives) enhance autophagy\textsuperscript{24}. Therefore, although autophagy is a known PCD pathway, it can also play a role in cell survival, including cancer cell survival\textsuperscript{170}.
As yet, the effect of UVB on autophagy is relatively unexplored. Many of the papers exploring the role of UV in autophagy have used sources producing UVC light\textsuperscript{248,249}, or the source of UV is unclear\textsuperscript{141}. UVB increases the concentrations of ROS and ROS have been shown to play an in autophagy control\textsuperscript{172,224,250}. We therefore hypothesized that using a compound containing powerful antioxidants may have an effect upon autophagy at a resting state and in the response of cells to UVB.

Indeed, BRE did have an effect upon autophagy. At a resting state, BRE not only affected autophagy in 308 cells, it affected autophagy in JB6 cells. BRE increased Beclin-1 at 48 hours in JB6 cells, and decreased it in 308 cells at the same time point. The effects continued in cells exposed to UVB. BRE changed the expression of autophagy proteins to one very similar to JB6 cells unexposed to UVB. This may show that BRE is able to return transformed cells to programmed cell death control.

These findings are further supported by the data showing that 308 cells grown in the presence of BRE exhibit a reduction in cell number compared to 308 cells grown in normal media. The initial growth inhibition may be due to apoptosis, but the continued decrease is likely due to autophagy, as we saw no significant amounts of activated caspase 3 at any time points. JB6 cells did not show a similar reduction in cell number, suggesting that BRE preferentially affects transformed cells. This data agrees with findings from other groups\textsuperscript{42,45,46}.

In tumors, BRE significantly increased the expression of both Beclin-1 and Atg7, while there was no change in LC3B expression. This data mirrors the \textit{in vitro} data, in that it may be showing the ability of BRE to return tumor cells to the at least partial control of programmed cell death. Beclin-1 has been shown to be a haploinsufficient
tumor suppressor\textsuperscript{141}. Elevating Beclin-1 may explain, in part, the ability of BRE to reduce the carcinogenic effects of chronic exposure to UVB. Atg7 has also been shown to be a tumor suppressor\textsuperscript{25}, and impairment of Atg7 in breast cancer cells reduces the ability of resveratrol to induce cell death\textsuperscript{142}. The lack of an increase in LC3B may suggest that although autophagy is initiated, there is a lack of recycling in tumor cells. A recent report shows that anthocyanins isolated from Mediterranean fruits are able to block autophagy in an \textit{in vitro} model of hepatocellular carcinoma\textsuperscript{251}. This shows that the anthocyanins present in the BRE may also be able to block the cell survival aspects of autophagy, and return tumor cells to the control of programmed cell death. This may be another mechanism through which BRE was able to reduce UVB-induced carcinogenesis (see Chapter 4).

Phosphate and tensin homologue (PTEN) is a well-known tumor suppressor\textsuperscript{228,247,252}. A majority of cancers have decreased expression of activated (phosphorylated) PTEN\textsuperscript{252-254}. In tumors taken from mice treated with BRE, phospho-PTEN is significantly increased, compared to vehicle-treated controls. Combined with the Beclin-1 data, we postulate that BRE may be able to protect tumor suppressors from inactivation. We cannot say whether this is a direct effect, or if the protection comes from the ability of BRE to reduce DNA damage in mice chronically exposed to UVB.

In conclusion, we have shown that BRE is able to alter the production of cytokines and may return tumor cells to the control of programmed cell death. We have also proposed that BRE may protect the expression of tumor suppressors in transformed cells. These data may provide the base into the exploration for the use of natural products in combating cancer.
In this work, we have shown that the drug chosen for immunosuppression affects UVB-induced inflammation and carcinogenesis (Chapter 2). Cyclosporine (CsA) exacerbates both responses, but is brought back to vehicle levels with the addition of mycophenolate mofetil (MMF). MMF alone is similar to vehicle in its effects on UVB-induced inflammation and carcinogenesis. These data may aid physicians in the management of post-transplant malignancies.

There are marked changes in keratinocytes as they progress to a pre-malignant phenotype (Chapter 3). Inflammatory cytokines and chemokines were more pronounced in pre-malignant 308 papilloma cells than in immortalized JB6 keratinocytes. 308 cells also exhibited differences in autophagy, before and after UVB. Beclin-1, a tumor suppressor, was down-regulated in unexposed 308 cells, as was LC3B. After UVB exposure, Beclin-1, LC3B and Atg7 were all increased in 308 cells. This data correlated well with results observed *in vivo* suggesting that the use of JB6 and 308 cells in vitro is an appropriate model to explore the changes in keratinocytes as they progress from normalcy to malignancy. Understanding the nuances of the transformation from normal to malignant cell will allow for the development of more effective therapies. One
possible therapy would be the use of a compound high in antioxidants that could block
the production of ROS and impair inflammatory cytokine production.

Towards that end, we tested a topical, post-exposure treatment derived from black
raspberries (BRE) to determine its effectiveness in reducing UVB-induced inflammation
and carcinogenesis (Chapter 4). BRE reduced all markers of inflammation, with the
exception of neutrophil infiltration. The reduction in inflammation may have led to a
reduction in carcinogenesis. We also proposed that the reduction in carcinogenesis may
be mediated by a reduction in tumor-infiltrating regulatory T cells, which may in turn be
a result in chronic inflammation. Further exploration of the mechanisms behind the
beneficial effects of BRE is warranted.

In Chapter 5, we showed that BRE is able to alter the cytokine and chemokine
production of JB6 and 308 cells, irrespective of UVB exposure. BRE treatment also
returned the expression of autophagy proteins in 308 cells to a pattern resembling JB6
cells. In vivo data confirmed some of these findings. This model could result in high-
throughput analyses to determine the toxicity, anti-inflammatory and anti-carcinogenic
effects of novel therapeutics in a skin-specific manner.

Overall, the data in this work give insight into the negative effects of UVB in the
general and immunosuppressed populations, and the mechanisms of action behind a
possible preventative agent. We also demonstrate an effective in vitro model for the
testing of novel therapeutics for the skin. It is the authors’ firm hope that this work will
provide new avenues for the treatment and prevention of non-melanoma skin cancers.
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