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Thermal Burn Injury Induced Microvesicle Particle Release

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THERMAL BURN INJURY INDUCED MICROVESICLE PARTICLE RELEASE

A thesis submitted in partial fulfillment of the
Requirements for the degree of
Master of Science

By

KATHERINE ERIN FAHY
B.S., Wright State University, 2015

2017
Wright State University
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Katherine Fahy ENTITLED Thermal Burn Injury Induced Microvesicle Particle Release BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Fahy, Katherine Erin. M.S. Department of Pharmacology and Toxicology, Wright State University, 2017. Thermal Burn Injury Induced Microvesicle Particle Release.

Microvesicle particles (MVP) are found to be important for cellular communication because they contain many bioactive proteins, lipids, cytokines, and nucleic acids. We have previously found that ultraviolet B radiation (UVB) and a Platelet-activating factor agonist (CPAF) can stimulate the release of MVP in keratinocytes. We hypothesized that there may also be an increase in MVP released after thermal burn and that could be involved in pathogenesis of the systemic effects found in some patients. In this thesis various keratinocyte cell lines, mice and human ex vivo skin were used as model systems to test our hypotheses. It was determined that thermal burn significantly increases the release of MVP compared to the untreated groups. UVB, CPAF and thermal burn all seemed to involve acid sphingomyelinase (aSMase), but different MAP kinase pathways. There was also a significant decrease in the cytokine concentration inside MVP after thermal burn, suggesting a possible defense mechanism to prevent cytokine storm.
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Figure 1: Laboratory members. From left to right: Dr. Eric Romer, Dr. Michael Kemp, Dr. Jeffrey B. Travers, Katherine E. Fahy, Christine Rapp, and Christina Borchers.
Chapter 1: Introduction

1.1 Statement of Problem:

Currently very little is known about keratinocyte-derived microvesicle particles (MVP). However, it has been reported that MVP released from other cell lines play important roles in cell to cell communication. Due to this important role, there has been increased interest in revealing the function of these MVP not only as biomarkers, also as important mediators in pathogenesis mechanisms and even potential targets for therapy. Injuries like UVB and burn affect the epidermis directly, but can also have indirect systemic effects. It is our belief that MVP are important in the cellular communication that results in these systemic effects.

1.2 Significance:

If the MVP are involved in the cellular communication that results in cutaneous inflammation, especially systemic effects induced by skin inflammation, a better understanding of the mechanisms involved can potentially improve the treatment strategies for inflammatory and/or toxic stimuli like thermal burn injury.
1.3 Statement of Purpose:

The goal of this study is to determine if various injuries, including thermal burn and UVB radiation, increase the release of MVP from keratinocytes. Specifically, do human keratinocytes release MVP in response to thermal burn injury in vitro? Also, is MVP release dependent upon PAF? Namely, can the human cell line KB cells that express the PAF receptor (KBP) release increased MVP in response to UVB and thermal burn compared to the KB cells that do not express the PAF receptor (KBM). Finally, does human skin release MVP in response to UVB radiation and thermal burn injury?

1.4 Null Hypotheses:

Our hypothesis is that thermal burn injury will increase MVP release in various keratinocyte cell lines, wild type mice, and human skin. We anticipate that the PAF-R will be involved in keratinocyte MVP release. We do not predict burn injury to increase MVP release in cells that do not express PAF-R or PAF-R -/- mice. We predict that when cells are pre-incubated with PAF antagonist, no significant increase will be observed after burn treatment. We also expect that these MVP will contain cytokine concentrations different from MVP released form keratinocytes under normal conditions. Finally, it is expected that when human skin is injected with MVP derived from HaCaT cells there will be an inflammatory response showing the functionality of these MVP. This inflammatory response will be measured by cytokine expression,
specifically IL-6, IL-8, and TNF-α, in burn induced MVP compared to MVP derived from untreated cells or saline injections.

Null Hypothesis 1:

\[ H_0: \mu_\varnothing = \mu_u = \mu_c = \mu_b: \text{The mean MVP release of no treatment groups equals the mean MVP release of UVB, CPAF and Burn treated groups.} \]

\[ H_a: \text{Not all means of MVP release are the same.} \]

Null Hypothesis 2:

\[ H_0: \mu_\varnothing_m = \mu_Um = \mu_{Em} = \mu_\varnothing_p = \mu_{Up} = \mu_CP = \mu_{BP}: \text{The mean MVP release of no treatment groups equals the mean MVP release of UVB, CPAF and Burn treated groups for cells that express or do not express the PAF receptor.} \]

\[ H_a: \text{Not all means of MVP release are the same.} \]

Null Hypothesis 3:

\[ H_0: \mu_\varnothing_C = \mu_uC = \mu_{cC} = \mu_{bC} = \mu_{pC}: \text{The mean concentration of the various cytokines for the no treatment group, vehicle, UVB, CPAF and Burn will be the same.} \]

\[ H_a: \text{The mean concentration of the various cytokines will not all be the same.} \]

Null Hypothesis 4:
\( H_0: \mu_\Phi = \mu_\theta = \mu_\zeta = \mu_{\Phi \zeta} = \mu_\beta = \mu_{\theta \beta} \): The mean concentration of MVP released from the no treatment groups, PAF-R antagonist alone, CPAF, CPAF + PAF-R antagonist, Burn, and Burn + PAF-R antagonist will be equivalent.

Ha: The mean concentration of the MVP release for these groups will not all be the same.

Null Hypothesis 5:

\( H_0: \mu_\Phi = \mu_i = \mu_\zeta = \mu_{\Phi \zeta} = \mu_u = \mu_{\Phi u} = \mu_b = \mu_{\Phi b} \): The mean concentration of MVP released from the no treatment groups, Imipramine alone, CPAF, CPAF + imipramine, UVB, UVB + imipramine, burn, and burn + imipramine will be equivalent.

Ha: The mean concentration of MVP for all groups will not be the same.

Null Hypothesis 6:

\( H_0: \mu_\Phi = \mu_{PDTC} = \mu_{PD98} = \mu_{SP} = \mu_{SB} = \mu_Y = \mu_\zeta = \mu_{CPDTC} = \mu_{CPD98} = \mu_{CSP} = \mu_{CSB} = \mu_Y = \mu_c = \mu_{PDTC} = \mu_{PD98} = \mu_{SP} = \mu_{SB} = \mu_Y = \mu_b \): The mean concentration of MVP release from no treatment groups, PDTC alone, PD98, 059 alone, SP600125 alone, SB203580 alone, Y-2632 alone, Z-VAD- FMK alone, CPAF, CPAF + PDTC , CPAF + PD98, 059, CPAF + SP600125, CPAF + SB203580, CPAF + Y-2632, CPAF + Z-VAD- FMK, UVB, UVB + PDTC , UVB + PD98, 059, UVB + SP600125, UVB + SB203580, UVB + Y-2632, UVB + Z-VAD- FMK, burn, and burn + PDTC , burn + PD98, 059, burn + SP600125, burn + SB203580, burn + Y-2632, burn + Z-VAD- FMK will be equivalent.

Ha: The mean concentration of MVP for all groups will not be the same.
1.5 Research Objectives:

- To test if there is an increase in the amount of MVP released in keratinocyte cell lines after a thermal burn.
- To test if there is an increase in the amount of MVP released in human and mouse skin after a thermal burn.
- To determine the mechanism of MVP release and the role of the PAF-receptor and MAP Kinase in the thermal burn injury induced MVP release.
- To determine if injecting MVP, derived from human keratinocytes cells, into human skin increases concentrations of inflammatory mRNAs specifically IL-6, IL-8, and TNF-α.
- To investigate the cytokine expression inside of burn induced MVP compared to MVP released from cells under normal conditions.

1.6 Definitions:

**Abdominoplasty:** A surgical procedure where extra skin and/or adipose tissue is removed from the patient’s abdomen.

**Acid Sphingomyelilase (aSMase):** An enzyme that catalyzes the breakdown of sphingomyelin into ceramide. ASMase is a member of the sphingomyelinase family unique in its optimal acidic pH of 4.5—5.0.

Dimethyl sulfoxide (DMSO): A universal solvent with the chemical formula (CH₃)₂SO. When compounds are dissolved in this solvent they can easily penetrate the skin or cell membrane.

Dulbecco's Modified Eagle Medium (DMEM): A common cell culture media to grow mammalian cells.

HaCaT: Human Keratinocyte cell line. These cells were originally spontaneously transformed keratinocytes from histologically normal skin.

Hanks’ Balanced Salt Solution (HBSS): A buffer solution that contains calcium and magnesium to support cell adhesion.

Human Keratinocyte Growth Supplement (HKGS): An ionically balanced supplement that contains bovine pituitary extract. HKGS is intended to use with Epi-Life media for growing human keratinocyte cell lines.

Intradermal Injection: An injection into skin between the epidermis and the dermis.

KBM: A cell line derived from human, nasopharyngeal carcinoma. These cells were transcribed with a blank vector, does not express the PAF-R.

KBP: A cell line derived from human, nasopharyngeal carcinoma. These cells were transcribed with functional PAF-R.

Knockout (KO): When referring to a mouse, a knockout mouse has been genetically altered to not express a protein on interest.
**MAPK/ERK kinase (MEK):** The MAP kinase-kinase that activates ERK 1/2 in the RAS-RAF-MEK-ERK signaling pathway.

**Microvesicle particle (MVP):** Also named as microvesicles and microparticles, are small membrane-bound particles with a diameter between 100-1000 nm that can be shed from the surface of virtually all eukaryotic cells in an active energy-dependent process.

**Mitogen-activated protein kinase (MAPK):** A type of protein kinase. The MAPK cascades regulate a diverse array of cellular programs including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis.

**nTERT:** Immortalized human keratinocyte cell line expressing TERT.

**PAF-receptor (PAF-R):** G-protein coupled receptor which binds to PAF.

**Phosphate Buffered Saline (PBS):** A buffer solution used for a variety of cell culture applications, such as washing cells before passaging, transporting cells or tissue, diluting cells for counting, and preparing reagents.

**Platelet-Activating Factor (PAF):** Acetyl-glyceryl-ether-phosphorylcholine, potent phospholipid activator and mediator of many leukocyte functions, platelet aggregation and degranulation, inflammation, and anaphylaxis. PAF is produced by a variety of cells, but especially those involved in host defense, such as platelets, endothelial cells, neutrophils, monocytes, and macrophages.
**Reactive Oxygen Species (ROS):** Chemically reactive molecules that contain oxygen, examples include superoxide, singlet oxygen, peroxides, and hydroxyl radical.

**Ultraviolet B (UVB):** A range of electromagnetic waves that have wavelengths ranging between 290-320 nm. UVB radiation is absorbed by the epidermis and cannot penetrate appreciably into the dermal layer of the skin.

**Vascular endothelial growth factor (VEGF):** A growth factor that stimulates the growth of new blood vessels.

1.7 Assumptions:

In this project it is assumed that cells will react similarly to the various treatments as a cell in a living organism. It is also assumed that there were no outside influences on the cells reactions. For skin samples it is assumed that all skin samples from the different patients will react similarly to the treatments. It is assumed that age, gender, and ethnicity will not affect MVP release from the various treatments. With the mouse model it was assumed that the mice would react severally to the various treatments. It was also assumed that age and gender did not affect the MVP response to the treatments.
1.8 Summary:

MVP appear to be very important in the transportation of various mRNA, cytokines, and proteins. It is currently believed that they are involved in cell to cell communication. However, very little research has been done to investigate keratinocyte MVP. MVP may be important mediators in systemic effects caused by thermal burn injury. If MVP are increased after burn trauma and there is evidence for their functionality, this would indicate that MVP may be an important therapeutic target for patients with severe burn. In this case determination of a potent MVP inhibitor could be beneficial for these patients in preventing systemic effects.

It was hypothesized that there will be increased MVP release after burn injury in the various keratinocyte cell lines, mice and human ex vivo skin. We also hypothesized that MVP release will be able to be inhibited with the addition of various kinase inhibitors or imipramine. We expected the MVP increase in cells to be PAF receptor dependent. We expected to see increased levels of inflammatory cytokines in the burn treated MVP compared to the no treatment groups. We anticipated that when HaCaT derived MVP were injected into human abdominoplasty skin there would be an increase in the amount of inflammatory cytokine mRNA.
Chapter 2: Review of literature

2.1 Introduction

Epidermal damage from external stressors, for example UVB and thermal burn, are known to be able to cause both local and systemic effects in humans. However, since these stressors only directly affect the skin, the mechanism(s) by which these systemic effects are induced is not currently understood. We have previously reported that UVB-induced MVP release is dependent on the presence of the PAF-R (1). Therefore we believe this receptor may also be required for the increased production of burn induced MVP release.

2.2 Microvesicle Particles:

Microvesicle particles (MVP; microvesicles, microparticles) are small membrane-bound particles with a diameter between 100-1000 nm. In an ATP-dependent manner, MVP can be shed from the surface of almost all eukaryotic cell types (2). Originally it was believed that MVP were just cellular debris. However, studies have since found that MVP can contain many important bioactive proteins, lipids, cytokines, nucleic acids, membrane receptors and adhesion molecules. Though little is known about MVP released from keratinocytes, there has been research on MVP released from several
other cell lines. It was previously found that various cytokines are in the MVP. Among them, TNFα was derived from alveolar macrophages (3) and endothelial cells (4). Also, alarmin HMGB1 was found in MVP released from macrophages after exposure to cigarette smoke extract (5). It is very possible that numerous bioactive agents produced by burn can travel throughout the body via MVP.

After being released from the parental cell, MVP can transfer biological information between cell via direct fusion or internalization (2). Because of their role in intercellular communication, there has been increased interest in MVP as both a biomarker and mediators in pathogenesis mechanisms (6). MVP are also viewed as a potential target for therapy (7,8). Though, there is increased knowledge on the functions of MVP, the formation and releasing mechanisms of MVP are not completely understood. Several studies have found that the formation and release of MVP is associated with mitogen-activated protein kinase (MAPK) pathways (5,9), small GTPases (10,11) and ATP-dependent receptor P2X7R (9). MVP are released by cells in response to a variety of stimuli including serum withdrawal, inflammatory cytokines and shear stress (12). Interestingly, different pathways seem to be involved in the MVP releasing mechanisms for different stressor stimuli. In endothelial cells, TNFα-induced MVP release was able to be blocked by the addition of P38 MAPK and NF-κB inhibitors. However, shear stress-induced MVP release in endothelial cells was blocked by ERK 1/2 (P42/P44) MAPK and Rho kinase inhibitors (13). It appears that MVP can be produced in response to a wide range of stimuli, and these different stimuli release MVP via a variety of signaling pathways. These pathways found to be important for MVP generation in cell
lines are also activated in response to burn in keratinocytes. It is very possible that thermal burn injury could generate MVP in keratinocytes.

2.3 Platelet-Activating Factor:

The lipid mediator, platelet activating factor (PAF; 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is currently known to play a role in platelet aggregation, inflammation, and allergic response. Previously, it was determined that primary human keratinocytes, A-431, and HaCaT cell lines all express functional PAF-Rs (14). Under normal circumstances PAF is not found in the skin; however, keratinocytes can synthesize PAF in response to a variety of stimuli. It was determined that HaCaT cells produce PAF following various stimuli, i.e., ionophores, growth factors, PAF agonists, thermal burn, cold shock, oxidative stress, ultra-violet radiation and pro-oxidant lipid tertiary butyl hydroperoxide (15-17). The antioxidant, vitamin C, was found to be able to block PAF agonist’s metabolism after UVB irradiation of skin. This demonstrates that upstream reactive oxygen species (ROS) induces PAF production after UVB treatment (18).

PAF binds to a single, 7-transmembrane, G-coupled protein receptor, PAF-R (19). The PAF-R is constantly expressed by, leukocytes and endothelial cells and keratinocytes (14,20,21). Once activated, the keratinocyte PAF-R stimulates the production and release of the inducible form of cyclooxygenase (COX-2), tumor necrosis factor α (TNF-α), IL-6, IL-8, and PAF (22,23). This ultimately results in the activation of several signaling
pathways including phosphatidylinositol 3-kinase (PI 3’K), protein kinase C, phospholipases, and protein tyrosine kinases (24). It has also been found that the activation of the endothelial PAF-R causes the activation of ERK and p38 but not JNK MAP kinases (25). It is believed that the activation of these PAF-R mediated pathways in skin results in cutaneous inflammation.

The PAF-R is also activated by carbamyl-platelet-activating factor (1-hexadecyl-2-N-methylcarbamyl-glycero-3-phosphocholine; CPAF), a functional analog of PAF. However, unlike PAF, which is quickly broken down, CPAF is non-metabolizable (26). We have previously found that treating HaCaT cells with CPAF results in the release of MVP (1).

WEB 2086 (also known as Apafant) is a potent and selective PAF receptor antagonist (27). Previously in this lab, it was found that a 30 minute pre-incubation with 25 µM WEB 2086 resulted in a statistically significant decrease in α-toxin-induced arachidonic acid release in HaCaT cells and KBP cells but not KBM cells (28). This dose and incubation time were chosen for this experiment since it was determined to be effective at that dose on HaCaT cells in the past.

2.4 Ultraviolet B:

Ultraviolet (UV) radiation is sub classified into three categories based on the wavelength range: UVA (320–400 nm), UVB (290- 320 nm), and UVC (100–290 nm). UVA can penetrate the dermis of the skin, UVB can only penetrate the epidermis, and
UVC is blocked mostly by the ozone layer and thus does not appreciably reach the surface of the earth (29). Compared to UVA and UVC, UVB has been found to be the most biologically active. When exposed to UVB, the major cell line in the epidermis, keratinocytes, produce cytokines including: transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), macrophage- CSF, interferon gamma (INF-γ), human Interleukin-1 alpha (IL-1α), human Interleukin-1 beta (IL-1β), human Interleukin-3 (IL-3), human Interleukin-6 (IL-6), human Interleukin-8 (IL-8), granulocyte colony stimulating factor (G-CSF), and platelet-derived growth factor (PDGF) (29,30). It has also been noted that after UVB exposure antimicrobial peptides including human β-defensin-2, -3 and ribonuclease-7 and psoriascin (S100A7) are generated (31). The upregulation of these antimicrobial pesticides has been proposed as an explanation for why the use of this immunosuppressant on skin causes resistance to secondary infection (32). IT has also been found that UVB exposure upregulates alarmin high mobility group box 1 (HMGB1). HMGB1 acts via toll-like receptors (TLRs 2, 4 & 9) and the receptor of advanced glycation end products (RAGE) to generate an inflammatory response (33).

The production of many bioactive lipid like, prostaglandin E2 and PAF are also stimulated by UVB radiation (30,34). In Keratinocytes, UVB exposure has been shown to induce the synthesis of PAF and 1-alkyl glycerophosphocholines with sn-2 oxidatively modified fatty acids (ox-GPC) many of which can also act as PAF-R agonists (34,35). UVB irradiation of keratinocytes also induces the upregulation of several microRNAs (36). The UVB induced production of these bioactive proteins, lipids and nucleic acids can
result in local effects including inflammation, degenerative aging, erythema, and skin cancer (37,38). UVB irradiation also can have systemic effects like fever and immunosuppression (39,40). Because UVB is only absorbed by the epidermis it is believed that the bioactive agent effectors are released by the epidermis and induce the systemic effects. The mechanism by which keratinocyte-derived bioactive products can leave the epidermis and migrate though the body is currently unknown.

Recently our group has found that *in vitro* keratinocytes irradiated with UVB, produce increased MVP compared to untreated keratinocytes (1). It was found that the MVP generated by UVB treatment were released in a dose dependent manner and could be detected as early as one hour after UVB treatment. It is known that UVB generates PAF-R agonists in both human keratinocytes and skin (34,35) and that the augmentation of the PAF-R can block UVB-induced cytokine generation. It was also found that pretreatment with the antioxidants vitamin C and N-acetyl cysteine, given at doses previously determined to block UVB-mediated generation of PAF-R agonists (34,35), could block MVP release in response to UVB irradiation but not CPAF treatment. This indicates that ROS plays an important role in UVB-mediated PAF-R MVP release. Competitive PAF-R antagonists are currently available, however these antagonists are not as potent as the native ligand (41). To develop a PAF receptor negative ligand human epithelial cell model, a PAF-R- negative human endothelial cell line (KB) was transfected with a functional leukocyte PAF-R (KBP) or a MSCV2.1 blank retroviral vector (KBM) (28). Using the KBP/KBM model, it was found that UVB irradiation of the cells
only generated MVP in KBP cells, not KBM cells. This suggests that the PAF-R seems to be necessary for the release of MVP from keratinocytes exposed to UVB.

2.5 Thermal Burn:

A thermal burn is any type of burn that results from direct contact with hot objects, like boiling water, steam, and fire. A scald is a thermal burn injury that is caused by contact with very hot water or steam. Scalds are the most common type of thermal burn injury for children. For adults, the most common type of thermal burn is from fire. Thermal burn injury can cause both local and systemic effects. The local wound occurs because of heat induced necrosis. After injury, there is an immediate inflammatory response to the localized area (42). After thermal burn injuries to localized areas, the release of cytokines and inflammatory mediators cause systemic effects to multiple organ systems throughout the body. These systemic effects usually peak around 5-7 days after injury (43-45).

The systemic response from thermal burn injuries is known to affect the cardiovascular system, the pulmonary system, the renal system, the gastrointestinal system and the immune system. In all systems, there is an acute (resuscitative phase) that lasts for roughly 48 hours and a hypermetabolic phase which is more delayed. Immediately after the trauma there is decreased blood flow to tissues and organs, decreased cardiac output, and increased capillary permeability (46,47). Then, during the hypermetabolic phase patients show increased blood flow to the tissues and organs and
increased core temperature (48). It has also been shown that even in the absence of smoke there are significant changes in the lungs after burn injury in animals and humans (49,50). Also, within the a few hours of burn injury, oxidants initiate lung inflammation and lipid peroxidation (49,51). For the renal system, there is decreased glomerular filtration rate (GFR) and renal blood flow during the acute phase of burn injury (52). During the hypermetabolic phase GFR and renal blood flow are increased (53).

Gastrointestinal responses to burn trauma includes adynamic ileus, gastric dilatation, increased gastric and duodenal ulcer incidence, gastrointestinal hemorrhage and local and general distribution of the blood flow with a decrease of metabolic flow (54-56). Finally, during the acute phase after burn injury the release of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) activate the pro-inflammatory cascade and lead to the body entering an immunosuppressed state that predisposes patients to sepsis and multiple organ failure (57-60).

After local or systemic burn injury, one of the main inflammatory mediators released is PAF (61). In a study on rats, it was determined that the PAF was an important mediator for gastric damage after burn injury (62). It was also found that in rats the pre-incubation of a PAR-R antagonist before a thermal burn injury reduced the hypotension and vasoconstriction effects of PAF. As PAF is an important bioactive product released after burn injury and was essential for UVB induced MVP release, it may also be important for the release of MVP released after thermal burn injury.
Inhibitors

Several inhibitors have been found to block MVP generated from various stimuli in different cell lines. For the current study the inhibitors used in Vion and colleagues (PDTC, PD98,059, SP600125, SB203580, and Y27632) were chosen to look at the importance of the various MAP kinases on the production of MVP in human keratinocytes (13). Also, the aSMase inhibitor, imipramine, used in Serban, et. al, was used in this study because it was found to be a very effective inhibitor of cigarette smoke induced MVP release in endothelial microparticles (63). Finally, the pan-caspase inhibitor, Z-VAD-FMK was used as a positive control because in Vion, et. al it was also found that the use of this caspase inhibitor did not have any effect on the MVP release (13).

2.6.1 PDTC:

NF-kappaB (NF-κB) proteins are involved in the mediation of a large number of normal cellular and organismal processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis (64-66). Chronic activation of NF-κB is found in several disease states including: asthma (67), arthritis (68), neurodegenerative diseases (69), heart disease (70), and cancer (71). NF-κB can be activated by two different pathways, the canonical pathway and the alternative pathway (72,73). In the canonical pathway, microbial products and cytokines (especially pro-inflammatory cytokines, TNFα and IL-1) activate complexes containing RelA- or cRel- (74). The alternative pathways can be activated by CD40 ligand (CD40L and TNFSF5)
(75), lymphotoxin β (TNFSF3) (75,76), receptor activator of NF-κB ligand (RANKL and TNFSF11) (77) and B cell activating factor (BAFF and TNFSF13B) (78). The activation of the alternative NF-κB pathway results in the activation of RelB- /p52 complexes (79). Ammonium pyrrolidine dithiocarbamate (PDTC; 1-Pyrrolidinecarbodithioic acid ammonium salt) is a well-known, selective NF-κB inhibitor and an antioxidant. PDTC inhibits the translation of nitric oxide synthase mRNA to prevent induction (80,81).

2.6.2 PD98,059:

Human extracellular-signal-regulated protein kinases 1 and 2 (ERK1/2) kinases are 84% structurally identical and are almost, if not entirely, functionally identical (82). ERK 1/2 is known to play an important role in cell survival, cell cycle progression, metabolism, migration, differentiation, cell adhesion, proliferation, and transcription (83). It has been found that ERK 1/2 signaling can be activated by a variety of stimuli including bradykinin, growth factors, insulin, cytokines, and osmotic stress (84,85). PD98,059 is a potent, selective and cell-permeable MAPK/ERK kinase (MEK) inhibitor. MEK is the upstream kinase that activate ERK1/2 (83). It has previously been determined that shear stress induced MVP release in endothelial cells can be inhibited when the cells are pre-incubated with PD98,059 (13).
2.6.3 SP600125:

C-Jun N-terminal protein kinase (JNK) is an important cell mediator in several important processes including differentiation, proliferation and apoptosis (86-88). It has been found that JNK is activated by external stimuli such as UVB (89), growth factors (90), TNF-α (91), and TPA (92). Once activated JNK can either result in cell proliferation or apoptosis (86). It has been found particularly that JNK plays a significant role in cellular apoptosis after UVB exposure (89). SP600125 (1,9-Pyrazoloanthrone) is a potent and selective inhibitor of the phosphorylation of JNK1, -2, and -3. It was found that the use of this inhibitor was able to block tobacco smoke extract induced MVP release from macrophages (93).

2.6.4 SB203580:

P38 (also called RK or CSBP) plays an essential role in the regulation of several important cellular functions like regulating inflammation (94), cell death (95), differentiation (96), senescence (97), and tumorigenesis (98). P38 can be activated by numerous external stimuli including UV radiation (99), heat shock (100), TNF-α (101), IL-1 (101,102), CSF-1 (103), and osmotic shock (100). Through the addition of a p38 inhibitor, It was found this kinase is required for the increased release of MVP from macrophages when exposed to tobacco smoke (93). SB203580 (4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl] pyridine) is a highly specific, potent, cell-permeable, reversible, and ATP-competitive inhibitor of p38 (104). As PAF signaling is
dependent on the activation of p38 kinase, the inhibition of this kinase also blocks PAF effects (25).

### 2.6.5 Y27632:

Rho-associated coiled-coil kinase family of kinases (Rho-kinase, ROCK, ROK) that includes the ROCK1 and ROCK 2 kinases. Like many other kinases, Rho-kinase is a serine/threonine kinase (105). Rho-kinase functions in the regulation of morphology (106), smooth muscle contraction, cytoskeletal reorganization (107), cell differentiation (108), and cell division (109). ROCK1 also plays a key role in the inflammatory response by suppressing the migration of inflammatory cells by the phosphorylation of PTEN (110). It has been found that Rho-kinase is involved in several disease states including nerve injury (111), vasospasm (112), glaucoma (113), and pulmonary hypertension (114). Y27632 is a selective and potent inhibitor of ROCK1 (p160ROCK; 4-[(1R)-1-aminoethyl]-N-pyridin-4-ylcyclohexane-1-carboxamide) (115). It has previously been determined that shear stress induced MVP release in endothelial cells can be inhibited when the cells are pre-incubated with y27632 (13).

### 2.6.6 Z-VAD-FMF:

Members of the caspase enzyme family play important roles in the regulation of both inflammation and apoptosis. Caspases are highly conserved endoproteases that
hydrolyze peptide bonds resulting in the activation of pathways leading to controlled
cell death or the activation of pro-inflammatory cytokines (116). Z-VAD-FMK
(carbobenzyox-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) is a highly potent,
cell-permeable, non-specific, general caspase inhibitor. Z-VAD-FMK binds to the catalytic
site of active caspase inside cells, forming unbreakable covalent bonds, inhibiting
apoptosis (117-119).

2.6.7  Imipramine:

Imipramine is a common nerve pain medication and antidepressant. Imipramine
has also been shown to be a potent inhibitor of acid sphingomyelinase (aSMase) activity
(120). ASMase is encoded by the gene sphingomyelin phosphodiesterase 1 (SMPD1)
located on chromosome 11 (121). ASMase is a major component of all mammalian cells,
and can be found in two forms, lysosomal aSMase and secretory aSMase (120). ASMase
is an enzyme, characterized by its optimum acidic pH of 4.5-5.0, that catalyzes the
hydrolysis of sphingomyelin to ceramide (122). ASMase is normally activated by
stressors like oxidative stress and cigarette smoke (63). It has also been found that PAF
increases extracellular ASMase activity (123). ASMase has been found to play an
important role in the development of lung injury (123), lung emphysema (124), cystic
fibrosis (125), Wilson’s disease (126), and atherosclerosis (127). A deficiency in aSMase
causes the rare autosomal recessive inherited lysosomal storage disorder, Niemann-Pick
Disease (128,129). ASMase KO mice, a model for Niemann-Pick Disease, has been
found to be resistant to radiation (130) and stress induced apoptosis, like Fas/CD95 (131), ischemia (132), radiation (133), chemotherapy (134), and tumor necrosis factor-alpha (TNF-α; 135).

Once formed, ceramide plays an important role with both pro-apoptotic (136) and inflammatory responses (137). Ceramide is a bioactive lipid made up of long-chain sphingoid base backbone, an amide-linked long-chain fatty acid, and a hydroxyl head group (120). The fatty acid can be monounsaturated or saturated and 2-28 carbons in length (138). The physical properties of ceramide depends on the length of the fatty acid. Mammalian cells usually contain ceramides with fatty acyl chains of 16-24 carbons in length. Long chains of fatty acids (12 carbons or longer) are characterized as non-swelling amphiphiles (139). In contract, smaller fatty acid chains (less than 12 carbons) swell in water (140). Ceramide can be produced by two methods. The first is sphingomyelinase dependent; this pathway is rapid and localized to the plasma membrane. The second method of ceramide production is through a de novo synthetic pathway. This pathway is very slow and is intracellular, mainly in the endoplasmic reticulum or mitochondrial-associated membrane (141-144). Ceramide initiates signaling for numerous stress stimuli including TNF-α, Fas ligand, ionizing radiation, heat shock, ultraviolet light, and oxidative stress (131,145-150).

It was found that the addition of imipramine to endothelial cells was able to block cigarette smoke-induced MVP release. This study also found, that when exposing cigarette smoke to aSMase KO mice there was no increase in MVP release (63).
2.7 Cytokines, Chemokines and Growth factors

Cytokines, Chemokines and Growth Factors are proteins that play important roles in the growth, proliferation and differentiation of cells. Cytokines are secreted by many cell types including immune cells, endothelial cells, keratinocytes, and fibroblasts (151). These secreted proteins play important roles in both the induction and effector phases of the inflammatory and immune responses. Cytokines also have specific effects on cellular interactions, communication, and death (152). Chemokines are a subgroup of cytokines that function in the recruitment of white blood cells to location of the infection (153). This is achieved by inducing chemotaxis, where the attracted cells move in the direction of the higher concentration of chemokines. This process can be induced by inflammation or just maintenance of homeostasis (154). Like cytokines, growth hormones are signaling molecules that mediate cellular activities by intracellular communication. Growth factors play important roles in multiple processes including cellular proliferation, differentiation, angiogenesis, metabolism, wound healing, homeostasis, and morphogenesis (155-161). In this project, 27 different cytokines, chemokines and growth factors were analyzed: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, FGF basic, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1(MCAF), MIP-1α, PDGF-BB, MIP-1β, RANTES, TNF-α, and VEGF.
2.7.1 Interleukin 1 beta

Interleukin 1 beta (IL-1β) is a member of the IL-1 family of cytokines. Originally there were thought to be only two members in this family, IL-α and IL-β. However, currently there are now 11 members total (162). IL-1β is produced from monocytes, macrophages, dendritic cells, B-lymphocytes and NK cells (163). IL-1β is a potent pro-inflammatory cytokine that is associated with both acute and chronic inflammation (164). For patients with auto-inflammatory diseases, IL-1β inhibitors are now a standard therapeutic agent to reduce their symptoms (162).

2.7.2 IL-1 Receptor Antagonist

IL-1 receptor antagonist (IL-1Ra) is also called by its generic name anakinra (162). Anakinra is currently an approved drug for symptom management of rheumatoid arthritis. It also is known to decrease the progression of joint destruction in rheumatoid arthritis (162). IL-1Ra was proposed as a possible therapeutic agent for patients with sepsis. However, in three clinical trials the reduction in in 28-day all-causes mortality was not significant from patients who received a placebo (165).

2.7.3 Interleukin 2

Interleukin-2 (IL-2) plays an important role in the development and death of T cells (166). IL-2 also has a critical role in the down regulation of immune responses to
prevent autoimmunity. In animal models that do not express IL-2 it has been found that the absence of this cytokine results in severe autoimmunity because activated T cells can’t be eliminated (167-169). Under normal conditions IL-2 is produced by CD4+ T helper (TH) cells and CD8+ T cells, natural killer cells and natural killer T cells (170). However, IL-2 can also be produced by activated dendritic cells and mast cells (171,172).

2.7.4 Interleukin 4

Interleukin-4 (IL-4) is an anti-inflammatory cytokine that is released from mast cells, eosinophils, Th2 cells, and basophils (173). This cytokine is known to be an important in the growth and differentiation of CD4+ T lymphocytes into Th2 cells (174,175). IL-4 is also an essential mediator for leukocyte survival (176,177).

2.7.5 Interleukin 5

Interleukin 5 (IL-5) is a pro-inflammatory cytokine that can be produced from T cells, granulocytes, and natural helper cells (178). IL-5 has a variety of effects on cells. One of these effects is that IL-5 has an important role in the migration of eosinophils to inflammation sites (179). IL-5 also is important for the development, function, and metabolism of basophils (180). Finally, IL-5 is important mediator in the regulation of the differentiation, growth, activation, recruitment, and survival of eosinophils (181,182).
2.7.6 Interleukin 6

Interleukin 6 (IL-6) is a pleiotropic cytokine that has both pro-inflammatory and anti-inflammatory effects (183). IL-6 is produced in response to a variety of stimuli such as Toll-like receptor ligands, IL-1, IL-17, TNF-α, physical exercise, lipopolysaccharide, anxiety, and PAF (184,185). Once activated, IL-6 has the ability to promote T cell activating, B cell differentiation, population expansion and regulate acute-phase response (186).

2.7.7 Interleukin 7

Interleukin 7 (IL-7) is a pro-inflammatory cytokine that is produced primarily from non–marrow-derived stromal and epithelial cells (187). This cytokine is important in the regulation of T- and B-cell development and T-cell homeostasis (188-190). IL-7 is critically important for T-cell development in humans.

2.7.8 Interleukin 8

Interleukin 8 (IL-8), also known as CXCL is a pro-inflammatory cytokine that mediates the activation and migration of neutrophils (191,192). IL-8 has been found to be both produced from monocytes and macrophages (192). It has been determined that
the release of IL-8 is regulated by a variety of stimuli including TNF-α, IL-1β, chemotherapy agents, hypoxia, androgens, estrogens, and dexamethasone (193).

2.7.9 Interleukin 9

Interleukin 9 (IL-9) is a pro-inflammatory cytokine that was originally cloned as a growth factor for T cells. T Lymphocytes are the main cell line that produce this cytokine, though it can be produced by other cells. IL-9 has both effects on mast cell and B cell growth and function. IL-9 has also been found to promote the development of Th17 cells (194).

2.7.10 Interleukin 10

Interleukin 10 (IL-10) is an anti-inflammatory cytokine that is released from most myeloid and lymphoid cells (195). This cytokine seems to function mainly by blocking the production of other cytokines and chemokines from dendritic cells and macrophages (196). It has also been found that IL-10 functions by inhibiting Th1 cell activity (195).
2.7.11 Interleukin 12

Interleukin-12 (IL-12) is a pro-inflammatory cytokine that is produced by inflammatory myeloid cells. One of its main functions is stimulating the development of TH1 cell (197). IL-12 also stimulates the release of IFN-γ and can increase the cytotoxicity of natural killer cells (198).

2.7.12 Interleukin 13

Interleukin 13 (IL-13) is an anti-inflammatory cytokine produced from T cells and dendritic cells (199). It has been found that IL-13 is important in the differentiation and proliferation of B cells (200). IL-13 also blocks the production of inflammatory cytokines and is involved in the regulation of the explosion of parasites in the gastrointestinal tract, inflammation caused by allergies, production of IgE antibodies, and remodeling of tissues (201).

2.7.13 Interleukin 15

Interleukin 15 (IL-15) is a pro-inflammatory cytokine that regulates the activation and proliferation of T cells and natural killer cells (202,203). IL-15 is produced mainly during the innate immune response by macrophages and monocytes (204). Mice that are deficient in IL-15 (knockouts) were considered to be overall healthy. However these mice are lymphopenic (205).
2.7.14 Interleukin 17

Interleukin 17 (IL-17) is a pro-inflammatory family of cytokines composed of six ligands that can bind to 5 ubiquitously expressed receptors. IL-17 can be secreted by many different cell lines including dendritic cells, natural killer T cells, macrophages, natural killer cells and γδ-T cells. IL-17 is known to be involved in the mediation of most autoimmune diseases like rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, and psoriasis (206).

2.7.15 Eotaxin

Eotaxin is a chemokine that is constitutively produced by many cell types including epithelial tissues, but can be upregulated during allergic reactions. It has been determined that one of its main functions appears to be the regulation of eosinophil trafficking (207). Exotoxin signals via a single receptor, CCR3, which is expressed on eosinophils, basophils, mast cells, and Th2 lymphocytes (208).

2.7.16 Granulocyte-Colony Stimulating Factor

Granulocyte-colony stimulating factor (G-CSF) is a growth factor that is released from several cell types including monocytes, lymphocytes, fibroblasts, endothelial cells,
astrocytes and bone marrow stromal cells. One of G-CSF’s main functions is to regulate the differentiation and proliferation of neutrophil precursors. G-CSF also regulates several functions of mature neutrophils like cell survival, release of superoxides, degranulation of cells, and neutrophil trafficking (209).

2.7.17 Interferon-gamma

Interferon-gamma (IFN-γ) is a member of the IFN cytokine family. The family is divided into two classifications: type I and type II. IFN-γ is the only member of the type II classification. Unlike the type I members, IFN-γ has a completely different receptor it binds to and is encoded in a different chromosomal region. IFN-γ is secreted by several cell lines including Th1 lymphocytes, CD8+ cytotoxic lymphocytes, natural killer cells, B cells, natural killer T cells, and professional antigen-presenting cells (210). IFN-γ is known to have a variety of functions including activating the innate immune system, mediating the balance between Th1 and Th2 cells, controlling cellular proliferation, regulating apoptosis, and activating macrophages.

2.7.18 Macrophage Inflammatory Protein 1 Alpha

Macrophage inflammatory protein 1 alpha (MIP-1 α) is a pro-inflammatory chemokine that is released from various cell lines including monocytes, fibroblasts, macrophages, Langerhans cells, and T lymphocytes. MIP-1 α has a variety of functions
including regulating the migration of both monocytes and T lymphocytes. MIP-1 α also plays a role in the degranulation of mast cells and can stimulate the production of other cytokines, specifically TNF-a, IL-1 and IL-6 (211).

2.7.19 Platelet-Derived Growth Factor-BB

The Platelet-Derived Growth Factor (PDGF) is a family of growth factors consisting of three proteins: PDGF-AA, PDGF-AB, and PDGF-BB. During early development PDGF are important in the migration and angiogenesis of endothelia progenitor cells and Mesenchymal stem cells. However, there is not significant evidence of any normal functions in adults. In several diseases it has been found that there is increased PDGF activity. Some of these conditions include pulmonary hypertension, retinal vascular disease, and dermal fibrosis (212).

2.7.20 Regulated on Activation, Normal T Cell Expressed and Secreted

Regulated on Activation, Normal T Cell Expressed and Secreted (RANTS), also called CCL5, is a chemokine that can be released from cells like epithelial cells and platelets. RANTS is currently known to play an important role in the migration of monocytes, natural killer cells, T cells, eosinophils, and dendritic cells (213-215).
2.7.21 Tumor Necrosis Factor Alpha

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine produced mainly by macrophages; however, it can also be produced by fibroblasts, monocytes, T-cells, adipocytes and smooth muscle cells (216). TNF-α is also one of the cytokines that is important for acute phase reaction (217).

2.7.22 Monocyte Chemoattractant Protein-1

Monocyte Chemoattractant Protein-1 (MCP-1; also called CCL2) is a chemokine that is important in the regulation of the migration and infiltration of monocytes and macrophages (218). MCP-1 can be synthesized and released from smooth muscle cells, fibroblasts, mononuclear cells, endothelial cells, keratinocytes and epithelial cells (219).

2.7.23 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) is a growth factor produced from cells, like tumor cells, macrophages, platelets, keratinocytes, and renal mesangial cells. Along with the critical role VGEF plays in angiogenesis, it also functions in wound healing, bone formation, development, and hematopoiesis (220).
2.7.24 Fibroblast Growth Factors

Fibroblast Growth Factors (FGF) is a highly conserved family of growth factors currently comprised of 22 members. FGF are expressed by most if not all tissues but the sub classes of VGF vary on when they are expressed. For example, some sub classes of FGF are only expressed during embryotic development, like FGF3, FGF4, FGF8, FGF15, FGF17 and FGF19. The function of FGF also changes depending on the developmental stage of the organism. While the organism is an embryo, FGF functions in mediating cell proliferation, differentiation, and migration. However, when the organism is an adult, FGF functions in tissue repair (221).

2.7.25 Granulocyte Macrophage Colony-Stimulating Factor

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a growth factor that also has prominent pro-inflammatory functions. GM-CFS is produced from many cell lines, including T cells, B cells, macrophages, endothelial cells, fibroblasts, neutrophils, eosinophils, epithelial cells, mesothelial cells, chondrocytes, Paneth cells, and tumor cells. This growth factor is known to stimulate the proliferation and differentiation of bone marrow precursor cells and myeloid progenitor cells, as well as promote the survival of mature myeloid cells (222,223).
2.7.26 Interferon-γ Inducible-Protein-10

Interferon-γ inducible-protein-10 (IP-10) also referred to as CXCL10, can be released from several cell lines including epithelial cells, endothelial cells, keratinocytes, leukocytes, eosinophils, monocytes, and neutrophils. IP-10 is important in the migration of monocytes, T cells and NK cells. IP-10 also plays an important role in the regulation of angiostatic effects, apoptosis, and cell growth (224).

2.7.27 Macrophage inflammatory protein

Macrophage inflammatory protein (MIP) contains two different chemokines, the MIP alpha protein (MIP-1α) and the MIP beta protein (MIP-1β). MIP-1α can be released from many cell types including monocytes, T lymphocytes, B lymphocytes, NK cells, dendritic cells, neutrophils, basophils and fibroblasts. MIP-1β can be released from several cells like Monocytes, T lymphocytes, B lymphocytes, NK cells, dendritic cells, microglia, and neutrophils. It has been determined that MIPs are involved in the migration of monocytes, T lymphocytes, natural killer cells, neutrophils, eosinophils, and basophils (225).
2.7 Summary

MVP are small membrane-bound particles that seem to play an important role in intercellular communication. Small amounts of MVP are released under normal conditions and is increased by a variety of stimuli including serum withdrawal, inflammatory cytokines and shear stress. Little is currently known about MVP released form keratinocytes. However it has been determined that MVP shed from other cell types contain many important bioactive proteins, lipids, cytokines, nucleic acids, membrane receptors and adhesion molecules. These MVP may carry bioactive agents produced by thermal burn injury throughout the body causing the various systemic effects associated with burn injury. If MVP are regulators for the systemic effects observed, MVP may be an important therapeutic target. Some of the important agents carried in MVP are cytokines, chemokines and growth factors. These agents are important in the growth, proliferation and differentiation of cells. Importantly, these are biologically active agents that may be being transported though out the body and causing systemic effects.

PAF is a lipid mediator that is important in the regulation on platelet aggregation, inflammation, and allergic response. PAF is not found in the skin under normal conditions, however it is synthesized in response to a variety of stimuli like PAF agonists, thermal burn, and UV radiation. PAF binds to a single G-coupled protein receptor, and once activated the PAF-R stimulates the production and release of COX-2, TNF-α, IL-6, IL-8, and PAF. CPAF is a functional analog of PAF that is metabolically stable, unlike PAF and related ox-GPCs which are rapidly inactivated by aecylhyrolases.
Previously in our lab we have determined that treating HaCaT cells with CPAF resulted in the increased release of MVP.

UVB is a sub type of ultra-violet light that can only penetrate the epidermis. When keratinocytes are exposed to UVB radiation they produce cytokines including TGF-α, TGF-β, TNF-α, INF-γ, IL-1α, IL-1β, IL-3, IL-6, IL-8, G-CSF, and PDGF. One important systemic effect of UVB radiation is immunosuppression. Because UVB can only penetrate the epidermis the bioactive proteins would have to be carrier throughout the body. We believe that MVP may fill this role.

Like UVB, thermal burn injury is known to can cause both local and systemic effects. In localized areas heat induces necrosis almost immediately and there is an immediate inflammatory response. Then around 5-7 days after the burn injury patients experience systemic effects on the cardiovascular system, the pulmonary system, the renal system, the gastrointestinal system and the immune system.

It has been previously determined that both UVB and CPAF were able to increase the MVP release in keratinocyte cells. As MVP can carry various bioactive proteins and lipids, it is reasonable to believe that they may play an important role in the systemic effects of UVB and burn injury. Previous papers have found that in other cell lines, various inhibitors have been effective at inhibiting the increase in MVP. If the MVP are important in regulating the systemic effects though carrying biologically active agents throughout the body, these inhibitors could be potentially important therapeutic agents.
Chapter 3: Materials and Methods

3.1 Introduction

This chapter discusses the model systems, procedures, and analysis performed to acquire the relevant data to answer the experimental question of this study. Three model systems were used in this study: in vitro human cell lines, in vivo mouse model, and ex vivo human skin.

3.2 Cell Culture

The commonly used spindle shaped cell line, HaCaT, is derived from spontaneously immortalized, human keratinocytes (226). Like primary keratinocytes, HaCaT cells do exhibit basal cell properties and still respond to various inducers of cell differentiation, for example calcium and high cell density (227). NtErT are an immortalized primary keratinocyte cell line expressing telomerase hTERT (228). KB cells were originally derived from a patient with a nasopharyngeal carcinoma (229). These cells are a model for human keratinocytes that lack PAF-R. The KB cells were transduced with the empty MSCV2.1 retrovirus containing the PAF-R (KBP). Control cells (KBM) were transduced with the control MSCV2.1 retrovirus. The functionality of these cells were demonstrated previously (17,22). Primary keratinocytes were isolated by Dr. Kemp
following the ThermoFisher Scientific protocol for the isolation and culture techniques of Human Keratinocytes (230). These keratinocytes were isolated from the donated skin of a female 32 year old abdominoplasty patient.

3.2.1 Media:

HaCaTs and KBP/ KBM cells were grown in DMEM media. Media consisted of a 500 mL bottle high glucose DMEM containing 100 U penicillin/ 0.1 mg/mL streptomycin (5mL), 2 mM glutamine (5 mL), and 50 mL 10% FBS FetalClone III. For NtErTs and primary keratinocytes use EpiLife Growth media with human keratinocyte growth supplement (HKGS) 100x and 100 U penicillin/ 0.1 mg/mL streptomycin (5mL).

3.2.2 Cells Passaging:

Media was removed from cells (KBP, KBM, HaCaT) and plates were washed 3 times with 10 mL of PBS 1X. 2 mL of 0.25% Trypsin-EDTA 1X was then added to each plate and placed in the incubator for 10 minutes. Then, 8 mL of media was added to each plate to bring it back to 10 mL and cells were triturred. For KBP/KBM cells 6 drops of the cells suspended in media was added to a new plate with 10 mL of fresh media. For HaCaT cells 2.5 mL of cells was added to a new plate with 10 mL of media.

Before passaging the primary keratinocytes, cell culture plates were coated with Gilbco* diluting medium (67 µL per cm²) and concentrated coating matrix (0.67 µL per
cm²) for keratinocytes. This coating was left on the plates for 1 hour and discarded before passaging cells (note: pre coated plates were only used for primary keratinocytes).

For NtErT and primary keratinocytes cells, plates were washed three times with 10 mL PBS 1X. Then, 2 mL of 0.05% Trypsin-EDTA 1X was added to each plate and placed in the incubator for 10 minutes. High glucose DMEM (6 mL) was then added to the plates and triturated.

3.2.3 Changing media:

To change the media on the cells, the old media was removed and the plate was washed 3 times with 10 mL of Hanks balanced salt solution. Then 10 mL of fresh media was added. This was performed roughly every 2 days between cell passaging.

3.2.4 Cell Count:

To count cells, first cell culture plates were passaged following the procedure above. Once the trypsinized cells are suspended in fresh media, 200 µL of passaged cells were added to 800 µL of PBS 1x in a 1.5 mL Eppendorf® tube and use a Millipore Scepter handheld automated cell counter.
3.2.5 Bringing Up Cells from Liquid Nitrogen:

To bring up frozen cells, the vial was removed from liquid nitrogen. The vial was warmed in hand and cells were immediately added to a new culture dish containing 15 mL of fresh media. The cells were fed the next day and checked every day for two weeks.

3.2.6 Inhibitors:

After plates were washed three times with HBSS, HBSS+ bovine serum albumin (BSA) was added to the plates for a final total volume up to 2.5 mL. Then the inhibitors were added at the following volumes and concentrations: 0.05 mL of 100µM imipramine, 0.5 mL 20 uM PDTC, 0.5 mL 20 µM PD98,059, 0.5 mL 2 µM SP600125, 0.5 mL 20 µM SB203580, 0.5 mL 2 µM Y27632 and 0.5 mL 48 µM Z-VAD-FMA. The inhibitors were incubated for 1 hour before treatments.

3.2.7 Treatments:

Before cells plates can be treated all culture plates received HBSS+ BSA for an end volume of 2.5 mL per plate. Sham culture plates received no treatment and were left in the culture incubator for the duration of the incubation time with 2.5 mL of HBSS+ BSA. CPAF treated cells received 1 mL of 100 nM CPAF and 1.5 mL HBSS + BSA. PMA treated cells were diluted from stock solutions to a final volume of 100nM. Cells
received 1 ML of 100 nM PMA and 1.5 mL HBSS + BSA. UVB treated cells received 3,600 J/m² with lids removed. Burn treated culture plates were placed in a 90° C water bath for 8 seconds, 30 seconds, or 45 seconds. Cells then were incubated for 1, 2, or 4 hours.

3.2.8 MVP Extraction:

After the designated incubation time, 2 mL of the HBSS +BSA from the treated cell culture plates were moved to a 2 mL Eppendorf® tubes and centrifuged with a Eppendorf® centrifuge 5810R at 2,000 x g for 20 minutes at 10° C. The supernatant was then transferred to a new tube and centrifuged at 20,000 x g at 10° C for 70 minutes. Then that supernatant was discarded and pellet was re-suspended in 100 µL of 1x PBS. The samples were diluted appropriately to be within the threshold of the NanoSight NS300 and this instrument was used count MVP concentration.

3.2.9 Analysis:

The concentration obtained from the NanoSight NS300 was multiplied by the dilution rate, divided by the cell count and then multiplied by 100,000 cells. Standard error was calculated for all treatments and graphed. On GraphPad Prism 5 an ANOVA was performed on samples to show significance with a 95% confidence interval.
3.3 Skin

Abdominoplasty, brechioplasty, and gynecomastia skin were graciously donated from Sycamore Medical Center (Miamisburg, OH). Patients (n=25) were between the ages of (23-65).

3.3.1 Treatment:

Skin was treated with either 90% DMSO/10% EtOH vehicle, 0.539 ng CPAF made in vehicle, 1,000 J/m² UVB, 2,500 J/m² UVB, or 90°C ddH₂O was poured into a funnel and held on the skin for 5 seconds. UVB treatment was performed using a Philips F20T12/UVB lamp as previously described (6). Sham skin and blisters received no treatment. To prevent migration each treatment was performed on separate pieces of skin.

3.3.2 Punch Biopsies:

After treating the skin. The skin pieces were placed in a 37°C water bath in individual culture dishes. Then 2-6 mm punch biopsies were taken from the center of each piece of skin. The fat was removed for the biopsies and they were individually weighed and placed in separate Eppendorf® tubes. Collagenase dispase (1 mL of 5mg/mL made in 1:1 H₂O and Trypsin-EDTA (0.5%), no phenol red) was added to each tube. The
tissue was then cut up finely in the Eppendorf® tube and digested overnight in a shaker at 37°C.

3.3.3 MVP extraction for Punch Biopsies:

After the overnight digestion the tissue was filtered and rinsed with an additional 1 mL of PBS (1x). Then, 1 mL of the fluid was then collected and moved to a new Eppendorf® tube and centrifuged at 500 x g for 30 minutes. Supernatant was moved to a new tube and centrifuged at 2,000 x g for 20 minutes. The supernatant was moved again to a new tube and centrifuge at 20,000 x g for 70 minutes. Supernatant was then discarded and pellet was re-suspended in 100 µL filtered PBS (1x). Samples were placed in -80 freezer until ready to analyze.

3.3.4 Suction Blisters:

Skin was rinsed, excess fat removed, and placed in a glass pan containing 37° C PBS (1X) to cover the bottom of the pan to prevent the skin from drying out. Vacuubrand® vacuum pump, 100 mbar, was attached to the barrel of a 20 mL syringe with plastic tubes. The barrels were placed against the skin and the pump was turned on to form a strong suction on the skin. Heating pads and a portable heater were used to keep the skin warm while blisters formed. Skin had high variability on blister formation,
15 minutes to 3 hours. Once blisters had formed the barrels were removed and the blisters were treated.

Figure 2: Suction blisters forming on abdominoplasty skin.
Figure 3: Formed blisters on skin divided into UVB treatment groups.

3.3.5 Inhibitors:

All inhibitors were purchased from Sigma (USA). After skin was washed and dried, the various inhibitors were applied topically in 90% DMSO/10% EtOH (1.64 µg PDTC, 2.67 µg PD98,059, 0.22 µg SP600125, 3.77 µg SB203580, 0.32 µg Y27632, or 11.22 µg Z-VAD-FMK). The inhibitors were incubated for 1 hour before proceeding to treatments above.
3.3.6 MVP Extraction for Suction Blisters:

A 30 ga needle on a 1 ml syringe were used to extract the blister fluid into tared tubes and weighed. Blister fluid weight was recorded for each sample to normalize data. PBS (1X) was added to each tube to bring the volume up to 0.5 mL (assuming 1 µg blister fluid = 1 µL). Samples were then centrifuged at 2,000 x g for 20 minutes at 10° C. Supernatant was then transferred to a new tube and centrifuged at 20,000 x g at 10° C.

*Figure 4: Topical application of inhibitors to blisters on abdominoplasty skin.*
for 70 minutes. Supernatant was discarded and pellet was re-suspended in 100 µL of 1x PBS. Samples were stored in -20 freezer until ready to analyze. (26).

3.3.7 Analysis:

After MVP were extracted from the blister fluid. The samples were diluted appropriately to be within NanoSight (NanoSight Ltd®) threshold. The concentrations of MVP in each sample was then multiplied by dilution rate and were normalized by dividing by the weight of the blister fluid. Standard error was calculated for all treatments and graphed. On GraphPad® Prism 5 an ANOVA was performed to show significance with a 95% confidence interval.

*Figure 5: Blister fluid being removed by syringe.*
3.4 RNA Isolation and Analysis from Human Skin Ex Vivo

3.4.1 MVP:

MVP were obtained from HaCaT cell line. Cells were washed 3x with HBSS and then received either no treatment, 3,600 J/m² UVB, or a 30 second thermal burn. Cell culture plates were then placed in an incubator for 4 hours and at that time 2 mL of supernatant was removed from each plate and pipetted into a 2 mL Eppendorf® tube. The samples were then centrifuged at 2,000 x g for 20 minutes at 4°C. The supernatant was poured into a new Eppendorf® tube and was centrifuged again at 20,000 x g for 70 minutes at 4°C. Supernatant was then discarded and the pellet was re-suspended in 200 µL filtered PBS. The samples were divided into two tubes, one for counting MVP concentration and one for injections. Samples were stored at -20°C until ready to use.

3.4.2 MVP injection:

10⁶ and 10⁷ MVP from each group (diluted in saline to have a final volume for all samples of 100 µL) was injected subcutaneously into a small pieces of skin. For a positive control 100 µL of 100 nM CPAF was injected subcutaneously into the skin. Also, for a negative control saline solution was also injected into the skin. Skin samples were placed in cell culture dishes with 1x PBS to prevent the skin from drying out. The samples were then incubated for 4 hours. After that, skin was frozen with a liquid nitrogen gun and epidermis was removed with a curette. Epidermis was then placed in
250 µL RNAlater® in an RNA free Eppendorf® tube. Samples were kept at 4° C until ready to perform RNA isolation.

3.4.3 RNA isolation:

RNA was isolated using Qiagen RNeasy® Plus Universal Mini Kit. A sonicator was used to homogenize the skin tissue, besides that all steps were followed as directed in the kits manual.

3.4.3 RNA Quantification

To determine the amount of RNA in the sample the Thermo Scientific NanoDrop One UV-Vis Spectrophotometer was used. System was blanked with 2 µL of RNase- free water. Then, 2 µL of sample was added and measurement was written on each tube. Between each sample, both sides of the reader where cleaned with a Kimwipe.

3.5 Mice

C57BL/6 EGFP Wild type mice were obtained from Indiana University–Purdue University Indianapolis (IUPUI). Mice were housed together, provided with water and food ad libitum in a 12 hour light/dark cycle room. The protocol for experimental use of
animals was approved by Wright State University, School of Medicine’s Laboratory Animal Care and Use Committee (LACUC).

3.5.1 Treatment:

Mice were IP injected with ketamine/ xylazine, shaved, and mice were given buprenorphine for pain. Mice then received an 8 second burn on their back from a 2 cm by 2 cm stainless steel iron, heated in a 90° C water bath. The mice were then placed in individual cages for a 2 hour incubation time period.

3.5.2 Mouse Epidermis Separation

After 4 hours of incubation, mice were euthanized. Skin was removed and flash frozen to an index card. Then the skin was transferred to a 60°C water bath for 10 seconds and then immediately moved to an ice bath for 15 seconds. Skin was then dried with paper towels and the connective tissue and dermis were gently scraped off with forceps. For RNA extraction samples were then placed in 250 µL RNAlater and stored at 4°C until ready to isolate. Skin samples for MVP counts were weighed in a pre-tared Eppendorf® tube. 1 mL of 5 mg/mL collagenase dispase, made up in 1:1 ddH₂O and Trypsin-EDTA (0.5%) no phenol red, was added to each tube. Tissue was then finely cut up and digested in a Rotisserie Hybridization Oven (Hybaid® HS9320) overnight at 37°C.
3.5.3 MVP extraction for Punch Biopsies:

After the incubation time period 4-6 mm punch biopsies were taken from the burnt area of the back and two punch biopsies were taken from the un-burnt stomach.

Mouse skin biopsies were individually weighed and placed in separate Eppendorf® tubes. 1 mL of 5mg/mL collagenase dispase made in 1:1 H2O and Trypsin-EDTA (0.5%), no phenol red was added to each tube. The tissue was then cut up finely in the Eppendorf® tube and digested overnight in a shaker at 37°C. After the overnight digestion, tissue was filtered by gravity with Pierce® Tissue Strainers. The tissue was then rinsed with 1 mL of PBS (1x). Strainer was discarded and 1 mL of solution was taken from each tube and placed in a clean Eppendorf® tube. Samples were then centrifuged at 500 x g for 30 minutes. Supernatant was moved to a clean Eppendorf® tube and then centrifuged at 2,000 x g for 20 minutes. Again, the supernatant was moved to a clean Eppendorf® tube and then centrifuged at 20,000 x g for 70 minutes. Supernatant was then discarded and the pellet was re-suspended in 100 µL filtered PBS (1x). Samples were kept at -80°C until analysis.

3.6 Cytokine Expression Assay:

Using HaCaT cells with a 4 hour incubation time frame, MVP were extracted from no treatment, vehicle, CPAF, UVB, and burn treated culture plates. MVP samples were then analyzed for total protein content with a BIORad assay and MVP
concentration was calculated with the NanoSight instrument. Sample were then given to Dr. Cool to run a Bio-Plex Pro™ Human Cytokine 27-plex Assay to analyze MVP contents for human cytokine expression. 27 cytokines were analyzed: FGF basic, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF.

3.7 Analysis:

Mean and significance were determined and graphs were made using GraphPad® Prism 5 (GraphPad Software; La Jolla, CA). Data is expressed as mean ± standard error. Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. Notation within figures include P<0.05 (*), P<0.01 (**), and P<0.001 (***).
Chapter 4: Results

4.1 Cell Culture:

Our first studies were designed to determine if the vehicle control caused an increase in MVP release. To that end, various levels of DMSO (1 µL, 5 µL, 10 µL) and 5 µL ethanol was added to 2.5 mL HBSS+BSA on the cell culture plates (Sup. Figure 22). It was determined that at these concentrations there was no increase in MVP release induced by DMSO compared to the cell culture plates that received no treatment (only 2.5 mL HBSS+BSA).

4.1.1 HaCaT:

To show that thermal burn injury (burn) can induce MVP and find the ideal incubation time and dose of burn, HaCaTs received CPAF, UVB and Burn (8 second, 30 second and 45 second; Figure 6) treatments and were then incubated for 1 hours, 2 hours and 4 hours (Figure 7). UVB has been previously found to induce MVP release in HaCaTs (1). Of interest, the 30 second burn was significant from the no treatment at all three incubation time periods. At the 2 hour and 4 hour incubation time periods the CPAF, UVB, and 30 second burn are all significant. Also, the 30 and 45 second burn groups, exhibited much higher MVP release than the UVB- and CPAF-treated. We then investigated the effects of the different stimuli on the cells viability to determine in MVP
release is associated with cell death. It was determined that there was no decrease in cell viability in cell culture plates treated with UVB, CPAF, 8 second burn or 30 second burn (Figure 8). However, there was a significant decrease in viability in cell culture plates that received 45 second burn. Since, the CPAF UVB, and the lower intensity burn treatments did not affect the cell viability it can be assumed that not all MVP release is associated with cell death.

**Figure 6:** Thermal burn injury induced MVP release dose response curve for HaCaT cells. Cell culture plates either received no treatment, CPAF (53.9 ng), 3,600 J/m2 UVB, 8 second thermal burn, 30 second thermal burn or a 45 second thermal burn and were incubated for 4 hours. The amount of MVP released after CPAF, UVB and 8 second burn were roughly 5-6x the amount of MVP released from the cells that received no treatment. The 30 and 45 second burn samples had an even larger amount of MVP released from the cells (15x the basal level). The data depicted are mean ± SE MVP per mL per 100,000 cells (average of n=4). Groups were compared using one-way ANOVA. Differences in samples were considered significant id the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).
**Figure 7:** Time response curve of MVP release from HaCaT cells. Cell culture plates either received no treatment, CPAF (53.9 ng), 3,600 J/m2 UVB, 8 second, or 30 second thermal and were incubated for 1, 2, or 4 hours. At one hour both the CPAF and burn treatment groups were significant from the no treatment group at two hours all three treatments are significant UVB, CPAF, and burn. Then at 4 hours, the amount of MVP released from the treated groups was even higher UVB, CPAF, burn. The data depicted are mean ± SE MVP per mL per 100,000 cells (average of n=4). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).
Figure 8: Cell viability for HaCaT cells after injury. Cells were treated with CPAF (53.9 ng), 3,600 J/m² UVB, 8 second burn, 30 second burn, 45 second burn, or received no treatment. Cells count and viability was performed using a Beckman Coulter Vi-Cell™ cell viability analyzer. Data is represented as the percent viability (average of n=2) with cell counts between $1.32 \times 10^6$ and $1.98 \times 10^6$. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).

Then, to test the potential involvement of MAP kinases on MVP release, various inhibitors were used on HaCaT cells after CPAF (Sup. Figure 23), UVB (Sup. Figure 24), and Burn (Figure 9). It was found that the MVP release induced by the different treatments was blocked by the addition of different MAP kinases. All three treatments showed significantly less MVP release when exposed to ERK ½ inhibitor (PD98,059), Jun-kinase inhibitor (SP600125), and p38 MAP kinase inhibitor (SB203580). However, CPAF induced MVP release was also blocked by the addition of the NF-κβ inhibitor (PDTC) and Rho kinase inhibitor (Y-27632) (Sup. Figure 23). UVB was inhibited by the Rho kinase inhibitor (Y-27632) (Sup. Figure 24). Finally, burn-induced MVP release was blocked pre-
incubation with NK-κβ inhibitor (PDTC) (Figure 9). These findings suggest there may be slightly different pathways involved in the MVP release in response to various treatments.
**Figure 9:** Inhibitor effect on MVP release after thermal burn injury of HaCaT cells. The various inhibitors (1.64 µg PDTC, 2.67 µg PD98,059, 0.22 µg SP600125, 3.77 µg SB203580, 0.32 µg Y-27632, or 11.22 µg Z-VAD-FMK) were added 1 hour before 30 second thermal burn injury. Cells were then incubated for 4 hours. *The addition of the NF-ƙβ inhibitor (PDTC), ERK ½ inhibitor (PD98,059), Jun-kinase inhibitor (SP600125) and p38 MAP kinase inhibitor (SB203580), all blocked MVP release after burn treatment (a-d). Burn induced MVP release was not affected by the addition of the p38 MAP kinase inhibitor (SB203580), Rho kinase inhibitor (Y-27632) and a general caspase inhibitor (Z-VAD-FMK) (e, f).* The data depicted are mean ± SE MVP per mL per 100,000 cells (average of n=4). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***)..

It has been previously reported that a common antidepressant drug, imipramine blocked MVP release through its ability to act as an inhibitor of aSMase (63). Thus, our next studies were designed to test the ability of imipramine to modulate MVP release in our system. First, CPAF imipramine was added one hour before CPAF treatment or at the same time as CPAF treatment. Compared to the CPAF alone group, pretreatment with imipramine blocked CPAF-induced MVP release. The CPAF + imipramine one hour before group also was not significant from the control plates. It was also found that the addition of imipramine one hour before UVB and burn injury was able to significantly reduce the amount of MVP release to similar levels as the imipramine alone control treated plates (Figure 10).
Figure 10: MVP release is inhibited in HACaT cells with the addition of the aSMase inhibitor (imipramine) for burn, CPAF and UVB. Cell culture plates were pre-incubated with imipramine (6.34 µg) or vehicle 1 hour before treatments. Then, the cells either received no treatment, CPAF (53.9 ng), 3,600 J/m2 UVB, or 30 second thermal burn, and were incubated for 4 hours. For all treated groups (CPAF, UVB and thermal burn) imipramine was a very potent inhibitor of the stimulus induced MVP release. The data depicted are mean ± SE MVP per mL per 100,000 cells (average of n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***)

We also looked at a PAF-R antagonist (WEB 2086) to see if the PAF-R was involved in the mechanism of burn-induced MVP release from human keratinocytes. Cells treated with CPAF had a significant decrease in MVP release when pre-incubated with the antagonist, no effect was noted in the 30 second Burn sample (Figure 11). However there was a significant decrease in the MVP release after 45 second burn when pre-incubated with the antagonist (Figure 12).
Figure 11: PAF-R antagonist (WEB 2086) effect on various injury induced MVP release. Cell culture plates were pre-incubated with WEB 2086 or vehicle 30 minutes before treatments. Then, the cells either received no treatment, CPAF (53.9 ng), TPA (61.68 ng), or 30 second thermal burn, and were incubated for 4 hours. As expected the CPAF treated group was blocked by the pre-incubation with the PAF-R antagonist. However, there was no significant decrease in the burn group pre-incubated with WEB 2086 compared to the burn alone or burn with vehicle groups. The data depicted are mean ±SE MVP per mL per 100,000 cells (n=1). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).
**Figure 12**: PAF-R antagonist (WEB 2086) effect on various injury induced MVP release. Cell culture plates were pre-incubated with WEB 2086 or vehicle 30 minutes before treatments. Then, the cells either received no treatment, CPAF (53.9 ng), TPA (61.68 ng), or 45 second thermal burn, and were incubated for 4 hours. As expected the CPAF treated group was blocked by the pre-incubation with the PAF-R antagonist. However, there was no significant decrease in the burn group pre-incubated with WEB 2086 compared to the burn alone or burn with vehicle groups. The data depicted are mean ± SE MVP per mL per 100,000 cells (n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***)

### 4.1.2 NtErTs:

The next studies were designed to test a non-cancerous keratinocyte-derived cell line. As shown in Figure 13, NtErT cells released MVP in response to treatment with CPAF, UVB and burn injury. These studies indicate that like the HaCaT cell lines, NtErTs also exhibited increased MVP release from CPAF, UVB and Burn treatments.
**Figure 13:** MVP release in NTERT cell line. Cell culture plates either received no treatment, CPAF (53.9 ng), 3,600 J/m2 UVB, or 30 second thermal burn, and were incubated for 4 hours. For all treated groups (CPAF, UVB and thermal burn) there was a significant increase in MVP release compared to the No Treatment group. The data depicted are mean ± SE MVP per mL per 100,000 cells (average of n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***)

### 4.1.3 Primary Keratinocytes:

Our next studies examined primary keratinocytes for their ability to respond to various stimuli similar to immortalized cell lines. To that end, we were by Dr. Michael Kemp provided human keratinocytes isolated from the abdominoplasty skin of a 32 year old African American Female following Thermo Fisher Scientific's protocol (230). It was noted that like the other cell lines, burn treatment resulted in a very large increase in MVP release compared to the cells that did not receive any treatment (Figure 14).
Figure 14: MVP release from keratinocytes. Cell culture plates either received no treatment, CPAF (53.9 ng), 3,600 J/m2 UVB, or 30 second thermal burn, and were incubated for 4 hours. After thermal burn injury, there was a significant increase in MVP release compared to the no treatment and vehicle groups. The data depicted are mean ± SE MVP per mL per 100,000 cells (n=1). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***)

4.1.4 KBP/KBM:

Given our group’s previous studies involving UVB, it was also of interest to see if the PAF-R was involved in burn-induced MVP release. To do this KBP (cells that over express the PAF-R) and KBM cells (cells that do not express the PAF receptor) were used. As expected only KBP cells showed increased MVP release after CPAF exposure. As shown in Figure 15, burn-induced MVP release seems to be partially dependent on the PAF-R. There was a nonsignificant reduction in MVP for the mild
and moderate burns (8 second and 30 second burns) in the absence of the PAF-R. However, for the 45 second burn there was a drastic reduction in MVP release in the KBM cells compared to the KBP cells. After UVB treatment there was also a decrease in the amount of MVP released in the absence of the PAF-R. This is consistent with our previous findings (1).

Figure 15: MVP release in cells that express the PAF-R (KBP) and cells that do not express the PAF-R (KBM). KBP and KBM cells either received no treatment, PMA (61.68 ng), CPAF (53.9 ng), 3,600 J/m² UVB, 8 second burn, 30 second burn, or 45 second thermal burn, and were incubated for 4 hours. In the KBP cell line all treatment groups (PMA, CPAF, UVB, 8 second burn, 30 second burn, and 45 second burn) had significant increase in MVP release compared to the no treatment group. However, in the KBM cells only the PMA, 30 second and 45 second thermal burn had significant MVP release. There was noted to be significant reduction in MVP release in the absence of the PAF-R for the CPAF, UVB, and 45 second burn. The data depicted are mean ± SE MVP per mL per 100,000 cells (average of n=4). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).
4.2 Skin:

Outside of cell culture, mammalian cells do not always behave the same way. To evaluate if MVP are released from keratinocytes in human skin similarly to stress induced MVP release from keratinocytes in cell culture, abdominoplasty skin was obtained from Sycamore Hospital. MVP were separated from skin punch biopsies of treated areas of skin. First, to determine if there was incision-induced MVP release, near the incision were compared to punch biopsies taken near the middle of the piece of skin. All skin was untreated and there was no real difference between the two punch locations (Sup. Figure 25). However, in all future experiments, punch biopsies were taken from the center of the skin explant, away from the cut edges. Similar to the cell culture results, all groups except the vehicle control (PMA, UVB, CPAF, and Burn) exhibited increased MVP release compared to the non-treated skin (Figure 16).
**Figure 16:** MVP release from skin punch biopsies. Skin was cut into small pieces and either received no treatment, topical DMSO, topical CPAF (5.39 g), 2,800 J/m² UVB, or 5 second thermal burn and were incubated for 4 hours. Compared to the no treatment and the DMSO vehicle, CPAF, UVB, and thermal burn treatments all significantly increased the amount of MVP released. The data depicted are mean ± SE MVP per g of tissue (average of n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).

Next, to determine if the MVP present in the skin biopsies treated with various stressors are derived from the epidermis, a novel suction blister protocol to separate epidermis from dermis was designed. UVB, CPAF and burn only effect the top layer of skin. When the epidermis and dermis are separated by blister fluid, this provides evidence that any increase in MVP concentration in the blister fluid of treated skin would suggest that it was derived from the epidermis. Also, the presence of the MVP in the blister fluid shows that MVP were moving from the epidermis to the dermis. It was
found using this methodology, that treatment-induced MVP release followed similar trends to the cell culture results and the punch biopsy results (Figure 17).

Figure 17: Stress induced MVP release from blisters on human abdominoplasty. Skin was cut into small pieces and with the use of a suction pump attached to 20 mL syringe barrels, blisters were formed on the skin. The blisters then received either received no treatment, topical DMSO, topical PMA (2.5 µg), 1,000 J/m² UVB, 2,500 J/m² UVB, topical CPAF (5.39 g), or 5 second burn and skin was then incubated for 4 hours. Compared to the no treatment and the DMSO vehicle, all treatment groups (PMA, UVB 1,000 J/m², UVB 2,500 J/m², CPAF, and thermal burn) had a significant increase in the amount of MVP released. The data depicted are mean ± SE MVP per g of tissue (average of n=11). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***)

The various inhibitors, used in cell culture, were then topically applied to small skin pieces prior to treatment to test if intact skin followed the same trend as the in vitro HaCaT cell results. The CPAF induced increase in MVP release was able to be blocked by the ERK ½ inhibitor (PD98,059), Jun-kinase inhibitor (SP600125), p38 MAP
kinase inhibitor (SB203580), and Rho kinase inhibitor (Y-27632). Unlike the cell culture results, PDTC did not exert any inhibitory effect on the MVP concentration in the blister fluid (Sup. Figure 26).

Jun-kinase inhibitor (SP600125), p38 MAP kinase inhibitor (SB203580), and Rho kinase inhibitor (Y-27632) all had inhibitory effects on the MVP concentration in the skin biopsy after 2,800 J/m² UVB injury (Sup. Figure 27). These findings are consistent with the inhibitor studies on the HaCaT cell line. However, unlike the cell culture results, there did not appear to be any effect on UVB-induced MVP release in the skin after pre-incubation with the ERK ½ inhibitor, PD98,059. NF-κβ inhibitor (PDTC), ERK ½ inhibitor (PD98,059), Jun-kinase inhibitor (SP600125), and p38 MAP kinase inhibitor (SB203580) all had inhibitory effects on the MVP concentration in the punch biopsy after 5 second burn treatment (Figure 18). These findings are consistent with the inhibitor studies on the HaCaT cell line.
**Figure 18:** Inhibitor effect on burn induced MVP release in human abdominoplasty skin punch biopsies. Skin was cut into small pieces and the various inhibitors (1.64 µg PDTC, 2.67 µg PD98,059, 0.22 µg SP600125, 3.77 µg SB203580, 0.32 µg Y-27632, or 11.22 µg Z-VAD-FMK) were topically applied 1 hour before thermal burn injury. The skin pieces then received either no treatment, topical DMSO vehicle, or 5 second burn and skin was then incubated for 4 hours. The addition of the NK-κB inhibitor (PDTC), ERK ½ inhibitor (PD98,059), Jun-kinase inhibitor (SP600125), and p38 MAP kinase inhibitor (SB203580) all blocked MVP release after burn treatment (a-d). Burn induced MVP release was not affected by the addition of the Rho kinase inhibitor (Y-27632) and a general caspase inhibitor (Z-VAD-FMK) (e, f). The data depicted are mean ± SE MVP per g of tissue (average of n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***)

Our next studies tested the ability of topical imipramine to modulate MVP release in response to CPAF, UVB and burn treatment in human skin. As depicted in Figure 19, imipramine pretreatment blocked MVP release in response to all of the stressors tested. This data is consistent with the cell culture results. To further investigate this aSMase inhibitor in a potentially clinically-relevant model, we also tested the effects of adding topical imipramine after the injuries. To that end, we applied imipramine 30 minutes and 1 hour after burn, UVB and CPAF (Figure 20). It was found that for all three treatments there was a significantly less MVP released if imipramine was topically applied 30 minutes after injury but not 1 hour after.
**Figure 19:** Topical imipramine inhibits MVP release in human abdominoplasty skin. Skin was cut into small pieces and was pre-incubated with either topical imipramine (1.58 µg) or DMSO vehicle. Then, the skin either received no treatment, CPAF (5.39 ng), 2,500 J/m² UVB, or 5 second thermal burn, and the skin was then incubated for 4 hours. For all treated groups (CPAF, UVB and thermal burn) imipramine was a very potent inhibitor of the stimulus induced MVP release. The data depicted are mean ± SE MVP per g tissue (average of n=4). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***)
Figure 20: Imipramine Inhibits Burn, UVB and CPAF induced MVP released up to 30 minutes after damage. Skin was cut into small pieces and was pre-incubated with either topical imipramine (1.58 µg) or DMSO vehicle. Then, the skin either received no treatment, CPAF (5.39 ng), 2,500 J/m2 UVB, or 5 second thermal burn, and the skin was
then incubated for 4 hours. Then 30 minutes and 1 hour after the various treatments topical imipramine was applied. For all treated groups (CPAF, UVB and thermal burn) topical imipramine was effective at blocking MVP release up to 30 minutes after treatment. The data depicted are mean ± SE MVP per g tissue (average of n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), P<0.001 (***)

4.3 Mice:

To show that living models also have increased MVP release after thermal burn injury WT mice were burnt for 8 seconds on their back. Burn samples were compared to their skin tissue that did not receive a thermal burn. Compared to the un-burnt skin, the tissue that received the thermal burn had a significant increase in MVP (Figure 21).

![Graph](image.png)

**Figure 21:** MVP release in mice skin increases after thermal burn injury. The data depicted are mean ± SE MVP per g tissue (representative of n=2). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***)
4.4 MVP Cytokine Expression:

Given our findings that multiple stressors triggered MVP, the next studies were designed to test if keratinocyte-derived MVP contained biologically active cytokines. Isolated MVP samples were analyzed by Dr. David Cool for the expression of 27 human cytokines, chemokines, and growth factors, listed previously. Data was normalized to the MVP content in the sample by pg/10^6 MVP. As shown in Table 1, we were surprised to find that there was a significant decrease in the concentration of all the cytokines, chemokines, and growth factors in the MVP released from cells treated with CPAF, UVB and burn compared to the concentrations in MVPS released from cells that received no treatment or the vehicle control. The only cytokine that did not follow this trend, IL-5, was below threshold for all samples.
<table>
<thead>
<tr>
<th></th>
<th>No Treatment</th>
<th>Vehicle</th>
<th>CPAF</th>
<th>UVB</th>
<th>Burn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu IL-1b</td>
<td>90.8 ± 7.9</td>
<td>87.6 ± 4.6</td>
<td>12.6 ± 0.24</td>
<td>OOR&lt;</td>
<td>0.61 ± 0.61</td>
</tr>
<tr>
<td>Hu IL-1ra</td>
<td>206 ± 17</td>
<td>199 ± 9</td>
<td>26.2 ± 1.09</td>
<td>19.8 ± 13.2</td>
<td>4.34 ± 0.21</td>
</tr>
<tr>
<td>Hu IL-2</td>
<td>19.9 ± 1.8</td>
<td>19.3 ± 1.4</td>
<td>2.60 ± 0.17</td>
<td>1.74 ± 1.16</td>
<td>0.35 ± 0.25</td>
</tr>
<tr>
<td>Hu IL-4</td>
<td>1.36 ± 0.13</td>
<td>1.31 ± 0.1</td>
<td>0.16 ± 0.02</td>
<td>0.10 ± 0.07</td>
<td>OOR&lt;</td>
</tr>
<tr>
<td>Hu IL-5</td>
<td>OOR&lt;</td>
<td>OOR&lt;</td>
<td>OOR&lt;</td>
<td>OOR&lt;</td>
<td>OOR&lt;</td>
</tr>
<tr>
<td>Hu IL-6</td>
<td>30.2 ± 2.7</td>
<td>29.0 ± 2.1</td>
<td>3.99 ± 0.14</td>
<td>2.87 ± 1.91</td>
<td>0.61 ± 0.02</td>
</tr>
<tr>
<td>Hu IL-7</td>
<td>191 ± 10</td>
<td>184 ± 10</td>
<td>26.0 ± 13.0</td>
<td>OOR&lt;</td>
<td>1.27 ± 1.27</td>
</tr>
<tr>
<td>Hu IL-8</td>
<td>29.9 ± 2.2</td>
<td>26.2 ± 0.8</td>
<td>3.09 ± 0.22</td>
<td>1.31 ± 0.87</td>
<td>0.10 ± 0.07</td>
</tr>
<tr>
<td>Hu IL-9</td>
<td>23.2 ± 1.6</td>
<td>22.4 ± 1.4</td>
<td>2.71 ± 0.28</td>
<td>2.11 ± 1.41</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>Hu IL-10</td>
<td>36.6 ± 4.0</td>
<td>34.2 ± 1.5</td>
<td>3.95 ± 0.74</td>
<td>2.34 ± 1.56</td>
<td>0.45 ± 0.09</td>
</tr>
<tr>
<td>Hu IL-12</td>
<td>48.4 ± 3.7</td>
<td>46.7 ± 0.7</td>
<td>5.58 ± 0.68</td>
<td>3.35 ± 2.23</td>
<td>0.29 ± 0.29</td>
</tr>
<tr>
<td>Hu IL-13</td>
<td>8.09 ± 0.75</td>
<td>7.85 ± 0.4</td>
<td>1.06 ± 0.04</td>
<td>0.70 ± 0.5</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>Hu IL-15</td>
<td>35.3 ± 3.7</td>
<td>35.3 ± 1.6</td>
<td>4.57 ± 0.37</td>
<td>3.04 ± 2.03</td>
<td>0.75 ± 0.11</td>
</tr>
<tr>
<td>Hu IL-17</td>
<td>52.2 ± 3.9</td>
<td>51.2 ± 4.1</td>
<td>6.35 ± 0.71</td>
<td>4.19 ± 2.79</td>
<td>0.90 ± 0.07</td>
</tr>
<tr>
<td>Hu Eotaxin</td>
<td>19.0 ± 1.4</td>
<td>19.1 ± 2.2</td>
<td>2.24 ± 0.24</td>
<td>1.81 ± 1.21</td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>Hu FGF basic</td>
<td>70.7 ± 6.2</td>
<td>68.2 ± 3.6</td>
<td>9.79 ± 0.17</td>
<td>7.33 ± 4.89</td>
<td>1.62 ± 0.10</td>
</tr>
<tr>
<td>Hu G-CSF</td>
<td>17.2 ± 2.4</td>
<td>16.0 ± 2.2</td>
<td>2.26 ± 0.58</td>
<td>2.52 ± 1.68</td>
<td>0.3 ± 0.09</td>
</tr>
<tr>
<td>Hu GM-CSF</td>
<td>48.0 ± 4.1</td>
<td>46.4 ± 1.6</td>
<td>5.93 ± 0.40</td>
<td>4.15 ± 2.76</td>
<td>0.9 ± 0.02</td>
</tr>
<tr>
<td>Hu IFN-g</td>
<td>30.9 ± 2.9</td>
<td>29.4 ± 1.9</td>
<td>3.96 ± 0.21</td>
<td>2.59 ± 1.72</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td>Hu IP-10</td>
<td>18.3 ± 1.6</td>
<td>18.0 ± 0.5</td>
<td>2.24 ± 0.01</td>
<td>1.39 ± 0.93</td>
<td>0.18 ± 0.10</td>
</tr>
<tr>
<td>Hu MCP-1</td>
<td>46.4 ± 3.7</td>
<td>44.4 ± 3.4</td>
<td>5.48 ± 0.49</td>
<td>3.74 ± 2.49</td>
<td>0.75 ± 0.08</td>
</tr>
<tr>
<td>Hu MIP-1a</td>
<td>0.89 ± 0.09</td>
<td>0.83 ± 0.07</td>
<td>0.11 ± 0.01</td>
<td>0.07 ± 0.04</td>
<td>OOR&lt;</td>
</tr>
<tr>
<td>Hu PDGF-bb</td>
<td>22.1 ± 1.7</td>
<td>21.5 ± 1.6</td>
<td>2.76 ± 1.39</td>
<td>1.98 ± 1.32</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>Hu MIP-1b</td>
<td>61.8 ± 5.4</td>
<td>59.7 ± 3.2</td>
<td>8.57 ± 0.15</td>
<td>6.42 ± 4.28</td>
<td>1.42 ± 0.09</td>
</tr>
<tr>
<td>Hu RANTES</td>
<td>17.2 ± 1.6</td>
<td>16.0 ± 0.9</td>
<td>2.26 ± 0.01</td>
<td>1.58 ± 1.05</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>Hu TNF-a</td>
<td>43.0 ± 3.3</td>
<td>42.3 ± 2.8</td>
<td>5.61 ± 0.30</td>
<td>4.07 ± 2.72</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>Hu VEGF</td>
<td>77.2 ± 6.7</td>
<td>73.8 ± 4.1</td>
<td>10.5 ± 0.1</td>
<td>7.53 ± 5.02</td>
<td>1.65 ± 0.09</td>
</tr>
</tbody>
</table>

Table 1: Cytokine, Chemokine, and growth factor expression in MVP released from cells that received no treatment or were treated with vehicle, CPAF (53.9 ng), 3,600 J/m² UVB, or a 30 second thermal burn IL-5 was below detection level in all samples. The other 26 cytokine concentrations were significantly decreased after CPAF, UVB and burn treatments compared to the cytokine concentrations of untreated cells and vehicle control cells. Data is represented in $10^9$ pg/10⁶ MVP. Groups were compared using one-way ANOVA. Significance ($P<0.05$) is represented by grey boxes.
Chapter 5: Discussion

5.1 Summary:

Thermal burn injury is a very common environmental stressor to the skin that can result in systemic effects. The mechanism by which this local injury can induce systemic effects is currently an important unanswered question. This study was designed to see if epithelial cells release MVP in response to burn injury. It is currently believed that the shedding of MVP from various cells in response to stressors are involved in cellular communication. For the in vitro studies, the human cell lines HaCaT, NtErT, primary keratinocytes, and KBP/KBM were either burned in a 90° water bath, received CPAF, or treated with UVB radiation. After incubation, the MVP were isolated and counted and analyzed using the NanoSight NS300. These studies demonstrated that thermal burn injury, UVB, and CPAF all increased MVP release from human keratinocyte cells in vitro.

Since UVB induced MVP were previously reported to be dependent on the PAF-R, we also wanted to determine if the PAF-R was involved in the generation of MVP after burn injury (1). To do this we first tested effects of a PAF-R antagonist. This data indicated that the PAF receptor was not likely involved in the short term, 30 second burn. We then used a cell line that does not express the PAF receptor (KBM) to further confirm these results. Looking at all three burn injuries (8 second, 30 second, and 45 second), only the long, 45 second injury, demonstrated a partial inhibition in MVP
release compared to the cells that expressed the PAF-R (KBP). These findings suggest that it is unlikely at low doses that the PAF-R is involved in thermal burn injury. However, MVP release from more aggressive burns may involve the PAF-R, yet is not totally dependent on its activation.

We then tested human abdominoplasty skin ex vivo to see if keratinocytes in the skin reacted the same as in cell culture. After taking punch biopsies and isolating the MVP, we determined that there was increased MVP release after topical CPAF, UVB, and burn injury. Then, to define if the MVP being released were derived from the epidermis, we instituted a novel methodology where suction blisters were induced on the human skin. After the thermal burn, UVB, or topical CPAF treatment, the blisters were incubated and the fluid was obtained from the blisters for MVP analysis. Again, UVB, burn and CPAF treatment induced increased MVP release.

We then investigated the possible mechanism by which MVP are released after burn injury. Various inhibitors were used in the cell culture and abdominoplasty skin, including imipramine, a potent aSMase inhibitor previously reported to inhibit MVP release in other cell lines. The UVB, CPAF, and thermal burn injury seemed to be blocked by different inhibitors. In both the HaCaT cell line and the explant tissue, thermal burn injury induced MVP release was able to be inhibited by the pre-incubation with an NF-κβ inhibitor, ERK ½ inhibitor, Jun-kinase inhibitor or a p38 inhibitor. CPAF stimulated MVP release was able to be blocked by the NF-κβ inhibitor, ERK ½ inhibitor, Jun-kinase inhibitor, p38 inhibitor, or the Rho kinase inhibitor. Finally, the UVB induced release of MVP was able to be blocked with a Jun-kinase inhibitor, p38 inhibitor, or a Rho kinase
inhibitor. Also, the general caspase inhibitor, Z-VAD-FMK, was unable to block MVP released from CPAF, UVB or burn treatments. Because caspases induce apoptosis, this shows that the MVP release was not due to cell death. This provides evidence that the MVP release for these stressors may be controlled by different pathways. However, the MVP released from all three treatments were able to be blocked by imipramine up to 30 minutes after injury. Since imipramine is effective at blocking MVP topically after the injury, this shows there is therapeutic potential for imipramine at blocking MVP release.

We also investigated if living models respond similarly to thermal burn injury as the keratinocyte cells and the explant tissue. As expected there was a significant increase in MVP release two hours after thermal burn in mice. This agrees with the other models and supports our hypothesis that thermal burn injury increases MVP release in mice.

These results agree with our original hypothesis that thermal burn injury increases MVP release in keratinocytes. As MVP often contain signaling molecules, these findings suggest that MVP could be involved in the human response pathway to stressors like thermal burn. If so, this data shows therapeutic potential for imipramine after thermal burn injury if applied within a short time after the burn.

Surprisingly we noted a very dramatic decrease in the concentrations of cytokines and growth factors in the MVP of cells that were treated with CPAF, UVB and burn. This did not agree with our original hypothesis that the cytokine concentrations in MVP would be greatly increased compared to the concentrations in MVP released under normal conditions. However, this data may show a novel defense mechanism to protect
the body from a cytokine storm. We believe that in some conditions this defense mechanism may be repressed causing a significant amount of cytokines to be concentrated in the MVP. If so, this increase in cytokines could cause the systemic effects and severe outcomes in some patients. In a specific case study a woman had a mild drug hypersensitivity reaction and tried to self-treat though a tanning bed. However, the combination of the UV light from the tanning bed and the drug hypersensitivity caused the patient to progress into Toxic Epidermal Necrolysis (231). In cases like this it shows that there could be some situations, like combined stressors, which can disrupt the body’s defensive mechanism to block cytokines in MVP. This would lead to a cytokine storm in the body and cause progression of diseased states.

5.2 Proposed Model:

As stated before it is likely that in most healthy people a low level of MVP are released from keratinocytes. These MVP contain cytokines, bioactive proteins and lipids, and nucleic acids. The amount of MVP released increases after stimulation from various factors like UVB, CPAF and burn. These stimuli seem to induce the MVP though different pathways. For burn injury the NF-kβ, ERK 1/2, Jun-kinase and p38 MAP kinase as well as the enzyme acid sphingomyelinase all seem to be essential for the increased release of MVP. The body then seems to have a protective measure to block cytokines from entering MVP. However, in some situations this protective mechanism is inhibited and patients experience cytokine
storm. It is possible that this is caused by the combination of multiple stressors and this overwhelms the cells.

5.3 Limitations and Future Studies:

One important limitation in this study is there is no evidence of MVP function in this data. This should be further investigated potentially though injecting MVP directly into an animal model (or in human skin ex vivo) and analyzing inflammation (or upregulation of cytokines). A second limitation is that the ex vivo skin does not have blood flow, limiting the movement of the MVP. This data did show that the MVP could travel from the epidermis to the dermis because the UVB treatment only effects the epidermis and MVP were found in the blister fluid between the epidermis and the dermis. However, this does not show that these MVP can migrate throughout the body. This can be further investigated though looking at animal models to see if there is increased MVP in the blood after UVB light treatment, compared to mice that received no treatment.

Also in future studies it is recommended to look into dosing the same cells with multiple stimuli to see if there are synergistic effects on the MVP release. This potential study could also investigate if there are increased cytokine concentrations in MVP when the system is overloaded with cytokines. Finally, it might also be good to further investigate imipramine by looking at the topical effects on a living mouse model, WT compared to aSMase knockout mice.
5.4 Conclusions:

In conclusion this data provides strong evidence that thermal burn injury does increase MVP release in keratinocytes. These MVP seem to be released though an NF-κβ, ERK 1/2, Jun-kinase and p38 MAP kinase dependent pathway. Low dose burn (8 second and 30 second) induced MVP release does not seem to involve the PAF-R. However, there seems to be a partial dependence on the PAF receptor for the increase of MVP in the high dose burn (45 seconds). Also, aSMase seems to be essential in the release of burn induced MVP. From a clinical perspective imipramine can significantly reduce burn, UVB and CPAF induced MVP release up to 30 minutes after the injury. This study shows that MVP may be involved in the pathophysiological effects of thermal burn injury. The function of these MVP, the effect of multiple stimuli, and the potential of imipramine should be investigated further.
Figure 22: DMSO vehicle effect on keratinocyte cell line in vitro. 1-10 µL DMSO vehicle has no effect on the MVP release in HaCaT. Positive control plate received 53.9 ng CPAF. The data depicted are mean ± SE MVP per mL per 100,000 cells (n=1). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).
Figure 23: Inhibitors effect on MVP release from CPAF stimulus in HaCaT cells. The various inhibitors (1.64 µg PDTC, 2.67 µg PD98,059, 0.22 µg SP600125, 3.77 µg SB203580, 0.32 µg Y-27632, or 11.22 µg Z-VAD-FMK) were added 1 hour before CPAF
treatment (53.9 ng). Cells were then incubated for 4 hours. The addition of the NF-κB inhibitor (PDTC), ERK ½ inhibitor (PD98,059), Jun-kinase inhibitor (SP600125), p38 MAP kinase inhibitor (SB203580), and Rho kinase inhibitor (Y-27632) all blocked MVP release after CPAF treatment (f-e). CPAF induced MVP release was not affected by the addition of a general caspase inhibitor (Z-VAD-FMK) (f). The data depicted are mean ± SE MVP per mL per 100,000 cells (average of n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).
Figure 24: Inhibitors effect on MVP release from UVB radiation in HaCaT cells. The various inhibitors (1.64 µg PDTC, 2.67 µg PD98,059, 0.22 µg SP600125, 3.77 µg SB203580, 0.32 µg Y-27632, or 11.22 µg Z-VAD-FMK) were added 1 hour before UVB
treatment (3,600 J/m2). Cells were then incubated for 4 hours. The addition of the Jun-kinase inhibitor (SP600125), p38 MAP kinase inhibitor (SB203580), and Rho kinase inhibitor (Y-27632) all blocked MVP release after UVB treatment (c-e). UVB induced MVP release was not affected by the addition of NK-κb inhibitor (PDTC), ERK ½ inhibitor (PD98,059), and a general caspase inhibitor (Z-VAD-FMK) (a, b, f). The data depicted are mean ± SE MVP per mL per 100,000 cells (average of n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).

Figure 25: The basal level of MVP release in non-treated abdominoplasty punch biopsies is the same for punches taken in the middle and punches taken near the incision. The data depicted are mean ± SE MVP per g tissue (n=1). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).
MVP Concentration (Particles/ g)

No Treatment
Vehicle
PDTC
CPAF
CPAF + PDTC

0.0
5.0 \times 10^{11}
1.0 \times 10^{12}
1.5 \times 10^{12}
2.0 \times 10^{12}
2.5 \times 10^{12}

***
***

MVP Concentration (Particles/ g)

No Treatment
Vehicle
SP600125
CPAF
CPAF + SP600125

0.0
5.0 \times 10^{11}
1.0 \times 10^{12}
1.5 \times 10^{12}
2.0 \times 10^{12}

**
***

MVP Concentration (Particles/ g)

No Treatment
Vehicle
SB203580
CPAF
CPAF + SB203580

0.0
5.0 \times 10^{11}
1.0 \times 10^{12}
1.5 \times 10^{12}
2.0 \times 10^{12}

**

MVP Concentration (Particles/ g)

No Treatment
Vehicle
Y-27632
CPAF
CPAF + Y-27632

0.0
5.0 \times 10^{11}
1.0 \times 10^{12}
1.5 \times 10^{12}
2.0 \times 10^{12}

**

MVP Concentration (Particles/ g)

No Treatment
Vehicle
Z-VAD-FMK
CPAF
CPAF + Z-VAD-FMK

0.0
5.0 \times 10^{11}
1.0 \times 10^{12}
1.5 \times 10^{12}
2.0 \times 10^{12}

**
Figure 26: Inhibitor Effect on CPAF induced MVP release in Human Abdominoplasty Skin Punch Biopsies. Skin was cut into small pieces and the various inhibitors (1.64 µg PDTC, 2.67 µg PD98,059, 0.22 µg SP600125, 3.77 µg SB203580, 0.32 µg Y-27632, or 11.22 µg Z-VAD-FMK) were topically applied 1 hour before CPAF treatment. The skin pieces then received either received no treatment, topical DMSO vehicle, or 5.39 ng CPAF and skin was then incubated for 4 hours. The addition of the NK-ƙβ inhibitor (PDTC), ERK 1/2 inhibitor (PD98,059), Jun-kinase inhibitor (SP600125), p38 MAP kinase inhibitor (SB203580), and Rho kinase inhibitor (Y-27632) all blocked MVP release after CPAF treatment (a-e). CPAF induced MVP release was not affected by the addition of the general caspase inhibitor (Z-VAD-FMK) (f). The data depicted are mean ± SE MVP per g tissue (average of n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant id the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).
Figure 27: Various Inhibitor effect on MVP release from UVB treated punch biopsies. Skin
was cut into small pieces and the various inhibitors (1.64 µg PDTC, 2.67 µg PD98,059, 0.22 µg SP600125, 3.77 µg SB203580, 0.32 µg Y-27632, or 11.22 µg Z-VAD-FMK) were topically applied 1 hour before UVB treatment. The skin pieces then received either received no treatment, topical DMSO vehicle, or 2,800 J/m² UVB and skin was then incubated for 4 hours. UVB induced MVP release was able to be blocked by Jun-kinase inhibitor (SP600125), p38 MAP kinase inhibitor (SB203580), and Rho kinase inhibitor (Y-27632) (c-e). Also, UVB induced MVP release was not affected by the addition of NK-ƙβ inhibitor (PDTC), ERK ½ inhibitor (PD98,059) and a general caspase inhibitor (Z-VAD-FMK) (a, b, f). The data depicted are mean ± SE MVP per g tissue (average of n=3). Groups were compared using one-way ANOVA. Differences in samples were considered significant if the P value was less than 0.05. P<0.05 (*), P<0.01 (**), and P<0.001 (***).
Appendix 2 - Calculations:

A.2.1 MVP Calculations:

A.2.1.1 Equation 1 MVP Concentration in Human Skin Samples:

\[
\frac{(MVP \text{ Concentration} \times \text{Dilution Rate})}{\text{Weight of Tissue or Blister Fluid}} = MVP \text{ Concentration} \frac{\text{particles}}{g \text{ or mL fluid}}
\]

Example:

\[
\frac{(7.11 \times 10^8) \times 35}{0.0292 \text{ g}} = 8.522 \times 10^{11} \frac{\text{particles}}{g \text{ or mL fluid}}
\]

A.2.1.2 Equation 2 MVP Concentration in Cells:

\[
\frac{(MVP \text{ Concentration} \times \text{Dilution Rate})}{\text{Cell Count}} \times 100,000 \text{ Cells} = MVP \text{ Concentration} \frac{\text{particles}}{100,000 \text{ Cells}}
\]

Example:

\[
\frac{(4.69 \times 10^8) \times 7}{5.88 \times 10^6 \frac{\text{cells}}{\text{mL}}} \times 100,000 \text{ Cells} = 5.588 \times 10^7 \frac{\text{particles}}{100,000 \text{ Cells}}
\]
A.2.2 Calculating ng of CPAF from nMoles:

A.2.2.1 *Equation 3 Calculating CPAF Concentrations:*

\[
\frac{\text{nMoles Added}}{1000 \text{ mL}} \times \text{mL added} \times \frac{\text{Molecular Weight (539 ng)}}{1 \text{ nM}} = \text{ng of CPAF added to sample}
\]

Example:

\[
\frac{10 \text{ nMoles}}{1000 \text{ mL}} \times 0.1 \text{ mL} \times \frac{539 \text{ ng}}{1 \text{ nM}} = 0.539 \text{ ng of CPAF Added to Sample}
\]

A.2.3 Diluting inhibitors:

A.2.3.1 *Equation 4 Calculating Inhibitor Dilutions:*

\[
\frac{\text{Desired Concentration (mM)}}{1000 \text{ mL}} \times \frac{\text{Molecular Weight of Inhibitor}}{1 \text{ mMole}} = \text{mg/mL}
\]

Then solve for x

\[
\frac{\text{mg Found Above}}{1 \text{ mL}} \times \frac{\text{Desired Weight or Total Weight in Bottle}}{x}
\]

Example:
\[
\frac{10 \text{ mM}}{1000 \text{ mL}} \times \frac{377.4 \text{ g}}{1 \text{ mole}} = 3.774 \text{ mg/mL}
\]

\[
\frac{3.774 \text{ mg/mL}}{1 \text{ mL}} \times \frac{1 \text{ mg}}{x} = \frac{0.265 \text{ mL}}{x}
\]

\[x = 0.265 \text{ mL of solvant to make a 10 mM solution}\]
Appendix 3- Equipment Pictures:

*Figure 28: Philips F20T12/UVB lamp.*
Figure 29: Water bath used for cell culture.
Figure 30: Cell Culture Incubator.
Figure 31: Laminar airflow hood used for cell culture to maintain a sterile environment.
Figure 32: 37° water bath for cell culture.

Figure 33: Rotisserie oven used for overnight digestion of punch biopsy samples.
Figure 34: Centrifuge used for the separation of MVP.

Figure 35: Syringe barrels attached to the suction pump for blister formation.
**Figure 36:** Syringe barrels pulling blisters on abdominoplasty skin (≈1-4 hours)

**Figure 37:** Vacuubrand® vacuum pump, 100 mbar for pulling suction blisters.
Figure 38: Nanosight for counting concentration of MVP in samples.
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