CHEMOKINES FROM MELANOMA UPREGULATE MMP2 WITHIN THE TUMOR MICROENVIRONMENT

A Thesis in
Bioengineering

by
Chong Haw Kwang

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The thesis of Chong Haw Kwang was reviewed and approved* by the following:

Cheng Dong  
Professor of Bioengineering  
Thesis Advisor

Peter J. Butler  
Associate Professor of Bioengineering

Gail L. Matters  
Assistant Professor of Biochemistry and  
Molecular Biology

Herbert H. Lipowsky  
Professor of Bioengineering  
Head of the Department of Bioengineering

*Signatures are on file in the Graduate School.
ABSTRACT

The goal of this work is to investigate the role of MMPs (Matrix Metalloproteinases) in melanoma invasion. MMPs have long been implicated in cancer progression and are found in greater concentrations and activation in malignant melanoma than normal or benign tissues. However, most current work focuses on MMPs’ role in angiogenesis and MMPs secreted by the cancer cells themselves. In this study, we examined the expression of MMPs that result from the dynamic interaction between the endothelial and the melanoma cells. We found that upon co-culture, MMP2 showed an increase in expression. There was also an increase in the activated form of MMP2.

We also investigated the role cytokines play in the changes in MMP expression and how the interactions between the two cell types affect their expression. We report that IL8 (Interleukin 8) and MCP1 (Monocyte Chemotactic Protein) that were secreted from the Lu1205 melanoma caused the MMP2 upregulation and activation. At the same time, the levels of TIMP2 (Tissue Inhibitor of MMP) increased while the levels of MMP14 decreased. Furthermore, we discovered that contact co-culture of HUVEC (Human Umbilical Vein Endothelial Cell) and Lu1205 melanoma resulted in a significant increase in expression of MCP1 and IL8 chemokines. The increased levels of MCP1 is mediated by MCAM (Melanoma Cell Adhesion Molecule) expressed on the surface of the endothelial and melanoma cells, while the receptors for the increase of IL8 remains unknown.

To study the role that MMP2 plays in melanoma invasion, we developed a transendothelial migration assay. The Boyden chamber was modified to allow HUVEC to be grown in a monolayer atop a Matrigel coated micropore filter with minimal migration of HUVEC across the filter. When the melanoma cells are placed on the top well, it allows for dynamic interaction between the HUVEC and the melanoma cell, upregulating and activating MMP2 for invasion. We showed that blocking IL8 and MCP1 with antibodies successfully reduced the transendothelial migration of melanoma cells.
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<td>ABTS</td>
<td>2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid</td>
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<tr>
<td>APMA</td>
<td>4-aminophenylmercuric acetate</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECGS</td>
<td>endothelial cell growth supplement</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HMVEC</td>
<td>Human micro vascular endothelial cell</td>
</tr>
<tr>
<td>HPMVEC</td>
<td>human pulmonary microvascular endothelial cells</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin -1</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin -8</td>
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<tr>
<td>JNK</td>
<td>c-Jun amino terminal kinase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MCAM</td>
<td>Melanoma cell adhesion molecule, also known as MUC18 or CD146</td>
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<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
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<td>Full Form</td>
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<tr>
<td>MEK</td>
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<td>MMP</td>
<td>matrix metalloprotease</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAR-1</td>
<td>proteinases activated receptor 1</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline Tween-20</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SiRNA</td>
<td>short interfering RNA</td>
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<tr>
<td>TCM</td>
<td>Tumor conditioned media</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Vascular Cell Adhesion Molecule -1</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
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Chapter 1

Introduction

1.1 Background

1.1.1 Cancer as a disease

Cancer is an ancient condition, well known since recorded history (Karpozilos and Pavlidis, 2004). It is a disease associated with tissue renewal and is intrinsic within our biological systems. It accounts for 13% of all deaths and a rising trend is expected, with 9 million people projected to be dying from cancer in 2015 (WHO, 2006). Cancer cells arise as a consequence of random mutations of the DNA (Deoxyribonucleic Acid) when normal cells divide and as such, it is not an infectious disease. However, because our bodies accumulate these mutations over our lifetimes, it does have a positive correlation with age (Cancer-Research-UK, 2007).

While mutations from DNA repair and replication occur spontaneously due to fundamental limitations of the mechanism, environmental factors have been known to contribute to the causation of cancer. These include ionizing radiation and chemical carcinogens such as tobacco smoke. Due to the genetic nature of cancer, family history is also an important consideration. No single mutation alone is the cause of a cell becoming cancerous – it is a series of accumulated mutations that enable cells to overcome the body’s natural checkpoints and defense. It is estimated that a cell would need at least 3-6
accumulated mutations over the course of several years to become cancerous (Alberts, 2004). These mutations provide the cells a competitive edge over neighboring cells, allowing them to proliferate faster or survive longer. When they proliferate, they pass these acquired mutations to their daughter cells, which are free to develop another chance mutation in a process similar to natural selection. Ultimately, the cells reach final state of a malignant cancer cell.

Although cancer cells acquire different combinations of abilities, they can be broadly defined as having six main properties (Hanahan and Weinberg, 2000): First, they must have the acquired ability for self sufficiency in growth signals. Most cells rely on neighboring cells for signals, either chemically or through physical contact of cell surface receptors. These signals dictate the cells’ response, such as growth, apoptosis or sending out other signals to affect other cells. Cancer cells must be able to be independent of extra-cellular signals for such growth. As an example, mutation of the Ras gene in melanoma cells, allows them to proliferate continuously, among other abilities. Second, cancer cells are able to override the apoptosis internal checkpoint. Normal cells, when damaged beyond repair or suffer chromosome breakage will activate the intracellular cell-death program and undergo apoptosis. This mechanism involves p53, a checkpoint mechanism within the cell. 50% of all human cancers have a mutation of the p53 gene. Third, cancer cells have an unlimited proliferation capacity. Normal cells can only proliferate a limited number of times because when they divide, the telomere lengths at the ends of the chromosomes shorten, and ultimately, the lengths become too short and proliferation stops. Cancer cells, on the other hand, are able to reactivate the production
of the telomerase enzyme and can maintain the lengths of the telomere. Fourth, due to unchecked proliferation and DNA replication, the cancer cells are genetically unstable. This is an advantage to the cancer cells as they are able to use this instability to become more malignant. Fifth, cancer cells are able to maintain sustained angiogenesis. Tissues rely on vasculature to supple oxygen and nutrients, obligating all cells to maintain a distance within 100μm to the nearest blood vessel. Blood vessel limitations can therefore limit the growth of highly proliferative cells. Cancer cells have been shown to be able to shift the balance of positive and negative signals to their advantage, for example, upregulating growth factors for angiogenesis and reduce secretion of angiogenesis inhibitors. Lastly, cancer cells have the acquired ability to metastasize into foreign tissue from their origin and proliferate in these distant locations. Normally, cells die when they are displaced from their original location but some cancer cells are able to intravasate from the origin, travel within the vasculature, all the while avoiding destruction from the body’s immune cells and extravasate into foreign tissue to form secondary tumors (Hanahan and Weinberg, 2000; King, 2000).

This work focuses on melanoma, which is a skin cancer that is associated with melanocytes, a type of pigment cell. They are located at the base of the skin’s epidermis and are responsible for generating melanin, a pigment that causes a tan to the skin when exposed to ultraviolet radiation. Malignant melanoma usually presents itself visibly on the skin and physicians use the following four visual criteria to ascertain its presence: It appears as an asymmetric lesion on the skin (1) with irregular borders (2). The lesion does not have a uniform coloration (3) and has a diameter of more than 6 millimeters (4).
Recent breakthroughs in melanoma research shows that 20% of melanoma suffered a mutation in the Ras gene and 40-60% undergo a mutation in the B-Raf gene (Lopez-Bergami et al., 2008). These breakthroughs led researchers to concentrate on the mitogen activated protein kinase (MAPK) signaling pathways in melanoma as they affect melanoma proliferation, angiogenesis, invasion and inhibition of apoptosis (Sharma et al., 2006).

1.1.2 Melanoma metastasis and tumor-host interaction

Cutaneous melanoma is highly invasive and statistics from the health bureau indicate 1 person out of 75 have a chance of contracting an aggressive form of melanoma in the United States in the year 2000. Malignant melanoma is incurable and the five year survival rate for melanoma is below 10% and median survival rate is between 6 to 12 months (Balch and Cascalini, 2001). Of all skin cancer deaths, it accounts for 75% (Jerant et al., 2000). Melanoma is a highly malignant disease that is characterized by aggressive invasion of the basement membranes and extracellular matrix. In the initial stage, tumor cells intravasate into the blood vessels deviating from the primary tumor site, followed by adhesion to endothelial cells further downstream before extravasating across the blood vessel wall into other tissues. At this point, the melanoma cell develops into a secondary tumor and it is these secondary tumors that develop in major organs (e.g. lungs) that cause mortality in patients.
Although it was initially thought that the participation of the host towards cancer metastasis and development was passive, recent research progress over the last decade has shown that the host actively and reciprocally communicates with the melanoma through cytokines or direct cell-cell contact. Indeed, melanoma cells have been found to secrete a whole host of growth factors, cytokines and chemokines, namely, platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 and -8 (IL-1, IL-8) and monocyte chemotactic protein-1 (MCP-1) among others (Hsu et al., 2002). These cytokines and growth factors generate active response from the host cells such as inflammatory cells, endothelial cells and fibroblasts, as shown in figure 1-1.

![Diagram showing the various interactions between melanoma cells, endothelial cells, inflammatory cells and fibroblasts that contribute to melanoma invasion and metastasis (Hsu et al., 2002)](image)

Figure 1-1: Diagram showing the various interactions between melanoma cells, endothelial cells, inflammatory cells and fibroblasts that contribute to melanoma invasion and metastasis (Hsu et al., 2002)
In melanoma metastasis, it was shown that melanoma cells were capable of using immune/inflammatory cells such as polymorphonuclear leukocytes (PMN) to adhere to the endothelium under shear forces (Slattery and Dong, 2003). IL-8 released by the melanoma causes positive feedback on the PMN, resulting in increased IL-8 secretion as well as Mac-1 (αMβ2) upregulation on the PMN that allows for increased adhesion to the endothelium, as shown in “Attachment to endothelium” in figure 1-2. Platelets are also capable of binding to the melanoma in the free stream and forming emboli. The platelets in the emboli can interact with proteoglycans on the endothelial cell surface via P-selectin. Tumor cells, when they express Very Late Antigen -4 (VLA4), are also capable of binding directly to Vascular Cell Adhesion Molecule -1 (VCAM1) on the activated endothelial surface.

The next stage of invasion is for the melanoma to interact with the endothelium and allow it a path for extravasation. Contact of melanoma to the endothelial cells triggered a Ca+ response that resulted in the breakdown of vascular endothelial (VE)-cadherin between the endothelial cells. Among the many proteins that have been upregulated from the interactions, one class of proteins, matrix metalloprotease (MMP) has been under intense study. MMPs work at the “invasion” stage of figure 1-2 and they act to degrade the extracellular matrix (ECM) to allow an invasion path for metastatic melanoma (Hofmann et al., 2005).
Figure 1-2: Stages of melanoma extravasation and invasion through the endothelial layer (King, 2000)
1.1.3 Matrix Metalloproteases

MMPs are extracellular proteinases that are physiologically used for development and remodeling of tissue. They are present pathologically in many diseases. Depending on metal ions for catalytic activity, they have the ability to cleave structural proteins in the ECM, cell surface molecules and pericellular non-matrix proteins. There are at present 25 identified MMPs and are identified with their common names (e.g. Gelatinase-A) or their sequential numeric nomenclature (e.g. MMP-2). Most MMPs are secreted, although there are some that are bound to the cell surface. Structurally, the pre-domain is followed by the propeptide “pro” domain. MMPs are secreted inactive and it is the presence of the pro domain that results in the inactivation. Removal or disruption of this domain activates the MMP. The catalytic domain follows the pro and contains the zinc binding region. For most MMPs, a hemopexin/vitronectin-like domain is connected to the catalytic domain by a linker region, as shown in figure 1-3.

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**Simple Hemopexin Domain-Containing MMPs**  
(MMP1/collagenase-1, MMP8/collagenase-2, MMP13/collagenase-3, MMP16/collagenase-4, MMP3/stromelysin-1, MMP10/stromelysin-2, MMP27, MMP12/metalloelastase, MMP19/RAS1-1, MMP20/enamelysin, MMP22/CMPMP)

**Gelatin-binding MMPs**  
(MMP2/gelatinase A, MMP9/gelatinase B)

Figure 1-3: Structure of some MMPs shown, showing the different domain regions (Sternlicht and Werb, 2001).
Natural, endogenous inhibitors for MMPs have been found. They are able to inhibit MMPs in a 1:1 stoichiometric ratio. They are 20-29 kDA and four of them have been described as TIMPs 1-4 (tissue inhibitor of metalloproteinases). Among the TIMPs, they inhibit different MMPs, although they do overlap, and differ in their gene regulation and expression. In non-pathological situations, the expressions of MMPs and TIMPs are intricately balanced and controlled. In these conditions, MMPs are involved in bone and vascular remodeling; they contribute to angiogenesis by allowing migration of endothelial cells through surrounding tissue and liberating angiogenic factors (Sternlicht and Werb, 2001).

1.1.4 Matrix Metalloproteases and Cancer

MMPs have been found in greater concentrations and activation in malignant melanoma than in normal or benign tissues (Sternlicht and Werb, 2001) as tumor invasion and metastasis require the controlled degradation of the ECM proteins and studies show that increased MMP expression correlates with poor prognosis in cancer. In fact, MMP levels have been indicated as an independent marker of overall survival of cancer patients (Sternlicht and Werb, 2001). When benign cells become malignant, MMP activity increases and TIMP activity decreases. It has also been found that in epithelial cancers, it is not the cancer cells but the surrounding stromal cells that express the MMPs, underscoring the active communication between cancer cells and their host in figure 1-1. Of the MMPs, the major ones that are implicated in cancer invasion include 1,2,3,9 and MMP14. As an example, activated MMP2 binds to $\alpha_v\beta_3$ on melanoma and
together with CD44, localize the MMP2 to the invasive front (Hofmann et al., 2000; Takahashi et al., 1999).

MMPs can affect cancer development at all stages. Studies show transgenic mice that overproduce MMP develop hyper proliferative lesions; these MMP overexpressing mice also develop more cancers than MMP knockout mice or mice that overproduce TIMP1 (Buck et al., 1999). In the invasive stages, extravasation and angiogenesis have been shown to involve MMP activity (Kim and Kim, 1999). Gelatinases MMP2 and MMP9 are found abundantly in malignant tumors; however, inflammatory cells such as PMNs also produce MMP9, and recruiting inflammatory cells to the site of tumor invasion can exacerbate the situation.

The above shows that while MMP does have a role in cancer invasion, the issue is not always very clear: TIMP1 overexpressing mice showed increase production of VEGF, growth of tumor size, vascularization and proliferation. B-cells show anti-apoptotic effect under the influence of TIMP1 but not a synthetic MMP inhibitor (Guedez et al., 1998). So while we can show in-vitro that TIMPs do inhibit MMPs, more work is also needed in-vivo to understand the complex mechanisms involved.

1.1.5 In Vitro Techniques in Cancer Research

In our laboratory, cancer research in Bio-Engineering focuses primarily on in-vitro techniques. In vitro techniques have the advantage in the ability to isolate and
control the key parameters of study and focus on them. Using these techniques, we are able to study melanoma cells in the process of invasion, which includes tethering and adhesion to the endothelium, the subsequent communication between the melanoma and host cells and migration through the endothelium to form the equivalent of distant metastases physiologically.

For invasion studies, we apply the Boyden chamber (Boyden, 1962). This assay employs a setup with two chambers separated by a micro-pore filter membrane with pores ranging from 5 to 8 μm in diameter. This assay uses the chemotactic ability of the cells to migrate towards the source of the chemotactic reagent. The reagent is added to the lower chamber and in the upper chamber, the cells are added. In cancer research, this assay simulates the invasion of the cancer cells from the blood vessels towards the extracellular matrix. Cells that have migrated to the underside of the filter are scored for analysis. Various treatments to the cells prior or during the migration allow us to study their effects on the invasiveness of the cells.

For the cells to invade across the endothelium, they must first arrest themselves onto the surface of the endothelium. The cancer cells can be trapped within the fine capillaries of the micro circulation (Chambers et al., 2000) or based on the receptors on the surface of the cancer and endothelial cell, tether onto the surface of the endothelium (Liang and Dong, 2008), or use another cell type, e.g. leukocytes, as a mediator for adhesion to the endothelium (Slattery and Dong, 2003). Adhesion and tethering of the cells are achieved under shear flow in the blood vessels. To simulate this in vitro, we use
a parallel plate chamber. This chamber is versatile as it can explore the adhesion characteristics under shear flow between any two cell types. One cell type, typically the endothelial cell, is grown on a Petri dish to form a confluent monolayer. The chamber is designed such that suspensions of the second cell type, typically the cancer cell, flows in a laminar profile over the monolayer of cells. The shear stress on the surface of the monolayer is controlled by varying the flow rate of the incoming cell suspension. Using this, we are able to characterize the strength of the bonds involved in the adhesion as well as discover new bonds that can cause cell adhesion.

An elegant combination of the Boyden chamber and parallel plate, the flow migration chamber, was developed by our laboratory (Slattery and Dong, 2003). This is due to the recognition that the Boyden chamber is static but studies migration only, while the flow chamber studies flow effects on adhesion but does not lead to migration. In a flow migration chamber, the concept of the bottom well with chemotactic agent is retained. However, the top well is replaced with a laminar flow of cell suspension that recirculates. The shear stress on the surface is again controlled by the volumetric flow rate. Using this assay, we can study both adhesion and migration that allows for a more physiologically representation of cancer invasion.

Melanoma cells have been found to interact with PMNs to act as a mediator for surface adhesion. To study the cell aggregation of the melanoma and PMN, the viscometer is used (Liang et al., 2008). The viscometer allows one to apply a uniform
shear over the entire sample in a time varying manner. The shear simulates the conditions in a blood vessel where the PMNs and melanoma cells flow and interact.

Fluorescence microscopy has also been used in our studies; cytokines secreted from tumor cells have been found to create gap junctions between the endothelial cells, through which we suspect melanoma cells migrate through. The sizes of the gaps are quantified under fluorescence microscopy after staining.

In this study, we have developed a modified the Boyden chamber to allow endothelial cells to be grown on filters that are coated with basement membrane extract. The melanoma cells will be able to dynamically interact with the endothelial cells which was a dimension that was lacking for the Boyden chamber assay. The melanoma cells will have to invade past the basement membrane and filter, towards the source of the chemotactic agent. This assay will allow experiments to control the factors involved in the communication (via cell surface receptors or cytokines) between the endothelial cells and melanoma cells. The development of this assay will be discussed in the later chapters.

1.2 Objective and Significance

While MMPs have been under intense study over the last decade, different studies focus on the various aspects of MMP involvement in the cancer metastatic process. In
this study, we focus on the interaction between melanoma and the endothelium, right at the incipient process of invasion.

The second chapter explores changes in MMP expression when the melanoma cells come in contact with the endothelial cells. The study covers the major MMPs associated with melanoma invasion: MMP 1, 2 and 9. The study involves three melanoma cell lines, of which two are considered highly metastatic and three types of endothelial cells. It will identify the interactions between the endothelial cells and melanoma cells with respect to MMPs 1, 2 and 9.

The third chapter will focus on one particular co-culture system that was found to positively generate and activate MMP2. This study will single out IL-8 as well as MCP-1 that are produced from the melanoma cells as the key antagonists in this MMP2 activation and upregulation. The status of TIMP2 and MT1-MMP in the co-culture system is discussed. The information reaped from this will allow us to identify key targets in cancer therapy.

The final chapter will focus on demonstrating the effect of increased MMP2 expression and activation on melanoma migration \textit{in-vitro}. The migration assay is novel in that, while being a transendothelial migration, the migration of the endothelial layer cultured on basement membrane is controlled so that the endothelial cells do not compete with the melanoma cells for available sites. In this way, it simulates the physiological
situation and one is able to better access the influence of MMP upregulation on melanoma migration.

1.3 Bibliography


Chapter 2

Investigating MMP expression change in Endothelial – Melanoma cell interactions

2.1 Introduction

This chapter examines how melanoma interactions with the endothelial cells induce changes in MMP expression. Three cell lines of melanoma will be studied: WM9, Lu1205 and A2058. Of the three, WM9 and Lu1205 are classified as highly metastatic while A2058 is moderately metastatic. The investigation will also focus on three MMPs that have been known in melanoma invasion: MMP1, 2 and 9.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Metastatic Potential</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM9</td>
<td>++</td>
<td>Metastatic Tumor site (lymph node)</td>
<td>(Juhasz et al., 1993)</td>
</tr>
<tr>
<td>Lu1205</td>
<td>+++</td>
<td>Metastatic variant of WM793 melanoma</td>
<td>(Juhasz et al., 1993)</td>
</tr>
<tr>
<td>A2058</td>
<td>+</td>
<td>Metastatic Tumor site (lymph node)</td>
<td>(Fabricant et al., 1977)</td>
</tr>
</tbody>
</table>

Table 2-1: Comparisons of metastatic potential of melanoma cells used. Each “+” represents a relative measure of metastatic potential.

MMP1 is expressed in a latent form at 52 kDa and when activated by cleavage of its pro domain is 42 kDa. Primarily, it targets interstitial collagen. MMP1 from melanoma and colon cancer cells can interact with specific proteinases activated receptor 1 (PAR-1) from endothelial cells, resulting in a pro-inflammatory state for the endothelium. This
inflammatory, cell adhesive state is characterized by intracellular calcium flux and secretion of IL8 and von Willebrand factor by the endothelial cells (Goerge et al., 2006). Using SiRNA (short interfering RNA) to knockdown MMP1 prevented the melanoma cells from metastasizing from the dermis spot to the lungs. At the same time, it also reduced tumor-induced angiogenesis \textit{in vivo} (Blackburn et al., 2007).

In clinical studies, overall survival of melanoma patients showed an inverse relationship with MMP9 level in their serum. MMP9 is also known as gelatinase-B and the molecular weight of the secreted form is 92 kDa while the activated form is 82 kDa. As the name suggests, the primary target for MMP9 is gelatin, a hydrolyzed form of collagen found in the ECM. Increased MM9 levels also correlated with bone metastasis (Clezardin, 2000). Increased levels of MMP1 in the serum, however, correlated with rate of progress of the invasion (Nikkola et al., 2005). While melanoma cells do produce MMPs, there is also evidence that in response to a tumor invasion, host cells produce MMPs that may aid the invasion process. Using MMP9 deficient mice, metastatic B16 murine melanoma were intravenously injected. These melanoma cells do not produce any MMP9 and in these mice, they found a decrease of 45% in metastatic colonies in the lung, showing that it was MMP9 in the host that was responsible for much of the metastasis (Itoh et al., 1999). In a related work \textit{in vitro}, mouse endothelial cells were co-cultured with various human cancer cells. Via Very Late Antigen-4 (VLA-4) ligands on the cancer cells interacting with Vascular Cell Adhesion Molecule-1 (VCAM-1) on the endothelial cells, they showed that MMP3, 9 and 10 were upregulated and expressed (Hasebe et al., 2007). This is strong evidence showing that melanoma cells have the
ability to harness the host’s signaling mechanism, either by cytokines or cell surface receptors, to their own advantage.

MMP2, or gelatinase-A, also targets gelatin. It is secreted at 72 kDa and when activated, becomes 62 kDa. The inactive, heavier form is denoted the Pro form and when activated is denoted the active form. The active form is lighter due to the cleavage of the Pro domain during activation. MMP2 appears to be another critical MMP with regards to melanoma. It was found that expression of activated MMP2 from the examined melanoma cells correlated with malignancy (Hofmann et al., 1999). Examining melanocytic lesions ranging from interdermal naeveus to dysplastic naeveus and finally to malignant melanoma, MMP2 expression was found to correlate with advancing structural disorganization and atypia of the lesion, suggesting a prognostic value (Vaisanen et al., 1998). In clinical trials, this same group later found that 64% of primary melanoma cases had MMP2 expressed in the tumor cells. The five year survival rate for patients with overexpressed MMP2 versus patients with lower MMP2 expression was 55% and 85% respectively (Vaisanen et al., 1998). As in MMP9, host derived MMP2 also factors in the melanoma invasion. After intravenously injecting the murine melanoma into mice, studies show that tumor volumes were decreased by 39% in MMP2 null or knockout mice compared to control mice 3 weeks after implantation. The number of lung colonies also fell by 54% (Itoh et al., 1998).
If tumor cells themselves produce MMPs, and at the same time is able to elicit MMP expression or activation in the host system, then it can create a potent situation that facilitates all aspects of melanoma metastasis.

2.2 Experimental Preparation and Procedures

2.2.1 Cell Culture

Human melanoma cell line, WM9, was provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Biosource, Inc, Camerillo, CA) supplemented with 10% by volume fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). Another human melanoma cell line, Lu1205 (provided by Dr. Gavin P Robertson, Penn State Hershey Medical Center, Hershey, PA) was cultured in Dulbecco’s Modified Eagle’s Medium F12 (DMEM F12), also supplemented with 10% FBS. The third melanoma cell line was A2058, obtained from NCI (NIH, Bethesda, MD) and cultured in DMEM (Biofluids, Inc Gaithersburg, MD), supplemented with 10% FBS.

Human umbilical vein endothelial cells (ATCC, America Type Culture Collection, Manassas, VA) were grown in Ham’s F12k media (Biofluids Inc, Gaithersburg, MD). The HUVECs are supplemented in the media by 10% FBS, 10ug/ml endothelial growth supplement (VWR, Westchester, PA) and 100ug/ml heparin (Sigma Chemical Co, St Louis, MO). All media contain 100 units/ml of penicillin/streptomycin
(Biofluids, Inc, Rockville, MD) and all cells were maintained under the conditions of 37 °C in an incubator with 5% carbon dioxide.

HPMEC STI.6 R (human pulmonary microvascular endothelial cells) was provided by Dr Kirkpatrick, Institute of Pathology, Johannes-Gutenberg University, Germany. They were maintained as outlined elsewhere (Krump-Konvalinkova et al., 2001).

HMVEC (Human micro vascular endothelial cell, Lonza, Walkersville, MD 21793-0127 USA) is a primary endothelial cell line from the lung. This was maintained in specialized media purchased from the vendor (Endothelial Cell Medium BulletKit, Lonza, Walkersville, MD 21793-0127 USA).

Cells were passed by first washing twice with DPBS, adding a thin coating layer of 0.5% trypsin (Gibco, Invitrogen, Carlsbad, Ca) for 5-10 minutes until the cells have shown detachment. The cells are then suspended in fresh medium and re-plated in new cell culture dishes.

2.2.2 Western Blot

Supernatant (2ml) is collected from cell cultures and transferred to a filter centrifuge device (Vivaspin Ultrafiltration, Sartorius Biotech) with a 3000 DA pore membrane and spun at 4°C at 3500 xg for 45 minutes. The retentate is adjusted with the
filtrate to concentrate the sample 40 times. It is then mixed in a 1:1 ratio with 2XSDS (4% SDS, 0.2% bromophenol blue, 100mM Tris at pH 6.8, 200mM DTT, 20% glycerol). The samples were boiled for 4 minutes in a water bath and then loaded at 15μl in each well of a 10% polyacrylamide SDS-PAGE gel. Electrophoresis of the gel was conducted at constant voltage of 100V for approximately 90 minutes. The gel is then removed and soaked in transfer buffer (48mM Tris, 39 mM glycine, 20% methanol) for 5 minutes. Using electroblotting (300mA at 15V for 30 minutes), the proteins are transferred onto a 0.2 μm nitrocellulose membrane (Protean, Whatman GmbH, Schleicher & Schuell, Germany). The effectiveness of the transfer was checked using Ponceau solution (Sigma) and washed with 0.1M sodium hydroxide to remove the Ponceau solution. The nitrocellulose membrane is then incubated with a blocking solution (5% milk powder in PBST [Phosphate Buffered Saline Tween-20]) for 2 hours at room temperature. The blocking solution was decanted and replaced with the blocking solution containing 1μg/ml of primary antibody. The primary antibodies used were either mouse anti-human MMP2 (R&D Systems, Minneapolis, MN), mouse anti-human MMP1 (R&D Systems, Minneapolis, MN) or mouse anti-human MMP9 (Santa Cruz Biotechnology, Santa Cruz, CA). This is rotated at 4°C overnight. The following day, the membrane was washed 3 times with PBST (8 minutes of rotation per wash). Secondary antibody (peroxidase conjugated goat anti mouse antibody, Sigma) was incubated with the membrane for 2 hours at room temperature. The membrane is then washed 3 times with PBST (8 minutes of rotation per wash) and temporarily stored in PBS. The proteins on the membrane were detected using Enhanced Chemiluminescence Detection System (Amersham, GE
Healthcare, Piscataway, NJ) exposed on photosensitive film (Kodak X-Omat LS film, Sigma, St Louis, MO).

### 2.2.3 Zymography

Samples, such as cell supernatant, were collected and mixed in a 1:1 ratio with SDS buffer (4% SDS, 0.2% bromophenol blue, 100mM Tris at pH 6.8, 20% glycerol). This is then added at 20μl per well in a 10% polyacrylamide SDS-PAGE gel containing gelatin (Novex Zymogram gels, Invitrogen, Carlsbad, Ca). The gel was run at a constant 100V for 90 minutes. The gel was removed from the casing and rocked in 1x renaturing buffer (Novex Invitrogen, Carlsbad, Ca) for 30 minutes a room temperature. The renaturing buffer was decanted and replaced with 1x developing buffer (Novex Invitrogen, Carlsbad, Ca) and rocked for another 30 minutes at room temperature. After rocking, the developing buffer was decanted and replaced with fresh developing buffer and incubated at 37°C for 24 hours. After decanting the developing buffer, the gel was rocked overnight at room temperature in Coomassie Blue solution (2.50 g Coomassie Brilliant Blue R-250, 455 mL ethanol, 455 mL deionized water, 90 mL glacial acetic acid) overnight. After staining, the gel was rocked in destaining solution (45% deionized water, 10% glacial acetic acid, 45% methanol) for approximately 30 minutes or until the blue intensity has reduced. The gel is then bagged in clear plastic and placed against a uniform light source and a photo was taken for analysis.
2.2.4 Heterotypic Cell Co-Culture

Endothelial cells (e.g. HUVEC) were plated onto 6 well plates and grown to confluence. One well is detached and the density of the endothelial cells determined. For contact co-culture, the wells were washed twice with DPBS and the same number of melanoma cells as endothelial cells was suspended in 3.5 ml of assay media (growth media respective to the endothelial cells). The wells are then incubated at 37°C for 24 hours then the media collected.

Samples were collected from the co-culture and were immediately incubated on ice. The supernatant was then spun at 3500 RPM for 5 minutes to pellet any floating cell debris. The clarified supernatant is then frozen or used for experiments.

2.3 Results

2.3.1 Changes in MMP Expression upon HMVEC Cultured with Melanoma

As detailed in Methods, melanoma cells and HMVEC cells were co-cultured under direct contact conditions for 24 hours. The supernatant was collected and probed for presence of MMP1 and 2. Figure 2-1 shows the results of the western blot for the co-culture between melanoma cell lines and HMVEC.

From figure 2-1 (A), it is observed that HMVEC alone already secretes a significant amount of MMP1, both in active and inactive forms. Of the 3 melanomas,
only Lu1205 does not show any MMP1 expression, while both A2058 and WM9 express MMP1, with the most expression coming from A2058. The co-culture of HMVEC with Lu1205 and WM9 did not see any appreciable change in MMP1 expression but co-culture with A2058 actually reduces MMP1 secretion. In figure 2-1 (B), melanoma co-culture with HMVEC is explored. HMVEC expresses a relatively high amount of inactive MMP2. However, co-culture of the endothelial cells to the various melanoma cells showed a decrease with respect to the basal level secreted by HMVEC alone.

Figure 2-1: A) MMP1 from melanoma/HMVEC co-culture B) MMP2 from melanoma/HMVEC co-culture. Lane 1: HMVEC only, 2: WM9 only, 3: HMVEC with WM9, 4: A2058 only, 5: HMVEC with A2058, 6: Lu1205 only, 7: HMVEC with Lu1205
2.3.2 Changes in MMP Expression upon HPMEC Cultured with Melanoma

Following the HMVEC study, melanoma cells co-cultured with HPMEC was performed and the results of the western blot are shown in figure 2-2. From figure 2-2 (A), it is shown that HPMEC, like HMVEC, will constitutively secrete MMP1, both pro and active forms. In this case, while WM9 secretes very low levels of MMP1 (below detection limit of this blot; Data not shown), the co-culture of WM9 and HPMEC reduces the overall expression of MMP1. A2058 secretes low levels of MMP1 and Lu1205 does not secrete any, but the co-culture of these 2 melanoma cell lines with HPMEC did not show significant change in MMP1 expression levels.

In figure 2-2 (B), it is observed that the endothelial cells do produce basal amounts of pro-MMP2. WM9 cells also express some MMP2, but with the co-culture we see a slight increase in both forms of MMP2. Increase in expression is also evident in co-culture with A2058 as well as Lu1205, with the greatest increase in active MMP2 expressed with Lu1205 co-culture.
2.3.3 Changes in MMP Expression upon HUVEC Cultured with Melanoma

Finally, the melanoma cell lines were co-cultured with HUVEC, as shown in figure 2-3. Figure 2-3 (A) shows that HUVEC does not express any MMP1 constitutively, unlike HPMEC and HMVEC. However, co-culture with all 3 melanoma cell lines induces no upregulation in MMP1 and with A2058, MMP1 is slightly downregulated. Figure 2-3 (B) shows the co-culture of melanoma cells with HUVEC for MMP2 - HUVEC expresses its own amount of MMP2 and the same is true for Lu1205. It is seen
Figure 2-3: A) MMP1 from melanoma/HUVEC co-culture B) MMP2 from melanoma/HUVEC co-culture. Lane 0: Media control, 1: HUVEC only, 2: WM9 only, 3: HUVEC with WM9, 4: A2058 only, 5: HUVEC with A2058, 6: Lu1205 only, 7: HUVEC with Lu1205

Figure 2-4: Zymography of melanoma cells co-cultured with HUVEC. Zymography result with inverted color. Pro-MMP2 is 72 kDa and active MMP1 is 62 kDa. Lane 1: 70kDa marker 2: HUVEC only, 3: WM9 only, 4: HUVEC with WM9, 5: A2058 only, 6: HUVEC with A2058, 7: Lu1205 only, 8: HUVEC with Lu1205
that MMP2 (both active and pro forms) is up-regulated in co-culture with all three types of melanoma, with the greatest expression in the Lu1205 co-culture. To confirm this upregulation in expression, a gelatin zymography was performed and is shown in figure 2-4.

2.3.4 MMP9 from the melanoma-endothelial interaction

MMP9 expression was also explored. However, it was found that MMP9 is secreted in concentrations so low or not at all that it was below the detection limit of the assays, especially the zymography which is a very sensitive assay for MMPs (data not shown). However, MMP9 has been found to be secreted from endothelial and melanoma cells in literature and will be discussed in the next section.

2.4 Discussion

In these experiments, we explored whether melanoma cells and endothelial cells will interact in such a way to increase the expression of MMP. MMPs play an important role in aiding melanoma invasion, and while much work has been done to show that MMP expression levels correlate with cancer prognosis as well as tumor formation and growth, this work explores MMP involvement at a very early stage of melanoma metastasis – at the incipient point of melanoma extravasation. The results of the study have been summarized into table 2-1.
Table 2-1: Summary of melanoma and endothelial co-culture: D: Detected, D(w): Detected weakly, ND: Not detected, NC: No Change, ↑: Increase in expression, ↓: Decrease in expression

### MMP1

<table>
<thead>
<tr>
<th></th>
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<th>WM9</th>
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<th>Lu1205</th>
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<tr>
<td>Only</td>
<td>D(w)</td>
<td>D</td>
<td>ND</td>
<td></td>
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<tr>
<td>Huvec</td>
<td>D(w)</td>
<td>NC</td>
<td>↓</td>
<td>NC</td>
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<tr>
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<td>D</td>
<td>↓</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Hmvec</td>
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### MMP2

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<tr>
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<td>ND</td>
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<tr>
<td>Huvec</td>
<td>D</td>
<td>↑</td>
<td>↑</td>
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<tr>
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<tr>
<td>Hmvec</td>
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### MMP9

<table>
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<td>Huvec</td>
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MMP1 was detected on all 3 of the endothelial cell lines and 2 of the melanoma cell lines. For the most part, there was no change in MMP1 expression upon co-culture. However, there were some instances of reduced expression, such as A2058 with HUVEC and WM9 with HPMEC. The reason for these downregulations is currently unclear. Endothelial cells are capable of expressing MMP9 although the basal expression may be rather low. In one study, the expressed levels of MMP9 by HUVEC is significantly lower when compared to the levels of MMP2 (Li et al., 2003). However, it has been found that when HUVEC cell are treated with conjugated eicosapentaenoic acid as well as vascular endothelial growth factor (VEGF), MMP9 expression is induced (Tsuzuki et al., 2007). In this study, it may be necessary to concentrate the collected supernatant and repeat the assays with the concentrate in order to give a more accurate status on the expression of MMP9 upon contact co-culture. The results in this chapter show that while MMP9 may be critical in angiogenesis and tumor formation, it may not be a significant factor in melanoma extravasation through the endothelium, a conclusion that was echoed in another study (Bjorklund and Koivunen, 2005).

From table 2-1, it is evident that MMP2 is a potential target for further study. Inactive MMP2 is upregulated in expression, together with increased concentrations of the active form with all 3 melanoma cell lines, with the greatest expression with Lu1205. It is noteworthy that of the 3 melanoma cell lines tested, Lu1205 has the highest metastatic potential. MMP2 is also upregulated when either HUVEC or HPMEC is in contact with melanoma cells. As of this point in the analysis, it remains to be explored if this upregulation is mediated by cell surface receptor-ligand or due to signaling through
soluble cytokines. It was shown that VLA-4 on the human lung cancer cells will interact with VCAM on endothelial cells to up-regulate the expression of MMP9 (Hasebe et al., 2007). Conversely, PMNs are able to release a soluble factor that activates MMP2 (Schwartz et al., 1998) indicating varied pathways of MMP regulation. The following chapter will further discover this up-regulation, especially MMP2.

2.5 Bibliography


3.1 Introduction

The previous chapter established that MMP2 was up-regulated and activated on contact co-culture of HUVEC with melanoma cells, most notably Lu1205. This chapter seeks to investigate if the signaling between the two cell types is mediated by cytokines or physical contact. We also look at the changes in TIMP2 expression as well as find some of the relevant cytokines that cause this up-regulation. We report a novel finding of the up-regulation of MCP-1 (Monocyte Chemotactic Protein 1) expression in the HUVEC-Lu1205 co-culture system.

MMP2 has been intensely studied in association with cancer progression. Eight different melanoma cell lines were examined for malignancy and analyzed for MMPs 1, 2, 3 and 9 expressions. TIMPs 1 and 2 were also examined. Of the MMPs, only MMP2 correlated with in vivo invasiveness (Hofmann et al., 1999). In a separate study, inhibiting MMP2 secreted from ovarian adenocarcinoma resulted in reduced invasion through HUVEC ECM in Boyden chamber migration assays (Leroy-Dudal et al., 2005). Although these studies show that aggressive melanoma cells secrete MMP2 constitutively, there is evidence that there are other sources of MMP2 secretion, most notably from the host stromal cells. Indeed, when examining tumors in mice, MMP2 was found at the invasive front, at the tumor stromal border, indicating that stromal cells are
the primary source (Hofmann et al., 2005). Furthermore, HUVEC is known to respond to soluble factors that can increase the expression. When PMN conditioned media (media enriched with the protein secretions of PMNs) was added to HUVEC, it was found that increased levels of inactive and active MMP2 was secreted into the supernatant (Schwartz et al., 1998). Significantly, MMP2 belongs to a group of MMPs that lack the TATA box and AP-1 and so are constitutively expressed (Vincenti and Brinckerhoff, 2007). HUVEC will shed vesicles from localized areas of the cell membrane. The vesicles are about 300-600nm and contain MMP2, MMP9, MT1-MMP, TIMP1 and TIMP2 (Taraboletti et al., 2002). MMP9 was only seen in the vesicles in detectable amounts after stimulation with fetal calf serum, fibroblast or vascular endothelial growth factors.

While active MMP2 is involved in melanoma progression, the protein is secreted in the inactive form. Much study has been devoted to understanding the activation of MMP2 and how it is able to create a path of invasion for the melanoma. Activation of MMP2 involves MT1-MMP and paradoxically, TIMP2 (Sternlicht and Werb, 2001). Initially, MT1-MMP on the cell surface binds to the N-terminal domain of TIMP2 and is also inhibited by it. The C-terminal domain of that TIMP2 bound to MT-MMP becomes a receptor for the hemopexin domain of inactive MMP2. An adjacent MT-MMP cleaves the pro domain of that MMP2 that is bound to the tethered TIMP2. This cleavage is not complete and has a residual portion, which is finally removed by another membrane-bound MMP2 to yield a free, active MMP2 molecule. TIMP2, however, can still bind with soluble active MMP2 to inhibit their activity. To create a path for invasion,
MMP2 can bind to $\alpha_\nu\beta_3$ integrin expressed on the surface of melanoma cells and angiogenic blood vessels. The integrin binds to vitronectin and is normally expressed on platelets but found to be expressed on melanoma cells as well. The C-terminus of the MMP2 interacts with $\alpha_\nu\beta_3$ and binds the MMP2 to the cell surface, retaining the proteolytic activity (Brooks et al., 1996).

Two chemokines, IL8 (CXCL8) and MCP1 (CCL2), were investigated in their ability to cause MMP2 up-regulation in the tumor microenvironment. We have found that the Lu1205 melanoma secretes them and have been shown to be involved in MMP2 expression (Luca et al., 1997; Werle et al., 2002). IL8 belongs to the CXC subfamily of chemokines in that two N-terminal cysteines in the structure are separated by one amino acid and MCP1 belongs in the CC subfamily as their amino terminus has two adjacent cysteines. Initially accepted as a chemotactic factor for neutrophils, IL8 was found to affect a wide range of targets, including induction of shape change, respiratory burst, release of lysosomal enzymes and generation of bioactive lipids on neutrophils. It also activates neutrophils to allow increased adhesion onto the endothelium and enhances the transmigration of neutrophils across the endothelial layer by inducing shedding of L-selectin (Mukaida et al., 1998). Conversely, MCP1 is a chemotactic factor for monocytes and is involved in the activation of monocytes, macrophages and lymphocytes. It induces the influx of intracellular calcium, respiratory burst, secretion of lysosomal enzymes and expression of adhesion molecules in monocytes. Under the influence of MCP1, monocytes will also express pro-inflammatory chemokines such as IL1 and IL6 which enhance the effectiveness of monocytes against some cancer cells. The receptors for IL8
are designated as CXCR1 and CXCR2, while the receptors for MCP1 are CCR2a and CCR2b.

Both chemokines (IL8 and MCP1) are important to cancer progression due to their association with inflammation. Recent evidence shows that chemokines and their receptors play critical roles in the metastatic process. They enable the melanoma cells to execute a variety of abilities such as, but not limited to, adhesion to the endothelium, survival, colonization and angiogenesis (Kakinuma and Hwang, 2006). There exist strong similarities between how melanoma take advantage of the chemokine receptors involved in the immune response and how immune cells function in respond to insult. Tumor-associated chemokines like IL8 and MCP1 can regulate inflammation within tumors and the environment such that it compromises the host’s ability to reject the tumor and allow the cancer cells to evade the immune system (Coussens and Werb, 2002).

3.2 Experimental Preparation and Procedures

3.2.1 Cell Culture

A human melanoma cell line, Lu1205 (a kind gift from Dr Gavin P Robertson, Penn State Hershey Medical Center, Hershey, PA), is cultured in Dulbecco’s Modified Eagle’s Medium F12 (DMEM F12, Gibco, Invitrogen, Carlsbad, Ca) supplemented with 10% FBS.
Human umbilical vein endothelial cells (ATCC, America Type Culture Collection, Manassas, VA) were grown in Ham’s F12k media (Biofluids Inc, Gaithersberg, MD). The HUVECs are supplemented in the media by 10% FBS, 10ug/ml endothelial growth supplement (VWR, Westchester, PA) and 100ug/ml heparin (Sigma Chemical Co, St Louis, MO). All media contain 100 units/ml of penicillin/streptomycin (Biofluids, Inc, Rockville, MD) and all cells were maintained under the conditions of 37°C in an incubator with 5% carbon dioxide.

Cells were passed by first washing twice with DPBS, adding a thin coating layer of 0.5% trypsin (Gibco, Invitrogen, Carlsbad, Ca) for 5-10 minutes until the cells have shown detachment. The cells are then suspended in fresh medium and replated in new cell culture dishes.

### 3.2.2 Western Blot

Supernatant (2ml) is collected from cell cultures and transferred to a filter centrifuge device (Vivaspin Ultrafiltration, Sartorius Biotech) with a 3000 DA pore membrane and spun at 4°C at 3500 xg for 45 minutes. The retentate is adjusted with the filtrate to concentrate the sample 40 times. It is then mixed in a 1:1 ratio with 2XSDS (4% SDS, 0.2% bromophenol blue, 100mM Tris at pH 6.8, 200mM DTT, 20% glycerol). The samples were boiled for 4 minutes in a water bath and then loaded at 15μl in each well of a 10% polyacrylamide SDS-PAGE gel. Electrophoresis of the gel was conducted at constant voltage of 100V for approximately 90 minutes. The gel is then removed and
soaked in transfer buffer (48mM Tris, 39 mM glycine, 20% methanol) for 5 minutes. Using electroblotting (300mA at 15V for 30 minutes), the proteins are transferred onto a 0.2 μm nitrocellulose membrane (Protean, Whatman GmbH, Schleicher & Schuell, Germany). The effectiveness of the transfer was checked using Ponceau solution (Sigma) and blots were washed with 0.1M sodium hydroxide to remove the Ponceau solution. The nitrocellulose membrane is then incubated with a blocking solution (5% milk powder in PBST [Phosphate Buffered Saline Tween-20]) for 2 hours at room temperature. The blocking solution was decanted and replaced with the blocking solution containing 1μg/ml of primary antibody. The primary antibodies used were either mouse anti-human MMP2 (R&D Systems, Minneapolis, MN), mouse anti-human MMP1 (R&D Systems, Minneapolis, MN) and mouse anti-human MMP9 (Santa Cruz Biotechnology, Santa Cruz, CA). This is rotated at 4°C overnight. The following day, the membrane was washed 3 times with PBST (8 minutes of rotation per wash). Secondary antibody (peroxidase conjugated goat anti mouse antibody, Sigma, St Louis, MO) was incubated with the membrane for 2 hours at room temperature. The membrane is then washed 3 times with PBST (8 minutes of rotation per wash) and temporarily stored in PBS. The proteins on the membrane were detected using Enhanced Chemiluminescene Detection System (Amersham, GE Healthcare, Piscataway, NJ) exposed on photosensitive film (Kodak X-Omat LS film, Sigma, St Louis, MO).
3.2.3 Zymography

Samples, such as cell supernatant, were collected and mixed in a 1:1 ratio with SDS buffer (4% SDS, 0.2% bromophenol blue, 100mM Tris at pH 6.8, 20% glycerol). This is then added at 20μl per well in a 10% polyacrylamide SDS-PAGE gel containing gelatin (Novex Zymogram gels, Invitrogen, Carlsbad, Ca). The gel is run at a constant 100V for 90 minutes. The gel was removed from the casing and rocked in 1x renaturing buffer (Novex Invitrogen, Carlsbad, Ca) for 30 minutes a room temperature. The renaturing buffer is decanted and replaced with 1x developing buffer (Novex Invitrogen, Carlsbad, Ca) and rocked for another 30 minutes at room temperature. After rocking, the developing buffer was decanted and replaced with fresh developing buffer and incubated at 37°C for 24 hours. After decanting the developing buffer, the gel was rocked overnight at room temperature in Coomassie Blue solution (2.50 g Coomassie Brilliant Blue R-250, 455 mL ethanol, 455 mL deionized water, 90 mL glacial acetic acid). After staining, the gel was rocked in destaining solution (45% deionized water, 10% glacial acetic acid, 45% methanol) for approximately 30 minutes or until the blue intensity has reduced. The gel is then bagged in clear plastic and placed against a uniform light source and a photo was taken for analysis.

3.2.4 ELISA

All ELISA (Enzyme-Linked ImmunoSorbent Assay) were performed in the NIH Cytokine Core Lab, Pennsylvania State University. Mouse anti human capture antibody for IL8 (MAB208, R&Dsystems, Minneapolis, MN) was diluted to 4μg/ml in coating
buffer (0.1M NaHCO$_3$) and added at 50μl to each well. The 96 well plate was placed in a humidified container for 4°C overnight. The plate was washed 4 times with PBST and blocking solution (1% BSA in PBS) at 200ul/well was added. It was blocked for 2 hours at room temperature, after which it was washed 3 times in PBST. The sample of interest in added at 100μl per well then incubated overnight at 4°C. The next day, the plate was washed 4 times and 100μl of biotinylated affinity purified goat anti-human detection antibody at 0.2μg/ml was added to each well and incubated for 2 hours at room temperature. The plate is then washed 6 times. 100μl of Streptavidin peroxidase at 1μg/ml was added into each well. The plate was sealed in a humidified container and further incubated for 30 minutes at room temperature. 100μl of ABTS (2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid], Sigma Chemical Co)/peroxide solution was added for 1 hour unsealed in the dark. The plate is then read in a microtiter plate reader (BioRad Laboratories, Hercules, CA) at a wavelength of 405nm.

3.2.5 TCM (Tumor Conditioned Media)

TCM is tumor conditioned media that was obtained from Lu1205 melanoma cells by using a 90mm cell culture dish and growing the cells to 90% confluency in normal growth media. The dish is then washed twice with DPBS and the media is replaced with 4.5 ml of Ham’s F12K media (Biofluids Inc, Gaithersburg, MD) supplemented with 10μg/ml endothelial growth supplement (VWR, Westchester, PA) and 100ug/ml heparin (Sigma Chemical Co, St Louis, MO). The dish is then returned to the incubator for exactly 24 hours. After the incubation, the media was collected and spun down at 2500
RPM for 5 minutes to pellet any debris, and then the collected TCM is aliquoted for experiments or frozen storage

3.2.6 Co-culture (HUVEC/Lu1205)

HUVEC was plated onto 6 well plates and grown to confluence. One well was detached and the density of the HUVEC determined. For contact co-culture, the wells were washed twice with DPBS and the same number of Lu1205 cells as HUVEC was suspended in 3.5 ml of assay media (Ham’s F12K media [Biofluids Inc, Gaithersberg, MD] supplemented with 10μg/ml endothelial growth supplement [VWR, Westchester, PA] and 100μg/ml heparin [Sigma Chemical Co, St Louis, MO]). The wells are then incubated at 37°C for 24 hours then the media collected.

For transwell co-culture, the wells were washed twice with DPBS. 2ml of assay medium was added to the wells and a 0.4μm pore filter transwell insert (Transwell Permeable Supports, Corning, NY) placed into the well. The same number of Lu1205 as the HUVEC was added in 1ml into each insert and the plate was incubated at 37°C for 24 hours.

For cytokine stimulation, after washing the wells, assay media was added to the wells with cytokines such as recombinant human IL8 (R&DSystems, Minneapolis, MN), or human MCP-1 (R&DSystems, Minneapolis, MN) at their respective concentrations. In the neutralizing cases, the assay media will contain 2μg/ml of anti-IL8 or 1.5μg/ml of
anti-MCP1 anti human monoclonal antibody (R&DSystems, Minneapolis, MN). As with the co-culture case, the 6-well plate is then incubated at 37°C for 24 hours and the media collected as described previously.

3.2.7 Cell Lysis

To collect the cell lysate, the cell culture media was aspirated and 50-100μl of cell lysis buffer (Sigma, St Louis, MO) was added to the base of the culture dish. A rubber scrapper was applied to scrap the cells together with the lysis buffer. The dish is then placed on ice for 30 minutes. The lysate is collected into a micro-centrifuge tube and spun at 10,000 RPM for 5 minutes to pellet any cell debris. The debris-free lysate is then obtained for frozen storage or for further experiments.

3.2.8 Flow cytometry

To detect surface expression of cell surface receptors, cells were incubated with 2μg/ml of antibody (for example, anti-MCAM, R&D Systems, Minneapolis, MN), for 30 minutes at 4°C in DPBS with 5% calf serum and 2% goat serum. They were then washed twice with DPBS containing the calf and goat serum. After an additional 30 minutes of incubation at 4°C with secondary TRITC labeled IgG antibodies (Jackson ImmunoResearch, West Grove, PA), the cells were washed twice and fixed in 2% formaldehyde solution. Detection was realized using a Guava personal cytometer.
(GUAVA Technologies Inc, Burlingame, CA). As a control, the cells were treated with primary IgG mouse antibodies.

3.2.9 Statistical Analysis

To test for statistical significance, assays were performed at least twice. The data is gathered and tested for significance using the 2 sample Student’s t-test with unequal variances (Excel, Microsoft, Redmond, WA). P-values are reported as a percentage, i.e., a p value of 0.05 is reported in the figures as 5%. Error bars shown in figures are standard error of mean (SEM).

3.3 Results

3.3.1 MMP2 up-regulation is cytokine dependant

The first step in understanding the melanoma-endothelial interaction is to explore if the MMP2 up-regulation is a result of a cell-surface receptor-ligand interaction or a result of chemokine signaling between the 2 cell types. To achieve this, the co-culture was performed using Transwell chambers that separate the 2 cell populations by a 0.4 micron pore filter. This prevents the cells from crossing the membrane for contact but allows diffusion of secreted chemokines to activate each other. This co-culture is also carried out for 24 hours and supernatant collected, and assayed via western blot for MMP2, as shown in figure 3-1.
Figure 3-1: Comparison of MMP2 up-regulation in contact and transwell co-culture. Pro-MMP2 is 72 kDa and active MMP1 is 62 kDa. A) Lanes 1, 2: HUVEC with Lu1205 contact co-culture, 3, And 4: HUVEC with Lu1205 transwell co-culture, 5: Lu1205 only, 6: HUVEC only, 7: recombinant MMP2 control. B) Zymography shown with relative intensity chart. HUVEC only case is incubated with 1mM APMA (4-aminophenylmercuric acetate) for 2 hours at 37°C to activate pro-MMP2. This indicates that the lower band on the Zymogram is indeed activated MMP2. Lanes 1, 2: HUVEC only; 3, 4: HUVEC after incubation with APMA; 5, 6: HUVEC with Lu1205 co-culture

From figure 3-1, we see that MMP2 was up-regulated in both contact and transwell co-cultures, indicating that this is a result of a soluble factor. Melanoma has been known to secrete various inflammatory cytokines to aid in survival and metastasis. In this case, we singled out 2 particular cytokines, IL8 and MCP1, to research if they can
be among the candidates that cause the MMP2 up-regulation. TCM, a concentrate of what is secreted by the melanoma only, is collected from Lu1205 and is used to stimulate HUVEC for MMP2 response. TCM allows us to amplify this signal which results in MMP2 up-regulation. The concentrations of IL8 and MCP1 were measured in TCM and compared to their respective concentrations in supernatant collected from Lu1205 control in co-culture experiments. The following table 3-1 shows there is significant amount of both IL8 and MCP1 in the co-culture to stimulate MMP up-regulation. Furthermore, Lu1205 itself secretes a significant amount of both IL8 and MCP1.

<table>
<thead>
<tr>
<th></th>
<th>Co-culture Supernatant</th>
<th>TCM from Melanoma</th>
</tr>
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<tbody>
<tr>
<td>IL8 pg/ml</td>
<td>730±150</td>
<td>15557±901</td>
</tr>
<tr>
<td>MCP1 pg/ml</td>
<td>490±11.7</td>
<td>3928±429</td>
</tr>
</tbody>
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Table 3-1: Concentrations of IL8 and MCP1 secreted from Lu1205 melanoma. Values with standard error are shown. The supernatant is collected from the co-culture experiments lasting 4 hours; TCM is collected over 24 hours but from a confluent monolayer of Lu1205 at 0.067ml/cm² of media per growth area.

3.3.2 Identification of IL8 and MCP1 as the chemokines that up-regulate MMP2

To investigate if the increase in MMP2 secreted is due to the presence of IL8 and/or MCP1 that is expressed by the melanoma, recombinant proteins of IL8 and MCP1 were used to stimulate the HUVECs. The concentration used reflects the amount that is present in the TCM. After 24 hours of stimulation, together with the controls, the
supernatant is collected as described and a zymography assay performed. Figure 3-2 shows the results of the assay. Although a zymography shows clear bands on a dark, blue stained background, the figure shown has been inverted to give dark bands against a clear background for better clarity.

Figure 3-2: Recombinant IL8 and MCP1 are used to stimulate HUVEC. Zymography assay is shown with the relative intensity chart. Pro-MMP2 is 72 kDa and active MMP1 is 62 kDa. Lanes 1, 2: HUVEC only, 3: Lu1205, 4: HUVEC with TCM, 5: HUVEC with IL8 15ng/ml, 6: HUVEC with MCP1 4ng/ml, 7: HUVEC with Lu1205 contact, 8: TCM only

From the figure, we can observe that TCM is a potent activator of MMP2. When HUVEC is stimulated with TCM, one can observe a strong increase in the amount of active MMP2 present and slight increase in the pro form of MMP2. When stimulated
with IL8 and MCP1, both pro and active forms are also increased. This shows that both IL8 and MCP1 are capable of eliciting increased MMP2 secretion. To further confirm the action of both these chemokines, we performed transwell co-culture with neutralizing antibodies to each of the chemokines. The result of the zymography is shown in figure 3-3.

Figure 3-3: Co-culture with neutralizing IL8 and MCP1. Zymography assay is shown above with the relative intensity chart. Pro-MMP2 is 72 kDa and active MMP1 is 62 kDa. Lanes 1, 2: HUVEC only, 3: Lu1205, 4: HUVEC with Lu1205 contact co-culture, 5, 6: HUVEC with Lu1205 transwell co-culture, 7: HUVEC with Lu1205 transwell co-culture with anti IL8, 8: HUVEC with Lu1205 transwell co-culture with anti MCP1, 9: HUVEC with Lu1205 transwell co-culture with anti MCP1 and anti IL8.

The figure shows that with either neutralizing IL8 or MCP1 there is reduced expression of active and inactive MMP2 compared to the positive control co-culture cases. Neutralizing both IL8 and MCP1 further reduced the expressed MMP2, however, the levels expressed are still slightly higher than basal levels. This may indicate the
presence of other cytokines that can also be responsible for the same response. To explore the sensitivity of HUVEC to MCP1, HUVEC was incubated with MCP1 at various concentrations for 24 hours and the results of the zymography are shown in figure 3-4. We see that even a concentration as low as 100pg/ml is sufficient to stimulate HUVEC expression and activation of MMP2.

Figure 3-4: Stimulation of HUVEC with MCP1 at increasing concentrations of MCP1. Zymography assay is shown with the relative intensity chart. Pro-MMP2 is 72 kDa and active MMP1 is 62 kDa. Lanes 1: HUVEC only, 2: Lu1205, 3: HUVEC with Lu1205 contact co-culture, 4: HUVEC with 0.1ng/ml MCP1, 5: HUVEC with 0.5ng/ml MCP1, 6: HUVEC with 1.0ng/ml MCP1, 7: HUVEC with 2.0ng/ml MCP1, 8: HUVEC with 4.0ng/ml MCP1
3.3.3 TIMP2 is up-regulated simultaneously with MMP2

With increasing levels of MMP2, we decided to investigate if the secreted levels of TIMP2, the natural inhibitor to MMP2, are affected by the co-culture. This is especially relevant given that TIMP2 also plays an important role in the activation of MMP2. The culture supernatant was collected as before for the MMP2 and concentrated in a filter centrifuge. The retantate is then used in a western blot to detect the presence of TIMP2, shown in figure 3-5A. The figure shows that HUVEC does secrete a basal level of TIMP2 while Lu1205 does not. However, the level of secreted TIMP2 rises sharply upon both contact and transwell co-culture, indicating that its increase is also, like MMP2, dependant on soluble factors. As MT1-MMP, or also denoted as MMP14, is also involved in MMP2 activation, we examined if there were any changes in expression. Figure 3-5B shows that MT1-MMP decreased in concentration upon contact co-culture.

Figure 3-5: Expression of TIMP2 and MT1-MMP after co-culture. A) TIMP2 is 21 kDA. Lane 1,2: HUVEC and Lu1205 transwell co-culture; 3,4: HUVEC and Lu1205 contact
co-culture; 5: Lu1205 only; 6: HUVEC only B) MT1-MMP is 66 kDA. Lane 1,2: HUVEC and Lu1205 co-culture; 3,4: Lu1205 only; 5,6: HUVEC only

3.3.4 Effect of HUVEC/Lu1205 co-culture on expression of IL8 and MCP1

Previous studies in the Dong laboratory have shown that IL8 is a potent chemokine in upregulating adhesion receptors on PMNs to allow tethering of melanoma and its subsequent migration. The studies also show that the IL8 that is endogenously produced by melanoma will stimulate further increase in secretion of IL8 from PMNs (Slattery and Dong, 2003). Knowing that these chemokines are responsible for MMP2 up-regulation, we decided to explore if their levels are similarly affected by the interaction that may lead to a further stimulus to effect MMP2 expression. The co-culture was performed and the supernatant collected to be analyzed by ELISA. The results are summarized in figure 3-6.
Figure 3-6 shows that both IL8 and MCP1 show a dramatic increase that is significant against the sum of the concentrations of HUVEC and Lu1205 individually. Clearly, the co-culture signaling results in the increase of the chemokines’ secretion. To further understand this, we investigated if this increase is contact dependant, similar to the study that was done with MMP2 and TIMP2. As before, contact co-cultures were performed with transwell co-cultures and the supernatant probed for MCP1 via ELISA. The results are shown in figure 3-7A. It is observed that the MCP1 up-regulation is only dependant on direct contact between HUVEC and Lu1205, as opposed to MMP2 and TIMP2. The kinetics of increase of MCP1 upon contact co-culture is also investigated and shown in figure 3-7B. We observe that the onset of MCP1 increase over the base levels of Lu1205 only occurs as early as 4 hours into the co-culture. Similarly, to see if the increase of IL8 is also contact dependant, a contact versus transwell co-culture was
performed. The results in figure 3-8 show that the increase of IL8 secretion is also dependant on direct contact between the HUVEC and Lu1205 melanoma.

Figure 3-7: MCP1 levels in contact versus transwell co-culture and time dependant increase of MCP1 in contact co-culture. A) The cell-cell contact dependence of MCP1 up-regulation is examined. B) Kinetics of the up-regulation of MCP1 on contact co-culture over 24 hours
Figure 3-8: Increase of IL8 is dependant on contact co-culture

Knowing that cell surface receptors control the up-regulation of MCP1 and IL8, MCAM (Melanoma Cell Adhesion Molecule, CD146, MUC18) was tried as a candidate to investigate if it played a role. The results in figure 3-9 show that when MCAM is blocked with antibodies, the up-regulation of MCP1 reduced by 42%. However, the increased levels of IL8 remain unchanged; suggesting that another cell surface receptor is responsible. As MCAM has been reported to form homotypic adhesion (Shih et al., 1997), the presence of MCAM on Lu1205 and HUVEC was explored. Figure 3-10 shows that MCAM is present on both Lu1205 as well as HUVEC.
Figure 3-9: Investigation of MCAM interactions in MCP1 and IL8 up-regulation. A) IL8 expression upon contact co-culture with MCAM antibodies; B) MCP expression upon co-culture with MCAM antibodies
Figure 3-10: MCAM expression from both Lu1205 and HUVEC. A) MCAM expression from Lu1205; B) MCAM expression from HUVEC, obtained from published literature (Sers et al., 1994). MUC18 refers to MCAM and UPC10 is a myeloma protein (IgG2A) that is used as a control.
3.4 Discussion

In this chapter, we established that both IL8 and MCP1, both of which are secreted in significant amounts by Lu1205, can affect the levels of secreted MMP2. Concentrations as low as 100pg/ml of MCP1 are able to cause MMP2 activation and up-regulation, highlighting the sensitivity of HUVEC to the chemokine. Neutralizing both the chemokines in the co-culture reduces the total amount of active and inactive MMP2. Simultaneously, secreted levels of TIMP2 are also raised and the response is dependant on cytokine signaling. Furthermore, we found that upon cell-cell contact, MCP1 and IL8 resulted in increased secretion. We also found that MCAM present on HUVEC and Lu1205 and it mediated the increase of MCP1, but not IL8.

The connection between IL8 and MMP2 secretion levels has been studied in cancer research. When non metastatic melanoma cells were transfected with IL8 cDNA, these cells became highly tumorigenic and increased their metastatic potential. They were highly invasive in in vitro invasion assays and were found to have increased MMP2 secretion (Luca et al., 1997). In another study, the authors found that HUVEC incubated with IL8 will increase secreted levels of both active and inactive MMP2 (Li et al., 2003). The study done here is unique in that we approached the investigation by examining interactions between the endothelial and melanoma cells, and found that MMP2, a potent protease in cancer progression, is up-regulated. This allowed us to isolate some of the responsible chemokines, such as IL8 and MCP1, which we found are secreted from the melanoma cells, giving us a better understanding of the physiological interactions that
occur during cancer metastasis. Currently our focus is on the MMP2 that is secreted from the cells into the supernatant. In the introduction of this chapter, it is stated that MMP2 will also interact with TIMP2 as well as MT1-MMP, especially during the activation phase. For now, we are taking the assumption that the secreted amount in the supernatant is proportional to the fraction bound to the cells. Since the cell-bound fraction of active MMP2 is just as important in melanoma invasion, future work can be directed to finding out the density of the surface bound MMP2, both active and inactive, upon endothelial and melanoma cell co-culture.

That HUVEC responds to the presence of IL8 is not unexpected. A study shows that human lung microvascular endothelial cells express CXCR1 and CXCR2, the receptors for IL8 (Schraufstatter et al., 2001). Similarly, a separate study found that HUVEC expresses CCR2, which is a receptor for MCP1 (Weber et al., 1999), and showed that MCP1 is involved in endothelial wound repair and inflammation. The authors found that MCP1, but not IL8, facilitates endothelial cell migration during repair from a wound injury and that it did not affect cell proliferation. The previous study mentioned (Li et al., 2003), however, showed that IL8 is responsible for regulating endothelial cell survival, proliferation and angiogenesis. Overall, it reinforces the concept that melanoma cells secrete these chemokines to utilize the existing mechanisms of inflammation and repair to their invasive advantage.

MCP1 was previously found to up-regulate expression of MMP2 from HUVEC (Werle et al., 2002). However, the study showed that the overall MMP2 concentration
was increased and did not explore the status of MMP2 activation. We showed that MCP1 was able to not only increase the overall secreted MMP2, but also resulted in significantly increased levels of active MMP2. The active form is potent as its presence allows matrix proteins to be digested for melanoma invasion. The study did provide some important insights; stimulation of HUVEC with MCP1 activated three mitogen activated protein kinase pathways (MAPK) – the extracellular signal-related kinase (ERK) pathway, c-Jun amino terminal kinase (JNK) pathway and p38 isoforms MAPK pathway. Treatment of the HUVEC with PD098059 (a specific MEK [MAPK/ERK Kinase] inhibitor) was shown to remove the increase of MMP2 levels. This is consistent with literature showing that mitogen activated protein kinase pathways are responsible for regulation of many MMPs (Westermarck and Kahari, 1999). It remains to be investigated if activation of the CXCR1/2 receptors by IL8 will also activate these same MAPK pathways or if there are other pathways that remain to be researched.

Initially, it appears counter intuitive that when there is increased levels of MMP2, the levels of TIMP2, which is supposed to counter and inhibit MMP2, is also increased. However, it was noted that TIMP2 plays a role in the activation of MMP2 (Sternlicht and Werb, 2001). This apparent conflict is resolved when it was found that while TIMP2 is required for MMP2 activation, higher concentrations of TIMP2 levels leads to inhibition of the activation (Bjorklund and Koivunen, 2005). In this case, we theorize that the levels of TIMP2 must lie within the “activation” zone for us to observe increased activated MMP2. The increase of TIMP2, like MMP2, is due to cytokine signaling between the 2 cell types. It remains to be investigated if the increase in expression is also due to IL8 or
MCP1. There is evidence that MMP2 and TIMP2 are secreted together within membrane vesicles in endothelial cells (Taraboletti et al., 2002), which would imply that they might share the same signaling pathways that regulate their expression.

MCP1 was discovered to be up-regulated upon contact co-culture. As no literature was found to link MCP1 expression to cell surface receptors, we decided to investigate the possible cell-cell interaction receptors such as VLA4/VCAM1, with VLA4 (α4β1) expressed on melanoma and VCAM1 on the endothelial, and MCAM, that binds homotypically and have been found on both melanoma as well as endothelial cells (McGary et al., 2002). Blocking the α4 subunit of VLA4 on the melanoma did not reduce the up-regulation of MCP1 (data not shown). Blocking MCAM, however, reduced the MCP1 up-regulation by 42%, giving clear evidence that MCAM is responsible for the increase in MCP1. As blocking did not remove all the up-regulation, it is still possible that other factors or receptors exist. MCAM is a transmembrane glycoprotein, belonging to the immunoglobulin superfamily. It is known to bind homotypically through heterotypic ligands although these heterotypic ligands are not yet identified (Melnikova and Bar-Eli, 2006). Significantly, metastatic melanoma is reported to have higher expression of MCAM and MCAM transfected cells displayed increased adhesion to human endothelial cells and increased MMP2 secretion (Kraus et al., 1997; Xie et al., 1997). Similarly, we found that upon contact co-culture, IL8 also had a significant increase in expression. However, the increased levels did not respond to anti-MCAM treatments, indicating that another cell surface receptor is responsible. Possibilities we
can try for future work are the VLA4/VCAM1 receptors that were investigated in the increased levels of MCP1.

This is relevant to melanoma metastasis as we know that a low concentration of MCP1 is sufficient to cause MMP2 secretion and activation. There are two possibilities: Melanoma cells are taking advantage of this interaction to generate more MCP1 and IL8 (and hence MMP2) for invasion, or endothelial cells, sensing the invasion of melanoma, releases MCP1/IL8 to attract monocytes to the site of invasion in a bid to neutralize the threat. Either way, it might be to the melanoma cells’ advantage as they can thrive in an inflammatory environment. Study is also underway to discover which of the two cell types is responsible for increase release of MCP1 and IL8.

3.5 Bibliography


Chapter 4

Involvement of MMP2 in Melanoma Invasion and Future Directions

4.1 Introduction

We have established in the previous chapter that MMP2 expression is regulated by IL8 and MCP1. As outlined in chapter 1, MMP2 plays several roles in cancer progression, including influencing angiogenesis and inflammatory response (Bjorklund and Koivunen, 2005). This chapter will explore the ability of MMP2, generated by endothelial and melanoma cell interaction, to aid in melanoma cell invasion.

It was suggested by the review cited above that extravasation, or the invasion of cancer cells from the circulatory system into foreign tissue, was not critically dependant on MMP activity. However, a study showed that invasion of human ovarian adenocarcinoma cells through the extracellular matrix involves participation of MMP2 (Leroy-Dudal et al., 2005). Another study transfected human melanoma cells with IL8 cDNA, which resulted in increased expression of MMP2 from the melanoma, and was shown to have increased invasive potential (Luca et al., 1997). Furthermore, MMP2 can localize to \(\alpha_\beta_3\) integrin expressed on the surface of invasive cancer cells; the C-terminus of the MMP2 binds directly to the integrin and results in a proteolytically active form on the cell surface (Brooks et al., 1996). Coincidentally, it was reported that the presence of
the $\alpha_v\beta_3$ integrin correlated with transendothelial migration ability of tumor cells (Bauer et al., 2007). The evidence suggests that MMP2 may play a role in melanoma invasion, especially after the cell tethers to the endothelial surface; MMP2 may be upregulated to allow the melanoma to carve a path of invasion across the endothelial cell and the underlying extracellular matrix. The extracellular matrix comprises glycosaminoglycans and numerous fibrous proteins including collagen, fibronectin, elastin and laminin (Alberts et al., 2002), all of which are substrates for active MMP2 (Sternlicht and Werb, 2001). We decided to investigate the possibility that the MMP2 upregulated by the melanoma-endothelial interaction may increase the invasive potential of melanoma cells.

Assays studying cell invasion in vitro have been widely reported in literature for the last 2 decades. To simulate the extracellular matrix, a reconstituted basement membrane extracted from murine Engelbreth-Holm-Swarm sarcoma is often used (Schuetz et al., 1988). The trade name for the basement membrane marketed by BD-Biosciences is Matrigel. Cells cultured on Matrigel are also able to demonstrate complex behavior that would not be observed on a tissue culture dish (Kleinman et al., 1987). As Matrigel is mainly composed of collagen and laminin, it is a good in vitro substitute for the extracellular matrix. Earlier designs of cellular invasion using Matrigel have endured until today. A two-chamber chemotaxis assay that was pioneered by Boyden (Boyden, 1962) is used, with Matrigel coating the micro-pore polycarbonate filters that divide the two chambers (Albini et al., 1987). However, this set-up is not appropriate to investigate MMP2’s role in melanoma invasion as the bulk of MMP2 is secreted from the endothelial cells and not from the invasive cells as in the studies mentioned above (Leroy-Dudal et
al., 2005; Luca et al., 1997). We decided to modify the set-up by attempting to culture HUVECs above the micropore filter to allow interaction with melanoma for MMP2 upregulation and activation. Matrigel was required both as a substrate for HUVEC to grow on and as a barrier for MMP2 to proteolyze. Ultimately, we are trying to achieve an in-vitro set-up that is described in recent literature as transendothelial migration. Although such a set-up has been used in recent studies, they were not appropriate for our use as some used a 5μm pore diameter filter membrane which was too small for migrating melanoma cells (Popovic et al., 2008; Zen et al., 2008). Using a larger pore diameter filter membrane results in HUVEC migration that can compete with the melanoma (Roomi et al., 2005). Although there are studies that circumvent the issue by doing away with the filter membrane altogether (Kusama et al., 2006; Ramjeesingh et al., 2003), we decided to apply the filter membrane but find a method to minimize or inhibit the migration of endothelial cells.

4.2 Experimental Preparation and Procedures

4.2.1 Cell culture

A human melanoma cell line, Lu1205 (a kind gift from Dr Gavin P Robertson, Penn State Hershey Medical Center, Hershey, PA), is cultured in Dulbecco’s Modified Eagle’s Medium F12 (DMEM F12, Gibco, Invitrogen, Carlsbad, Ca) supplemented with 10% FBS.
Human umbilical vein endothelial cells (ATCC, America Type Culture Collection, Manassas, VA) were grown in Ham’s F12k media (Biofluids Inc, Gaithersberg, MD). The HUVECs are supplemented in the media by 10% FBS, 10ug/ml endothelial growth supplement (VWR, Westchester, PA) and 100ug/ml heparin (Sigma Chemical Co, St Louis, MO). All media contain 100 units/ml of penicillin/streptomycin (Biofluids, Inc, Rockville, MD) and all cells were maintained under the conditions of 37°C in an incubator with 5% carbon dioxide.

Cells were passed by first washing twice with DPBS, adding a thin coating layer of 0.5% trypsin (Gibco, Invitrogen, Carlsbad, Ca) for 5-10 minutes until the cells have shown detachment. The cells are then suspended in fresh medium and replated in new cell culture dishes.

4.2.2 Transendothelial Migration

Growth factor reduced Matrigel (BD Biosciences, Franklin Lakes, NJ) was diluted 10 times with cold sterile distilled, deionized water and coated at 250μl per 8μm pore filter transwell insert (Transwell Permeable Supports, Corning, NY). The inserts were dried overnight in a laminar flow hood. If not used immediately, the filters were kept at 4°C. Prior to use, the filters were rehydrated with media with 2ml in the well and insert at 37°C. Confluent HUVEC cultures were detached into serum free media and rocked for 30 minutes in 2μM Cell Tracker Red CMPTX (Molecular Probes, Eugene, OR) at 37°C. The suspension was then centrifuged to pellet the cells and resuspended in
growth media (F12K media containing 0.5% FBS with ECGS and heparin). Growth media was replaced in the wells and 1ml of the prepared HUVEC suspension was added into the insert. This was left in the incubator for 2 days for the HUVEC to form a confluent monolayer.

After visual inspection of a confluent HUVEC monolayer, Lu1205 was detached and suspended in serum free media and rocked for 30 minutes in 0.5μM Cell Tracker Green CMFDA (Molecular Probes, Eugene, OR) at 37°C. The suspension is then centrifuged to pellet the cells and resuspended again in serum free media. The growth media in the well and inserts were aspirated and serum free media containing 100ug/ml of collagen IV (BD Biosciences, Franklin Lakes, NJ) is replaced in the well. Lu1205 cells at the concentration of 1 million/ml at 1ml volume was added into the insert. In the neutralizing cases, the assay media will contain 2μg/ml of anti-IL8 or 1.5μg/ml of anti-MCP1 anti human monoclonal antibody (R&DSystems, Minneapolis, MN). The set-up was returned to the incubator for 30 hours. After the incubation, the filters on the side of the inserts were wiped by wetted wipes twice and rinsed with DPBS. This ensured that only cells on the underside of the filter were left. Pictures of the remaining cells were taken under fluorescent microscopy and counted. 10-15 frames were taken per insert and the cells in the frames counted for analysis.
4.2.3 Cell Viability Assay

In designing the parameters for the transendothelial migration assay, cell viability of the HUVECs was determined. HUVECs were cultured on a 96-well flat bottom tissue culture plate under normal growth conditions at 100μl per well. After a confluent monolayer was formed, the media was replaced with media under experimental conditions and incubated an additional 24 hours. 20μl of MTS reagent (Promega, Fitchburg, Wisconsin) was added directly into each well and incubated for 1-4 hours at 37°C. The absorbance in each well was measured by a plate reader (Benchmark Plus Microplate Reader, BioRad, Hercules, CA) at 490nm.

4.2.4 HUVEC Migration Assay

Also included in designing the parameters for the transendothelial migration assay, we wanted to be able to control migration of HUVEC through the 8μm pore filter. Matrigel was coated on the filter as previously described for the transendothelial migration. HUVECs were detached and resuspended in growth media of varying conditions. This same growth media was also replaced in the wells. The cell suspension is added at 1ml into the insert and incubated at 37°C for 24 hours. After the incubation, the filters were cut from the insert and stained for 2 minutes in each of the 3 hematology stain solutions (Hemacolor, EMD Chemicals, Darmstadt, Germany). The filters were mounted on glass slides for analysis of cell migration.
4.3 Results

4.3.1 Development of Transendothelial Migration Assay

As literature on transendothelial migration assays suitable to study the MMP2 dependent migration is limited, we decided to modify the current assay widely used in literature. Matrigel was prepared as stated in the previous section and dried onto micropore filters. HUVECs were then seeded onto the filters to allow a confluent monolayer to be formed. However, we faced our first challenge when it was discovered that HUVECs have a tendency to migrate across the filter during the monolayer formation. This is undesirable because migration of HUVECs may lead to competition of available pores during the transendothelial migration of Lu1205, reducing the melanoma migration and affecting the final readout. After much experimentation, it was found that HUVEC migration is affected by the concentration of serum in the growth media. Figure 4-1 shows the stained filters of the HUVEC migration under different serum concentrations. After the formation of a monolayer, the filters were removed from the insert, stained and mounted onto the slides. The top layer of cells on the filter is removed and the remaining stain represents the migrated HUVEC. The intensity of the stain correlates to the number of HUVECs present.
Figure 4-1: Migration of HUVEC grown under different serum levels. The relative intensity chart is shown below which corresponds to the migration of HUVEC. A: HUVEC growth media with 10% FBS, B: Serum free growth media, C: Growth media with 2% FBS, D: Growth media with 0.5% FBS.

Figure 4-2: Cell viability of HUVEC under varying serum levels. HUVEC was seeded into 6-well plates and incubated under the varying serum conditions for 48 hours, reflecting the same length of time in forming a confluent monolayer on Matrigel.
From figure 4-1, we see that at 10% FBS, there is significant migration of HUVEC. The migration is controlled at 0.5% FBS and not present when the media is serum free. However, we are not able to use serum free media as serum is important for cell survival and proliferation. A cell viability assay was performed to assess the health of the HUVECs under the varying growth conditions with differing concentrations of FBS, as shown in figure 4-2. We can observe that cell viability takes a major toll when there is a complete absence of FBS. Hence for the transendothelial migration assay, we took the optimum compromise of using 0.5% FBS to culture the HUVEC monolayer. It gives the next lowest value (representing the optimum compromise) when one takes the ratio of the migration values and the relative intensity in the cell viability assay, as shown in table 4-1. Furthermore, there was a significant decrease in the migration of Lu1205 cells across the filter (without any endothelial cells) when Matrigel was coated compared to the standard Boyden Chamber with the filter only (data not shown). This shows that the Matrigel coated layer provided a higher resistance to migration compared to the resistance of the filter and should allow us a more accurate readout of the difference in melanoma invasion under the various treatment conditions.

<table>
<thead>
<tr>
<th>FBS%</th>
<th>Migration</th>
<th>Cell viability</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
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<td>30.60</td>
<td>0.65</td>
<td>47.24</td>
</tr>
<tr>
<td>0</td>
<td>0.00</td>
<td>0.28</td>
<td>0.00</td>
</tr>
<tr>
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<tr>
<td>0.5</td>
<td>3.65</td>
<td>0.43</td>
<td>8.43</td>
</tr>
</tbody>
</table>

Table 4-1: Ratio of the relative values of HUVEC migration and cell viability.
4.3.2 MMP2 and the Effect on Transendothelial Migration

As mentioned in the introduction, the \( \alpha_\text{v} \beta_3 \) integrin was shown to be able to localize MMP2 to the surface of cells to allow increased invasive characteristics. The expression of \( \alpha_\text{v} \beta_3 \) on Lu1205 was measured using flow cytometry and the results are shown in figure 4-3. From the figure, we can clearly observe that Lu1205 has a significant population of the integrin and lends itself to the hypothesis that it may bind MMP2 for increased invasiveness.

![Figure 4-3: Expression of the \( \alpha_\text{v} \beta_3 \) integrin on Lu1205 melanoma. Courtesy of Shile Liang.](image)

To test invasion of melanoma cells \textit{in vitro}, the transendothelial migration assay that was developed in the previous section was performed. Antibodies to IL8 as well as MCP1 were added to the media to inhibit upregulation and activation of MMP2. The
results of migration, shown in figure 4-4, indicate that inhibiting IL8 and MCP1 has a positive effect in reducing the number of migrated melanoma cells. The number of HUVEC that migrated at the end of the experiment was also quantified and was found to be less than 10% of the total number of cells on the underside of the filter (data not shown).

Figure 4-4: Transendothelial migration of Lu1205. Migration assay performed with antibodies against IL8 only (center column), or both IL8 and MCP1 (right column) to inhibit MMP2 upregulation.
4.4 Discussion

In this chapter, we developed a transendothelial migration assay to allow us to study the effects of MMP2 upregulation on melanoma invasion. We used the assay to show that inhibiting both IL8 and MCP1 reduced melanoma invasion. This indicates that MMP2 may play a role in melanoma invasion, especially in the light of the discovery that the $\alpha_v\beta_3$ integrin was found on the surface of the Lu1205 melanoma.

In the study mentioned previously (Brooks et al., 1996), it was shown that invasive melanoma expressed the $\alpha_v\beta_3$ integrin compared to less invasive primary melanomas. When cDNAs that encoded the integrin were transfected into noninvasive melanoma and implanted in vivo, stainings of the tumors showed extensive co-localization with MMP2. Furthermore, it was shown that the MMP2 that localized on the surface were functionally active. In another study that looked at 45 different tumor cell lines (Bauer et al., 2007) to correlate the expression of $\alpha_v\beta_3$ with transendothelial migration ability, they found that melanoma expressed high levels of the integrin and it correlated with invasive capacity. It is even suggested that $\alpha_v\beta_3$ may activate MMP2 (Yan et al., 2000; Yang et al., 2003). However, the exact mechanism of how $\alpha_v\beta_3$ localizes and activates MMP2 is currently unknown.

Reduction of transmigration with inhibition of IL8 and MCP1 shows the success of the assay. This assay is not limited to studying MMP expression on invasion. Given that it has the potential to allow dynamic interaction between the endothelial cell
(HUVEC or otherwise) and the migrating cell, it opens a wide field of possibilities to examine other proteases, cytokines or cell-surface receptors that may play a role in cancer cell invasion. It can also be applied to study leukocyte migration from the blood vessels. However, the assay may have its limitations. We may not be seeing the full extent of the difference in invasive ability as both the Matrigel and the micropore filter are barriers to the final readout. Most accurately, the difference in invasiveness lies in the cell count in the Matrigel layer just above the filter, but given the opaque and uneven quality of the filter, the cells are difficult to quantify using confocal microscopy. Also, the reduction in HUVEC viability using lower serum concentrations to grow a monolayer may be a concern. However, the assay also measures proliferation, as the absorbance of the reagent changes with total mitochondria activity. In this case, it is possible that the cells in higher serum concentrations proliferate faster and they reflect a higher reading as opposed to actual cell death in the HUVEC grown at 0.5% FBS. It is possible that growing the HUVEC at 0.5% FBS may affect their response compared to HUVEC grown with the usual 10% FBS. Future experiments to observe MMP2 up-regulation can be performed with HUVEC grown under these conditions to ensure that the lower FBS concentration agrees with the results found in the previous chapters. An additional alternative to the current assay design is to use Calcein acetoxymethylester (Calcein AM, BD Biosciences, Bedford, MA) in the lower wells after the transmigration. It is initially non-fluorescent but can be hydrolyzed by intracellular esterases into fluorescent Calcein and the absorbance can be detected using a micro-plate reader. This would be a faster and potentially more accurate method to quantify the number of cells invaded through the filter.
While the reduction of transmigrated melanoma suggests a role for MMP2 in invasion, we have not confirmed if inhibition of IL8 and MCP1 may affect other protein expressions which may also affect the invasion and the result is the sum of the various responses under the inhibition. Additionally, it implies that inhibiting IL8 alone appears to be as effective at reducing transmigration as blocking both IL8 and MCP1 together. Certainly, we have reason to conclude that MMP2 activation could be one of the major contributing factors, however, there may be other mechanisms at involved to we have yet to fully elucidate. To discover MMP2’s exact contribution, it may be necessary to use SiRNA to knockdown the MMP2 expression from both HUVEC and Lu1205.

4.5 Future Research Directions

The overall study examined the interactions between HUVEC, a model endothelial cell, and Lu1205, a highly metastatic melanoma, with regard to cytokines involved and the subsequent effect on invasion. That tumor cells elicit the host endothelium’s responses for invasion has been under intense study. This is not limited to MMPs, such as the involvement of MMP9 in breast cancer metastasis (van 't Veer et al., 2002) or MMP2 in ovarian adenocarcinoma (Leroy-Dudal et al., 2005). It includes other mechanisms such as contact of melanoma with endothelial cells results in breakdown of endothelial cell junctions that facilitate invasion (Peng et al., 2005). This study not only contributes knowledge in that respect but is unique as it focuses on the role of MMP2 in invasion. While most literature examining the role of MMPs in cancer focuses on angiogenesis, recent literature is beginning to examine MMPs with respect to invasion.
We showed that MCP1 secreted by melanoma cells had the ability to upregulate and activate MMP2. It was also reported that MCP1 could activate the MAPK pathways in HUVEC to cause upregulation of MMP2. However, it remains to be studied if IL8, through the CXCR1/2 receptors, will also use the same pathway for MMP2 upregulation. Furthermore, TIMP2 was also discovered to be upregulated and the controlling mechanism remains to be examined. However, it is likely that TIMP2 secretion should follow the same mechanism as MMP2 secretion as it was reported that they were secreted together from the same membrane vesicles in endothelial cells (Taraboletti et al., 2002).

On contact co-culture of HUVEC and Lu1205, we discovered that MCP1 undergoes an at least 3 fold increase in secretion. This is a potentially important result as we shown how sensitive MMP2 activation and upregulation is to even 0.1ng/ml of MCP1. Further work is required to elucidate this source of the MCP1 as well as the underlying mechanisms. Just as significant, we also showed that MCAM is involved in this upregulation. Currently, the function of MCAM is still poorly understood and under study. A recent study reported that MCAM has the ability to induce micro-villi and hence VCAM1 on the endothelial cell surface, reducing the rolling velocities of lymphocytes under shear stress on the surface of the endothelial cell monolayer (Guezguez et al., 2007). If this is also the case for melanoma cells, then MCAM may be responsible for inducing rolling and adhesion of melanoma on the endothelial cell surface, then inducing MCP1 which results in MMP2 expression and activation, facilitating cell invasion. To further this study, exploration into the biochemical and mechanical effects of MCAM
would be worth conducting. On the other hand, we also discovered that IL8 had increased levels of expression upon contact co-culture with HUVEC and Lu1205 as well, but the cell surface receptors remain unknown. Further work can be done to find the receptors involved.

Finally, this study took a model endothelial cell, the HUVEC, and a highly aggressive melanoma for studies into melanoma invasion. As melanoma has a known affinity to metastasize in lung tissue, among others, the scope of the exploration can be widened to include other types of endothelial cells and melanoma. If we can show that the metastatic invasion of melanoma correlates with expression of MCAM on the cell surface as well as the ability to activate MMP2 for invasion, then there is great therapeutic potential.

4.6 Bibliography


