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THE EFFECTS OF THE TUMOR MICROENVIRONMENT ON ENDOTHELIAL CELLULAR ADHESION MOLECULE EXPRESSION

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ABSTRACT

The goal of this work is to evaluate the how tumor cells interact with and, in effect, change endothelial cells. Through the development of a contact coculture assay, tumor cells and endothelial cells could be cultured separately while still allowing for some direct cell-to-cell contact. This allowed for the measurement of endothelial cellular adhesion molecule expression level changes due to direct cell-to-cell contact with melanoma cells. It was found that direct cell contact resulted in significant increases in ICAM-1 expression levels on HUVEC following a 4 hour coculture. In comparison, HUVEC exposed to only tumor-secreted cytokines did not have the same increase in ICAM-1. Evaluation of cytokine levels following coculture showed increases in IL-8, IL-6, and Gro-α following coculture, though not enough to induce ICAM-1 expression. The results point to cell-to-cell contact as the trigger for increased ICAM-1 expression. Through cross-linking of certain other cellular adhesion molecules on endothelial cells, it was determined that increases in ICAM-1 expression can occur as a result of receptor-ligand interactions between cell types, most likely involving P-Selectin and E-Selectin. Further research needs to be done to determine the specific mechanism of how these interactions specifically cause ICAM-1 upregulation. However, the establishment of cell contact as the cause of rapid ICAM-1 upregulation provides a substantial foundation for further research into the mechanics of melanoma metastasis, and down the line, potential treatment methods.
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Chapter 1

INTRODUCTION

1.1 Cancer and Melanoma

Cancer is a disorder involving the uncontrolled growth of abnormal cells. In the United States, cancer ranks as the second leading cause of death behind heart disease, and about one third of the entire human population will develop some form of cancer during their lifetime (Dittmar, 2008). In fact, 1,479,350 new cancer cases and 562,340 deaths due to cancer are expected in 2009 (Jemal et al., 2009). Cancer is a complicated disorder with approximately 200 different varieties, but all start out as abnormal cells. Unlike normal cells, which grow, die off, and are replaced, these cells are able to grow and replicate at uncontrollable rates. Due to some form of DNA damage or mutation, a cell may develop this ability for uncontrolled growth and will begin to produce more cells like itself. The result of this process is a tumor: a mass of cells whose growth does not match surrounding normal tissue and continues without inciting stimuli (McKinnell, 1998). Many tumors are classified as benign in that they are slow growing and self-contained. These can often be easily removed through surgery. However, tumor cells undergo constant change over the course of the disease, and tumors that were once benign can quickly become malignant and lethal (Fidler, 2002).

Melanoma is a specific form of skin cancer affecting melanocytes. Though it accounts for only a small portion of all skin cancer cases, it is by far the most lethal, accounting for the vast majority of skin cancer deaths due to its malignant nature (McKinnell, 1998). It is one of the few types of cancers whose incidence rate is currently
increasing, with more than 68,000 new cases and nearly 9,000 deaths expected to have in 2009 (Jemal et al., 2009). Melanocytes are cells located in the epidermis that secrete melanin, a pigment that helps protect skin from ultraviolet radiation. The main cause of melanoma, and all other types of skin cancer, is exposure to sunlight, particularly ultraviolet radiation. This exposure is especially damaging to people who burn easily, tan easily, and freckle. Ultraviolet radiation can cause breaks in DNA strands which presumably lead to the development of abnormal cancerous cells. Melanomas often develop in pigmented nevi (also known as moles) which are nests of mature melanocytes (Johnson, 1999). When cells first become cancerous, they grow primarily in a horizontal direction staying near the epidermis. Malignant melanoma legions are characterized by vertical growth downward into the dermis, signaling a change in the nature of the tumor cells. The vertical depth of these lesions is often the best indicator of the current stage of the cancer in its development. A depth of even 1.5mm can be prove to be fatal, and a less than 15% of patients with lesions which invade the subcutaneous tissue can be fully cured (McKinnell, 1998). Figure 1.1 shows the course of melanoma development in the vertical direction labeled according to Clark’s levels of melanoma invasion. The higher the level, greater the invasion into the tissue and the greater the risk of melanoma recurrence (Schofield and Robinson, 2000). Melanoma is known as a particularly fast-developing, lethal form of cancer with some malignant forms taking as little as a few months to cause death. This lethality stems from the ability of melanoma cells to develop the capability to travel from the primary tumor to other areas of the body in a process called metastasis (McKinnell, 1998).
Figure 1.1 Clark’s levels of melanoma invasion. Malignant melanoma lesions invade deeper and deeper into tissue over time. The deeper the lesion, the more likely cells will be able to detach from the lesion and metastasize (Schofield and Robinson, 2000).

1.2 Cancer Metastasis

It is through the process of metastasis that malignant cancer takes the lives of most victims. Metastasis is the spread of tumor cells from the original tumor site to other areas of the body where they form secondary tumors. These secondary tumors can greatly disrupt normal bodily function resulting in damage to organ systems and often death. The reason for the great lethality of metastatic cancer is that multiple secondary tumors often develop at the same time. And since cancer cells can metastasize from secondary tumors
as well, surgery is no longer an effective option for elimination of the cancer.

Chemotherapy provides the only real potential option for a cure (McKinnell, 1998).

The process of metastasis is made up of a series of interrelated steps, each of which can be rate limiting (Fidler, 2002). Figure 1.2 gives a detailed illustration of the series of steps tumor cells must accomplish in order to establish secondary tumors. As tumors develop, cells become more and more heterogeneous. This results in the development of aggressive cells that are able to detach from the primary tumor (McCarthy et al., 1991). In a process called invasion, these cells enter circulation via the capillary and lymphatic vessels. The small number of tumor cells that are able to survive this invasion and circulation throughout the body eventually arrest at a secondary site and extravasate into the surrounding tissue. The secondary tumors that result upset normal bodily functions and account for 90% of cancer-related deaths (Pardee, 2009). However, metastasis is a very difficult process for cells. Apoptosis due to loss of anchorage, mechanical damage due to shear stress and collisions, and attack by immune cells can all lead to damage of tumor cells attempting to metastasize to other areas of the body (Glinsky, 2006). Studies have shown that 24 hours after tumor cells are injected into circulation, less than 0.1% of the cells are still viable and less than 0.01% survive to produce metastases (Fidler, 1970). Though only a select few cells are capable of surviving the entire metastasis process, only a very small number of cells are actually needed to establish secondary tumors, making any level of metastasis very dangerous.
Figure 1.2 The major steps of cancer metastasis. Cancer cells grow and proliferate in the primary tumor. Some cells detach from the tumor and enter circulation which transports them to other organs. Cells eventually adhere to vessel walls and extravasate into tissue where they form secondary tumors (Fidler, 2002).

Due to its deadly nature, understanding metastasis is an important goal of cancer research today. It has been shown that many forms of cancer, including melanoma, tend to metastasize preferentially to certain areas of the body. Malignant melanoma most often forms secondary tumors in the lungs and lymph nodes with the brain, liver, and bones.
also being common sites (Thompson et al., 2003) Theories regarding this selective metastasis date back as far as 1889, when Paget introduced his “seed and soil” hypothesis. The theory postulated that tumor cells (“seeds”) can only proliferate in certain organ systems (“soil”) which provide a fertile environment (Paget, 1889). While Paget’s observations of organ specific metastasis have since been proven correct, there is contradicting evidence regarding the actual cause of this organ-specific homing. In opposition to the “seed and soil premise, one theory hypothesizes that the main causes of selective metastasis are constraints due to the sizes of tumor cell clusters and capillaries. One study in particular has shown through intravital videomicroscopy that tumor cells arrest in microcirculation due to size restrictions alone (Chambers et al., 1995). This theory has arisen primarily due to the observation that tumor cells tend to arrest primarily in pre-capillary vessels and capillaries. However, other studies have countered this argument by showing that mechanical trapping of tumor cells in capillaries will not occur when tumor cell-endothelial cell interactions are blocked (Glinsky, 2006). It has also been shown that melanoma sublines known to metastasize in the lung adhere more strongly to lung microvessel endothelial cells than do sublines known to metastasize to other areas of the body (Belloni, 1989). Additionally, tumor cells in circulation constantly interact with leukocytes and platelets, and this interaction can lead to formation of aggregates of the three cell types. These aggregates can help protect tumor cells from the mechanical stresses of circulation and stabilize tumor cells, enabling them to interact with endothelial cells.
Regardless of the actual mechanism by which tumor cells find their way to specific sites, it is only through interactions with the vascular endothelium that these cells are able to find their way out of circulation and into the surrounding tissue. In a process called extravasation, tumor cells adhere to the endothelial surface and transmigrate across the endothelium (Nicholson, 1989). It is believed that this process mirrors the process by which leukocytes move from circulation into tissue. For leukocytes in blood flow, the first step of the extravasation process involves the transient and reversible reactions between adhesion molecules on the leukocytes and the endothelium, particularly through the binding of selectins on the endothelium to ligands on leukocytes (Hoskins and Dong, 2006). These interactions result in the rolling of leukocytes along the endothelium at rates slower than the actual blood flow (Arfors et al. 1987). This rolling allows the leukocytes to stay in contact with endothelial cells for prolonged period, which eventually leads to the activation of certain adhesion molecules. The result is the firm adhesion and arrest of leukocytes on the endothelium. Transmigration of these cells across the endothelium soon follows (Ley, 1996).

Of particular importance to cancer metastasis research is the way leukocytes interact with and arrest on the vascular endothelium due to its resemblance to tumor extravasation. In particular, cellular adhesion molecules (CAMs) are known to play a prominent role in the extravasation process. Cellular adhesion molecules are receptors which mediate cell-cell and cell-substrate interactions (Albelda and Buck, 1990). These molecules are expressed on the cell surface and bind to specific surface molecules (ligands) on other cells. A given CAM can bind to itself (hemophilic interaction) or to an unrelated CAM (heterophilic interaction), and many can bind to various ligands
depending on the situation. CAMs play an important role in allowing a cell to sense and interact with its environment by providing a direct pathway for the exchange of information between cells (Cairns et al., 2003; Klymkowsky and Parr, 1995). Many endothelial CAMs, including the selectins, ICAM-1, and VCAM-1, are also known to be involved in the leukocyte extravasation process, and many of these molecules also play roles in tumor cell extravasation.

**Figure 1.3 PMN-mediated adhesion of melanoma cells to the endothelium.** PMN adhesion to the endothelium (EC) is mediated by selectin/selectin-ligand and ICAM-1/β2-integrin interactions. Melanoma cell (C8161) adhesion to PMN is also mediated by ICAM-1/β2-integrin interactions (Hoskins and Dong, 2006).

Recent research has shown that interactions between tumor cells and polymorphonuclear neutrophils (PMN) may assist in the progression of cancer
metastasis. In particular, recent studies have shown that while melanoma cells on their own cannot migrate through the endothelium in flow conditions, the presence of PMNs in addition results in melanoma cell adhesion and extravasation (Slattery et al., 2005). These results have led to the hypothesis that melanoma adhesion and extravasation may be mediated by PMNs. Extravasation of PMNs first involves the rolling of cells along the endothelium mediated by weak bonding between selectins on the endothelium and their ligands on PMNs. Firm adhesion after rolling occurs as a result of binding between β2-integrins on PMNs to ICAM-1 on endothelial cells (Hoskins and Dong, 2006). In comparison, melanoma cells have been shown to lack the proper ligands to facilitate adhesion to the endothelium under shear conditions. Melanoma cells do, however, express ICAM-1, and studies have shown that adhesion between melanoma cells and PMNs is decreased when β2-integrins or ICAM-1 are blocked (Slattery and Dong, 2003). These studies point to interactions between PMNs and melanoma cells as a potential mediator of melanoma cell adhesion and extravasation. Figure 1.3 shows the interactions between PMN and melanoma (C8161) and between PMN and the endothelial cell (EC). In addition, interactions between VLA-4 and CD44 on melanoma cells with VCAM-1 and selectins on the endothelium may also play important roles in the metastatic process (Qian et al., 1994; Konstantopoulos and Thomas 2009).
Chapter 2

EXPERIMENTAL METHODS

2.1 Cell Culture

Human melanoma cell lines WM35 and 1205Lu were provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia PA) and Dr. Gavin P. Robertson (Penn State Hershey Medical Center, Hershey, PA) respectively. WM35 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Biosource, Inc.) while 1205Lu cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM). All cells were grown in media supplemented with 10% fetal bovine serum (FBS; Biosource, Inc.) and 100 units/ml penicillin-streptomycin (Biosource, Inc.). Cells were cultured on tissue culture treated polystyrene Petri dishes in a 37°C, 5% CO₂ environment.

Tumor conditioned medium (TCM) was produced using 1205Lu cells. These cells were grown to confluency in T75 flasks (Becton Dickenson, Franklin Lakes, NJ). Standard medium was removed, and following washing with DPBS, 5mL of fresh serum-free media was added to the flask. Cells were incubated for 6 or 24 hours after which the media was removed. After centrifugation to remove cells and debris, the cell-free media was collected as TCM.

Human umbilical vein endothelial cells (American Type Culture Collection, Manassas VA) were cultured in Ham’s F12-K media (Biofluids, Inc., Gaithersberg, MD). Media was supplemented with 10% FBS, 100μg/ml heparin (Sigma Chemical Co., St. Louis, MO), 10μg/ml endothelial cell growth supplement (ECGS, Sigma Chemical Co.),
and 100 units/ml penicillin-streptomycin (Biosource, Inc.). Cells were cultured in passages 3~9 on tissue culture treated polystyrene Petri dishes in a 37°C, 5% CO₂ environment. Prior to experiments, all cells were detached using 0.05% trypsin.

2.2 Coculture Methods

For Transwell cocultures, endothelial cells were added to 6-well plates (Corning) at a concentration of 0.5 million cells/ml (0.375 million cells per well). Selected tumor cells were added to endothelial cells separated by Transwell inserts (3μm pore diameter; Corning) at a concentration of 0.25 million cells/ml (0.125 million cells per well).

Cross-linking studies were performed using. Cells were incubated in serum free media with 100ng/ml of both goat anti-mouse-Ig F(ab)₂ fragments (Jackson ImmunoResearch, Baltimore, MD) and mouse-anti-human antibody specific to the cellular adhesion molecule of interest for one hour. Following this incubation, media was aspirated out and fresh serum free media was added and cells were incubated for the allotted coculture period.

For contact cocultures, a Transwell insert (3μm pore; Corning) was inverted and endothelial cells were seeded on the upturned side of the membrane at a concentration of 0.5 million cells/ml (0.375 million cells). Cells were allowed to adhere to the membrane for 2 hours in cell culture conditions (37°C, 5% CO₂). The insert was then reverted and placed in the well of a 6-well plate (Corning) containing 2ml of HUVEC culture media. For tumor cell-endothelial cell cocultures, select tumor cells were seeded into the Transwell insert at a concentration of 0.25 million cells/ml (0.125 million cells).
For all cocultures, cells were incubated for approximately 12 hours to allow cells to reach confluency. Once cells were confluent, all media was aspirated out and replaced with coculture media (respective media for each cell type supplemented with 1% FBS, no other supplements). Cocultures were incubated in cell culture conditions for 0.5 or 4 hours. At the end of the coculture period, endothelial cells were lifted from the bottom well or the bottom Transwell membrane using 0.05% trypsin. Cells were washed with fresh cell culture medium and allowed to recover for 30 minutes while being rocked at 8rpm at 37°C. (See Appendix for detailed protocol for contact coculture)

2.3 Cross-linking Studies

Endothelial cells were incubated in serum-free media with 100ng/ml of both goat anti-mouse-Ig F(ab)2 fragments (Jackson ImmunoResearch, Baltimore, MD) and mouse-anti-human antibody specific to the cellular adhesion molecule of interest for one hour. Following this incubation, media was aspirated out and fresh serum-free media was added and cells were incubated for the allotted coculture period. At the end of the coculture period, endothelial cells were lifted using 0.05% trypsin. Cells were washed with fresh cell culture medium and allowed to recover for 30 minutes while being rocked at 8rpm at 37°C.

2.4 Flow Cytometry

For analysis of adhesion molecule expression levels, cells of interest were treated with murine anti-human CD marker primary antibodies (anti-ICAM-1 or anti-VCAM-1; 1µg Ab/10^6 cells) (CalTag Laboratories) for 1 hour at 4ºC. Cells were then treated with
secondary antibody: Alexa Fluor 546 goat anti-mouse IgG (Invitrogen) for 30 minutes. Cells were then analyzed using the Guava Personal Cell Analyzer (Guava Corp.) Appropriate IgG controls were used to determine background fluorescence. A positive control was established by incubating HUVEC in 25ng/ml TNF-α (Invitrogen) for 24 hours in serum-free media. The fold increase in expression was determined by dividing the geometric mean fluorescence of the cocultured endothelial cells by the geometric mean fluorescence of unstimulated endothelial cells. Hence, endothelial cells expressing twice as much ICAM-1 as unstimulated endothelial cells would have a fold increase in expression of 2.

### 2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Following certain cocultures, coculture supernatant was collected and centrifuged at 1500rpm for 5 minutes. The upper layer of the centrifuged supernatant was removed and stored at -20°C until ELISA was to be performed. ELISA detection of supernatant protein concentrations was performed at the Penn State General Clinical Research Center. 50μl of 2μg/ml mouse anti-human capture antibody specific to the cytokine of interest was added to each well of a 96-well ELISA plate and incubated at 4°C overnight. After washing the plate 4 times with 20% Tween 20 in phosphate-buffered saline (PBST) [pH 7.0], the plate was blocked with PBS containing 1% bovine serum albumin (BSA) and incubated at room temperature for 2 hours. Following __ washes with PBST, 100μl of samples or target cytokine standards were added to each well and incubated at 4°C overnight. The plate was then washed 4 times with PBST and 100μl of 0.2μg/ml biotinylated affinity purified goat anti-human polyclonal detection antibody was added to
each well and the plate was incubated for 2 hours at room temperature. After 6 washes with PBST, the 10μl of streptavidin peroxidase (1μg/ml, Sigma Chemical Co.) was added to each well and incubated for 30 minutes at room temperature. 100μl of 100 μl of 2,2’-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma Chemical Co.)/peroxide substrate solution was then added. After incubation for 1 hour at 4°C in the dark, the plate was read using a microtiter plat reader (Packard, Downers Grove, IL) at a wavelength of 405nm.
Chapter 3

RESULTS AND DISCUSSION

3.1 Results and Discussion

This study looks to characterize the effects of the tumor microenvironment on the endothelium. Interactions between tumor cells in circulation and vascular endothelial cells play an important role in the metastatic cascade and can be critical to the successful spread of tumor cells from the primary tumor to secondary tumor sites. In particular, interactions between these cell types are very important to the rolling and subsequent firm adhesion of tumor cells to the endothelium while in circulation. These interactions occur through two main mechanisms: receptor-ligand interactions via direct cell contact and exchange of soluble factors (cytokines) secreted by both cell types (Christofori et al., 1993). This study evaluates the effects of both of these types of interactions in their effects on endothelial cellular adhesion molecule expression. In particular, ICAM-1 was evaluated due to the crucial role it plays in cell-cell and cell-matrix adhesion (Roland et al., 2007). Since PMNs have been shown to aid tumor cells in firm adhesion to the endothelium and β2-Integrins bind to ICAM-1 on the endothelium, any change in endothelial ICAM-1 caused by the tumor microenvironment could play an important role in PMN-mediated tumor cell adhesion and subsequent extravasation (Slattery et al., 2005).

Through the development of a special protocol, direct cell-to-cell contact between tumor cells and endothelial cells was established through porous membrane inserts. This technique allowed for the specific analysis of endothelial cells separate from tumor cells.
In particular, the expression levels of cellular adhesion molecules on endothelial cells after contact with tumor cells could be quantified. To characterize these effects, two melanoma cell lines (WM35 and 1205Lu) were cocultured with HUVEC under various conditions. The use of these cell lines allowed for the characterization of the difference in effects caused by cells with high (1205Lu) and low (WM35) metastatic potential as reported in literature (Table 3.1, Peng et al., 2007). To evaluate effects of direct cell-to-cell contact between HUVEC and melanoma cells, cells were seeded on either side of a porous membrane. This orientation allowed for the establishment of direct contact between cell types while still keeping cells separated for analysis. HUVEC cultured in this contact coculture setup with either 1205Lu or WM35 showed a significant increase in endothelial expression following a 4 hour coculture as compared to unstimulated HUVEC (Figure 3.1). The increase in ICAM-1 expression following contact coculture with 1205Lu showed a dramatically higher increase in expression compared to coculture with WM35. Contact coculture for 0.5 hours resulted in no significant change in expression levels for both melanoma cell types.

Table 3.1 Metastatic potentials of melanoma cell lines used in this study.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Metastatic</th>
<th>Chemotactic (CIV)</th>
<th>Adhesion (ICAM-1)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1205Lu</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>Sharma et al., 2006</td>
</tr>
<tr>
<td>WM35</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Wistar Institute</td>
</tr>
</tbody>
</table>
Figure 3.1 ICAM-1 expression change following contact coculture. WM35 and 1205Lu melanoma cell lines were cocultured with HUVEC on either side of a porous membrane for 0.5 or 4 hours after which ICAM-1 expression levels were analyzed via flow cytometry. ICAM-1 expression rose significantly following 4 hours for both melanoma cell types but not after 0.5 hours. * P < 0.05 compared with unstimulated HUVEC case. Results are presented with mean ± SEM for n=3.

As a comparison to the contact coculture assay, HUVEC and melanoma cells were cocultured while separated by a Transwell insert. The Transwell insert allows for exchange of media and soluble factors between cell types, but in this case no cell-to-cell contact occurs. Resulting ICAM-1 expression levels following 4 hour coculture showed
no significant change compared to unstimulated HUVEC. In addition to Transwell cocultures, effects of soluble factors were also evaluated through coculture of HUVEC with TCM. Similar to the Transwell cocultures, coculture with TCM for 4 hours showed no significant change in ICAM-1 expression (Figure 3.2). However, coculture of HUVEC with TCM for 24 hours resulted in a significant increase in ICAM-1 expression along with changes in the expression levels of other cellular adhesion molecules, in particular CXCR1 and CXCR2 (Figure 3.3, Courtesy of Shile Liang). It has been well established that cytokines can affect endothelial cellular adhesion molecule expression levels. For example, cellular adhesion molecules such as E-Selectin and VCAM-1 require the presence of inflammatory cytokines (TNF-α, IL-1β, etc.) in order to be upregulated (Scholz et al., 1996).
Figure 3.2 ICAM-1 expression following Transwell or contact coculture. HUVEC were cocultured with 1205Lu cells for 4 hours either separated by a Transwell insert or on either side of a porous membrane. HUVEC were also cocultured TCM for 4 hours after which ICAM-1 expression levels were analyzed via flow cytometry. ICAM-1 expression changed significantly following contact coculture but not following coculture in Transwell or with TCM. TNF-α is reported as a positive control. * P < 0.05 compared with unstimulated HUVEC case. Results are presented with mean ± SEM for n = 3.
Figure 3.3 Changes in adhesion molecule expression following TCM treatment.

Confluent endothelial cell monolayers were cultured with TCM for 4 or 24 hours. Following treatment, expression levels of targeted adhesion molecules were evaluated via ELISA. * P < 0.05 compared with the respective unstimulated case. Results are presented as mean ± SEM for n ≥ 3 (Courtesy of Shile Liang).

Even though cytokines have been shown to have an effect on ICAM-1 expression after 24 hours, the fact that no change in expression occurred after 4 hours points to the significance of contact in this upregulation. There are 2 possible hypotheses that come from this evaluation: either contact between cells directly induces ICAM-1 expression or
causes increased release of cytokines which then induce ICAM-1. In investigation of this second hypothesis, enzyme-linked immunosorbent assays (ELISA) were run to quantitatively analyze the concentrations of various cytokines (IL-6, IL-8, IL-1α, IL-1β, TNF-α, MCP-1, and GRO-α) following both Transwell and contact cocultures. Concentrations were deemed significant increases if the concentration of a given cytokine following coculture was significantly greater than the sum of the constitutive levels secreted by unstimulated HUVEC and by melanoma cells. Of the cytokines tested, only IL-6, IL-8 and Gro-alpha showed significant changes in secretion following coculture of HUVEC with 1205Lu, and these increases were seen after both Transwell and contact cocultures (Figure 3.4). However, contact coculture resulted in significantly higher levels of secretion than Transwell cocultures. Coculture between WM35 and HUVEC (either Transwell or contact) showed no significant changes in the concentrations of these factors, and the levels were far lower than those from HUVEC/1205Lu cocultures. Resulting concentrations of IL-1α, IL-1β, TNF-α, and MCP-1 were low in all samples and coculture (either Transwell or contact) did not induce any changes (Data not shown).
**Figure 3.4 Concentrations of cytokines following coculture.** WM35 and 1205Lu melanoma cell lines were cocultured with HUVEC for 4 hours either separated by a Transwell insert or on either side of a porous membrane and ELISAs were run on the coculture supernatant. A, IL-8; B, IL-6; C, Gro-α. * P < 0.05 compared with sum of HUVEC and respective melanoma cell line concentrations. Results are presented with mean ± SEM for n = 3.

The increases in cytokine concentrations in coculture show that soluble factors do play a role in the interaction of melanoma cells and HUVEC. However, it does not prove that increase in ICAM-1 after coculture is a direct result of these elevated cytokine levels. To further examine this possibility, cytokine concentrations resulting from coculture were compared to concentrations of the same molecules present in TCM (Table 3.2, TCM data courtesy of Tara Yunkunis). Comparison shows the concentrations present in TCM are far greater than those found following 4 hour contact coculture with 1205Lu (the case resulting in the highest level of cytokine production). Seeing as it has been shown that coculture with TCM for 4 hours does not result in increased ICAM-1 expression, it is very unlikely that the cytokine levels seen in coculture (far less than those seen in TCM), could have caused the observed ICAM-1 increase. Additionally, the fact that contact coculture between HUVEC and WM35 elicited no change in cytokines but still caused a change in ICAM-1 expression further points to cytokines not playing a significant role in the expression change.
Table 3.2 Cytokine Concentrations in TCM and Coculture Supernatant

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>24hr TCM 1205Lu (ng/ml)</th>
<th>4hr Contact Coculture Supernatant 1205Lu (ng/ml)</th>
</tr>
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<tr>
<td>IL-8</td>
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<tr>
<td>IL-6</td>
<td>10</td>
<td>0.65</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.081</td>
<td>&lt;0.008</td>
</tr>
<tr>
<td>Gro-α</td>
<td>8.3</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Results of the cytokine studies point to direct cell-to-cell contact as the likely cause of the increased ICAM-1 levels seen following contact coculture. Many endothelial cellular adhesion molecules have been implicated in cancer metastasis, and in particular, interactions with tumor cells. Integrin VLA-4 (α4β1, CD49d/CD29), a molecule known to be expressed on many melanoma cell lines, has been shown to bind to vascular adhesion molecule-1 (VCAM-1) on endothelial cells. In addition, P-selectin and E-selectin have been reported as mediators of interactions between melanoma and endothelial cells, in particular in the capture of circulating tumor cells by the endothelium. Recently, CD44 has been shown to be a potential selectin ligand capable of mediating tumor cell rolling and adhesion to the endothelium (Konstantopoulos and Thomas, 2009).

To examine the potential link between receptor-ligand activation due to contact and ICAM-1 upregulation, antibody cross-linking was used to activate VCAM-1, E-Selectin, and/or P-Selectin. Cross-linking makes use of F(ab)2 fragments in addition to CAM specific primary antibody in order to mimic the clustering of CAM ligands. This will result in the clustering of CAMs simulating ligand-receptor bond formation (Van Wetering et al., 2003). Activation of VCAM-1, E-Selectin, or P-Selectin alone resulted in
no significant change in ICAM-1 expression (Figure 3.5). However, cross-linking of these molecules in tandem resulted in a significant increase in ICAM-1 expression following cross-linking of both E- and P-Selectin (Figure 3.6). These results point to the upregulation of ICAM-1 being caused by the synergistic activation of E-Selectin and P-Selectin.

**Figure 3.5 ICAM-1 expression following individual CAM cross-linking.** HUVEC were incubated in 100ng/ml of both goat anti-mouse-Ig F(ab)2 fragments (Jackson ImmunoResearch, Baltimore, MD) and mouse-anti-human antibody specific to the cellular adhesion molecule of interest for 1 hour. Cells were then cultured for 4 hours in serum-free media after which ICAM-1 expression levels were analyzed via flow
cytometry. TNF-α is reported as a positive control. * P < 0.05 compared with unstimulated HUVEC case. Results are presented with mean ± SEM for n = 3.

**Figure 3.6 ICAM-1 expression following tandem CAM cross-linking.** HUVEC were incubated in 100ng/ml of goat anti-mouse-Ig F(ab)₂ fragments (Jackson ImmunoResearch, Baltimore, MD) and 50ng/ml mouse-anti-human antibody specific to the each cellular adhesion molecule of interest for 1 hour. Cells were then cultured for 4 hours in serum-free media after which ICAM-1 expression levels were analyzed via flow cytometry. TNF-α is reported as a positive control. * P < 0.05 compared with both unstimulated HUVEC and F(ab)₂ control cases. Results are presented with mean ± SEM for n = 3.
Both E- and P-Selectin have been shown to bind to ligands on a variety of tumor cells, particularly during the rolling phase of tumor cell extravasation (Konstantopoulos and Thomas, 2009). The upregulation of ICAM-1, particularly if caused by rolling of melanoma cells via selectins, would be important to the successful adhesion and extravasation of melanoma out of circulation and into tissue. Since PMNs bind to the endothelium via endothelial ICAM-1, the upregulation of this molecule could result in increased attraction of PMNs and potential endothelial adhesion of PMN-melanoma cell aggregates. The activation of endothelial cells by melanoma cells via contact would then be an important component of the adhesion/extravasation process. In addition, cytokines, particularly IL-8, have been shown to play important roles in the attraction of PMN and PMN-mediated melanoma cell extravasation (Peng et al., 2007). Based off this, the increased secretion of cytokines resulting from contact between HUVEC and melanoma cells could also be important, not in ICAM-1 activation, but in the attraction of PMN to the activated endothelium.
Chapter 4

CONCLUSIONS

4.1 Further Directions

While this study has identified direct cell-to-cell contact between melanoma cells and HUVEC as the cause of upregulated endothelial ICAM-1, the actual mechanism has not yet been determined. Though receptor-ligand binding and activation through endothelial cellular adhesion molecules VCAM-1, E-Selectin, and P-Selectin have given some picture of what may be occurring, the results do not completely prove that these molecules are the ones involved in the upregulation of ICAM-1. The simplest potential method for potentially evaluating the effects of these endothelial cellular adhesion molecules would involve the blocking of these molecules or their respective melanoma cell ligands and run the same contact coculture assays. Such blocking, via blocking antibodies or siRNA knockout, would prevent binding and activation through these receptor-ligand pairs. By blocking individual cellular adhesion molecules on melanoma or endothelial cells in coculture and evaluating the resulting ICAM-1 expression, the direct effects of these molecules can be determined. The results of these experiments could point to other direction regarding further research.

One other area worth investigating would be the potential differences in response of other endothelial cell types to similar coculture procedures. Since melanoma is known to preferentially metastasize to lungs and lymph nodes, testing endothelial cells from these organs or tissues as well as from other areas of the body in similar fashion as in this study could provide valuable information in regards to melanoma’s preferential
metastasis pattern. If there were found to be specific differences in ICAM-1 or other cellular adhesion molecule expression levels, it could shed some light onto how and why melanoma travel to these sites preferentially and potentially provide basis for treatment strategies.

### 4.2 Conclusions

This study has shown the importance of direct contact between HUVEC and melanoma cells in the upregulation of endothelial ICAM-1 expression. ICAM-1 on HUVEC was shown to increase only following contact coculture with melanoma cells. Cross-linking of certain CAMs gave further evidence for this receptor-ligand effect seen in contact between cell types with results pointing towards P- and E-Selectin as likely endothelial receptors involved in the increased expression of ICAM-1. Melanoma cell secreted cytokines showed no effect on ICAM-1 over a 4 hour time period although it has been shown previously that these cytokines can have an effect over a longer period of time. Additionally, concentrations of certain cytokines were shown to increase significantly following HUVEC/melanoma cell coculture, pointing to a potential role of these molecules in the overall metastatic process. While the exact mechanism behind this ICAM-1 increase has not yet been fully established, experiments involving the blocking of specific melanoma CAMs could provide sufficient information to identify the actual method behind this contact-derived ICAM-1 increase.
References


Appendix

CONTACT COCULTURE PROTOCOL

Items Needed:
- Transwell inserts (24mm diameter) – 0.4µm or 3µm (preferably 3µm for max contact and polycarbonate membrane for best visualization)
- 2 cell types (for example: tumor cells and endothelial cells)
- 2 standard Petri dishes (do not have to be tissue culture treated) for every 3 Transwell Inserts to be used
- 6 well plates

1) Bottom Layer Preparation
- Use the bottoms of 2 Petri dishes to make a larger chamber by taping the dishes together (see diagram)

![Figure A.1: Assembly of Coculture Chamber](image)

- Place Transwell inserts upside-down (membrane facing up) in chamber (make sure inserts are level, up to 3 inserts can fit in one chamber, see Figure 2)
Follow regular cell culture protocol to lift cells that will be placed on bottom of membrane.

Note: It is best that the cell type to be tested be located on the bottom side of the membrane.

- Transfer lifted cells to centrifuge tube.
- Spin down cells at 1500rpm for 5min.
- Resuspend cells to a concentration of 5e5 cells per ml.
- Add ~500μL of suspended cell solution to upturned Transwell membrane. Repeat for each insert being used. (See Figure 3)

**Caution:** Do not add too much solution otherwise media will leak down sides of Transwell resulting in an insert that cannot be used. Add solution slowly and make sure to create a bead of solution that does not reach the edges of the membrane.)
• Close and secure chamber with tape and carefully place in incubator.
• Incubate for 1.5-2 hours allowing cells to settle on membrane.
• After incubation, carefully aspirate most of the media off of membrane (do not touch the membrane).
• Place insert right-side-up in a well of a 6-well plate containing 2ml of regular cell culture media.

2) Top Layer Preparation
• Follow regular cell culture protocol to lift cells that will be placed on top of membrane.
• Transfer lifted cells to centrifuge tube.
• Spin down cells at 1500rpm for 5min.
• Resuspend cells to a concentration of 2.5e5 cells per ml.
• Add ~500-750µL of suspended cell solution to Transwell chamber (top of membrane).
• Add enough culture media so that top chamber contains 2ml total.
  Note: Cell specific media should be used for the top and bottom sides of the membrane. For example, if HUVEC is being grown on the bottom side, F12K media should be used in the actual well, but if WM35 cells are being grown on the top side, RPMI media should be placed in the top chamber.
• Place plate in incubator and allow cells to culture together until confluent (approximately 12hrs).

3) Coculture Procedure
• Prepare coculture media
  o For tumor cells: tumor cell specific media (DMEM, RPMI, etc.) with 1% fetal bovine serum
  o For endothelial cells: cell specific media (e.g. F12K) with 1% fetal bovine serum and no added nutrients
• Aspirate out all media
• Remove Transwell
• Add 2ml of bottom side cell specific coculture media to well
• Replace Transwell
• Add 2ml of top side cell specific coculture media to top chamber of Transwell
• Place plate in incubator for the length of the coculture

4) Harvesting of Bottom Cells
• At the end of the coculture period, aspirate out all media
• Add ~2ml DPBS to well and allow Transwell to soak for 1-2 minutes
• Aspirate out DPBS
• Lift out Transwell, add 1.5ml of trypsin to well, and replace Transwell
• After ~2 minutes, tap plate firmly and observe well under microscope, cells should be visible floating freely.
- Every 30 seconds, tap plate firmly and observe cells. Repeat until tapping does not result in an increase in visibly floating cells (no more than 6 minutes after trypsin is added).
- Immediately remove Transwell and add 8ml cell culture media with 10% FBS (specific to bottom side cell type).
- Dip Transwell up and down in well, but do not allow media to leak over the sides into top chamber of Transwell.
- Pipette out resulting media/cell suspension from well into a centrifuge tube.
- Spin down cells at 1500rpm for 5min.
- Aspirate out media, resuspend in 4ml of cell culture media with 10% FBS.
- Allow cells to recover for 30-45 minutes while being rocked at 37°C.
- From this point, follow normal protocol for procedure to be performed (e.g. staining for flow cytometry).

**Alternate Protocol for 2) Top Layer Preparation**

Allowing cells to incubate together and reach confluency is the most effective way for performing this coculture. However, if altering one of the cell types (e.g. blocking of adhesion molecule), the protocol must be altered. This altered protocol will describe how to proceed if blocking an adhesion molecule on the cells on the top membrane, but this protocol can be altered to fit specific aims.

Note: only Step 2 is different, follow all other steps of the protocol as depicted above.

2) Top Layer Preparation (Alternate)

- Follow regular cell culture protocol to lift cells that will be placed on top of membrane.
- Transfer lifted cells to centrifuge tube.
- Spin down cells at 1500rpm for 5min.
- Resuspend cells in DPBS to a concentration of 5e5 cells per ml.
- For each coculture well to be run, transfer 1ml of cell suspension to an Eppendorf tube. Fill one extra tube to be used later.
- Spin down cells at 1500rpm for 5min.
- Resuspend cells in 500µl DPBS + 1% CS/GS.
- Add blocking antibody to suspension to each tube. No antibody needs to be added to extra tube.
- Incubate at 4°C for 1hr.
- Spin down cells at 1500rpm for 5min.
- Resuspend cells in 1mL cell culture media
- Add resulting cell suspension to top well of prepared coculture Transwell inserts.
- For extra tube, add cell suspension to one well of a 6 well plate. Observe cell suspension under microscope.
- Incubate plates in incubator.
• **Important:** Check extra cell plate every 20 to 30 minutes and observe what proportion of cells are still freely floating in media. When ~90% of cells have adhered to the bottom of the well (no longer floating around in solution), proceed immediately to Step 3 of Contact Coculture Protocol.
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