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Alphav Beta6 Integrin Expression in Inflamed & Malignant Prostate

Melissa Christine Hackett
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αvβ6 Integrin Expression in Inflamed & Malignant Prostate

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

Melissa Coonradt

April 27, 2006

APPROVED:

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ABSTRACT

Prostate cancer cell functions are regulated by elaborate signaling pathways activated by extracellular stimuli. The integrin family of cell surface receptors is known to activate intracellular pathways in response to signals generated via specific interactions with the extracellular matrix. Through the use of immunohistochemistry (IHC) and immunoblotting, this study shows that the $\alpha_v\beta_6$ integrin is predominately expressed in epithelial cells in human preneoplastic, prostate intraepithelial neoplasia (PIN), proliferative inflammatory atrophy lesions (PIA) and prostatic adenocarcinoma. The results indicate that $\alpha_v\beta_6$ is highly expressed in areas with infiltrating leukocytes, and is expressed focally in malignant and some benign prostate glands, but not in normal prostate tissue.
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BACKGROUND

Prostate Cancer

With the exception of skin cancer, prostate cancer is the most commonly diagnosed form of cancer in American men. It is estimated that 1 in 6 men will be diagnosed with this form of cancer at some point during his lifetime, although with early detection and treatment only 1 in 34 will die from it (http://www.cancer.org/). The American Cancer Society estimates that 232,090 men will be diagnosed with prostate cancer in 2005 alone. Despite treatment options and the benefits of early detection, prostate cancer is exceeded only by lung cancer as the leading cause of death among men in the United States, accounting for nearly 10% of all cancer-related deaths in men. In 2005 it was estimated that more than 30,000 would die from prostate cancer. The number of prostate cancer-related deaths remains high, but modern methods for detection and treatment have led to more effective therapy and thus a decline in death rate of nearly 3.5% per year (http://www.cancer.org/).

After a patient has been diagnosed and the tumor is staged according to a Gleason Score (Gleason, 1966) there are several options available for treatment: expectant management, surgery to remove the entire prostate and some surrounding tissue, radiation therapy, cryosurgery, androgen deprivation therapy, chemotherapy, treatment only for pain and other symptoms, or clinical trials (http://www.nccn.org). A successful therapeutic approach has not been identified making it likely that the cancer will metastasize to another area of the body. Ideally, a patient would have several options for treatment, each promising a future without ongoing tumor therapies. Doctors and
researchers are working to improve detection, diagnosis and treatment for prostate cancer, while exploring ways to prevent a tumor from ever occurring. Over the years many helpful discoveries have been made in this field, though there is still much that remains to be understood about how prostate cancer works before it can be cured or prevented.

Despite many theories, scientists have not pinpointed the exact cause of prostate cancer. Researchers have identified some risk factors, including lifestyle and diet. Several studies have indicated that a diet rich in animal fat can increase a man’s chances of developing prostate cancer (Bairati et al., 1998). It has also been hypothesized that high levels of testosterone may lead to an increase in a man’s risk of prostate cancer (Gann et al., 1996).

Prostate cancer development proceeds through a series of states that can be clearly defined. Of those marked states of progression, some of the most clearly defined are prostatic intraepithelial neoplasia (PIN), invasive cancer, and androgen-dependent or androgen-independent metastases (Scher and Heller, 2000; Nicholson and Theodorescu, 2003). Accumulating data suggest that an inflammation-carcinoma sequence has been invoked as a potential mechanism with regard to prostate carcinogenesis (Boudreau et al., 1995; Ruoslahti, 1997; Varner and Cheresh, 1996; Bogenrieder and Herlyn, 2003, Fornaro et al., 2001). Available data are consistent with the view that proliferative inflammatory atrophy (PIA), which shows a low apoptotic rate, may represent a precursor of high-grade PIN or prostate cancer (DeMarzo et al., 1999).
**Integrin and Integrin Functions**

Integrins are a group of heterodimeric transmembrane glycoproteins responsible for cell to cell interactions, as well as cell to extracellular matrix (ECM) interactions, and the modulation of a variety of cellular functions (Hynes, 2002). Their function is regulated at many levels, though the ligand specificity of a given integrin depends on which $\alpha$-chain associates with which $\beta$-chain (Figure 1). All human cells express one or more of the integrin heterodimers, with the exception of erythrocytes (Hynes, 1992). Integrins are important for the alteration of cellular growth and tumor progression through the regulation of apoptosis, cell adhesion, proliferation, gene expression and migration (Breuss et al., 1995). The variety of integrin functions is also what allows these cation-dependent receptors to control events that lead to malignant tumor growth (Fornaro et al., 2001).

Each is made up of one $\alpha$ subunit non-covalently bound to a $\beta$ subunit (Busk et al., 1992). Every integrin must have both subunits to be expressed on the cell surface. Without either than alpha or beta chain, the single subunit will be degraded before

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**Figure 1. Integrin Structure** (http://www.aw-bc.com/)

![Integrin Structure Diagram](http://www.aw-bc.com/)

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reaching the surface. To date, 18 α subunits and 8 β subunits have been found, for a total of 24 complexes that have been identified, with expression and function being characterized in a variety of cell types (Fornaro et al., 2001). In prostate cancer, tumor cells have a different surrounding matrix than normal cells; thus changes in the integrin profile may be functionally relevant and contribute to metastases establishment and growth (Bogenreider and Herlyn, 2003; Fornaro et al., 2001). A number of studies have reported changes in integrin expression profile as prostate cancer progresses to an advanced stage (Knox et al., 1994; Murant et al., 1997). This project focuses on the integrin αvβ6, a member of the integrin family of transmembrane protein receptors which mediate attachment of cells to the ECM through the binding of ligands such as latency-associated peptide (LAP) of transforming growth factor-β (TGF-β) (Munger et al., 1999), fibronectin (Busk et al. 1992), tenasin (Prieto et al., 1993) and vitronectin (Huang et al., 1998). αvβ6 is detectable exclusively in epithelial cells and observed primarily during embryonic development. In fully differentiated epithelia αvβ6 is barely detectable, but is strongly induced during wound healing, inflammation and tumorigenesis (Breuss et al., 1995). αvβ6 is frequently up-regulated in epithelial tumors. Research has shown the up-regulation of αvβ6 in lung (Smythe et al., 1995), breast (Arihiro et al., 2000), colon (Agrez et al., 1994), colorectal (Bates, 2005), oral (Regezi et al., 2002), ovarian (Ahmed et al., 2002) and pancreatic cancer (Sipos et al., 2004). A connection has also been identified between αvβ6 and metastasis in oral squamous cell carcinoma (Busk et al., 1992; Breuss et al., 1995; Jones et al., 1997) and ovarian cancer (Ahmed et al., 2002). Despite expression of αvβ6 in these forms of cancer, it is still not entirely understood how αvβ6 may influence tumor formation and metastasis.
**Integrins in Wound Healing**

Wound repair is one of the most important aspects of skin maintenance, involving complex interactions between the epidermis, dermis and immune cells (Hakkinen et al., 2004). To prevent infection, a wound must be covered as quickly as possible. This process, known as re-epithelialization, involves epidermal cells from the area immediately surrounding a wound. Once in place, epidermal cells can begin dividing and proliferating to cover the injured area. Integrins are an important factor in this process, as they facilitate the migration of epidermal cells, and contribute to cell-to-ECM or cell-to-cell interactions (Hakkinen et al., 2000). Integrins are able to affect cell motility because of their affinity for a given ligand, such as fibronectin and laminin. An integrin’s affinity for a ligand is not particularly strong, which means that several integrins must be localized at a focal contact in order to form an effective cell-to-cell or cell-to-ECM contact. An integrin expressed equally over the entire cell surface or a region of the cell surface will not attract its ligand (Cohen et al., 2004). Human epidermal cells have the ability to synthesize eight integrins capable of mediating cellular responses to ECM molecules (Larjava et al., 1996). For example, the integrin α6β4 is seen in epidermal cells that are bound to the basement membrane, though it facilitates cell migration after injury when distributed over the entire cell surface, as opposed to being focally expressed on the lower region of epidermal cells (Stepp et al., 1990). In addition to the normally expressed integrins, there are integrins that only appear during wound repair. For example, integrin α5β1 can be observed a short time after wounding and lasts only a few hours before it can no longer be detected at the wound site (Larjava
et al., 1993). Finally, as epidermal cells undergo re-epithelialization, the basement membrane is formed and the fusion of newly formed epidermal sheets is associated with higher levels of the integrin \( \alpha_v\beta_6 \). The molecule is a binding partner of some wound matrix molecules, although it is not involved with epidermal cell migration, as can be demonstrated by the fact that it appears in the later stages of wound repair after cell migration has stopped (Koivisto et al., 1999).

### Inflammation and Cancer

It has long been accepted that chronic inflammation can lead to some types of cancer. Most commonly, chronic inflammation of the gastrointestinal tract is associated with colon and / or colorectal cancer (Dalgleish and O’Byrne, 2006). In 1863 Rudolf Virchow first noted leukocytes in neoplastic tissue, making a

connection between inflammation and cancer. He hypothesized that the origin of cancer was at the site of chronic inflammation, and that some irritants, when combined with tissue injury and the resulting inflammation would result in the enhancement of cell proliferation (Coussens and Werb, 2002). Current research supports Virchow’s findings, as scientists seek to further understand the inflammatory microenvironment of malignant tissues

<table>
<thead>
<tr>
<th>Malignancy</th>
<th>Inflammatory stimulus / condition</th>
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<tr>
<td>Bladder</td>
<td>Schistosomiasis</td>
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<tr>
<td>Cervical</td>
<td>Papillomavirus</td>
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<tr>
<td>Ovarian</td>
<td>Pelvic Inflammatory Disease / talc / tissue remodeling</td>
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<tr>
<td>Gastric</td>
<td><em>H. pylori</em> induced gastritis</td>
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<td>MALT lymphoma</td>
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<td>Oesophageal</td>
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<td>Hepatocellular</td>
<td>Hepatitis virus (B and C)</td>
</tr>
<tr>
<td>Bronchial</td>
<td>Silica, asbestos, cigarette smoke</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>Asbestos</td>
</tr>
<tr>
<td>Kaposi’s sarcoma</td>
<td>Human herpesvirus type 8</td>
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</tbody>
</table>
(Balkwill and Mantovani, 2001). Above is a table taken from Balkwill and Mantovani’s review article titled “Inflammation and cancer: back to Virchow?” The authors cite an article which suggests that 15% of the global cancer burden can be attributed to infectious agents, of which inflammation is a major component. Balkwill and Mantovani also suggest that an increased risk of malignancy can be associated with chronic inflammation as a result of chemical and physical agents as well as autoimmune disorders and inflammatory reactions of uncertain etiology.

In order to understand how inflammation can lead to the formation of cancer, one must understand how inflammation functions and its contribution to both physiological and pathological processes such as wound healing and infection. In addition to the section above involving wound healing and integrins, there is the immune aspect of wound repair, which involves the activation and directed migration of leukocytes such as neutrophils, monocytes and eosinophils from the circulatory system to the wounded area. In a review article titled “Inflammation and Cancer” (2002) by Coussens and Werb, the authors explain how inflammatory cells are recruited to a wound where they form a provisional ECM, creating a base for fibroblasts and endothelial cells to proliferate, thus restoring the normal microenvironment of the skin.

In addition to the mechanism for recruitment of inflammatory cells is a family of chemotactic cytokines known as chemokines. Chemokines are able to attract specific leukocyte populations and have the ability to control the inflammatory response. Tumor necrosis factor-α and TGF-β1 are two proinflammatory cytokines that have the ability to control inflammatory cell populations and mediate other aspects of inflammation (Coussens and Werb, 2002). Generally speaking, normal inflammation is self-limiting,
that is, anti-inflammatory cytokines are active shortly after pro-inflammatory cytokines, preventing the continued recruitment of inflammatory cells to the site of a wound. Any disruption in this process can result in abnormalities and eventually pathogenesis.

Although there is evidence showing inflammation contributing to several types of cancer, as seen in Table 1, there is no evidence supporting a role for inflammation in prostate cancer progression. It has been hypothesized that PIA contributes to progression from preneoplastic to neoplastic phenotype in prostate, as shown in Figure 2.

![Figure 2. Progression of normal prostate epithelium into cancer when influence by inflammatory cells.](image)
PROJECT PURPOSE

For this project one specific cell surface integrin receptor, αvβ6, was examined to confirm its presence in malignant prostate tissue, as well as to explore the molecule’s possible function as a regulator mediating the prostate cancer progression. Results obtained through immunoblotting and immunohistochemistry show that αvβ6 is predominantly expressed in PIN, PIA, and adenocarcinoma in human prostate cancer tissue.
Antibodies

The following antibodies were used in this project: anti-human β6 integrin, 2A1 (Biogen Idec, Inc., Cambridge, MA) for immunohistochemical (IHC) staining, purified anti-mouse IgG (mIgG) or anti-rabbit IgG from Pierce (Rockford, IL); anti-Akt from Cell Signaling (Beverly, MA); and anti-β6 integrin (B1) (a gift from Dr. Dean Sheppard, UCSF, San Francisco, CA).

Cells and Culture Conditions

RWPE-1 and LNCaP cells were purchased from ATCC. LNCaP stable cell lines expressing full-length β6 integrin. RWPE-1 cells were cultured in a defined keratinocyte serum free medium supplemented with pituitary extracts, 100 units/ml penicillin and 100 µg/ml streptomycin (all from GIBCO, Invitrogen, Grand Island, NY). LNCaP cells were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), 100 units/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate and 1 mM HEPES (all from Invitrogen). Transfected LNCaP cells were cultured in the same medium as LNCaP cells with 1 mg/ml G418 (Invitrogen).

Human Tissue Specimens

Specimens from 48 human radical prostatectomies performed for prostatic adenocarcinoma from the Cooperative Human Tissue Network (CHTN) (Vanderbilt
University, Nashville, TN) and the Department of Pathology, University of Massachusetts Medical School (Worcester, MA) were processed according to Review Board – approved protocols. Tissues were fixed in neutral-buffered formalin and embedded in paraffin. Hematoxylin and eosin sections were reviewed, and the tumor grade, according to Gleason’s criteria (Gleason, 1966) and the stage of tumor, according to TNM system (Sobin, 1997) were estimated in each tumor sample.

**Immunoblotting**

Cells were lysed in lysis buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 10 mM NaF, 1 mM NaVO4, 1 mM Na4O7P2, 2 µM leupeptin, 2 µM aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were cleared by centrifugation at 14,000 rpm for 15 minutes at 4°C, and total protein was quantified using a BCA Protein Assay (Pierce Biotechnology, Inc, Rockford, IL). Total protein was resolved by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with pAb to Akt (0.1 mg/ml), mAb to β6 integrin (1:10). Proteins were visualized using ECL reagent and developed using autoradiography (Kodak X-Omat AR film). To determine Akt activation in β6 LNCaP cells, cells were serum starved for 12 hr and plated on mIgG (2.5 µg/ml), β6 integrin antibody 10D5 (2.5 µg/ml), BSA (1%), or LAP-TGF-β (0.5 µg/ml) coated 60 mm plates at the density of 1 X 10⁶ cells per plate. After incubation for 30 min or 1 hr, cells were lysed in a lysis buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 10 mM NaF, 1 mM NaVO4, 1 mM Na4O7P2, 2 µM leupeptin, 2 µM aprotinin, and 1 mM phenylmethylsulfonyl fluoride
(PMSF). Proteins (50 µg) were separated by 10% SDS-PAGE under reducing conditions and immunoblotted with an Ab specific to Akt as a loading control.

**Immunohistochemistry (IHC)**

All IHC staining was performed on 4 µm sections prepared from paraffin-embedded blocks and placed on charged glass slides. The sections were first deparaffinized in two changes of xylenes, and then rehydrated in ethanol and distilled water. For β6 integrin staining, the sections were incubated in 3% hydrogen peroxide (H₂O₂) in methanol to remove endogenous peroxidase activity. Antigen retrieval was performed by incubating the sections with pepsin at 37°C for 5 min. Following two washes in PBS, the slides were blocked for biotin activity with Avidin/Biotin Blocking Kit (Vector) at room temperature. After rinsing, the sections were blocked with 0.25% casein (SP 5020, Vector Laboratories) in PBS for 15 min at room temperature and then incubated with primary antibody overnight at 4°C. Human prostate sections were stained with 0.5 µg/mL mouse mAb 2A1 (Biogen Idec, Inc., Cambridge, MA). A mouse-IgG, 0.5 µg/ml in 1% BSA in PBS, was substituted for the antibody as negative control. After washing with PBS, the biotinylated secondary IgG antibody was applied for 30 min at room temperature. Immunoperoxidase staining was performed using the Vectastatin Elite ABC kit (6200 Vector kit for human prostate tissue; Vector Laboratories, Inc., Burlingame, CA). The signal was amplified using DAB Substrate-Chromogen System (K0367, Dako Cytomation). Finally, the slides were counterstained with Mayer’s hematoxylin and dehydrated. Tissue sections were examined on an Olympus BX41 microscope and photographed using an Olympus DP12 camera. The immunostaining results were independently evaluated by Dr. Z. Jiang, Dr. L.R. Languino, and Dr. J.Li
(University of Massachusetts Medical School Departments of Pathology and Cancer Biology) and scored in two classes by percentage (positive “+” and negative “-“ immunoreactivity) of glands over the total glands of tissue.
RESULTS & ANALYSIS

Immunoblotting Analysis of β6 Expression in Human Tissue Lysates

Immunoblotting analysis of β6 subunit expression in benign and tumor prostate tissue as well as RWPE cell lysate and non-tumorigenic LNCaP prostate cells was performed. It is important to also note that detection of the β6 subunit includes the presence of αv, as β6 is only able to bind αv. Without the alpha subunit, β6 would be degraded. Therefore, where it is stated that β6 is present one can assume αv is also present. Results obtained using 2A1 monoclonal antibody (mAb) against β6 showed expression of the β6 subunit at 90-110 kDa under non-reducing conditions for both benign and malignant prostate tissue (Figure 3). RWPE-1 cells, which are known to express αvβ6 integrin shows strong β6 expression using 2A1 mAb under non-reducing conditions. RWPE-1 was used as a positive control for all immunoblot analysis. Non-tumorigenic LNCaP cells do not express αvβ6, as demonstrated in Figure 3 (lane 11) where no expression can be seen in the lane containing 20 µg of LNCaP lysate. These findings show that β6 is expressed in both tumor and benign prostate tissue. Later, unpublished, findings revealed that αvβ6 is not present in normal prostate tissue. When combined with the evidence of strong β6 expression in the presence of infiltrating leukocytes (Figure 5), it is possible that the expression of β6 in immunoblotting is the result of leukocytes in the tissue lysates. Given the distribution of β6 observed in immunostained sections of prostate (shown in subsequent figures) it is difficult to analyze the results shown in Figure 3. As a result, the information obtained in Figure 3 is strictly
verification that the 2A1 antibody does, in fact, specifically bind a protein of the correct size.

Expression of Integrin αvβ6 in Human Prostate Tissue

In addition to the results obtained through immunoblotting, we were able to show the focal expression of β6 at sites of inflammation (Figure 5), proliferative inflammatory atrophy (PIA) and prostate intraepithelial neoplasia (PIN) in benign and malignant specimens (Figure 4). Strong reactivity was observed in malignant prostate sections stained with monoclonal 2A1 antibody against the β6 integrin subunit (Figure 4A). The specificity of the 2A1 antibody was verified using β6-transfected SW480 cells, shown in Figure 4C, when compared with the negative control (Figure 4D) stained with mouse
IgG. No expression of β6 was observed in normal human prostate tissue obtained from a biopsy (Figure 4E). The β6 subunit was found to be focally expressed in benign tissue sections, that is, areas of “normal” tissue within a pathogenic prostate (Figure 4F).

Strong immunoreactivity was consistently observed in glandular tissue surrounded by inflammatory cells (Figure 5). The expression of β6 in these areas appeared to be cell-type specific, as it was observed in epithelial cells but not in stromal cells or the infiltrating inflammatory cells. Based on the above results, the conclusion is that αvβ6 expression is inducible in adenocarcinoma tissue while normal tissue shows little
expression. Additionally, β6 expression can be detected in response to cancer-associated lesions of inflammation, PIA and PIN.

**Figure 5. Expression of β6 at sites infiltrated by leukocytes**

Immunohistochemical staining shows expression of β6 in areas where high concentrations of leukocytes can be seen. Panel A shows β6 expression within glands being infiltrated by leukocytes. Staining was done using monoclonal 2A1 (0.5 μg/ml) antibody against β6. B shows a negative control for panel A, stained with mouse IgG (0.5 μg/ml). C, D, E, F show expression of β6 in the presence of leukocytes (as shown by the arrows).
DISCUSSION

Prostate cancer cell functions are regulated by elaborate signaling pathways activated by extracellular stimuli. The integrin family of cell surface receptors is known to activate intracellular pathways via interactions with specific ligands in the tumor microenvironment. Among others, the $\alpha v\beta 6$ integrin appears to be a key player in mediating cancer progression given its ability to mediate signals originating in the tumor microenvironment via activation of latent TGF-$\beta$. An increasing amount of data suggests that the expression of $\alpha v\beta 6$, which is not typically found in the healthy adult, is associated with neoplastic and metastatic phenotypes in colorectal, lung, oral, ovarian, breast, and pancreatic cancers. Research for this project focused on the expression of $\beta 6$ (a subunit of the $\alpha v\beta 6$ heterodimer) in human prostate cancer tissue specimens. Results show that while $\alpha v\beta 6$ is not expressed in normal human prostate, it is induced in human prostatic intraepithelial neoplasia (PIN), proliferative inflammatory atrophy (PIA) and prostatic adenocarcinoma.

The research done for this project is only a small portion of the entire report being compiled for publication, which ultimately found that $\alpha v\beta 6$ regulates survival and proliferative pathways, and consequently, prostate tumor growth, via modulation of AKT and androgen receptor activity. My work on this project helped lead the lab to the finding that integrins inhibit androgen receptor activity, suggesting a novel integrin-mediated mechanism of prostate cancer progression. In addition to my analysis with prostate tissue using immunohistochemistry, later work was done in mice to show that the
expression of $\alpha v\beta 6$ in LNCaP prostate cancer cells results in increased tumor volume in SCID mice, as compared to expression of a different $\alpha v$–containing integrin $\alpha v\beta 3$.

Further analysis of the mechanism by which $\alpha v\beta 6$ increases tumor growth shows that it inhibits androgen receptor transcriptional activity via activation of the PI3-kinase/AKT-pathway. Additionally, results show that $\alpha v\beta 6$ inhibition of androgen receptor (AR) activity results in up-regulation of an anti-apoptotic molecule, survivin, known to promote prostate cancer progression and radio/chemo-resistance (unpublished data). The above findings have important implications for prostate cancer therapy, since altered AR activity is believed to be the main contributing factor leading to the development of prostate cancer. Understanding of an $\alpha v\beta 6$-mediated signaling pathway which controls AR activity is therefore important in understanding the molecular mechanism leading to prostate cancer progression. The future direction of this project will be to determine what induces $\alpha v\beta 6$ in the prostate. This will be done through the screening of a number of cytokines to find which ones have the ability to up-regulate the expression of $\alpha v\beta 6$ in prostate cell lines. Ultimately, the goal is to find what causes increased inflammation in the prostate, which may be a precursor to prostate malignancies and then to find a way to prevent the inflammation from occurring, theoretically preventing some forms of prostate cancer from ever developing.
REFERENCES


