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The Laminin Binding Integrin $\alpha 6 \beta 1$ in Prostate Cancer Perineural Invasion

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Abstract

Metastasizing prostate tumor cells invade along nerves innervating the encapsulated human prostate gland in a process known as perineural invasion. The extracellular matrix laminin class of proteins line the neural route and tumor cells escaping from the gland express the laminin binding integrin $\alpha 6 \beta 1$ as a prominent cell surface receptor. Integrin $\alpha 6 \beta 1$ promotes aggressive disease and supports prostate tumor cell metastasis to bone. Laminins and their integrin receptors are necessary for the development and maintenance of the peripheral nervous system, indicating the potential role for integrin receptors in directing prostate tumor cell invasion on nerves during perineural invasion.

A general model for metastasis consists of a multi-step process involving tumor cell interactions with the surrounding environment. These steps include a loss of cellular adhesion and detachment, local invasion of the host extracellular matrix, intravasation into the vasculature or lymphatics, and extravasation into the parenchyma of distant tissues. Neurotropic cancers however, including prostate, pancreatic, head and neck, and colorectal cancer exhibit remarkable affinity for and utilization of the complex neuroanatomy of highly innervated organs as a means for primary tumor cell escape (Liebig et al., 2009). Prostate tumor cell migration and invasion in prostatic nerves and neurovascular bundles, a process known as perineural invasion (PNI), is a major route for extracapsular extension during prostate cancer metastasis (Villers et al., 1989). Accordingly, the clinical significance of perineural invasion as a major factor contributing to prostate cancer metastasis and as a predictor for poor patient outcome has been documented (Harnden et al., 2007; Lee et al., 2007; Liebig et al., 2009).

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Prostate cancer is multifocal and heterogeneous, and is a slow growing solid tumor (Johansson et al., 1997, 2004). Confined and localized prostate cancer is considered curable while invasion through the prostate capsule and development of metastases is associated with poor prognosis. Current diagnostic procedures do not provide accurate early detection or define individuals who will develop aggressive and incurable metastatic disease. This is a significant clinical problem considering prostate carcinoma is the most commonly diagnosed visceral neoplasm and second leading cause of death in men (Jemal et al., 2007). Discovery of novel prognostic indicators or molecular targets which directly relate to tumor cell metastasis along nerves will enable a dramatic shift in the approaches used to currently treat the disease, including potentially improving nerve sparing radical prostatectomy approaches.

The laminin class of extracellular matrix proteins are present in the neural route (Ryschich et al., 2009) and prostate tumor cells expressing the $\alpha 6 \beta 1$ integrin invade along prostatic nerves. The laminin receptor integrin $\alpha 6 \beta 1$ is essential during peripheral nerve development and our group has demonstrated its major role in regulating prostate tumor cell migration and invasion (Pawar et al., 2007) and prostate tumor metastasis to bone (Ports et al., 2009; Sroka et al., 2009). This review will discuss the novel role of integrin $\alpha 6$ in directing prostate tumor cell adhesion and invasion on nerves during perineural invasion. Establishing a comprehensive understanding of the interdependence between increased laminin dependent invasion of tumor cells and nerves during PNI will provide framework for developing pertinent biomarkers or targeted therapies for isolating and treating patients exhibiting aggressive disease. Inhibiting tumor cell escape along nerves in the prostate will be instrumental for future clinical control of the disease.

Laminin Adhesion and Prostate Cancer Progression

Integrins are non-covalently bound heterodimeric cell surface receptors which play a major role in cell adhesion and migration. The integrins are heterodimer receptors consisting of 24 members comprised of 18 α and 8 β subunits all encoded by distinct genes (van der Flier and Sonnenberg, 2001; Hynes, 2002). These receptors are tissue specific and regulate cell locomotion and intracellular signaling events by binding to their respective ligands in the extracellular environment while simultaneously interacting with intracellular signaling components. The laminin binding integrin alpha subunits including $\alpha 6$ (CD49f), $\alpha 3$ (CD49C), and $\alpha 7$ are among the most highly conserved of all integrin family receptors (Hughes, 2001; Huhtala et al., 2005) and play a significant role in both normal and pathological conditions.

The role of laminin binding integrins and laminin extracellular matrix proteins in regulating cell adhesion and migration events during prostate cancer progression has been extensively studied by our group and others. Altered expression or function of extracellular matrix and adhesion molecules including laminins and integrins is accompanied by acquisition of motility and invasiveness in prostate cancer and is therefore a significant event during the natural progression of the disease (Nagle et al., 1995). The major expression patterns of integrin family members and their role in directing cellular signaling in normal and neoplastic prostate glands have been reviewed (Goel et al., 2008, 2009). We and others have shown that several integrins including the $\alpha 2 \beta 1$ collagen receptor, $\alpha 3 \beta 1$ and $\alpha 6 \beta 1$ laminin

receptors, the $\alpha 5 \beta 1$ fibronectin receptor, the $\alpha v \beta 1$ vitronectin receptor and the $\alpha 6 \beta 4$ hemidesmosome laminin receptor are all expressed in normal prostate glands (Bonkhoff et al., 1993; Knox et al., 1994; Nagle et al., 1995; Davis et al., 2001), while only the laminin binding integrins $\alpha 6 \beta 1$ and $\alpha 3 \beta 1$ are expressed in prostate carcinoma (Schmelz et al., 2002). Attachment of epithelial cells to the substratum in normal prostate cells primarily occurs via integrin $\alpha 6 \beta 4$ to laminin-332 in highly adhesive hemidesmosome structures. The disruption of $\alpha 6 \beta 4$ adhesions between quiescent prostatic basal cells and the substratum in prostate cancer occurs from the absence of the integrin $\beta 4$ subunit. This enables engagement of the $\alpha 6 \beta 1$ and $\alpha 3 \beta 1$ laminin receptors and the pro-migratory phenotype of luminal epithelial cells during prostate cancer progression.

Coincident with expression changes observed with laminin binding integrins, the laminin extracellular matrix proteins are also modified during prostate cancer progression. Laminins are a family of heterotrimeric glycoproteins consisting of one α , β , and γ subunit. In vertebrates, five α , three β , and three γ chains have been identified and all isoforms are encoded by distinct genes. The α chains are encoded by the LAMA gene family, the β chains by the LAMB family and the γ chains by the LAMC family. Our group has identified that laminin-332 ($\alpha 3 \beta 3 \gamma 2$) and -511 ($\alpha 5 \beta 1 \gamma 1$) are expressed in normal prostate glands while only laminin-511 persists in prostate cancer (Hao et al., 1996; Bair et al., 2005). The crucial role of laminin-332 in maintaining basal lamina integrity and epidermal adhesion was initially identified in the severe and lethal blistering skin disease Herlitz's junctional epidermolysis bullosa (JEB) (Aberdam et al., 1994; Pulkkinen et al., 1994; Uitto et al., 1997; Nakano et al., 2000). Specific mutations in any of the three laminin-332 genes (LAMA3 ($\alpha 3$), LAMB3 ($\beta 3$), and LAMC2 ($\gamma 2$)) results in complete loss of laminin-332 leading to defective adhesion structures and the blistering phenotype (Pulkkinen and Uitto, 1999). The decrease in laminin-332 and integrin $\beta 4$ expression and the persistent expression of its ligand laminin-511 illustrate the significance of $\alpha 6 \beta 1$ regulated laminin adhesion events in prostate cancer invasion.

The retention of $\alpha 6 \beta 1$ expression in human prostate cancer implicates a potential role for laminin dependent adhesion in prostate cancer progression. Indeed, our group has identified a novel structural variant of $\alpha 6 \beta 1$, $\alpha 6 p \beta 1$, specific to human cancer tissue and tumor cell lines (Davis et al., 2001; Demetriou et al., 2004). The structural variant is formed on the surface of tumor cells through proteolytic cleavage of the extracellular laminin binding domain by the serine protease urokinase plasminogen activator (uPA) at amino acid residues R594 and R595 (Davis et al., 2001; Demetriou and Cress, 2004; Demetriou et al., 2004; Pawar et al., 2007). This post-translational modification of integrin $\alpha 6 \beta 1$ increases prostate tumor cell migration and invasion on laminin substrates (Pawar et al., 2007) and promotes metastasis to the laminin rich bone microenvironment (Ports et al., 2009). Extracellular domain cleavage of $\alpha 6$ to $\alpha 6 p$ is necessary for normal development in *Xenopus laevis* (Demetriou et al., 2008) implicating the role for post-translational cleavage of $\alpha 6$ in both normal and pathological conditions.

Laminin Receptors and the Peripheral Nervous System

Substantial evidence exists for the role of laminins and laminin receptors in the peripheral nervous system (PNS) (Colognato et al., 2005; Feltri and Wrabetz, 2005). Laminins are one of the most abundant extracellular matrix proteins in the PNS. Specific laminin family members play a significant role in neuronal regeneration and development. Studies have identified laminin-211, -411, and -511 expression in the PNS and each are required for peripheral nerve fiber formation (Feltri and Wrabetz, 2005). Laminins initiate signaling and regulate cellular functions on Schwann cells which sort and myelinate neurons for proper function of the nervous system (Chernousov et al., 2008). Laminin $\alpha 4$ deficient mice demonstrate defects in hypomyelination and axonal sorting of peripheral nerves, and mutations in the laminin $\alpha 2$ gene cause peripheral neuropathy (Wallquist et al., 2005; Yang et al., 2005). It has also been shown that inhibition of Schwann cell specific expression of the laminin $\gamma 1$ chain in an in vivo model disrupts Schwann cell myelination and axonal sorting (Chen and Strickland, 2003).

Engagement of laminin through integrin receptors is a requirement for Schwann cell regulated myelination of axons in the peripheral nervous system during various stages of development (Yu et al., 2007). Specifically, the $\alpha 6 \beta 1$ and $\alpha 6 \beta 4$ integrins are laminin receptors in Schwann cells and the nerve sheath (Niessen et al., 1994; Terpe et al., 1994; Dubovy et al., 1999) and the $\alpha 6 \beta 1$ receptor is necessary for axonal sorting by Schwann cells (Feltri and Wrabetz, 2005) and early nervous system development (Lallier et al., 1996). Studies using Schwann cell specific knock out of the $\beta 4$ subunit in vivo demonstrated that the receptor is necessary for nerve regeneration and myelination (Van der Zee et al., 2008). Interestingly, recent studies have shown that a significant percentage of human neural progenitor cells express the $\alpha 6$ integrin subunit (Flanagan et al., 2006; Hall et al., 2006; Mueller et al., 2006), and the significance of $\alpha 6 \beta 1$ and laminin dependent adhesion of neuronal stem cells and neural progenitor expansion has also been reported (Ma et al., 2008). In this study, human embryonic cell-derived progenitor expansion, migration and differentiation was most responsive to laminin extracellular matrix and was abrogated when $\alpha 6$ integrin function was blocked.

Perineural Invasion in Prostate Cancer

The human prostate gland is a complex tubulo-alveolar gland with regions defined by concentric zones including the anterior fibromuscular stroma, the central zone, the peripheral zone, and the transition zone (McNeal, 1981; Timms, 2008). The prostate gland is completely surrounded by a smooth muscle casing known as the prostate capsule. The duct-acinar system of the prostate is comprised of luminal, neuroendocrine and underlying basal cells surrounded by a thin basal lamina (Ware, 1994). The substratum surrounding normal acini is comprised of laminin-332, laminin-511, collagen IV, and collagen VII, and forms a contiguous lining which surrounds the basal cell layer (Knox et al., 1994; Nagle et al., 1995). The smooth muscle stroma of the human prostate gland is permeated by the cavernous nerve and neurovascular formations of the pelvic plexus which are comprised of autonomic nerves (Dunzendorfer et al., 1976; Vaalasti and Hervonen, 1980; Gil-Vernet, 1996; Schwalenberg et al., 2009). Anatomic variation does exist regarding the

neuroanatomical structures of the male pelvis and prostate gland (Schwalenberg et al., 2009). However, it has been identified that there is significant nerve innervation in the peripheral zone of the prostate gland, which is the predominant site for prostate tumor development (Chang et al., 1998; Powell et al., 2005).

Perineural invasion is a major route for extraprostatic extension during prostate cancer metastasis (Liebig et al., 2009; Marchesi et al., 2010). Prostate tumor cells migrate and invade along all three structural components of the prostatic nerves, including the perineurium, epineurium and the innermost endoneurial sheath during PNI (Fig. 1). The clinical implication of PNI as a reliable prognostic indicator for disease recurrence has been noted (Freeman, 2009) and several studies have identified PNI as an independent and significant prognostic factor for the presence of tumor cell extension beyond the prostate (Ukimura et al., 1998; Vargas et al., 1999; de la Taille et al., 1999). Recent evidence has also shown that patients with prostate cancer have increased axonal and neurite outgrowth in cancerous regions, implying that tumor cell motility and invasiveness along nerves is not a tumor cell-autonomous process, but a reciprocal relationship between nerves and tumor cells to promote invasion (Ayala et al., 2008).

Since the description by Walsh et al. (1983) of anatomical nerve sparing radical retropubic prostatectomy the ability of urologists to preserve erectile function has improved. The posterolateral and extraprostatic location of the neurovascular bundles should not compromise surgical margins in cases in which the cancer is truly organ confined. Pathological evidence of cancer extending through the prostatic capsule (pT3) is associated with an increased risk of positive surgical margins, and potential disease recurrence. Thus in some pT3 cases preservation of the neurovascular bundles may adversely affect surgical margin status. Examination of the prognostic role of perineural invasion in prostatectomy specimens has demonstrated a significant correlation between perineural invasion and extraprostatic extension (Vargas et al., 1999). While the clinical utility of this prognostic information remains controversial, many urologic surgeons are using this information to determine extent of resection at the time of prostatectomy.

Molecular Mechanisms of Prostate Cancer Perineural Invasion

Detailed molecular mechanisms describing the characteristics of tumor cells and nerves in the prostate cancer PNI microenvironment have been hampered due to difficulties in creating an accurate representation of the disease state in in vitro and in vivo models. However, several groups have established unique model systems in which analyses of tumor and nerve interactions can be observed. Ayala et al. established that nerves and prostate tumor cells exhibit increased growth and reciprocal interactions in a co-culture model (Ayala et al., 2001). In this model, prostate tumor cells were grown in Matrigel with adult mouse dorsal root ganglia. Directed neurite outgrowth toward tumor cells and tumor cell migration along penetrating neurite extensions was observed. Their group also identified increased expression of anti-apoptotic regulators such as nuclear factor kappa β and Pim-2 in prostate tumor cells growing adjacent to nerves (Ayala et al., 2004; Dai et al., 2005) in prostate tumor cells growing adjacent to nerves. Further work by Cornell et al. identified the requirement of prostate stroma in mediating reciprocal interactions between malignant prostate cells and

nerves during PNI using the mouse dorsal root ganglia and Matrigel model (Cornell et al., 2003). Other groups have identified increased expression of the mir-244 miRNA in tumor cells associated with prostatic nerves (Prueitt et al., 2008) and increased expression of the chemokine CXCL12 and its associated receptor CXCR4 (Zhang et al., 2008).

The nerve rich microenvironment may also provide a niche for tumor cells to adapt and alter expression of genes necessary for maintaining survival and an aggressive phenotype prior to executing the metastatic program. Indeed, a recent study has shown that prostate tumor cells undergoing active PNI harbor a specific transcriptome signature (Prueitt et al., 2008). The gene expression changes noted in this study associated with PNI were related to changes in cell metabolism and mitochondrial function, while earlier studies demonstrate decreased apoptosis and increased survival in tumor cells undergoing PNI (Yang et al., 1996; Ayala et al., 2004, 2006a). Specific gene expression changes have also been noted during PNI in pancreatic cancer using both in vitro and in vivo models (Koide et al., 2006; Dai et al., 2007). These studies along with the finding that prostate cancer leads to increased neurogenesis (Ayala et al., 2008) established that the PNI microenvironment facilitates a growth and survival advantage for both nerves and prostate tumor cells migrating along nerves.

Cell Adhesion Molecules and Perineural Invasion

The role of tumor cell adhesion molecules in regulating PNI during disease progression has been most extensively studied in pancreatic PNI models. Recent work by Marchesi et al. (2008) identified the role of the transmembrane chemokine CX3CL1 and its receptor CX3CR1 in the adhesion and migration of pancreatic tumor cells on nerves during pancreatic cancer perineural invasion. In this study, activation of $\beta 1$ integrins and focal adhesion kinase (FAK) was necessary for CX3CR1 mediated pancreatic tumor cell neurotropism. Additional work by Gil et al. (2010) determined the role of the glial cell-derived neurotrophic factor (GDNF), a chemoattractant secreted by nerves, in regulating pancreatic tumor cell adherence and invasion on nerves. The transmembrane mucin known as MUC1 expressed on pancreatic tumor cells also plays a role in pancreatic tumor cell adhesion during PNI by binding to myelin-associated glycoprotein (MAG) on Schwann cells (Swanson et al., 2007). Ryschich et al. identified expression of laminin in the perineural space of nerves in pancreatic cancer and increased migration and invasion of pancreatic cells on laminin substrates was observed during pancreatic cancer PNI (Ryschich et al., 2009). Recent work has also demonstrated that inhibition of melanoma cell adhesion molecule (MCAM or CD146) using short hairpin silencing RNA in an adenoid cystic carcinoma cell line decreased perineural invasion in vitro (Chen et al., 2009).

Studies specifically defining the role of adhesion molecules in prostate cancer PNI are needed. We hypothesize that prostate tumor cells depend on tumor cell adhesion to laminin rich nerves during PNI for promoting tumor cell invasion on nerves. Sinha et al. previously demonstrated laminin expression in prostatic nerves in human prostate cancer tissue (Sinha et al., 1989). In Table 1, we also summarize our findings regarding laminin chain expression in human prostate, including normal, cancer, and regions of PNI. Interestingly, the prominent laminin chains expressed in neoplastic lesions and PNI comprise the ligands of

integrin $\alpha 6$, the laminin $\alpha 5$, $\alpha 4$, and $\gamma 1$ chains (Table 1). Further, we have identified remarkable expression of the integrin $\alpha 6$ receptor in tumor cells actively invading prostatic tumors during PNI (Figs. 1 and 2A,B). Importantly, Figure 2A,B illustrates remarkable differences in the manner in which tumor cells actively invade along the prostatic nerves. Tumor cells can be observed invading both the endoneurium and perineurium (2A) and integrin $\alpha 6$ expression is observed in tumor cells undergoing both perineural and endoneural invasion. It is currently unknown whether tumor cell invasion in the perineural versus endoneural regions of nerves reflects differences in their metastatic phenotype. We speculate that the metastatic advancement of more differentiated glandular structures (Fig. 2B) varies greatly from less differentiated tumors which invade both the endoneurium and perineurium (Fig. 2A). Future studies to dissect the importance of the variability in how tumor cells invade along nerves and the role laminin dependent adhesion plays in this process will be the focus of ongoing studies in our laboratory.

Concluding Remarks

The mechanisms directing perineural invasion in neurotropic cancers are currently poorly defined although the clinical significance of the disease has been established (Liebig et al., 2009). Determining how tumor and nerve interactions orchestrate and promote metastatic disease is necessary for combating extraprostatic extension during prostate cancer. The laminin rich prostatic nerves and the persistent expression of the pro-metastatic laminin binding $\alpha 6$ integrin in prostate tumor cells undergoing PNI may be potential targets for therapeutic intervention or diagnostic procedures. The deleterious effects of metastatic prostate cancer warrant increased study in the field of perineural invasion.

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Fig. 1.

The prostatic nerve plexus breaches the prostate capsule and penetrates into the peripheral zone of the prostate gland where most prostate tumors develop. The layers of the nerve sheath include the epineurium, perineurium and endoneurium from outside in, respectively. The perineurium encompasses a bundle of nerves called a fascicle. Perineural and endoneural invasion of prostate tumor cells along the nerve route promotes extra prostatic escape. Inset: During tumor invasion on prostatic nerves, human prostate tumor cells express high levels of the $\alpha 6$ integrin (brown). Note the absence of cancer cell (CA) invasion along vessels (V) when compared to significant invasion of the nerve (N). Integrin $\alpha 6$ is expressed around vessels as expected. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.

Fig. 2.

Integrin $\alpha 6$ expression in endoneural and perineural invasion in human prostate cancer.

Human prostate cancer tissue was stained with the AA6NT polyclonal antibody specific for the $\alpha 6$ integrin (Ports et al., 2009) (brown) and the neural specific marker PGP 9.5 (red). A: Tumor cell (C) invasion of the endoneurium (E) and perineurium (P) of nerves (N). B: Higher magnification of the $\alpha 6$ integrin in PNI during prostate tumor cell invasion of the perineurium of nerves (P). Note the polarized glandular structures developed by tumor cells invading the perineurium in (B) versus the less differentiated structures observed in tumor cells invading the endoneurium in (A). Whitebar, 80 μm (A) and 40 μm (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 1

Laminin chain expression in prostate cancer and PNI

Laminin chain	Normal prostate glandular tissue	Prostate cancer	PNI prostatic nerves	Antibody clone
$\alpha 3$	+++	++	ND	BM165
$\alpha 4$	+++	++	+++	FC10
$\alpha 5$	+++	+++	+++	4C7
$\beta 2$	++++	–	–	C4
$\beta 3$	++	–	–	Clone 17
$\gamma 1$	+++	++	+++	2E8
$\gamma 2$	+++	–	–	GB3

Code: +++ high expression, ++ moderate expression, – absence of expression, ND, not determined. Frozen human prostate cancer specimens were reacted with laminin chain specific antibodies as indicated followed by incubation with Alexa Fluor secondary antibodies (Molecular Probes, Eugene, OR). Immunofluorescence was observed with a confocal laser scanning microscope (Carl Zeiss, Germany). The laminin $\alpha 3$ chain BM165 antibody has been described previously (Sroka et al., 2008), the $\alpha 4$ chain monoclonal antibody FC10 was a kind gift from Dr. I. Virtanen (University of Helsinki, Finland). The $\alpha 5$ (4C7), $\beta 2$ (C4) and $\gamma 1$ (2E8) antibodies were kind gifts from Dr. E. Engvall (The Burnham Institute, La Jolla, CA). The $\gamma 2$ monoclonal antibody GB3 was from Serotec (Oxford, UK) and the $\beta 3$ chain monoclonal antibody Clone 17 was from BD Bioscience (San Jose, CA).