

Original Article

$\alpha_v\beta_6$ integrin expression is induced in the POET and Pten^{pc/-} mouse models of prostatic inflammation and prostatic adenocarcinoma

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Received March 19, 2012; accepted April 6, 2012; Epub April 10, 2012; Published April 30, 2012

Abstract: Chronic inflammation is proposed to prime the development of prostate cancer. However, the mechanisms of prostate cancer initiation and development are not completely understood. The $\alpha_v\beta_6$ integrin has been shown to play a role in epithelial development, wound healing and some epithelial cancers [1, 2]. Here, we investigate the expression of $\alpha_v\beta_6$ in mouse models of prostatic inflammation and prostate cancer to establish a possible relationship between inflammation of the prostate, $\alpha_v\beta_6$ expression and the progression of prostate cancer. Using immunohistochemical techniques, we show expression of $\alpha_v\beta_6$ in two *in vivo* mouse models; the Pten^{pc/-} model containing a prostate-specific Pten tumor suppressor deletion that causes cancer, and the prostate ovalbumin-expressing transgenic (POET) inflammation mouse model. We show that the $\alpha_v\beta_6$ integrin is induced in prostate cancer and inflammation *in vivo* in these two mouse models. $\alpha_v\beta_6$ is expressed in all the mice with cancer in the Pten^{pc/-} model but not in age-matched wild-type mice. In the POET inflammation model, $\alpha_v\beta_6$ is expressed in mice injected with activated T-cells, but in none of the control mice. In the POET model, we also used real time PCR to assess the expression of Transforming Growth Factor Beta 1 (TGF β 1), a factor in inflammation that is activated by $\alpha_v\beta_6$. In conclusion, through *in vivo* evidence, we conclude that $\alpha_v\beta_6$ integrin may be a crucial link between prostatic inflammation and prostatic adenocarcinoma.

Keywords: Prostate cancer, PIN lesion, chronic inflammation, TGF β 1, $\alpha_v\beta_6$ integrin, Akt

Introduction

Many treatment options exist for prostate cancer and newer targeted treatments are being developed based on current research [3, 4]. However, prostate cancer mechanisms of initiation and progression are not completely understood.

The idea that inflammation may be a precursor

to cancer has been proposed for many types of cancer. Epidemiological evidence supports this theory in the respiratory, genitourinary, reproductive and gastrointestinal systems [5, 6]. Chronic inflammation occurs due to macrophages, lymphocytes and plasma cells infiltrating damaged tissue [5]. All of these leukocytes can release potentially harmful products such as reactive oxygen species (ROS), cytokines, and other mediators [6], which may contribute

to the onset of cancer [5].

Prostatic inflammation has been linked to prostate cancer through epidemiological, histopathological and molecular pathological studies [7]. The morphologic entity known as prostate inflammatory atrophy (PIA) may be a precursor lesion to the onset of prostate cancer. In some cases, PIA can progress to prostatic intraepithelial neoplasia (PIN) and invasive cancer; however, not all cases of prostate cancer are preceded by PIA or PIN [8].

Integrins are transmembrane heterodimers consisting of α and β subunits [9-11]. They bind extracellular matrix (ECM) proteins and may, in some instances, mediate cell-cell contacts [12]. Integrins activate many signaling pathways, including the PI-3kinase/Akt pathway [13] and CDKs [14, 15], which affect proliferation and the cell cycle. Different integrins have been shown to be both over- and under-expressed in different cancers [16] and the role of integrins in the progression of prostate cancer has been recently reviewed [17]. Among others, the $\alpha_v\beta_6$ integrin is a potential target that may link inflammation and cancer. This integrin has been sequenced in humans, guinea pigs [18] and mice [19] and has been shown to pair with only one alpha subunit (α_v) and to bind fibronectin, tenascin, vitronectin and osteopontin [20, 21]. Additionally, β_6 integrin subunit is only expressed in epithelial cells and is not detectable in most resting, fully differentiated epithelia, including those in the lungs and skin [22]. However, this integrin is shown to be expressed in wound healing and some cancers [1, 2, 23, 24].

Here, we have evaluated $\alpha_v\beta_6$ integrin expression in two *in vivo* murine models of prostate diseases. Both models utilize the Cre-Lox system with the prostate-specific promoter ARR₂PB originally developed by X. Wu and co-workers [25]. This system has been used to conditionally delete the Pten tumor suppressor in the prostate of mice [26]. This tumor suppressor is often inactivated in human prostate cancer [27, 28]. These mice develop PIN at an early age and then, later, invasive adenocarcinoma. The other model, the prostate ovalbumin-expressing/transgenic (POET) model [29], used this system to express a gene of a membrane-bound, ovalbumin-transferring, receptor fusion protein in the prostate. This model is used to mimic inflammation of the prostate and these mice have

not been reported to develop cancer of the prostate.

Materials and methods

Cells, reagents and antibodies

PC3 cells were cultured in RPMI medium containing 5% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). TGF β 1 was from R&D System Inc. The antibodies used were: rabbit antibodies against ERK1/2 from Santa Cruz Biotechnology (Santa Cruz, CA), p-Akt (ser473) and Akt from Cell Signaling Technology (Beverly, MA) and monoclonal mouse antibody 2A1 to β_6 for immunoblotting [30, 31]. β_6 siRNA duplexes (IDT, Inc.) were: D2 (sense) 5'-ACC ACG GGA ACG GCU CUU UCC AGT G-3' and (antisense) 5'-CAC UGG AAA GAG CCG UUC CCG UGG UGA-3'. A non-silencing siRNA duplex, 5'-CUU CCUCUC UUU CUC UCC CUU GUG A-3' and 5'-UCA CAA GGG AGA GAA AGA GAG GAA GGA-3', was used as a control. siRNA duplexes were transfected using oligofectamine at a final concentration of 20 nM. Twenty-four hours after two round-transfections, cells were harvested to test expression of the interested proteins.

Immunoblotting (IB)

Cell lysates were prepared, separated by SDS-PAGE gel and analyzed by IB as previously described [20]. Frozen tumor tissues collected from a bone injection site were homogenized as previously described [32].

Generation of mouse models

All Pten^{pc/-} mice were generated in Dr. Pradip Roy-Burman laboratory. All POET-3 mice were generated in Dr. Timothy L. Ratliff laboratory. All β_6 -null murine tissues were provided by Drs. Shelia Violette and Dean Sheppard. We bred Pten^{pc/-} mice as described by S. Wang et al. [26]. Prostate tissue was collected at 7/8, 12, 20, 28, and 44 weeks of age. The POET-3 inflammation model was developed as previously reported [29]. Inflammation was induced by the injection of 5×10^6 ovalbumin specific T-cells (OT-I cells) and prostate tissue was collected after 7 days. Animal studies were conducted in accordance with approved Institutional Animal Care and Use Committee protocols and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunohistochemistry (IHC)

IHC was performed as previously described in Hahm et al [33], employing the monoclonal antibody ch2A1 directed against $\alpha_v\beta_6$ integrin while a class-matched monoclonal human IgG antibody was used as a control for immunostaining. All slides were reviewed by microscopy using an Olympus BX41TF optical microscope equipped with an Evolution MP 5.0 RTV digital camera attached to a computer. Digital images were captured using QCapture Pro software. Immunohistochemically stained slides were reviewed and prostate gland lobes (dorsolateral, ventral and anterior) were scored individually for the presence of invasive adenocarcinoma, as well as for the percentage of neoplastic cells that expressed $\alpha_v\beta_6$ integrin when adenocarcinoma was present.

Real time PCR (RT-PCR)

RNA used for RT-PCR was harvested from up to 20 mg of tissue using the RNeasy Mini Kit® (Qiagen; Valencia, CA) and accompanying Qias shredder® (Qiagen) and RNase-Free DNase Set (Qiagen). Yield was determined by UV spectrophotometry. RNA was added to 50 ng of Random Hexamer/pd(N)6 (Roche, Basel, Switzerland) to a volume of 10 μ l in RNase-free water. RNA and Oligo dT were heated to 65°C for 5 minutes and immediately placed on ice. The RNA/random hexamer mix was added to 10 μ l of a RT reaction mix ((2 \times First Strand Buffer; Invitrogen; Carlsbad, CA), 10 mM dithiothreitol (DTT), 2 mM dinucleotide triphosphate (dNTP) mix, 40 U RNasin® (Promega Biosciences, San Luis Obispo, CA), and 200 U Superscript II® Reverse Transcriptase (Invitrogen)) and incubated in sequence at 25°C for 10 minutes, 50°C for 50 minutes, 85°C for 5 minutes, and stored at 4°C. Two μ l of the RT reaction were used in a 25 μ l total PCR reaction containing: 0.5 μ M TGF β primers (TaqMan gene expression assay Mm01178820_m1 from Applied Biosystems), 200 μ M dNTP mix, 1 \times PCR Buffer (Roche), 2.5 U Taq Polymerase (Roche).

Statistical analysis

Mann-Whitney test was used to analyze the significance of TGF β 1 RNA expression fold changes in POET mice as compared with C57BL/6 wild-type mice, using a p-value < 0.05.

Results

The $\alpha_v\beta_6$ integrin is expressed in prostatic adenocarcinoma in the Pten^{pc/-} mouse model

In the Pten^{pc/-} mouse prostate using immunohistochemical analysis, we detect expression of the $\alpha_v\beta_6$ integrin in PIN (data not shown) and invasive adenocarcinoma (**Figure 1**), whereas $\alpha_v\beta_6$ expression is undetectable in the normal prostate gland of age-matched wild-type mice (**Figure 1**). More than 90% of the animals between seven and 46 weeks of age have evidence of adenocarcinoma in the prostate gland most notably in the dorsolateral lobe (**Table 1**). In all animals where adenocarcinoma presented, $\alpha_v\beta_6$ staining is seen within the adenocarcinoma in at least one lobe (**Table 1**). As expected, expression of $\alpha_v\beta_6$ is readily detectable in malignant epithelial cells, but is never detected within the stroma, or in the epithelium of wild-type prostates (**Figure 1**).

The $\alpha_v\beta_6$ integrin is induced during inflammation of the prostate

In the POET-3 mouse model, we also show focal expression of $\alpha_v\beta_6$ within epithelial cells of inflamed glands (**Figure 2**). Flow cytometric analysis shows strong prostate infiltration of leukocytes in ventral, dorsolateral and anterior prostate (data not shown). No staining occurred in the control group, indicating that the positive staining was a result of inflammation (**Figure 2**). The number of mice injected with activated T-cells and positive staining for $\alpha_v\beta_6$ expression in the prostate gland over time are presented in **Table 2**. These data demonstrate a strong association in the POET model between activated T-cells and $\alpha_v\beta_6$ expression that decreases over time.

TGF β 1 mRNA expression is increased in the POET model

The expression of TGF β 1 mRNA is increased during inflammation in the POET model as determined by quantitative RT-PCR (**Figure 3**). **Figure 3** shows that the TGF β 1 mRNA expression in prostate tissues from the POET-3 mice is greater than that seen in prostate tissues isolated from C57BL/6 mice receiving OT-I cells. The increase is seen as early as 7 days post injection and remains elevated 14 days post OT-I-injection (**Figure 3**). The increased expression

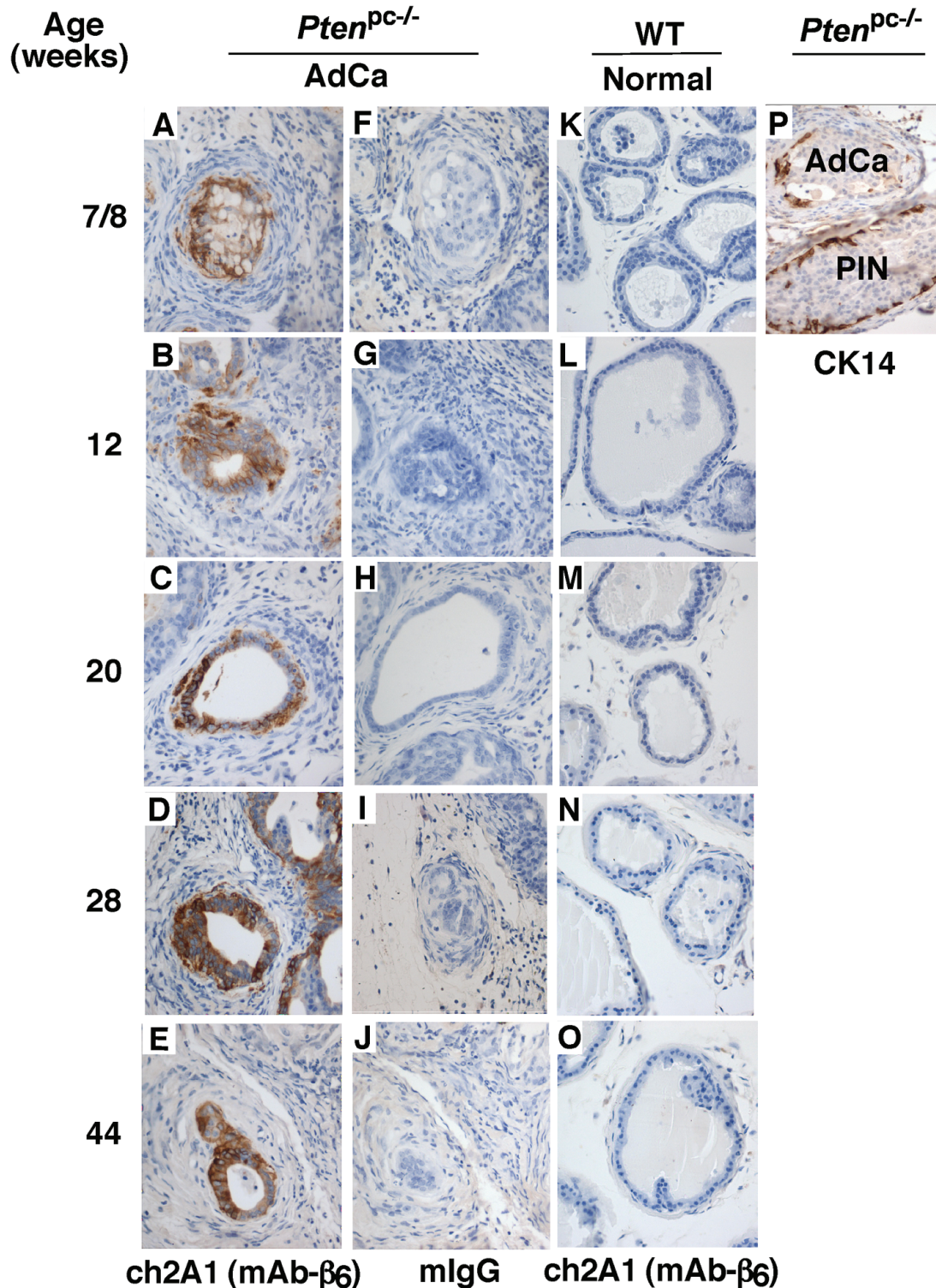


Figure 1. $\alpha_v\beta_6$ Expression in the *Pten*^{pc/-} Mouse Model of Prostate Cancer. *Pten*^{pc/-} mice at ages 8 to 44 weeks show strong expression of $\alpha_v\beta_6$ in malignant epithelial cells of invasive adenocarcinoma (A, B, C, D, E) as evaluated using ch2A1, a monoclonal antibody specific for $\alpha_v\beta_6$; non-immune mouse IgG were used a control (F, G, H, I, J). $\alpha_v\beta_6$ is not expressed in prostate epithelial cells from *Pten* wild type (WT) littermate controls (K, L, M, N, O). CK14 is a basal marker whose expression is lost during cancer; this marker is shown to be more heavily expressed in PIN than in cancer. The dorsolateral lobes are shown in this Figure.

Table 1. Distribution of $\alpha_v\beta_6$ integrin in PIN and Adenocarcinoma in the Pten^{pc/-} Model. The percentage of PIN and adenocarcinoma cells exhibiting positive staining for $\alpha_v\beta_6$ integrin is shown by prostate lobe. The dorsal and lateral lobe values were combined. AdCa, adenocarcinoma. AP, anterior prostate. DLP, dorso-lateral prostate. VP, ventral prostate. -, not available, mo, months.

| Age (mo) | Tissue | % AdCa Cells Stained | | Age (mo) | Tissue | % AdCa Cells Stained |
|----------|--------|----------------------|--|----------|--------|----------------------|
| 1.7 | AP | - | | 7 | AP | 0 |
| 1.7 | DLP | - | | 7 | DLP | 75 |
| 1.7 | VP | - | | 7 | VP | 100 |
| 2 | AP | - | | 8.5 | AP | 0 |
| 2 | DLP | 100 | | 8.5 | DLP | 100 |
| 2 | VP | - | | 8.5 | VP | - |
| 3.1 | AP | - | | 9.4 | AP | - |
| 3.1 | DLP | 100 | | 9.4 | DLP | 100 |
| 3.1 | VP | 100 | | 9.4 | VP | 0 |
| 3.9 | AP | - | | 10 | AP | - |
| 3.9 | DLP | 75 | | 10 | DLP | 5 |
| 3.9 | VP | 0 | | 10 | VP | 0 |
| 5 | AP | - | | 11 | AP | 100 |
| 5 | DLP | 100 | | 11 | DLP | 100 |
| 5 | VP | 0 | | 11 | VP | 100 |
| 5.1 | AP | 0 | | 11.2 | AP | 60 |
| 5.1 | DLP | 100 | | 11.2 | DLP | 75 |
| 5.1 | VP | - | | 11.2 | VP | 50 |
| 5.8 | AP | 100 | | 11.6 | AP | 75 |
| 5.8 | DLP | 80 | | 11.6 | DLP | 100 |
| 5.8 | VP | 50 | | 11.6 | VP | 0 |

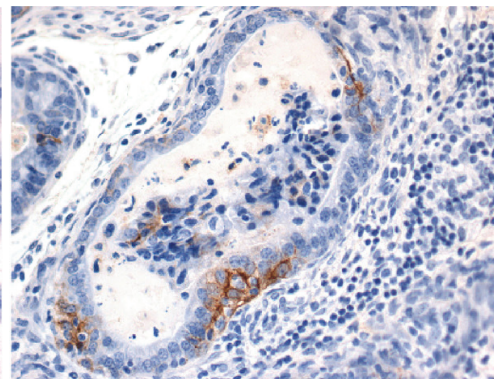
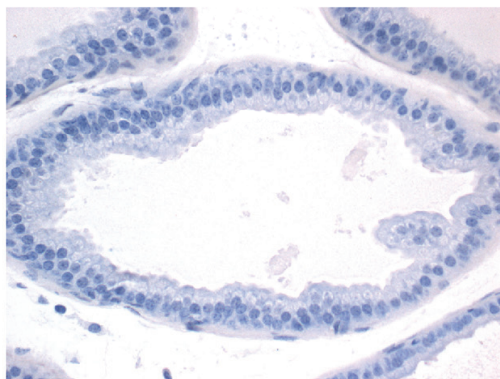
POET-3 mouse model

activated T-cells:

-

+

ch2A1
(mAb- β_6)



VP

Figure 2. $\alpha_v\beta_6$ Expression in the POET Model of Prostate Inflammation. $\alpha_v\beta_6$ Expression in the POET Model of Prostate Inflammation. The expression of $\alpha_v\beta_6$ integrin is seen focally in glandular epithelium of the ventral prostate in association with interstitial inflammation (right panel), as evaluated using ch2A1, a monoclonal antibody specific for $\alpha_v\beta_6$. No inflammation or $\alpha_v\beta_6$ integrin expression is seen where there are no activated T-cells (left panel). Tissues were collected 14 days post-injection. VP, ventral prostate.

Table 2. Number of Mice Injected with Activated T-cells and $\alpha_v\beta_6$ Integrin Staining in the POET Model. The number of mice injected with activated T-cells and positive staining for $\alpha_v\beta_6$ are shown at 8, 14, and 21 days post-OT-I injection. The control group of three mice did not receive the OT-I injection and did not exhibit T-cell activation or positive staining for $\alpha_v\beta_6$.

| Time (days) | case | Activated T-cells | Positive Staining for |
|----------------|------|-------------------|-----------------------|
| Post-Injection | (n) | | |
| 8 | 9 | 9 | 8 |
| 14 | 9 | 7 | 4 |
| 21 | 12 | 7 | 2 |
| control | 3 | 0 | 0 |

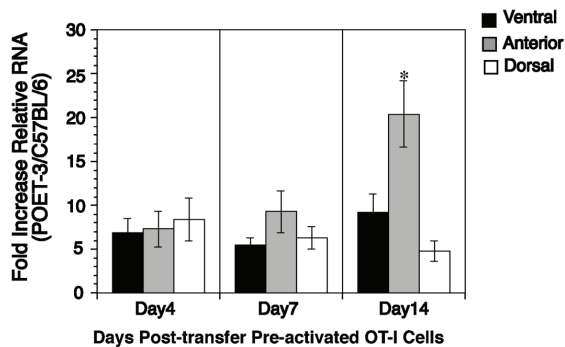


Figure 3. TGF β 1 mRNA levels in the Inflamed POET-3 Prostate. Ventral, anterior, and dorso-lateral prostate lobes were harvested from C57BL/6 and POET-3 mice at 4, 7 and 14 days after transfer of pre-activated OT-I cells. Prostate tissues were homogenized and RNA was isolated, reverse transcribed and real time PCR was performed with primers for TGF β 1. $P < 0.05$, Mann-Whitney test.

of TGF β 1 is most prominent in the anterior prostate (Figure 3).

$\alpha_v\beta_6$ is not required for normal prostate gland development

Hematoxylin and eosin stained sections of each prostate gland lobe from β_6 -null mice were examined to evaluate whether constitutive expression of the β_6 integrin subunit is necessary for prostate gland development. The results show that the morphology of prostate glands from mice that lack the β_6 integrin and wild-type animals was similar (Figure 4). Normal morphology is seen in all four lobes of β_6 -null mice (Figure 4), indicating that this integrin is not essential for proper morphological maintenance of the prostate.

$\alpha_v\beta_6$ downregulation does not affect Akt activa-

tion in PC3 cells

$\alpha_v\beta_6$ integrin expression has been shown to be associated with neoplastic and metastatic phenotypes [34]. To investigate if the β_6 integrin has an effect on the Akt pathway, known to promote androgen-independent survival and growth of prostate cancer cells [35], we down-regulated β_6 in PC3 cells using β_6 siRNA and measured activation of Akt using antibody specific to phospho-Akt. The results show that downregulation of β_6 does not have an effect on activation of Akt (Figure 5A). Downregulation of β_6 integrin was confirmed by immunoblotting (Figure 5B). Our results suggest that expression of β_6 integrin in prostate cancer cells does not regulate the Akt signaling pathway, which appears to be constitutively activated in this *in vitro* system.

Discussion

In this study, we show that the $\alpha_v\beta_6$ integrin is expressed in both the Pten^{pc/-} and the POET-3 mouse models of, respectively, cancer and inflammation. In the Pten^{pc/-} mouse model, we show, in PIN and invasive adenocarcinoma, epithelial-specific $\alpha_v\beta_6$ integrin expression which is instead undetectable in the normal prostate gland of age-matched wild-type mice. In the POET-3 mouse model, we show $\alpha_v\beta_6$ expression within epithelial cells of inflamed prostate glands, but not in the control group. In addition, we show that constitutive expression of the β_6 integrin is not necessary for prostate gland development, since the prostate gland morphology of mice that lack the β_6 integrin and wild-type animals are comparable. This integrin, and its downstream signaling, may be a link between inflammation and cancer in the prostate.

$\alpha_v\beta_6$ integrin expression during inflammation

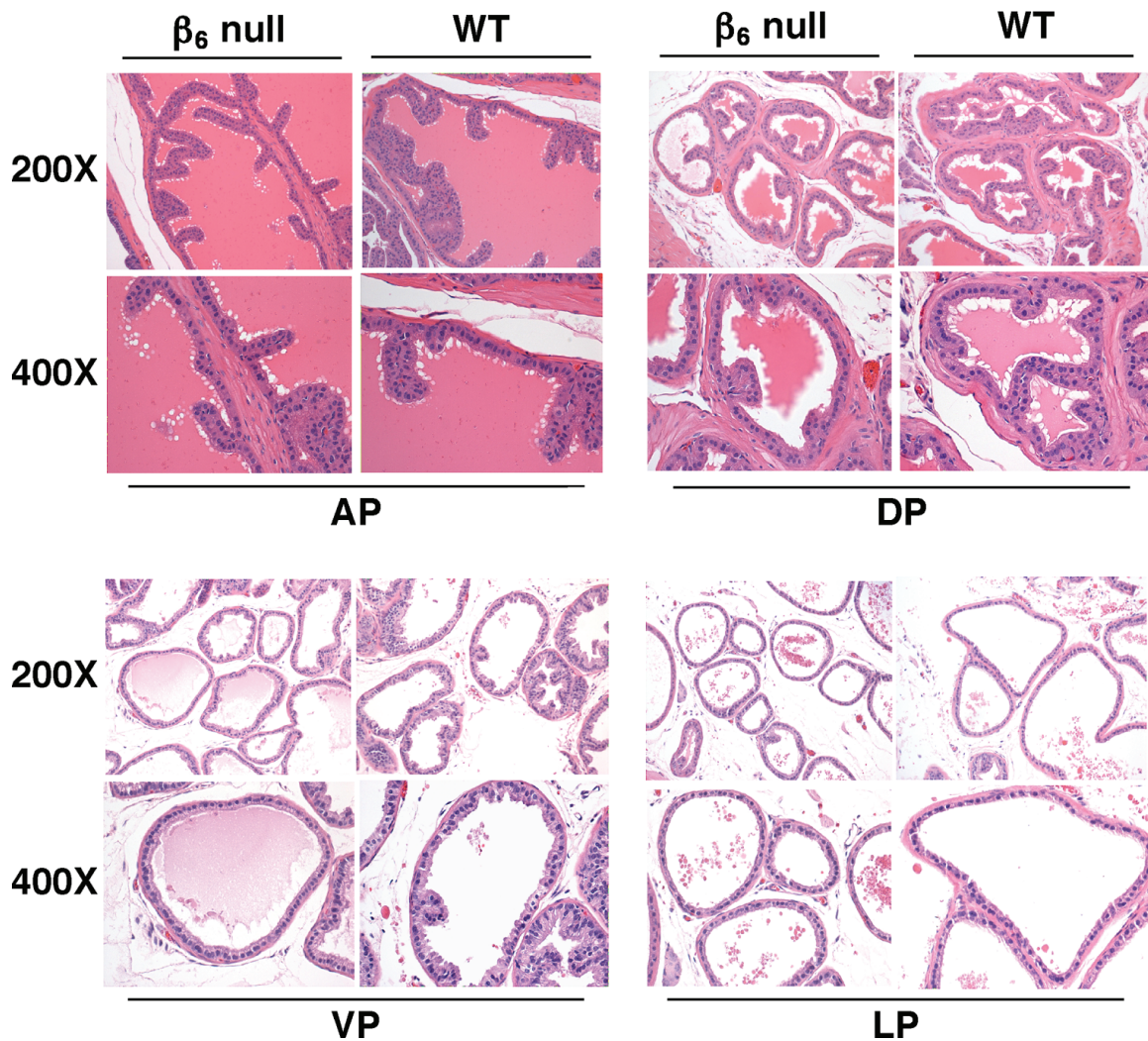


Figure 4. Histological Evaluation of Murine Prostate from β_6 -null and Wild-type Mice. β_6 -null mice have normal prostate morphology compared with prostates isolated from wild type mice. Normal morphology is seen in all lobes. AP, anterior prostate. DP, dorsal prostate. LP, lateral prostate. VP, ventral prostate.

and adenocarcinoma in the mouse models employed for this report adds to the current evidence supporting inflammation as a precursor to cancer in the prostate. However, due to the transient nature of inflammation in the POET model, inflammation is not sustained for a period of time sufficient to induce adenocarcinoma; in our system, the levels of $\alpha_v\beta_6$ staining decrease between 14 and 21 days post-injection even though the number of activated T-cells remained the same. Further research with an *in vivo* model of chronic inflammation may confirm these results. In the $Pten^{PC/-}$ model, PIN develops as a precursor to malignancy-as is believed to be the case in humans. In this

model, mice containing the conditional deletion of $Pten$ develop adenocarcinoma and show increased expression of $\alpha_v\beta_6$ in association with invasive adenocarcinoma. However, PIN and adenocarcinoma develop at an early age-as young as 7 weeks-making it hard to determine if inflammation occurs prior to PIN and adenocarcinoma in this model. In humans, prostate cancer does not develop until later in life, allowing time for chronic inflammation to possibly play a role in the progression to cancer.

At the molecular level, some similarities between inflammation and cancer of the prostate may exist. For example, the enzyme COX-2,

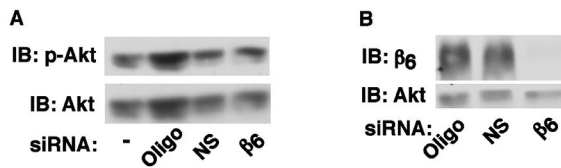


Figure 5. Downregulation of $\alpha_v\beta_6$ Does Not Affect Akt-Phosphorylation. PC3 cells, which contain endogenous β_6 integrin, were transfected with β_6 integrin siRNA duplexes. After transfection, cells were harvested and the interested proteins in cell lysates were separated by using 10% polyacrylamide gel and detected using immunoblotting probed with specific antibodies. Activation of Akt was determined using antibody specific to phospho-Akt (A). The downregulation of β_6 integrin was confirmed (B). Akt was used as a loading control. Oligo, oligofectamine; NS, non silencing.

which is involved in the breakdown of arachidonic acid, has been implicated in the progression of inflammation to cancer [36]. Some studies have shown this enzyme to be over-expressed in PIN [37] and prostate cancer [37-41]. However, a recent study suggests that over-expression of COX-2 occurs only during inflammation and not during cancer [42]. One group found that neither genetic nor therapeutic inhibition of COX-2 affected prostate carcinogenesis in TRAMP mice [Wang et al, personal communication]. Some genetic mutations in this gene have been shown to affect prostate cancer risk [43] and inhibition of COX-2 has been shown to limit the effects of $\alpha_v\beta_6$, including preventing Rac-1 activation in an oral squamous cell carcinoma cell line [44]. Both COX-2 and ROS have been proposed to explain the progression from inflammation to cancer in many models. Some of the genetic mutations found in prostate cancer also support this theory; for example, lack of expression of the immune response proteins RNASEL and MSR1 can lead to chronic inflammation due to loss of, or alteration of, immune functions [45].

We also show transcriptional upregulation of TGF β 1 in the *in vivo* model of prostatic inflammation. The interaction of TGF β 1 with $\alpha_v\beta_6$ integrin may play a key role in the molecular signaling associated with inflammation and progression to cancer in the prostate gland. TGF β 1 is known to interact with, and become activated by, the $\alpha_v\beta_6$ integrin. This growth factor is also important in inflammation as it is synthesized, and released, by active T-cells [46-48]. TGF β 1

can induce apoptosis and regulate cell growth, as well as function as a tumor suppressor [49]. Other studies have also linked TGF β 1 over-expression to poor clinical prognosis [49]. TGF β 1 can actually support metastasis and tumor invasion [49], thus $\alpha_v\beta_6$ activation of TGF β 1 reveals a possible role for $\alpha_v\beta_6$ in metastasis. A potential correlation between levels of the $\alpha_v\beta_6$ integrin and of activated TGF β 1 in human prostate cancer will need to be addressed in future *in vivo* studies.

Acknowledgements

We would like to thank Dr. Marco Trerotola (Thomas Jefferson University) and Dr. Guoqiang Zheng (University of Massachusetts Medical School) for assistance. We thank Joseph A. Thomas and Genna Viozzi (Thomas Jefferson University) for helping with the preparation of this article. This research was supported by NIH grants: R01 CA-89720, R01 CA-109874, R01 CA-59705, P01 CA140043, R01 DK084454; Our Danny Cancer Funds: P00010003300000, P60010008300000. Research in this publication includes work carried out using the Kimmel Cancer Center Facilities, which are supported in part by NCI Cancer Center Support Grant P30 CA56036. This work was also supported in part by U. Massachusetts Center Grant DK3250 and DK084454.

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