

Integrin A6 Cleavage in Mouse Skin Tumors

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Abstract: We have previously identified a structural variant of the $\alpha 6$ integrin (Laminin receptor) called $\alpha 6p$. The $\alpha 6p$ variant is a 70 kDa form of the full-length $\alpha 6$ integrin (140 kDa) that remains paired with either the $\beta 1$ or $\beta 4$ subunit on the cell surface. $\alpha 6p$ is produced by urokinase-type plasminogen activator (uPA), which removes the extracellular β -barrel domain while the receptor is on the cell surface. The $\alpha 6p$ integrin was present in human prostate cancer tissue but not in normal tissue and the cleavage of the $\alpha 6$ integrin extracellular domain promotes tumor cell invasion and migration on laminin. The objective of the present study was to determine whether the $\alpha 6p$ integrin is observed in other models of carcinogenesis. Our results indicate detectable low levels of $\alpha 6p$ in normal mouse skin, and comparatively elevated levels in mouse papillomas and squamous cell carcinomas induced by DMBA, TPA and MNNG treatments. Furthermore, we have found that $\alpha 6p$ was present at high levels in skin melanomas of transgenic mice that over express activated Ha-ras under the control of the tyrosinase promoter. Finally, subcutaneous injection into athymic nude mice of a malignant mouse keratinocyte derived cell line (6M90) that is $\alpha 6p$ negative, results in the development of tumors that contain $\alpha 6p$ integrin. The latter results indicate that $\alpha 6p$ is induced *in vivo* suggesting that the tumor microenvironment plays a major role in the production of $\alpha 6p$. Taken together, these data suggest that the cell surface cleavage of the $\alpha 6$ integrin may be a novel mechanism of integrin regulation and might be an important step during skin tissue remodeling and during carcinogenesis.

INTRODUCTION

Integrins are heterodimeric cell surface proteins composed of α and β subunits. Each $\alpha\beta$ combination has its own binding specificity and signaling properties [1, 2]. Different integrins recognize different extracellular matrix (ECM) proteins [1, 2]. They are involved in many processes including cell migration, differentiation, blood clotting, tissue organization and cell growth. Integrins generally contain a large extracellular domain (α subunit ~1000 residues, and β subunit ~750 residues), a transmembrane domain and a short cytoplasmic domain (~50 residues or less) with the exception of $\beta 4$, whose cytoplasmic domain is large (more than 1000 residues) [3, 4].

Our previous work has shown that a structural variant of the $\alpha 6$ integrin called $\alpha 6p$ exists [5]. This variant is missing the extracellular β -propeller domain associated with ligand binding [5]. We have also shown that $\alpha 6p$ is produced by proteolytic cleavage of the $\alpha 6$ integrin by Urokinase-type Plasminogen Activator (uPA) [6]. The $\alpha 6p$ integrin is 70 kDa protein, and mass spectrometry analysis showed that the NH₂ terminal end of the molecule contains at least amino acids starting at arginine 595 [5]. Using a multiple sequence alignment tool, this position in the $\alpha 6$ integrin lies within an accessible loop upstream from the genu region described for the αV integrin subunit [7]. Using site-directed mutagenesis we have identified residues R594 and R595 as essential for cleavage [8].

The protease that cleaves the $\alpha 6$ integrin, uPA, is a secreted 54-kDa serine protease which cleaves plasminogen as a primary substrate [9]. uPA has also been shown to catalyze the proteolytic cleavage of the extracellular matrix protein fibronectin [10], hepatocyte growth factor/scatter factor (HGF/SF) [11] and macrophage-stimulating protein (MSP) [12]. The expression of uPA has been shown to be correlated with many cancers. In addition, TPA treatments have been shown to increase uPA levels in cell lines and mouse skin [6, 13, 14]. Moreover, we have previously shown that TPA induces a uPA dependent $\alpha 6$ integrin cleavage in normal breast cells MCF10A [6]. Also, MNNG treatments have been shown to induce uPA expression in different cell lines [15].

The objective of the present study was to determine if $\alpha 6p$ integrin was present in other models of carcinogenesis, to examine if the $\alpha 6p$ integrin is present in mouse skin tumors, and to determine whether $\alpha 6p$ could be induced *in vivo*. We have analyzed normal mouse skin, skin tumors induced by DMBA, TPA and MNNG treatments, tumors from Ha-ras transgenic mice that were treated with DMBA, and tumors from subcutaneous injection of tumorigenic cell lines into athymic nude mice. Our results showed that $\alpha 6p$ is detectable in low levels in normal mouse skin and its expression is elevated in mouse skin tumors. These data suggest that the cleavage of the $\alpha 6$ integrin occurs in epithelial tissues other than prostate and can be induced *in vivo*.

MATERIALS AND METHODOLOGY

Reagents

The DU145 human prostate cancer cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL: Gaithersburg, MD, USA) plus 10% fetal bovine

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serum (FBS) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

The AA6A rabbit polyclonal antibody was raised and purified by Bethyl Laboratories Inc. (Montgomery, TX, USA). AA6A is specific for the last 16 amino acids (CI-HAQPSDKERLTSDA) of the human $\alpha 6$ A sequence [17], as described previously [18] and will recognize full-length $\alpha 6$ as well as full-length $\alpha 3$ due to amino acids QPS. Since $\alpha 3$ is not cleavable, $\alpha 6$ p can be readily detected at 70kD on the western blot. DMBA was purchased from Sigma (Sigma, St. Louis, MO), and TPA from Alexis Corp. (Alexis Corp., San Deigo, CA).

Western Blotting

Protein samples (50ug for tissues or 20ug for cell lines) were diluted in 2X non-reducing sample buffer and the samples were boiled for 5 min, and after a quick chill on ice, they were loaded onto 7.5% SDS-PAGE. Proteins resolved in the gel were electrotransferred to Millipore Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), incubated with primary antibody and after washing with secondary antibody conjugated to horseradish peroxidase and visualized by chemiluminescence (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL, USA).

Mouse Tissue Analyses

A single initiating dose of 100 μ g DMBA (Sigma, St. Louis, MO) in 0.2 ml of acetone was applied topically to the shaved backs of female ICR mice (Harlan Sprague Dawley, Indianapolis, IN). It was followed by bi-weekly application of 5 nM TPA (Alexis Corp., San Deigo, CA) in 0.2 ml of acetone for 21 weeks. At week 22, TPA applications were stopped and replaced with weekly application of 120 μ g MNNG in 0.2 ml of acetone for a total of 40 weeks. For normal tissue, untreated mice were either unshaved and the whole skin was removed and frozen in liquid nitrogen, or they were treated with hair removing cream to remove the hair and the skin was harvested and frozen in liquid nitrogen. The tumors were collected from the treated mice and were frozen in liquid nitrogen. The samples were homogenized by mortar and pestle and lysed in RIPA buffer. The samples were centrifuged at 14000RPM at 4°C for 15 minutes and the supernatants collected and sonicated. The resulting sample (50ug) was analyzed on a 7.5% SDS-PAGE gel, and a Western blot was performed using the AA6A antibody.

The transgenic mice that express an activated Ha-ras gene in their pigmented cells were described previously [19]. Melanoma tumors in different stages from these mice that were treated with DMBA were isolated and protein was extracted using RIPA lysis buffer. The samples were then analyzed by western blotting for the $\alpha 6$ or $\alpha 3$ and $\alpha 6$ p integrins using the AA6A rabbit polyclonal antibody.

The 6M90 cells were injected subcutaneously in athymic nude mice and the tumors were isolated and protein was extracted using RIPA lysis buffer. The samples were then analyzed on an SDS-PAGE gel and the $\alpha 6$ or $\alpha 3$ and $\alpha 6$ p integrins were detected using the AA6A antibody.

RESULTS

Detection of $\alpha 6$ p in Normal Mouse Skin, Papillomas and Squamous Cell Carcinomas

We have previously shown that $\alpha 6$ p exists in human prostate cancer tissue but not in normal prostate tissue. This prompted us to investigate the $\alpha 6$ p expression in other tissues. We first analyzed normal mouse skin isolated from C57BL6 and ICR CD1 mice. We analyzed unshaved skin as a non-treated control and shaved skin that was treated with hair removing cream to remove the hair from mouse backs. Our results in Fig. (1) indicated that normal mouse skin contains low but detectable levels of $\alpha 6$ p integrin.

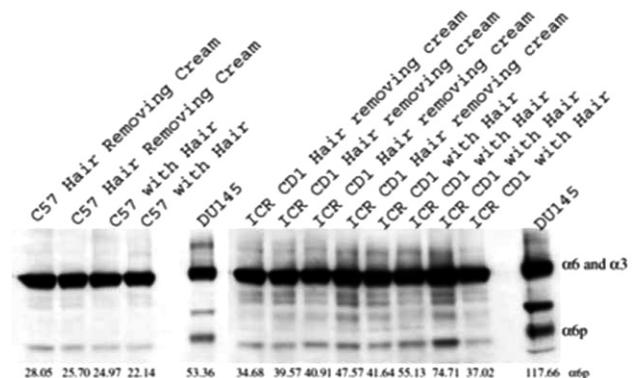


Fig. (1). Detectable expression of $\alpha 6$ p in normal mouse skin. Normal ICR and C57 mouse skin was analyzed for $\alpha 6$ or $\alpha 3$ and $\alpha 6$ p levels by western blotting. Two C57 and four ICR untreated mice were shaved using hair removing cream, and the whole skin was removed and frozen in liquid nitrogen. Another two C57 and four ICR untreated mice were left with the hair and the skin was then removed, and frozen in liquid nitrogen. The samples were processed and analyzed by western blotting. The $\alpha 6$ or $\alpha 3$ and $\alpha 6$ p integrin levels from DU145 cells are shown for comparative purposes. The relative numeric values of densitometric analysis are shown.

Since TPA and MNNG induce uPA expression in other systems, we utilized ICR mice in a tumor promotion model to analyze $\alpha 6$ p levels in resulting tumors (Fig. 2). A single initiating dose of 100 μ g DMBA in acetone was applied topically to the shaved backs of female ICR mice. It was followed by bi-weekly application of 5 nM TPA in acetone for 21 weeks. At week 22, TPA applications were stopped and replaced with weekly application of 120 μ g MNNG in acetone for a total of 40 weeks. The tumors were isolated and first analyzed by H&E staining (data not shown) and classified by a pathologist as papillomas and squamous cell carcinomas (SCC). The tumors isolated from untreated control mice were analyzed by SDS-PAGE analysis for $\alpha 6$ or $\alpha 3$ and $\alpha 6$ p integrin expression (Fig. 2). The results showed that $\alpha 6$ p was present in both papilloma and SCC tissues. Interestingly two of the papilloma tissue samples showed complete conversion of the $\alpha 6$ integrin to $\alpha 6$ p. The presence of $\alpha 6$ p without detecting the full length $\alpha 6$ or $\alpha 3$ has not been observed previously.

$\alpha 6$ p is Present in Melanomas of Transgenic Mice Expressing Activated Ha-Ras

In Fig. (3), we analyzed tumors isolated from transgenic mice that express an activated Ha-ras gene in their pig-

mented cells that were described previously [19]. Melanoma tumors in different stages from these mice that were treated with DMBA were isolated and protein was extracted using RIPA lysis buffer. The results showed that all melanomas tested expressed high $\alpha 6p$ levels.

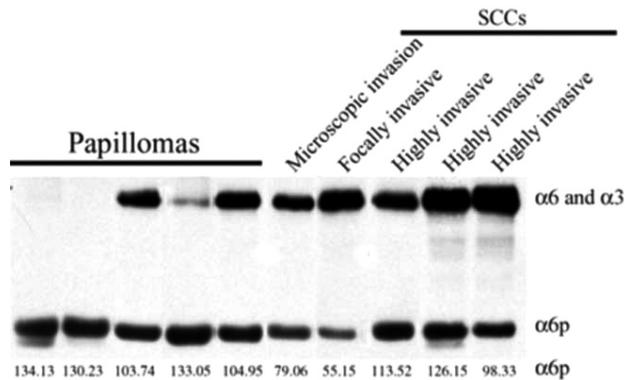


Fig. (2). $\alpha 6p$ is present in mouse skin papillomas and squamous cell carcinomas. Papillomas and SCCs from ICR mice treated topically with a single initiating dose of DMBA, followed by twice weekly application TPA for 21 weeks and finally, at week 22, weekly application of MNNG for a total of 40 weeks, were collected and frozen in liquid nitrogen. The samples were homogenized by mortar and pestle, lysed in RIPA buffer and analyzed by western blotting. The relative numeric values of densitometric analysis are shown.

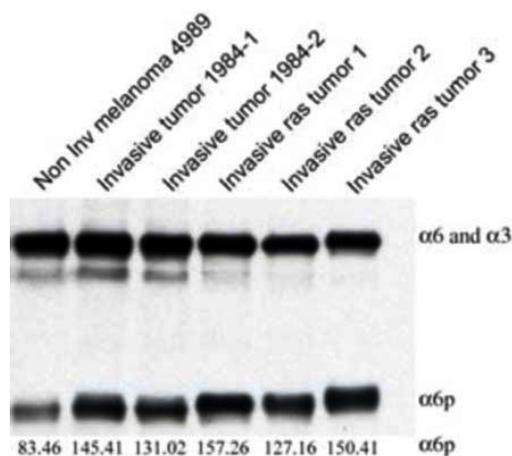


Fig. (3). $\alpha 6p$ is present in melanomas from transgenic mice expressing an activated Ha-ras. Tumors in different stages were treated with DMBA, isolated and protein extracted using RIPA lysis buffer. The samples were then analyzed on an SDS-PAGE gel and the $\alpha 6p$ integrin was detected by western blotting. The relative numeric values of densitometric analysis are shown.

Induction of $\alpha 6p$ In Vivo

The next experiments were designed to determine whether cells growing as a tumor mass within an animal resulted in $\alpha 6p$ production. To accomplish this, we tested whether $\alpha 6p$ could be induced in tumors established by subcutaneous injection of a malignant cell line that does not contain endogenous $\alpha 6p$ under tissue culture conditions. The establishment of the malignant keratinocyte cell line (6M90) was described previously [16]. These cells were injected subcutaneously into athymic nude mice and the resulting

tumors were isolated and protein was extracted using RIPA lysis buffer. In addition, RIPA lysis buffer was used to produce whole cell lysate from the 6M90 cell line. The samples were then analyzed by western blotting. The results showed that $\alpha 6p$ is present in all the tumors but absent in the cell line grown in tissue culture (Fig. 4).

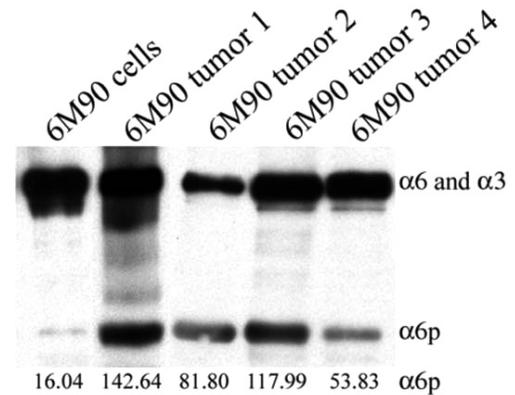


Fig. (4). The $\alpha 6p$ variant is induced *in vivo*. The establishment of the malignant keratinocyte cell line (6M90) was described previously [16]. The 6M90 cells were injected subcutaneously in athymic nude mice and the tumors were isolated and protein was extracted using RIPA lysis buffer. In addition, RIPA lysis buffer was used to produce whole cell lysate from the 6M90 cell line. The samples were then analyzed by western blotting. The relative numeric values of densitometric analysis are shown.

DISCUSSION

Our previous work has shown that the $\alpha 6p$ integrin is produced by uPA cleavage on the cell surface and that this cleavage is important in invasion and migration on laminin [6, 8]. In addition, we have shown that $\alpha 6p$ is present in human prostate cancer tissue [6]. In this study we wanted to determine the $\alpha 6p$ integrin expression in normal mouse skin and skin tumor tissues. Several pieces of evidence indicate that uPA expression is induced after TPA treatments in different cell lines and on mouse skin [6, 13-15]. In addition, MNNG treatment has been shown to induce uPA expression in different cell lines [15, 20, 21]. Collectively, the above evidence led us to investigate the expression of $\alpha 6p$ in a mouse skin carcinogenesis model. Tumors from mice that were treated with DMBA, followed by TPA and then MNNG were analyzed for $\alpha 6p$ expression. The results showed that mice topically treated with DMBA, TPA followed by MNNG developed papillomas and squamous cell carcinomas that contained $\alpha 6p$ in high levels suggesting the involvement of $\alpha 6p$ during carcinogenesis. A very interesting finding was the fact that two of the papillomas showed complete conversion of the $\alpha 6$ integrin to $\alpha 6p$. This phenomenon has never been seen before *in vivo* or *in vitro*. Papillomas can progress, regress or remain stable. The observed variability in full length $\alpha 6$ and $\alpha 3$ expression in these samples could be due to a difference in the probability of progression. In addition, the results showed that $\alpha 6p$ is present at low but detectable levels in normal mouse skin and this suggests that $\alpha 6$ integrin cleavage may be involved in normal skin tissue remodeling. Collectively these data suggest that $\alpha 6p$ may be involved in normal skin tissue remodeling and during skin carcinogenesis.

We then analyzed melanoma tumors from transgenic mice that express an activated Ha-ras gene in their pigmented cells. All the tumors analyzed contained high $\alpha 6p$ levels. These data provide additional support to the emerging view that expression of $\alpha 6p$ is not simply a tissue culture phenomenon and is found expressed in tumor types other than prostate cancer.

Our next objective was to determine whether the tumor microenvironment is important in the production of $\alpha 6p$ *in vivo*. We utilized a mouse keratinocyte cell line, 6M90, that does not express $\alpha 6p$ *in vitro*. Upon subcutaneous injection of the 6M90 cell line into athymic nude mice, the mice developed tumors. Analysis of these tumors by western blotting indicated that $\alpha 6p$ was induced. This suggests that growth of the tumor cells as a tumor mass will result in $\alpha 6p$ production, perhaps through the stimulation of uPA activity within the tumor microenvironment.

Collectively, these data suggest that $\alpha 6p$ may be involved in both normal skin processes and during skin carcinogenesis. It is important to understand the function of $\alpha 6p$ during these processes. Our studies so far with integrin $\alpha 6$ mutants, incapable of cleavage, have implicated $\alpha 6p$ in promoting tumor cell invasion and migration on laminin [8]. More recent work by others has proposed that $\alpha 6$ integrin is an adhesion molecule on progenitor or stem cells [23]. This raises an interesting possibility that cleavage of the ligand binding domain of $\alpha 6$ while preserving heterodimer formation may provide a mechanism of release within a stem cell like compartment. Testing of these ideas awaits further experimentation.

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