Investigation into the Mechanism of the Loss of Laminin 5 ($\alpha 3\beta 3\gamma 2$) Expression in Prostate Cancer

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Laminin 5 is a pivotal hemidesmosomal protein involved in cell stability, migration, and anchoring filament formation. Protein and gene expression of the $\alpha 3$, $\beta 3$, and $\gamma 2$ chains of laminin 5 were investigated in normal and invasive prostate carcinoma using immunohistochemistry, Northern analysis, and *in situ* hybridization. Laser capture microdissection of normal and carcinomatous glands, in conjunction with RNA amplification and reverse Northern analysis, were used to confirm the gene expression data. Protein and mRNA expression of all three laminin 5 chains were detected in the basal cells of normal glands. In contrast, invasive prostate carcinoma showed a loss of $\beta 3$ and $\gamma 2$ protein expression with variable expression of $\alpha 3$ chains. Despite the loss of protein expression, there was retention of $\beta 3$ and $\gamma 2$ mRNA expression as detected by *in situ* hybridization, Northern and reverse Northern analysis. Our findings imply that an altered mechanism of translation of $\beta 3$ or $\gamma 2$ mRNAs into functional proteins contributes to failure of anchoring filaments and hemidesmosomal formation. The resultant hemidesmosome instability or loss would suggest a less stable epithelial-stromal junction, increased invasion and migration of malignant cells, and disruption of normal integrin signaling pathways. (*Am J Pathol* 2001, 158:1129–1135)

Laminins are components of the extracellular matrix that contribute to the architecture of the basal lamina (BL), and mediate cell adhesion, growth, migration, proliferation, and differentiation.¹ These molecules are heterotrimers consisting of α , β , and γ chains. Given the existence of 11 genetically distinct chain forms ($\alpha 1$ to $\alpha 5$, $\beta 1$ to $\beta 3$, and $\gamma 1$ to $\gamma 3$),^{2–4} different combinations of chains could result in up to 45 different heterotrimeric isoforms. Only 13 laminin isoforms (laminins 1, 2, 4 to 12, 14, 15), however, have thus far been convincingly demonstrated.^{3–6} Some laminins, such as laminin 10 ($\alpha 5\beta 1\gamma 1$), are ubiquitously present in BL, whereas other laminins demonstrate considerable tissue specificity. Laminin 5 ($\alpha 3\beta 3\gamma 2$), for example, is restricted to

the BL of stratified and certain other epithelia, and is one of the primary hemidesmosomal proteins.² The N-terminus of the $\alpha 3$ chain serves as the collagen VII attachment site for anchoring fibril formation.⁷ The globular domain of the laminin 5 $\alpha 3$ chain is the putative binding site for the $\alpha 6\beta 4$ and $\alpha 3\beta 1$ integrin receptors. The $\alpha 6\beta 4$ integrin/laminin 5 complex is essential for signal transduction.^{8–11} In addition to monomeric molecules, laminin 5 is frequently found covalently associated with laminin 6 ($\alpha 3\beta 1\gamma 1$) and laminin 7 ($\alpha 3\beta 2\gamma 1$).¹²

Direct evidence for the crucial role of laminin 5 in maintaining the integrity of the BL has come from the identification of mutations in the laminin 5 genes observed in the Herlitz's variant of junctional epidermolysis bullosa, a blistering and usually lethal skin disease caused by disruption of the epidermal-dermal junction. In Herlitz's variant of junctional epidermolysis bullosa, genetic disruptions associated with the observed pathology have been associated with the presence of premature stop codons, or frameshift mutations on both alleles of any of the three genes encoding the chains of laminin 5.^{13,14} Mutations resulting in the failure of expression of any of these three chains results in a complete absence of laminin 5 immunoreactivity and to ultrastructural changes in hemidesmosomes.¹⁵

Laminin 5 has revealed variable patterns of expression in tumors derived from different tissues. In gliomas and carcinomas of the colon, stomach, and squamous epithelium, laminin 5 has been observed to be highly expressed and located at the invasive edge of the tumor.^{16–19} In sharp contrast, laminin 5 expression is down-regulated in basal cell and squamous carcinomas, as well as in carcinomas of breast and prostate.^{20–25}

We have shown that the BL circumscribing normal prostate glands differs from the BL surrounding prostate carcinoma in that several extracellular matrix proteins are not detected in carcinoma.^{26,27} Prostate tumor progression probably involves changes that occur within the *de novo* synthesized extracellular matrix of the BL of prostate carcinoma. These changes include the loss of laminin 5 expression,^{23,26} which leads to cytoplasmic membrane instability of its integrin receptor $\alpha 6\beta 4$,²⁸ and to alterations in cell signaling. We have shown a simultaneous loss of laminin 5,

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Table 1. Antibodies Used in this Study

Specificity	Clone	Type	Dilution	Source
$\alpha 3$ of L5	BM2(BM165)	mAb	1:100	Dr. R.E. Burgeson, Harvard Medical School
$\beta 3$ of L5	6F12(BM140)	mAb	1:100	Dr. R.E. Burgeson, Harvard Medical School
$\gamma 2$ of L5	GB3	mAb	1:15	Accurate Chemical & Scientific Corp
$\gamma 2$ of L5	J20	Polyclonal	1:100	Dr. Jonathan Jones, Northwestern University, Chicago, IL
$\gamma 2$ of L5	D4B5	mAb	1:100	Chemicon International, Inc.

collagen VII, and $\beta 4$ -integrin protein expression in prostate carcinoma.²⁸ We conducted this study to clarify the mechanism explaining the loss of laminin 5 protein expression in prostate carcinoma. Immunohistochemistry, Northern analysis, and *in situ* hybridization were used to investigate gene expression. Moreover, individual selected normal and carcinoma glands underwent LCM. RNA isolated from these glands was amplified and examined by reverse Northern analysis.

Materials and Methods

Immunohistochemistry

Freshly obtained surgical samples of normal and malignant human prostate tissue were snap-frozen in an isopentane bath cooled by Freon, sectioned, and examined using hematoxylin and eosin (H&E) staining to select normal areas and invasive carcinoma. Frozen 5- μ m sections of human prostate samples containing both normal tissue and carcinomas were reacted with primary antibodies followed by binding with secondary antibodies conjugated to biotin. Streptavidin-diaminobenzidine was subsequently used to detect the antibody complex. Sources and dilutions of antibodies against the $\alpha 3$, $\beta 3$, and $\gamma 2$ chains of laminin 5 are listed in Table 1.

Cell Culture

Normal human prostate epithelial cells (PrE4428) were obtained from Clonetics Corp. (San Diego, CA). The cultured cells were maintained in PrEGM medium from Clonetics Corp. at 37°C in a humidified atmosphere of 95% air, 5% CO₂.

Preparation of Riboprobes

To construct sense and antisense probes for the $\alpha 3$, $\beta 3$, and $\gamma 2$ chains of laminin 5, chain-specific cDNA clones (Table 2) were subcloned into the pBluescript SKII transcription vector (Stratagene, La Jolla, CA). The se-

quences of the subclones were confirmed by DNA sequencing. The plasmids were then linearized and sense and antisense probes were synthesized using either T3 or T7 RNA polymerase, respectively. For *in situ* hybridization, the antisense and sense probes were labeled with digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN) as previously described.²³

In Situ Hybridization

In situ hybridization with digoxigenin-labeled RNA probes was performed as previously described.²³

Northern Analysis

Total RNA was isolated from PrEC4428 cells as well as from microdissected areas of primary prostate carcinoma and adjacent normal prostate from three different patients. RNA was isolated using TRIzol reagent (Life Technologies, Inc, Gaithersburg, MD). Approximately 20 μ g of total RNA was loaded in each lane, separated on a 1.2% agarose/formaldehyde gel, and transferred to nylon membrane (Life Technologies, Inc.). The blots were probed with α^{32} P-dCTP-labeled cDNA probes specific for the $\beta 3$ and $\gamma 2$ chains of laminin 5, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Total RNA from PrEC4428 was used as a positive control for probe specificity, and GAPDH hybridization was used as a loading control. Hybridization was performed at 42°C in 50% formamide, 2 $\times$ standard saline citrate (SSC), 5 $\times$ Denhardt's, and 20 μ g/ml of salmon sperm DNA. Post-hybridization washes were performed in 2 $\times$ SSC/0.1% sodium dodecyl sulfate (SDS) at 50°C for 30 minutes, followed by 0.1 $\times$ SSC/0.1% SDS at 55°C for 30 minutes.

Laser Capture Microdissection (LCM) and Reverse Northern Analysis LCM and RNA Isolation

Five-micron cryostat sections of snap-frozen prostate tissue samples were applied to positively charged glass

Table 2. Probes Used in this Study

cDNA clones	Specificity	Regions as probes	Sources
EP-1	$\alpha 3$ of Lm-5	1183–1701 bp	Dr. M.C. Ryan, Seattle, WA
5-5	$\beta 3$ of Lm-5	–171–2202 bp	Dr. D. Gerecke, Charlestown, MA
92-1	$\beta 3$ of Lm-5	2005–3751 bp	Dr. D. Gerecke, Charlestown, MA
pL4	$\gamma 2$ of Lm-5	583–2527 bp	Dr. K. Tryggvason, Oulu, Finland

Figure 1. Differential expression of laminin 5 chains by human prostate. **A–C:** Immunohistochemical staining using mAb BM165 ($\alpha 3$), BM140 ($\beta 3$), and polyclonal antibody J20 ($\gamma 2$). Note in **A** the protein for the $\alpha 3$ chain of laminin 5 is retained by both normal prostate epithelium (n) and variably by prostate carcinoma (c), but the $\beta 3$ and the $\gamma 2$ chains were not detected in invasive carcinoma (**B** and **C**). Original magnification, $\times 100$.

slides. Sections were stained by H&E according to slightly modified National Institutes of Health protocols (<http://www.arctur.com> for hematoxylin and eosin staining protocols; <http://dir.nichd.nih.gov/lcm/LCMTAP.htm> for LCM preparation and analysis). Laser capture of target cells was performed using the PixCell II image archiving system (Arcturus Engineering, Mountain View, CA) as previously described.²⁹ Total RNA was extracted from laser-captured cells using the Micro RNA isolation kit (Stratagene, San Diego, CA).³⁰ Image archiving and LCM recovery of ~ 1500 cells per sample from prostatic carcinoma and adjacent normal glandular epithelium was found to provide sufficient mRNA to detect, using two rounds of amplification.

RNA Amplification and Reverse Northern Blot Analysis

The purified RNA sample underwent amplification using $0.5 \mu\text{g}/\mu\text{l}$ of T7-oligo dT primer, Superscript II reverse transcriptase (Life Technologies, Inc.), and Ampliscribe T7 transcription kit (Epicentre Technologies, Madison, WI) as previously described.³⁰ Ten μl of purified, resultant amplified RNA (aRNA) underwent second-round amplification using pdN6 random hexamers ($1 \mu\text{g}/\mu\text{l}$; Pharmacia, Piscataway, NJ) during first-strand synthesis, and T7-oligo dT primer for the second-strand reaction, as previously described.³⁰

Second-round aRNA specimens were synthesized into randomly labeled $\alpha^{32}\text{P}$ -dCTP probes using pdN6 random

hexamers ($1 \mu\text{g}/\mu\text{l}$; Pharmacia) and the Superscript pre-amplification system reagents (Life Technologies, Inc.) as previously described.³¹ Target cDNA ($0.5 \mu\text{g}$) was cross-linked to the blot membrane with a UV Stratalinker 2400 (Stratagene). The positive control for each blot consisted of $0.5 \mu\text{g}$ of GAPDH. The negative control was single-strand antisense laminin 5 $\beta 3$ cDNA.

Reverse Northern blots were performed as previously described.³¹ In brief, 500 ng of target cDNAs including controls were denatured, combined with $111 \mu\text{l}$ of sterile water, $80 \mu\text{l}$ of 1 mol/L NaOH, and $4 \mu\text{l}$ of 0.5 mol/L ethylenediaminetetraacetic acid, and cross-linked to an Ambion Bright Star Plus positively charged nylon membrane (Ambion Inc., Austin, TX) using a Minifold II slot-blot apparatus (Schleicher and Schuell, Keene, NH). The membranes were prehybridized at 68°C for 45 minutes in Perfect Hyb Plus solution (Sigma, St. Louis, MO). Radio-labeled cDNA probe was denatured at 100°C for 10 minutes, added to the prehybridization mixture, and hybridized overnight at 68°C in Perfect Hyb Plus solution. The membrane was washed twice for 20 minutes each in $2\times \text{SSC}/0.1\% \text{SDS}$ at 68°C for 15 minutes. Higher stringency washes were performed in sequence as necessary to remove background signals from the membrane, including two $0.2\times \text{SSC}/0.1\% \text{SDS}$ washes at 68°C for 15 minutes, and two $0.1\times \text{SSC}/0.1\% \text{SDS}$ washes at 68°C for 15 minutes. The blots were then exposed on phosphorimaging screens (Molecular Dynamics, Sunnyvale, CA). Each blot was measured and plotted with a Phosphorim-

Figure 2. *In situ* hybridization using chain-specific laminin 5 probes. *In situ* hybridization was performed using digoxigenin-labeled antisense RNA probes of the laminin 5. **A:** $\alpha 3$. **B:** $\beta 3$. **C:** $\gamma 2$. The mRNAs of the three chains are present in normal glands (n) as well as in the invasive carcinoma (c). **D:** Higher magnification of the area of C designated by the **black box** showing that the level of expression of the $\gamma 2$ message appears to be higher in the carcinoma cells than in normal glandular epithelium. Original magnifications, $\times 100$ (A–C) and $\times 400$ (D).

age 445SI scanner (Molecular Dynamics). Blots were stripped and reprobed up to four times.

Results

Laminin 5 Protein Expression

Twenty-five surgical specimens containing invasive carcinomas and adjacent nonmalignant glands were examined by immunohistochemistry. Monoclonal antibodies BM165 and 6F12, and polyclonal antibody J20 enabled the detection of $\alpha 3$, $\beta 3$, and $\gamma 2$ chains of laminin 5, respectively. An additional five cases were stained with the g2 chain-specific antibody (Chemicon,

Figure 3. Northern analysis of laminin 5 $\beta 3$ and $\gamma 2$ chain expression in human prostate. Total RNA was isolated from the normal prostate epithelial cell line, PrEC 4428, and from three cases of prostate carcinoma and adjacent normal tissue. The Northern blot was probed with ^{32}P -dCTP-labeled cDNA probes specific for the $\beta 3$ and $\gamma 2$ chains of laminin 5, and GAPDH. Total RNA from normal prostate epithelial cell line, PrEC 4428, was used as positive control, and GAPDH hybridization was used as a loading control. Northern analysis confirmed the presence of the $\beta 3$ and $\gamma 2$ mRNAs of laminin 5 in both normal prostate and prostate carcinomas.

Temecula, CA). The results showed variable protein expression of the $\alpha 3$ chain in both normal and neoplastic prostate epithelium, but the $\beta 3$ and $\gamma 2$ chains were not detected in invasive carcinoma (Figure 1).

Laminin 5 mRNA Expression

In situ hybridization experiments performed on six cases of prostate carcinoma showed the presence of mRNAs for the $\alpha 3$, $\beta 3$, and $\gamma 2$ chains of laminin 5. The mRNAs were present in all of the carcinomas studied, as well as in the basal cells of normal glands (Figure 2; A–C). The level of expression of the $\gamma 2$ message seemed to be higher in the carcinoma cells than in normal glandular epithelium (Figure 2, C and D). Adjacent serial sections reacted with control sense probes were consistently negative. Northern analysis confirmed the presence of the $\beta 3$ and $\gamma 2$ mRNAs of laminin 5 in both normal prostate and prostate carcinomas (Figure 3).

Samples of normal glands from two patients and malignant glands from five patients with adenocarcinoma of the prostate were isolated by LCM (Figure 4). Figure 5A presents a representative reverse Northern blot of one case of malignant tissue. Figure 5B summarizes the quantitation of cDNA band phosphor intensities from the two normal samples (N1 and N2) and five malignant samples (C1 to C5). Individual band

Figure 4. LCM of prostate carcinoma. Laser capture of target cells was performed using the PixCell II image archiving system on 5- μ m cryostat sections of snap-frozen prostate tissue stained by H&E. **A:** H&E-stained section before LCM. **B:** Target areas identified as carcinoma by H&E staining are designated by **black boxes**. Normal glands targeted for capture are surrounded by **white boxes**. **C:** White areas of H&E section are the holes left after carcinoma glands have been laser-captured and then transferred to cap surface (**D**). Original magnification, $\times 100$.

intensities were expressed as a percentage of the total intensity of all seven possible bands on a single blot. Standardized amounts of target cDNA and identical conditions for probe and target cDNA production enabled band intensity data to be used as relative indicators of the amount of mRNA present in the laser-captured samples before amplification.

Inspection of this data revealed that mRNAs for the laminin 5 $\alpha 3$, $\beta 3$, and $\gamma 2$ chains, $\alpha 6$ and $\beta 4$ integrins, and GAPDH were present in both the normal and malignant prostate glands studied. These findings were consistent with *in situ* and Northern data. The data also shows that the band intensities of laminin 5 chains and $\alpha 6$ integrin were comparable between normal and malignant tissue samples, suggesting that each maintained a low level of synthesis of these chains *in vivo* at the time of tissue sampling. The interesting exception was the $\beta 4$ integrin that exhibited consistently higher band intensity than the other cDNAs. These results were substantiated by the dominant intensity of the positive control GAPDH, a ubiquitous housekeeping gene, in each blot. Negative control findings were also significant, because they indicated that data distortion because of nonspecific binding of radiolabeled materials on each blot had minimal effect on sample measurements.

Discussion

Prostate cancer is the most common visceral neoplasm in men, and a major health concern of the aging male population. The mechanism of initiation and progression of this disease is not well understood, but the progression is highly variable and is associated with alterations in the composition of the prostatic extracellular matrix. We have shown previously that the BL circumscribing normal prostate glands differs from the BL surrounding prostate carcinoma.^{23,26–28} There seems to be a simultaneous loss of laminin 5, collagen VII, and $\beta 4$ -integrin protein expression in prostate and basal cell carcinoma.^{24,28,32–35} These findings suggest that $\alpha 6\beta 4$ integrin expression may be dependent on the presence of its extracellular ligand. The loss of laminin 5 expression may be functionally important for tumor progression in the prostate and could lead to cytoplasmic membrane instability of its integrin receptors $\alpha 3\beta 1$ and $\alpha 6\beta 4$ and altered signaling.²⁸

This study used four independent techniques to explore the mechanism of loss of laminin 5 expression in the prostate. Immunohistochemistry showed that the protein for the $\alpha 3$ chain of laminin 5 is variably expressed by both normal and neoplastic prostate epithelium. The $\beta 3$ and $\gamma 2$ chains, however, were not detected in invasive carcinoma. It is known that the $\alpha 3$ chain can persist by trimer

formation with the $\beta 1$ or $\beta 2$ and $\gamma 1$ laminin chains (laminin 6 or 7) in the absence of $\beta 3$ and $\gamma 2$,¹² which most likely explains the continuing expression of the $\alpha 3$ chain observed in this study.

Despite the absence of the $\beta 3$ and $\gamma 2$ proteins, the carcinoma cells expressed substantial amounts of both messages for these genes. The $\beta 3$ and $\gamma 2$ mRNAs were detected by *in situ* hybridization and Northern analysis, and their presence in neoplastic glands was confirmed using LCM coupled with RNA amplification and reverse Northern analysis. Although we reported that certain prostate cell lines synthesize but fail to secrete individual laminin chains,³⁶ at that time we did not examine specific laminin 5 chains. Recently, we have shown that LNCaP cells fail to synthesize the $\beta 3$ chain of laminin 5 (unpublished result). These data suggest that human prostate carcinoma cells exhibit posttranscriptional defect(s) in protein translation of the $\beta 3$ and $\gamma 2$ chains of laminin 5.

The loss of laminin 5 is not simply because of the loss of basal cells because the mRNAs for the $\beta 4$ integrin and the three chains of laminin 5 are made by the carcinoma cells. The loss of laminin 5 is not universal because other tumors continue to express laminin 5 chains.^{16–21} Several studies

have correlated prognostic significance of laminin 5 $\gamma 2$ chain expression in tumors.^{25,37} Ono and colleagues²⁵ have shown that laminin 5 $\gamma 2$ chain expression was a significant factor associated with poor prognosis in squamous cell carcinoma of the tongue. The amount of the $\gamma 2$ chain expressed in pancreatic adenocarcinomas, however, was not associated with the patients' prognosis.³⁷ The $\gamma 2$ chain of laminin 5 has been shown to be up-regulated in gliomas and colonic, gastric, and squamous cell carcinomas.³³ In contrast, decreased expression of the $\gamma 2$ chain has been observed in other types of carcinomas.^{22–24,26,33} The $\gamma 2$ chain of laminin 5 has in fact been proposed as a marker of increased invasiveness of certain tumor types.¹⁸ The nature of the laminin 5 defect in prostate carcinoma is currently unknown. It is apparent that prostate carcinoma is different from other tumors in which $\gamma 2$ expression is up-regulated. Whether the loss of $\gamma 2$ expression in prostate carcinoma explains its slow progression remains to be studied. Invasive prostate cancer creates a new BL and presumably, reestablishes survival signaling contact through $\alpha 6 \beta 1$ and other adhesion molecules, which react with the components of the *de novo* synthesized BL. The nature of these signaling pathways and their effects on gene transcription are unknown but will require further investigation and will possibly reveal new therapeutic applications.

One question addressed by this study is whether or not the loss of protein expression in prostate carcinoma is a posttranscriptional event. Analysis of mRNAs and proteins from carcinoma and adjacent normal glands, supports the idea that constitutive production of mRNA occurs, but with altered translation into protein under conditions of malignancy. The loss of laminin 5 expression in prostate carcinoma apparently is a posttranscriptional event. There are several ways in which translation may be affected including mutations that result in premature stop codons or frame-shifts, or failure of initiation or elongation of protein synthesis. Interestingly, the presence of a defect in an elongation factor in prostate carcinoma has been found,^{38,39} suggesting a more general defect in protein translation. Regardless of the mechanism, the altered translation of $\beta 3$ or $\gamma 2$ mRNAs into functional proteins contributes to failure of anchoring filament and hemidesmosomal formation. The resulting hemidesmosome loss would predict a less stable epithelial-stromal junction, increased invasion and migration of malignant cells, and disruption of normal integrin signaling pathways.

In summary, protein and mRNA expression of the $\alpha 3$, $\beta 3$, and $\gamma 2$ chains of laminin 5 were investigated in normal prostate and invasive prostate carcinoma using immunohistochemistry, *in situ* hybridization, LCM, and Northern and reverse Northern analysis. Protein and mRNA expression of all three laminin 5 chains were detected in the basal cells of normal glands. In contrast, invasive prostate carcinoma showed a loss of $\beta 3$ and $\gamma 2$ protein expression with variable expression of $\alpha 3$ chains, but retention of $\beta 3$ and $\gamma 2$ mRNAs as detected by *in situ* hybridization, and Northern and reverse Northern analysis. The loss of laminin 5 protein expression in prostate carcinoma thus seems to be a posttranscriptional event.

Figure 5. Reverse Northern blot of laser-captured prostate glands. **A:** Reverse Northern slot blot of one representative case of malignant prostate tissue. Probe samples were obtained by laser-captured microdissection of ~1500 cells from sections of snap-frozen prostate tissue. GAPDH and antisense laminin 5 $\beta 3$ sequences served as positive and negative controls, respectively. Band intensity serves as a relative indicator of the amount of message RNA present in the laser-captured sample before amplification. **B:** Summary of reverse Northern blot data showing comparative quantitation of cDNA probe phosphor intensities of normal (NL) and carcinoma (Ca). Two representative normal samples (N1 and N2) and five malignant samples (C1 to C5) are arranged by individual (rows) and type of target cDNA (columns). Individual band intensities are expressed as a percentage of the intensity of all seven possible bands on the blot from which it came.

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