Laminin 5 is a pivotal hemidesmosomal protein involved in cell stability, migration, and anchoring filament formation. Protein and gene expression of the α3, β3, and γ2 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 β3 and γ2 protein expression with variable expression of α3 chains. Despite the loss of protein expression, there was retention of β3 and γ2 mRNA expression as detected by in situ hybridization, Northern and reverse Northern analysis. Our findings imply that an altered mechanism of translation of β3 or γ2 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)

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collagen VII, and β4-integrin protein expression in prostate carcinoma. 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-μm 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 α3, β3, and γ2 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 α3, β3, and γ2 chains of laminin 5, chain-specific cDNA clones (Table 2) were subcloned into the pBluescript SKII transcription vector (Stratagene, La Jolla, CA). The sequences 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.

**In Situ Hybridization**

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

**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 μg 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 α32P-dCTP-labeled cDNA probes specific for the β3 and γ2 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× standard saline citrate (SSC), 5× Denhardt’s, and 20 μg/ml of salmon sperm DNA. Post-hybridization washes were performed in 2× SSC/0.1% sodium dodecyl sulfate (SDS) at 50°C for 30 minutes, followed by 0.1× 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...
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/LCM(TAP).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. Total RNA was extracted from laser-captured cells using the Micro RNA isolation kit (Stratagene, San Diego, CA). 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 μg/μl of T7-oligo dT primer, Superscript II reverse transcriptase (Life Technologies, Inc.), and Ampliscribe T7 transcription kit (Epicentre Technologies, Madison, WI) as previously described. Ten μl of purified, resultant amplified RNA (aRNA) underwent second-round amplification using pdN6 random hexamers (1 μg/μl; Pharmacia) and the Superscript pre-amplification system reagents (Life Technologies, Inc.) as previously described. Target cDNA (0.5 μg) was cross-linked to the blot membrane with a UV Stratalinker 2400 (Stratagene). The positive control for each blot consisted of 0.5 μg of GAPDH. The negative control was single-strand antisense laminin 5 β3 cDNA.

Reverse Northern blots were performed as previously described. In brief, 500 ng of target cDNAs including controls were denatured, combined with 111 μl of sterile water, 80 μl of 1 mol/L NaOH, and 4 μl 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). Radiolabeled 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× SSC/0.1% 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× SSC/0.1% SDS washes at 68°C for 15 minutes, and two 0.1× SSC/0.1% 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 1.** Differential expression of laminin 5 chains by human prostate. A–C: Immunohistochemical staining using mAb BM165 (α3), BM140 (β3), and polyclonal antibody J20 (γ2). Note in A the protein for the α3 chain of laminin 5 is retained by both normal prostate epithelium (n) and variably by prostate carcinoma (c), but the β3 and the γ2 chains were not detected in invasive carcinoma (B and C). Original magnification, ×100.
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 $\gamma_2$ chain-specific antibody (Chemicon, 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
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.\(^\text{23,26–28}\) There seems to be a simultaneous loss of laminin 5, collagen VII, and \(\beta_4\)-integrin protein expression in prostate and basal cell carcinoma.\(^\text{24,29,32–35}\) 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.\(^\text{28}\)

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 β1 or β2 and γ1 laminin chains (laminin 6 or 7) in the absence of β3 and γ2,18 which most likely explains the continuing expression of the α3 chain observed in this study.

Despite the absence of the β3 and γ2 proteins, the carcinoma cells expressed substantial amounts of both messages for these genes. The β3 and γ2 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 β3 chain of laminin 5 (unpublished result). These data suggest that human prostate carcinoma cells exhibit posttranscriptional defect(s) in protein translation of the β3 and γ2 chains of laminin 5.

The loss of laminin 5 is not simply because of the loss of basal cells because the mRNAs for the β4 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 γ2 chain expression in tumors.25,37 Ono and colleagues25 have shown that laminin 5 γ2 chain expression was a significant factor associated with poor prognosis in squamous cell carcinoma of the tongue. The amount of the γ2 chain expressed in pancreatic adenocarcinomas, however, was not associated with the patients’ prognosis.37 The γ2 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 γ2 chain has been observed in other types of carcinomas.22–24,26,33 The γ2 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 γ2 expression is up-regulated. Whether the loss of γ2 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 α6β1 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 β3 or γ2 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 α3, β3, and γ2 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 β3 and γ2 protein expression with variable expression of α3 chains, but retention of β3 and γ2 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.
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