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Laminin-511, inducer of hair growth, is down-regulated and its suppressor in hair growth, laminin-332 up-regulated in chemotherapy-induced alopecia

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Abstract

Background—Chemotherapy-induced alopecia (CIA) has a devastating cosmetic effect, especially in the young. Recent data indicate that two major basement membrane components (laminin-332 and -511) of the skin have opposing effects on hair growth.

Objective—In this study, we examined the role and localization of laminin-332 and -511 in CIA.

Methods—We examined the expression of laminin-332 and -511 during the dystrophic catagen form of CIA induced in C57BL/6 mice by cyclophosphamide (CYP) treatment.

Results—Our data indicate that both laminin-332 and its receptor $\alpha 6\beta 4$ integrin are up-regulated (both quantitatively and spatially) after mid to late dystrophic catagen around the outer root sheath (ORS) in the lower third of hair follicles in CIA. This up-regulation also occurs at the transcriptional level. In contrast, laminin-511 is down-regulated after mid dystrophic catagen at the protein level, with transcriptional inactivation of laminin-511 occurring transiently at the early dystrophic catagen stage in both epidermal and ORS keratinocytes. Laminin-511 expression correlates with expression of $\alpha 3$ integrin in CIA and we also demonstrate that laminin-511 can up-regulate the activity of the $\alpha 3$ integrin promoter in cultured keratinocytes. Injection of a laminin-511 rich protein extract, but not recombinant laminin-332, in the back skin of mice delays hair loss in CYP-induced CIA.

Conclusions—We propose that abrupt hair loss in CIA is, at least in part, caused by down-regulation of laminin-511 and up-regulation of laminin-332 at the transcriptional and translational levels.

Keywords

A549 cells; Chemotherapy; Extracellular matrix; Hemidesmosome; Integrin; Outer root sheath cells

1. Introduction

Chemotherapy-induced alopecia (CIA) occurs frequently in patients using anticancer agents [1]. For example, the incidence of CIA in patients treated with paclitaxel (PTX), docetaxel (TXT) and etoposide (VP-16) is 87.7, 78.4, and 75.7% respectively [2]. These chemotherapeutic drugs are known to lead to hair loss in anagen (anagen effluvium) by inducing apoptosis of hair matrix cells [3–6].

Previous studies have identified two distinct chemotherapy-induced hair follicle dystrophies, termed the dystrophic catagen and the dystrophic anagen pathways [7]. The dystrophic catagen pathway occurs in response to a higher dose of chemotherapeutic drugs than the dystrophic anagen pathway. Hair follicles that have progressed along the dystrophic catagen pathway exhibit abnormal distributions of melanin pigments. The hair roots in the affected follicles taper off and are without the typical club-shaped morphology [8]. The dystrophic anagen pathway is triggered by lower doses of chemotherapeutic drugs and consequently results in a milder impairment in hair elongation. During the dystrophic anagen pathway the hair shaft is shed and the follicle undergoes a so-called “incomplete recovery”. The follicle regenerates but with a faulty hairshaft and then undergoes a complete catagen-telogen transition to enter a “secondary recovery” phase [9]. In the latter pathway, miniaturization of the hair, atrophy of hair matrix, and abnormality in the distribution of melanin pigments have been observed [8].

A number of procedures and reagents have been used in attempts to ameliorate the effects of CIA. These include scalp tourniquets, scalp cooling, scalp hypothermia and treatment with tocopherol [10–12] and minoxidil [13–17]. Unfortunately, the success of these treatments is quite limited. Thus, there remains a need for new therapies for patients afflicted with CIA. The development of such new therapies would be facilitated by a better understanding of the molecular mechanisms that underlie hair loss in CIA. To this end we have analyzed the expression of certain extracellular matrix proteins in a mouse model of CIA. In our study, we focused on laminin-332 and -511 since there is accumulating evidence that these laminin heterotrimers are key regulators of hair growth [18]. Specifically, it has been reported that the skin of day 16.5 embryonic mice lacking the $\alpha 5$ subunit of laminin-511 contains fewer hair germs compared to wild type controls [19]. In addition, Gao et al. [20] have presented data indicating that laminin-511 is essential for the transduction of crucial morphogenetic signals that regulate ciliary function and dermal papilla cell maintenance during hair downgrowth. Moreover, we have presented evidence that laminin-511 supports hair growth while laminin-332 antagonizes such effects in an in vitro model [18]. The data we present here indicate that chemotherapeutic agents affect the dynamics of laminin-511 and -332 in the hair growth cycle. Furthermore, our data indicate that intradermal injection of laminin-511 may prevent CIA.

2. Materials and methods

2.1. Antibodies

The rabbit polyclonal antiserum termed J18 generated against intact rat laminin-332, rabbit polyclonal antiserum prepared against mouse recombinant $\alpha 5$ laminin, and a rabbit polyclonal

antiserum against mouse $\alpha 3$ integrin have been described by others [21–23]. J18 recognizes rat, human and mouse laminin-332 but in mouse it appears to preferentially recognize the $\gamma 2$ subunit. In this regard, the homology of laminin-332 subunits in mouse and rat is as follow: $\alpha 3$ 90%, $\beta 3$ 93%, $\gamma 2$ 90%. Anti-human $\alpha 5$ laminin antibody (4C7) and anti-human $\beta 1$ laminin antibody were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). 4C7 recognizes the G domain of the laminin $\alpha 5$ subunit [24]. 4C7 recognizes human but not mouse protein consistent with the homology of the human and mouse laminin $\alpha 5$ subunit G domain being only 78%. The rat anti- $\gamma 1$ laminin monoclonal antibodies were obtained from Millipore (Billerica, MA). Anti-human laminin $\alpha 1$ antibody was purchased from R&D Systems (Minneapolis, MN). A rabbit polyclonal serum against human laminin 5 (Abcam, MA) was used for detection of recombinant human laminin-332 which was injected into the back skin of CIA mice. Rat anti-mouse $\beta 4$ integrin MAb was obtained from BD Pharmingen (San Diego, CA). Monoclonal rat anti-mouse Ki-67 antigen was purchased from Dako (Carpinteria, CA).

Horseradish peroxidase (HRP)-conjugated goat anti-rat IgG antibody was obtained from Millipore (Billerica, MA). Horse anti-goat IgG antibody conjugated with HRP was from VECTOR Laboratory (Burlingame, CA). FITC-conjugated goat anti-rat IgG and HRP-conjugated goat anti-rabbit IgG were purchased from Zymed Laboratories (South San Francisco, CA). Alexa Fluor 594-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse IgG were obtained from Molecular Probes (Eugene, OR).

2.2. C57BL/6 CIA mouse model and determination of hair growth stage

We followed a method previously reported by Hendrix et al. [9]. Briefly, anagen was induced by the depilation of the back hair in 7-week-old female mice (purchased from Japan SLC). In this treatment, synchronized hair growth was induced as previously described. Then, 9 days after depilation (anagen stage VI), 150 mg/kg body weight of cyclophosphamide, kindly provided by Shionogi pharmaceutical company, was injected peritoneally. Back skin samples were then collected on days 0–7 after the injection of drug. We analyzed tissue from at least three animals for each time point.

Tissue samples were dissected out, divided into four pieces, and then processed for paraffin embedding, cryosectioning, protein analyses, or real-time quantitative RT-PCR. The CIA stage was assessed in HE sections according to the procedure described by Hendrix et al. [9]. Briefly, samples with the following characteristics were associated with early dystrophic catagen; melanin clumps in the bulb IRS, ORS and proximal CTS, condensed onion-shaped or ball-like dermal papilla, more than 10 apoptotic cells in the hair bulb, with remnant of hair shaft in the proximal hair canal, widened distal hair canal, follicular distortion and smaller distal hair bulb. Samples with the following characteristics were associated with mid dystrophic catagen; melanin clumps in the bulb IRS, ORS and proximal CTS, round, ball-shaped dermal papilla, remnant of hair shaft in the proximal hair canal, small CTS tail proximal of dermal papilla, widened distal hair canal, follicular distortion and no club hair formation. Samples with the following characteristics were associated with late dystrophic catagen; melanin clumps in the ES, proximal CTS, trailing CTS distal of DP, ball-shaped DP, remnant of hair shaft in the proximal hair canal, widened distal hair canal and substantial shortening of the hair follicle. Finally, samples with the following characteristics were associated with dystrophic telogen; melanin clumps around DP in the hair germ capsule and perifollicular dermis, ball-shaped DP resides in the dermis, abnormally wide open hair canal, without club hair and without CTS tail proximal of DP. All animal and human studies were approved by the Osaka City University Medical School Committee on Research Animal Care and were conducted in accordance with the principles of the Declaration of Helsinki.

2.3. Semiquantitative RT-PCR

Skin specimens were processed for semiquantitative RT-PCR as previously described [18]. Briefly, skin specimens were homogenized in ISOGEN (Nippon Gene; Tokyo, Japan). Addition of chloroform to these lysates enabled RNA rich aliquots to be extracted. Following precipitation with isopropyl alcohol and 70% ethanol, RNA samples were isolated. Levels of mRNAs encoding the subunits of laminin-332 ($\alpha 3$, $\beta 3$, $\gamma 2$), the subunits of laminin-511 ($\alpha 5$, $\beta 1$, $\gamma 1$), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each of our samples were measured by semiquantitative RT-PCR using an ABI PRISM 7700 sequence detector (PE Applied Biosystems; Foster City, CA). RT-PCR conditions were as follows: 42 °C for 20 min; 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Taqman reaction reagents were obtained from TOYOBO (Osaka, Japan). Taqman probes and primers were purchased from Sigma-Genosys (Hokkaido, Japan). mRNA levels were normalized to *gapdh* mRNA. Data were analyzed for statistical significance by using Fisher's Protected Least Significant Difference in a modified ANOVA test. Nucleotide sequences for primers or probes in the Taqman reaction for *Lama3a*, *Lamb3* and *Lamc2* subunits (laminin-332) and for *Lama5*, *Lamb1* and *Lamc1* subunits (laminin-511) were reported previously [18].

2.4. In situ hybridization

Detection of mRNA in tissue specimens was performed as described elsewhere [18]. Briefly, sense and antisense probes for the *Lama3a* subunit, *Lama5* subunit, and 28S ribosome RNA were purchased from Bex Co. (Tokyo, Japan). Nucleotide sequences for 28S and *Lama3a* subunit probes were reported previously. The nucleotide sequence for the *Lama5* subunit probes were as follows: *Lama5* sense probe: TTA TTA CTA TGG CTA TCC TAG CTG TCG CCC CTG CCA TGA GGC AGG CAC CAT GGC TAG CGT ATT ATT ATT; *Lama5* antisense probe: TTA TTA ACG CTA GCC ATG GTG CCT GCC TCA TGG CAG TCA CAG GGG CGA CAG CTA GGA TAG CCA TAG ATT ATT ATT. Probes were dimerized at the thymine–thymine (T–T) dimer sequence by using an ultraviolet lamp at 12,000 J/m². For in situ hybridization, fresh skin specimens were fixed in 4% paraformaldehyde in PBS at room temperature overnight, washed with distilled water, dehydrated in a graded ethanol series, and embedded in paraffin. Sections were cut, placed on glass slides, dewaxed, rehydrated, and then immersed in methanol for 15 min. After a 20-min incubation in 0.2M HCl, the sections were treated with 10 µg/ml proteinase K in PBS for 15 min, rinsed in PBS, and refixed with 4% paraformaldehyde in PBS for 5 min. Sections were rinsed in 2 mg/ml glycine in PBS, prehybridized with 4 × standard sodium citrate (SSC) containing 40% deionized formamide for 30 min, hybridized with 10 mM Tris–HCl, pH 7.4, 600 mM NaCl, 1 mM EDTA, pH 7.4, 1 × Denhart's medium, 0.25 mg/ml yeast tRNA, 0.125 mg/ml salmon sperm DNA, 2 µg/ml T–T dimerized probe in Tris–EDTA containing 40% deionized formamide at 37 °C overnight, washed five times with 2 × SSC/50% formamide at 37 °C for 1 h and twice with 2 × SSC at room temperature for 15 min, blocked with 500 µg/ml normal mouse IgG (Sigma–Aldrich, St Louis, MO), 5% bovine serum albumin (BSA), 100 µg/ml salmon sperm DNA, 100 µg/ml yeast tRNA at room temperature for 1 h, and then covered with anti-T–T dimer antibody, diluted 803 (KyowaMedic; Tokyo, Japan), 5% BSA, 100 µg/ml salmon sperm DNA, and 100 µg/ml yeast tRNA at 37 °C overnight. Unreacted antibodies were washed off the sections by rinsing four times with 0.075% Brij 35 in PBS at room temperature for 15 min and a further wash with PBS. Bound antibodies were visualized by treatment with 0.5 mg/ml DAB (Dojindo; Kumamoto, Japan), 0.025% cobalt chloride, 0.02% ammonium nickel(II) sulfate hexahydrate, and 0.01% hydrogen peroxide in 0.1 M phosphate buffer, pH 7.2, for 10 min.

2.5. Western blotting analyses

Proteins extracted from fresh skin specimens were reduced and separated on a 7.5% or 5–10% SDS–polyacrylamide gel to analyze laminin-332 or laminin $\alpha 5$ chain. We also extracted

proteins from the epidermis only. Isolation of epidermis was achieved by using a scalpel blade to cut through the skin at the dermo-subcutaneous fat interface. Separated proteins were transferred to nitrocellulose membranes (Protran BA 85 nitrocellulose; Schleicher and Schuell, Dassel, Germany). These membranes were processed with J18 rabbit polyclonal laminin-332 antiserum, anti-laminin $\alpha 5$, $\beta 1$ or $\gamma 1$ anti-tubulin or anti- β actin antibody (Ambion; Austin, TX), washed, and probed with HRP-conjugated goat anti-rabbit (for $\alpha 5$, J18 and tubulin), HRP-conjugated horse anti-goat ($\alpha 1$), HRP-conjugated goat anti-mouse antibody ($\gamma 1$), or HRP-conjugated goat anti-mouse antibody (for $\beta 1$, and β -actin). Signals were detected with an ECL Western blotting detection reagent (Amersham Pharmacia Biotech; Little Chalfont, UK). Bound antibodies were visualized with Supersignal West DURA Extended Duration Substrate (Thermo Scientific; Rockford, IL).

2.6. Histology and immunohistochemistry

For histochemical examination, skin specimens were fixed in 4% paraformaldehyde in PBS, dehydrated, and embedded in paraffin. Five- μ m-thick sections were cut and stained with hematoxylin and eosin (H&E). For IHC, fresh skin specimens were embedded in optimal cutting temperature compound (OCT; Sakura Fine Chemical, Tokyo, Japan) and frozen in liquid nitrogen. Cryosections were cut and air dried. To localize mouse laminin-332 and integrin $\beta 4$, tissue sections were treated with 1% Triton X-100 in PBS for 10 min and then fixed in 2% formaldehyde in PBS for 20 min at room temperature. For the localization of mouse $\alpha 3$ integrin and laminin-511, sections were treated with 1% Triton X-100 in PBS for 10 min and fixed in acetone at 4 °C for 2 min. To stain for human laminin-511, tissue sections were processed with acetone at 4 °C for 10 min. Primary antibodies were added at 37 °C for 1 h, and washed in PBS. Secondary antibodies in PBS at 37 °C were then added for an additional 1 h. Sections were washed in PBS, mounted, and examined by laser confocal microscopy (LSM510; Carl Zeiss AG, Oberkochen, Germany). For Ki67-immunohistochemistry, five- μ m-thick sections were deparaffinized and react with 3% H_2O_2 in water to inhibit endogenous peroxidases. The sections were treated with DaKo REAL Target Retrieval Solution (Dako, CA) and then incubated with rat anti-mouse Ki67 antibody (Dako, CA) at 37 °C for 30 min. We processed these with VECTASTAIN Elite ABC Rat IgG Kit (VECTOR LABORATORIES, INC, Burlingame). Bound antibodies were visualized by treatment with Dako liquid DAB Substrate chromogen system (Dako, CA).

2.7. Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling of fragmented DNA

For terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling of fragmented DNA (TUNEL) staining, five- μ m-thick sections were deparaffinized and treated with 20 μ g/ml proteinase K (Wako, Osaka). Subsequently, sections were reacted with 3% H_2O_2 in water as above and then processed using the In situ Apoptosis Detection Kit (TAKARA BIO INC, Otsu).

2.8. Preparation of laminin-511 rich protein extract from A549 cell conditioned media

Laminin-511 rich protein extract was prepared by Kitayama Labes (Nagoya, Japan). Briefly, human lung adenocarcinoma A549 cell lines, obtained from the JCRB Cell Bank (Osaka, Japan), were cultured in Dulbecco's modified essential media, supplemented with 15 mM HEPES and 10% fetal bovine serum. The conditioned medium from passage three cells was collected. We added ammonium sulfate into the conditioned media at 80% concentration and stirred. After 24 h, precipitates were collected by centrifugation at $18,000 \times g$ for 30 min at 4 °C. The precipitates were dialyzed against PBS containing 0.005% Brij35 and 0.1% CHAPS. Protein extracts were then processed for SDS-PAGE and transferred to PVDF membrane, as

outlined previously [18]. These membranes were processed with anti- $\alpha 5$, $\alpha 1$, $\beta 1$ and $\gamma 1$ antibodies for immunoblot described in Section 2.5.

2.9. Intradermal injection of laminin-511 rich protein extract and recombinant laminin 332 into the back skin of CIA mice

Laminin-511 rich A549 cell conditioned media protein extract (the protein concentration was 2.81 mg/ml in a volume of 50 μ l) or PBS (50 μ l) were intradermally injected daily into the back skin of CIA mice. Recombinant human laminin 332 was injected into back skin at the same day when cyclophosphamide was administrated (day 9 after depilation). Skin samples were collected five days after injection. Skin specimens were fixed in 4% paraformaldehyde in PBS, dehydrated, and embedded in paraffin. Five- μ m-sections were cut and stained with hematoxylin and eosin (H&E), used for Ki67-immunohistochemistry or TUNEL staining.

For morphometric and statistical analysis, we collected defined back skin areas of treated and untreated mice. We were able to assess the stages of at least 40 different hair follicles from one mouse by above-mentioned criteria [9]. Only every 10th cryosection was used for our analyses to exclude the repetitive evaluation of the same hair follicles, and three sections were assessed from each animal [25]. About 350 hair follicles in 9 mice were examined and compared among three groups, respectively. We analyzed all sections at $\times 200$ –400 magnification.

2.10. Human follicle organ culture with 4-Hydroperoxycyclophosphamide (4-HC) treatment

Anagen VI human follicles (HFs) were microdissected and organ-cultured as described previously [26–29]. In total 36 human anagen VI HFs were isolated from excess normal human temporal scalp skin obtained from a 44-year-old female patient undergoing routine plastic surgery. All experiments were performed according to Helsinki guidelines with Institutional Research Ethics Committee permission (University of Lübeck) and patient consent. Isolated HFs were maintained in serum-free William's E medium (Biocrom, Cambridge, UK) supplemented with 2 mmol/L L-glutamine (Invitrogen, Paisley, UK), 10 ng/ml hydrocortisone (Sigma–Aldrich, Taufkirchen, Germany), 10 μ g/ml insulin (Sigma–Aldrich), and 1% antibiotic mixture (100 $\times$; Gibco, Karlsruhe, Germany). For 4-Hydroperoxycyclophosphamide (4-HC) (Niomech, Bielefeld, Germany) treatment, HFs were first incubated with supplemented William's E medium for 2 days without 4-HC. 30 μ M of 4-HC was added to each well and the HFs incubated for another 3 days. Medium was changed every other day. All HFs were incubated at 37 °C in a 5% CO₂ incubator. After cryoembedding the cultured HFs, 6 μ m thick sections were prepared for immunohistochemical evaluation. Frozen sections were fixed in cold acetone and rinsed with phosphate-buffered-saline (PBS). After the incubation with 5% of normal goat serum in PBS, the sections were incubated with mouse anti-human Laminin $\alpha 5$ antibody (Millipore Co, Birellica, MA, USA) (1:25 in PBS) at 4 °C for overnight. After the incubation with Alexa fluor 546 goat anti-mouse antibody (Invitrogen) (1:200 in PBS) at RT for 45 min, the sections were washed with PBS, mounted, and examined by laser confocal microscopy.

The immunoreactivity of LN $\alpha 5$ in defined reference areas was assessed by quantitative immunohistomorphometry [28,29] as indicated in the figure legends, using the ImageJ software (National Institutes of Health, Bethesda, MD).

Data were analyzed using t-test for unpaired samples (GraphPad Prism, GraphPad Software, San Diego, USA) and a *p* value of <0.05 was considered significant.

2.11. Luciferase assay

Human keratinocyte cells (HaCaT cells kindly provided by Dr Nobert Fusenig) were cultured in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% FBS (Invitrogen) and 50 U

ml⁻¹ penicillin at 37 °C in a humidified atmosphere containing 5% CO₂. Luciferase assays were performed according to previously reported methods [30,31]. A reporter plasmid containing approximately the 4.0 kb EcoRI/SacI fragment of the 5'-flanking region of the mouse $\alpha 3$ integrin gene linked to a luciferase gene-containing plasmid (pGL3-basic vector; Promega, Madison, WI) was prepared as described elsewhere [31]. Luciferase assay was conducted using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) along with reporter plasmids constructed in pGL3-basic plasmid. In brief, HaCaT cells were trypsinized and then transfected with a mixture of the plasmid construct in pGL3 vector (2.0 μ g) and pGL4.74 [hRluc/TK] (0.15 μ g) (used as an internal control) using Lipofectamine 2000 (Invitrogen) in serum-free media (OPTI-MEN, GIBCO) for 10 min with shaking. 5×10^5 cells were seeded in 22-mm dish, and subsequently cultured for 24 h. After the cells were harvested by scraping, the cell extracts were assayed for firefly luciferase activity with a luminometer. After quenching the firefly luciferase reaction, we simultaneously activated the Renilla luciferase reaction. Renilla luciferase activity was measured immediately. The firefly luciferase activity was normalized to Renilla activity.

3. Results

3.1. Histology of CIA model mice

We induced anagen in 7-week-old female C57BL/6 mice by depilating the hair from back skin. Nine days after depilation, we injected CYP peritoneally [9]. Hematoxylin–eosin staining of the back skin of the treated mice reveals that the hairs 12 days after depilation (3 days after peritoneal injection of CYP) are at an early stage of dystrophic catagen, as indicated by the following characteristics; melanin clumps, which were larger than the nucleus of keratinocytes in both the IRS and ORS, condensed onion-shaped or ball-like dermal papilla, the remnants of the hair shaft in the proximal hair canal, follicular distortion and smaller distal hair bulbs. The hairs in skin samples at 13–14 days after depilation (4–5 days after CYP treatment) appear to be in mid dystrophic catagen, as indicated by the presence of widened distal hair canals, the position of DP situated in the mid-region of subcutaneous fat and severe follicular distortion. Hairs in the skin samples at 15 days after depilation (6 days after CYP treatment) bear characteristics of late dystrophic catagen, due to the presence of melanin clumps in the trailing CTS distal of DP and the close proximity of DP to the subcutaneous fat-dermis border. Finally, hairs in the skin samples 16 days after depilation (7 days after CYP treatment) appear to be at a stage of dystrophic telogen, as indicated by ball-shaped DP located in the dermis with no existence of a CTS tail.

3.2. Immunohistochemical localization of laminin-332, laminin-511, $\beta 4$ integrin and $\alpha 3$ integrin in hair follicles in the CIA mouse model

We analyzed the localization of laminin-332 and laminin-511 in the back skin of mice treated with CYP using immunohistochemical techniques. In early dystrophic catagen, laminin-332 localizes along the BM zone of the epidermis and the upper two thirds of hair follicles, whereas there is limited or no expression of laminin-332 in the lower third of the hair follicles (Fig. 1, upper panel, green). However, during mid and late dystrophic catagen and dystrophic telogen, we noted an up-regulation in expression of laminin-332 around the lower third of the hair follicles (Fig. 1, upper panel, green).

Expression of laminin-511 is detected along the BM of the intrafollicular epidermis and the surrounding hair follicles at early dystrophic catagen (Fig. 1, lower panel, green). In contrast, expression of laminin-511 rapidly decreases at mid dystrophic catagen (Fig. 1, lower panel, green). Weak expression of these antigens at the above-mentioned location is also observed in the dystrophic telogen stage (Fig. 1, lower panel, green). Intriguingly, laminin-332 exhibits a similar localization pattern to that of integrin $\beta 4$ (Fig. 1, upper panel, red), while laminin-511

localization corresponds well with that of integrin $\alpha 3$ throughout each of the stages that we assessed (Fig. 1, lower panel, red).

3.3. Immunoblot detection of laminin-332 and laminin $\alpha 5$ chain reveals that laminin-332 is transiently up-regulated at mid to late dystrophic catagen, while laminin $\alpha 5$ chain is down-regulated after mid dystrophic catagen

To quantitatively determine laminin-332 and laminin-511 (laminin $\alpha 5$ chain) levels, we performed immunoblot analyses in the skin of mice (containing hair follicles) treated with CYP. The results reveal an up-regulation of $\gamma 2$ chain of laminin-332 during mid to late dystrophic catagen stages (Fig. 2, upper panel). On the other hand, laminin $\alpha 5$ levels were down-regulated after mid dystrophic catagen (Fig. 2, middle panel). These results are consistent with our immunofluorescence observations. Moreover, we checked the expression of laminin-511 and -332 in epidermal extracts derived from the skin of the mice at the same time points. These skin samples did not contain hair follicles. There was little change in expressions of laminin subunits in these epidermal extracts (Fig. 2, lower panel). This suggests that the changes we observe in expression of laminin proteins in the specimens of skin that contained hair follicles of the treated mice are most likely the result of changes in laminin proteins in these hair follicles (Fig. 2, lower panel).

3.4. In situ hybridization detection of *Lama3a* and *Lama5* indicates that the up-regulation of *Lama3a* and the down-regulation of *Lama5* occur at the transcriptional level

We next examined the localization of *Lama3a* mRNA, the α subunit of laminin-332, and *Lama5*, the α subunit of laminin-511, by in situ hybridization. *Lama3a* mRNA is detected throughout the basal epidermal, ORS, IRS and hair matrix cells at each of the dystrophic catagen stages and the dystrophic telogen stage (Fig. 3a–e). A signal for *Lama3a* signal is much more intense from mid to late dystrophic catagen (Fig. 3c–e). *Lama5* subunit mRNA is detectable in keratinocytes of the epidermal, IRS, ORS and hair matrix cells during late anagen (Fig. 3f). However, *Lama5* subunit mRNA levels diminish and no signal can be detected during early dystrophic catagen (Fig. 3g). *Lama5* subunit mRNA is rapidly recovered during mid to late dystrophic catagen (Fig. 3h–i). As a control, an antisense probe for 28S ribosomal RNA stains all cells discernible in the specimen, whereas sense probes were negative at all times in each of the specimens that we examined (Fig. 3k–t).

3.5. Semi-quantitative RT-PCR confirms an up-regulation of *Lama3a* and down-regulation of *Lama5* occur at the transcriptional level

To analyze the transcriptional expression of laminin-332 and -511, we performed semi-quantitative RT-PCR using Taqman probes, which were specific for each of the individual laminin subunits. Our data indicate that *Lama3a* mRNA is transiently up-regulated during mid dystrophic catagen and *Lama5* mRNA is transiently down-regulated during early dystrophic catagen (Fig. 4). In contrast, the expression levels of the *Lamb3* and *Lamc2* subunits of laminin-332, and *Lamb1* and *Lamc1* subunits of laminin-511 are stable throughout dystrophic catagen and dystrophic telogen (Fig. 4).

3.6. Injection of a laminin-511-rich protein extracts reverses CIA while recombinant laminin-332 does not

To assess whether the down-regulation of laminin-511 by CYP plays a functional role in hair loss in CIA, we injected human laminin-511-rich protein extracts into the back skin of CYP treated mice. The extract was characterized by immunoblotting using antibodies against laminin $\alpha 5$, $\beta 1$, $\gamma 1$, and $\alpha 1$ subunits (Fig. 5). We first assessed the fate of the exogenous human laminin-511 protein by performing immunofluorescence studies using an anti-human laminin $\alpha 5$ antibody (4C7) that fails to recognize mouse protein. Human laminin-511 localizes into the

BM around the hair follicles in the mice at 15 days after depilation (6 days after single CYP treatment) treated with the human protein extract. Arrowheads indicate the positive staining for human laminin-511 (Fig. 6c). In contrast, there is no staining with the same anti-human laminin $\alpha 5$ antibody in the depilated back skin of untreated mice, regardless of CYP exposure. Next, we evaluated the morphological effects of injection of the laminin-511-rich protein extract. Whereas there is a marked alopecia in CYP treated mice that were left uninjected, CYP treated mice injected with laminin-511-rich extract demonstrate elongated hair at 15 days after depilation (6 days after single CYP treatment) (Fig. 6a). We also examined the hair of the mice at the histological level. The hair in the depilated back skin of untreated, age matched control mice are at the anagen VI stage, hair in the CYP treated mice is at late dystrophic catagen, whereas hair in the mice treated with both CYP and laminin-511-rich protein appears to be at early to mid dystrophic catagen (Fig. 6b). Ki67-positive cells in the matrix keratinocytes below the distal end of the DP were much rarer in CYP treated mice than in untreated control mice and in CYP treated mice injected with laminin-511-rich extract. This result indicates that the injection of laminin-511-rich extract may prevent the inhibition of proliferation by CYP treatment (Fig. 6d). More than 10 TUNEL-positive cells were observed in the vicinity of the DP of CYP treated mice injected with laminin-511-rich extract (early to mid dystrophic catagen), whereas multiple TUNEL-positive cells are present in the epithelial strand of CYP treated mice (late dystrophic catagen) (Fig. 6e).

Quantitative morphometric and statistical analysis also revealed that treatment of human laminin-511-rich protein extract inhibits the progress of stages of dystrophic catagen in our animal CIA model. Specifically, all hair follicles in the group of mice treated with laminin-511-rich extract exhibited early or mid dystrophic catagen, whereas more than 80% hair follicles in untreated mice appeared in late dystrophic catagen (Fig. 6f).

We also injected human recombinant laminin-332 into the back skin of CYP treated mice and then performed the same studies as above (Fig. 7). There was no obvious difference between CYP treated mice injected with laminin-332 and control uninjected. In animals in both groups, more than 80% hair follicles appeared in late dystrophic catagen.

3.7. The expression of laminin-511 was down-regulated in HF organ culture following 4-Hydroperoxycyclophosphamide (4-HC) treatment

To analyze the expression of laminin-511 in the human hair follicle, we treated hair follicles in organ culture with 4-Hydroperoxycyclophosphamide (4-HC). Expression of laminin-511 is detected along the BM of the intrafollicular epidermis and the surrounding hair follicles in control specimens. In sharp contrast, the expression of laminin-511 is down-regulated in hair follicles treated with 4-HC (Fig. 8).

3.8. Laminin-511 up-regulates $\alpha 3$ integrin promoter activity

The localization studies we describe above demonstrate a correlation between laminin-511 and $\alpha 3$ integrin expression. To assess if expression of $\alpha 3$ integrin is regulated by laminin-511, we performed a luciferase assay to analyze $\alpha 3$ integrin promoter activity following laminin-511 treatment of keratinocytes. Laminin-511 treated HaCaT cells exhibit significantly higher levels of luciferase activity than untreated cells, indicating that laminin-511 can up-regulate the activity of the $\alpha 3$ integrin promoter (Fig. 9).

4. Discussion

Using a well-established mouse model of CIA the data we present here indicate that (1) laminin-332 and $(\alpha 6)\beta 4$ integrin are up-regulated at mid to late dystrophic catagen at both the translational and transcriptional level; (2) laminin-511 is down-regulated after mid dystrophic

catagen at the protein level. The same phenomenon was also observed in *in vitro* human hair follicle cultured specimens. Interestingly, the down-regulation of laminin-511 is a specific, characteristic phenomenon for CIA compared to natural hair cycle since in a recent publication. Tateishi et al. presented evidence that laminin-511 is expressed around the BM of hair follicles in normal catagen [32].

CIA is thought to be caused by apoptotic cell death in hair matrix cells. The signals leading to apoptosis in CIA are thought to be p53, Fas (APO-1, CD95), Fas-associated death domain, procaspase-8, caspase-3, cyclin-dependent kinase 2, Bcl-2 [33–36]. In this regard, the down-regulation of laminin-511 that is induced by CYP may well contribute to activation of apoptosis of hair matrix cells. In fact, Hendrix et al reported that in early and mid dystrophic catagen, TUNEL-positive cells appear around the DP, and after late dystrophic catagen, TUNEL-positive cells dramatically increased along the epithelial strand located in the lower part of hair follicle in the same CIA mouse model [9]. Cell-matrix interactions are essential for survival and proliferation of epithelial cells which undergo a specialized form of apoptosis termed anoikis when deprived for substrate attachment [37]. Thus, the loss of laminin-511 may trigger apoptotic pathways in hair matrix cells. In support of this possibility, here we have demonstrated that the number of TUNEL-positive cells in CYP treated mice is decreased by treatment with a laminin-511 rich protein extract.

The results we present are consistent with emerging data indicating that laminin-511 is an essential and primary factor for both hair morphogenesis and anagen hair growth. Moreover, here we show that the effects of CYP exposure in mice can be partially overcome by injecting a laminin-511-rich protein extract into the back skin of treated mice. This leads to an obvious question. How does laminin-511 drive/support hair growth? Conti et al. reported that hair cycle progression is altered in $\alpha 3$ -integrin-deficient grafted skin [38]. Thus one possibility is that laminin-511 may promote hair growth control through regulating the expression of $\alpha 3$ integrin. This notion is supported by our finding that the expression of laminin-511 was spatially and temporally well correlated with that of $\alpha 3$ integrin. Moreover, we also demonstrate that laminin-511 increase $\alpha 3$ integrin promoter activity in cultured keratinocytes. These findings lead us to speculate that the up-regulation of $\alpha 3$ integrin triggered by laminin-511 is inhibited by CYP treatment leading to CIA. In this regard, it should be noted that in our experiments, as laminin-332, a ligand for $\alpha 3\beta 1$ integrin, is up-regulated following CYP. However, our preliminary studies do not indicate a role for laminin-332 in CIA since laminin-332 neutralizing antibodies do not appear to inhibit CIA nor does recombinant laminin-332 enhance the process.

In summary, our data strongly suggest that laminin-511 is a useful adjuvant for the treatment of CIA. The direct application of laminin-511 or stimulation of laminin-511-mediated signaling pathways may serve as a possible treatment for CIA and be beneficial in the reduction of hair loss in cancer patients undergoing chemotherapy.

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Fig. 1.

Immunohistochemistry of laminin-332, laminin-511 and their receptor proteins integrin $\alpha 3$ and $\beta 4$ in the back skin of the CIA model mice. Skin samples were labeled with anti-laminin-332 (green)/anti- $\beta 4$ (red) or anti-laminin-511 (green)/anti- $\alpha 3$ (red). In early dystrophic catagen, laminin-332 and integrin $\beta 4$ localize along the BM zone of the epidermis and the upper two thirds of hair follicles, whereas there is limited or no expression of laminin-332 and integrin $\beta 4$ in the lower third of the hair follicles. However, during mid and late dystrophic catagen and dystrophic telogen, expression of laminin-332 and integrin $\beta 4$ around the lower third of the hair follicle is upregulated. Expression of laminin-511 and integrin $\alpha 3$ is detected along the BM of the intrafollicular epidermis and the surrounding hair follicles at early dystrophic catagen. In contrast, expression of laminin-511 and integrin $\alpha 3$ rapidly decreases at mid dystrophic catagen. Weak expression of these antigens at the above-mentioned location is also observed in the dystrophic telogen stage. The expressions of laminin-332 and integrin $\beta 4$, or laminin-511 and integrin $\alpha 3$ is in parallel throughout the catagen stages. Bar = 100 μ m.

Fig. 2.

Immunoblot detection of laminin-332 and laminin $\alpha 5$ chain in CIA model mice. Extracts of skin containing hair follicles were obtained from each stage in the dystrophic catagen pathway and were processed for immunoblotting with an antiserum against laminin-332 (upper panel) or anti-laminin $\alpha 5$ chain (middle panel). The expression of β -actin or tubulin is shown as loading controls. *Left*: molecular weight is indicated. The results reveal an up-regulation of the $\gamma 2$ chain of laminin-332 during mid to late dystrophic catagen stages, and a down-regulation of $\alpha 5$ chain of laminin-511 during mid to late catagen stages (stages are indicated beneath the blots). We show quantification of densitometric scans with data being expressed in arbitrary units (y-axis) from 3 individual experiments. Bars = $\pm$ SEM. $*p < 0.05$. Extracts of epidermis lacking hair follicles were also processed for immunoblotting. There was little change in expression of laminin subunits in these epidermal extracts.

Fig. 3.

In situ hybridization detection of *Lama3a* or *Lama5* in CYP-induced CIA in the mouse.

Lama3a mRNA distribution at each of the dystrophic catagen stages and the dystrophic telogen stage is shown (a–e: anti-sense probe for *Lama3a*, k–o: sense probe for *Lama3a*). *Lama5* is detected in keratinocytes of the epidermis, IRS, ORS and hair matrix cells after late anagen, mid to late dystrophic catagen and dystrophic telogen (k–t). However, this positive signal is transiently down-regulated at early dystrophic catagen (q). (f–j: antisense probe for *Lama5*, p–t: sense probe for *Lama5*). (a, f, k, p) late anagen. (b, g, l, q) early dystrophic catagen. (c, h, m, r) mid dystrophic catagen. (d, i, n, s) late dystrophic catagen. (e, j, o, t) dystrophic telogen. (u) 28S antisense probe. Arrows indicate positive staining in the epidermal and ORS keratinocytes, and arrowheads indicate melanin granules. Bar = 100 μm.

Fig. 4. Semiquantitative RT-PCR analyses of the subunits of both laminin-332 and -511. Transient up-regulation of *Lama3a* at mid dystrophic catagen and transient downregulation of *Lama5* at early dystrophic catagen is observed. Stages of CIA are indicated. Data are expressed in arbitrary units (y-axis) from 3 individual experiments. Bars = $\pm$ SEM. * $p < 0.01$. ** $p < 0.05$.

Fig. 5. Characterization of laminin-511 rich protein extract generated from A549 conditioned medium. The extract was prepared for immunoblotting using antibodies against the α5, α1, β1 and γ1 laminin subunits as indicated. The right most lane shows an amido black stain of the protein extract used for immunoblotting.

Fig. 6. Laminin-511 ameliorates the effects of CYP treatment. (a) Animals were treated with PBS, CYP, and PBS or CYP and laminin-511 as indicated. (b) Hematoxylin–eosin staining of sections of skin of the animals in (a). (c) Anti-human laminin-511 staining in hair follicle specimens in the animals as in a. Human laminin-511 is detected in the basement membrane surrounding the hair follicle (c: right) whereas no signal is observed in the untreated control (c: left) or CIA mice (c: center). Arrowheads indicate the positive staining for human laminin-511. (d) Ki67-immunohistochemistry. The positive cells in CYP treated mice (d: center) are less easily observed compared with the same age control (d: left) and CYP-treated mice following injection of laminin-511-rich extract (d: right). Bar = 100 μ m. (e) TUNEL

staining. More than 10 TUNEL-positive cells appear around the DP of CYP treated mice injected with a laminin-511-rich extract (early to mid dystrophic catagen), whereas multiple TUNEL-positive cells are present in the epithelial strand of CYP alone treated mice (late dystrophic catagen). (f) All hair follicles in the group of mice treated with laminin-511-rich extract appear in early or mid dystrophic catagen, although more than 80% of hair follicles in untreated mice are in late dystrophic catagen.

Fig. 7.

Laminin-332 has no effects to CIA. (a) Animals were treated with PBS, CYP, and PBS or CYP and laminin-332 as indicated. (b) Hematoxylin–eosin staining of the skin samples. (c) Anti-human laminin-332 staining. Human laminin-332 is detected in the basement membrane surrounding the hair follicle (c: right) whereas no signal is observed in the untreated control (c: left) or CIA mice (c: right). Arrowheads indicate the positive staining for human laminin-332. (d) Ki67-immunohistochemistry. The positive cells in CYP treated mice (d: center) and CYP-treated mice with recombinant human laminin-332 (d: right) are less easily observed compared with the same age control (d: left). Bar =100 μ m. (e) TUNEL staining. Multiple TUNEL-positive cells are present in the epithelial strand of CYP alone treated mice (e: center) and CYP treated mice with a recombinant human laminin-332 (e: right) (late dystrophic catagen). (f) More than 80% of hair follicles in CYP alone treated mice and CYP treated mice with recombinant human laminin-332 are in late dystrophic catagen.

Fig. 8. Laminin-511 was down-regulated in HF Organ Culture with 4-Hydroperoxycyclophosphamide (4-HC) treatment. HF organ cultures were processed for immunofluorescence with an anti serum against human laminin $\alpha 5$. Expression of laminin-511 is detected along the BM of the intrafollicular epidermis and the surrounding hair follicles in the control. In contrast, there is only a weak expression of laminin-511 in the HF treated with 4-Hydroperoxycyclophosphamide (4-HC). The immunoreactivity of LNa5 in the defined reference area was assessed by quantitative immunohistomorphometry using the ImageJ software (National Institutes of Health, Bethesda, MD) Data are expressed in arbitrary units (y-axis) from 18 individual experiments. Bars = $\pm$ SEM. * $p < 0.05$.

Fig. 9.

Promoter activity of $\alpha 3$ integrin is enhanced in HaCaT cells treated with a laminin-511-rich protein extract stimulation. Relative luciferase activity was determined following incubation with or without laminin-511-rich protein extract for 24 h. The luciferase activity was normalized to Renilla activity induced by cotransfection with pGL4.74 [hRluc/TK]. Cells treated with recombinant laminin-511 exhibit significantly higher levels of luciferase activity than control group. The assays were carried out in triplicate. Bars = $\pm$ SEM. * $p < 0.05$.