Impaired function of ECM1 underlies the pathogenic disorganization of vascular and basement membrane molecules in lichen sclerosis

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INTRODUCTION

Lichen sclerosis (LS) is a common acquired inflammatory mucocutaneous disease that mainly affects the anogenital area. Evidence have suggested an autoimmune basis to the disease; e.g. an association with HLA class II antigens DQ7-9 and an increased incidence of other autoimmune conditions, implicating the presence of a humoral immune response in the LS pathology.

IgG autoantibodies to extracellular matrix protein 1 (ECM1), a glycoprotein ubiquitously expressed in the skin components, have been detected in ~70% of LS sera (Oyama et al, *Lancet* 2003;362:118-23). Because of the low serum titer and/or affinity of anti-ECM1 IgG in LS sera, an ordinary indirect immunofluorescence (IF) study provides mostly negative results, and the pathogenic significance of anti-ECM1 antibodies in LS remains unclear.

OBJECTIVE

Our present study aimed to confirm: i) whether confocal laser scanning microscope (CLSM) is valuable for detecting IgG antibodies to ECM1 in LS sera, and ii) whether the anti-ECM1 antibody in LS sera is indeed accessible to the native in vivo skin antigen, subsequently affecting the expression of hemidesmosomal and dermal vascular antigens in the LS lesional skin, and iii) the landscape of transcriptome alterations in ECM1-knockdown human fibroblasts on cDNA microarray, to determine the disease-specific gene signature driven by ECM1 dysfunction. We further summarized to gain comprehensive understanding of molecular diversity into the LS pathology.

RESULTS

Immunosignals specific for ECM1 were detectable in an indirect IF-confocal laser scanning microscope (CLSM), but not an ordinary indirect IF

On CLSM, 9/23 LS sera (39.1%) with non-specific IF findings on an ordinary microscope exhibited intense immunoreactivity in the lower epidermis and dermal blood vessel walls of normal human skin substrate (Fig. 1). This immuno-labeling was consistent with the staining pattern of anti-ECM1 polyclonal antibodies raised against the COOH terminus of human ECM1 (Fig. 1, *rt. upper*) and affinity-purified IgG from LS serum (Fig. 1, *rt. lower*) (Oyama et al, *J Clin Invest* 2004;113:1550-9).

Fig. 1. Immunolabelling with LS sera between ordinary indirect IF and indirect IF-CLSM

Assessment of abundant ECM1 expression in cultured human dermal fibroblasts and its gene targeting by siRNA system

When transfected with different concentrations of two siRNA for ECM1 (#1 and #2 siECM1s) targeting the NH2- and COOH-terminal portions, respectively, the transfectants with 6 pmoL of each siECM1 yielded a significant decrease of ECM1 mRNA by quantitative real-time RT-PCR (~88.1% and ~84.7% decrease in #1 and #2 siECM1s, respectively). A time-course analysis revealed the maximum inhibition of ECM1 mRNA at 48h after transfection, consistent with that of ECM1 protein in both cell lysate and culture supernatant (Fig. 2, *lt. and right.*, respectively). The transfection condition was used in the following experiments.

To study the relationship between in vivo ECM1 dysfunction and molecular events in the LS skin, we generated human dermal fibroblasts with siRNA-knockdown for ECM1 and analyzed transcription profiles by cDNA microarray (Fig. 3, *lt.*). Comparison with mock fibroblasts identified 3035 differentially expressed genes. Functional assessment assigned that 1471 upregulated (Fig. 3, *rt. upper*) and 1,564 downregulated genes (Fig. 3, *rt. lower*) were related to "protein binding" with cellular components, divalent cations, enzymes, and nucleotides.

Fig. 2. Quantitation of ECM1 protein expression in cell lysates and cultured supernatants from fibroblasts transfected independently with two siECM1s on western blotting

Fig. 3. cDNA microarray of differentially expressed gene profiles in ECM1-knockdown fibroblasts

Upregulated gene profiles in ECM1-knockout fibroblasts identified gene signature characteristic for LS

To further address which upregulated genes are essential linking between ECM1 dysfunction and LS pathology, we employed 2 different arms of GO pathways based on the expression sites and predicted function of ECM1 in the skin. For the ECM1-expressing skin sites, we adopted 3 major criteria for the skin components that constitutively express ECM1, "epidermal or epithelial or epidermal basement membrane", "dermis or dermal components", and "vessels or vascular" (Oyama and Merregaert, *Open Dermatol J* 2013;7:29-41), accounting for 127, 52, and 62 upregulated genes, respectively (total 164 genes; Fig. 4, *lt.*).

Next, the 164 upregulated genes specific for the ECM1-expressing skin sites were over-crossed with another arm of GO pathway for proposed ECM1 function, consisting 3 keywords "cell assembly or disassembly", "structural molecules", and "ECM constituent"; each of which met 186, 40, and 4 genes, respectively (total 208 genes; Fig. 4, *rt.*). The quadruple comparison found 49 upregulated genes related to cell adhesion and migration, ECM organization, intracellular signaling, angiogenesis, and skin development (Fig. 5); one of which, RAPGEF2 gene, was duplicated with different primer sets in our microarray, identifying 48 mutual genes.

Of the 48 upregulated genes, 14 (29.2%) have been shown to share a pathogenic significance in LS, including KIT, CTGF, PDGF, SMAD7, TGFB3, MDMD2, FBN1, collagens (COL4A1, 4A5, 4A6, and 5A1) and laminins (LAMA2, 3, and 4). The remaining 34 upregulated genes that have yet to be characterized for the LS pathology may also explain the underlying cellular events in the disease; migration, attachment, and proliferation in a wide variety of cell types including malignancies. Realtime RT-PCR and ELISA validated the upregulation of paneled genes and corresponding proteins.

Fig. 4. Identification of target-specific upregulated gene profile in ECM1-knockout fibroblasts

Gene	Log2FC	P Value	Term	Cou nt	%	P Value
F11R	2.38000903	0.1747	cell adhesion	11	22.9	4.42E-07
KIT	1.74497911	0.1278	extracellular matrix organization	11	22.9	1.33E-10
CCDC103	1.73000949	0.232	positive regulation of GTPase activity	9	18.8	1.54E-04
COL4A5	1.60502015	0.0297	positive regulation of cell migration	9	18.8	3.90E-08
APCE	1.53500781	0.0435	signal transduction	8	16.7	4.13E-02
MAG12	1.5099998	0.2298	positive regulation of cell proliferation	8	16.7	2.92E-04
S1PR1	1.49998616	0.0592	angiogenesis	8	16.7	2.65E-06
PTK2B	1.4100154	0.2861	cell migration	8	16.7	4.66E-07
PIK3R1	1.34499888	0.0024	collagen catabolic process	7	14.6	2.29E-08
CDH2	1.33502584	0.1385	intracellular signal transduction	6	12.5	5.21E-03
LAMA3	1.27500705	0.0374	MAPK cascade	6	12.5	7.99E-04
CTGF	1.26999063	0.0994	regulation of cell proliferation	6	12.5	1.62E-04
SMAD7	1.24999137	0.0305	positive regulation of ERK1 and ERK2 cascade	5	10.4	1.43E-03
COL4A1	1.20501709	0.1226	response to hypoxia	5	10.4	1.35E-03
JAG1	1.18998265	0.3731	phosphatidylinositol-mediated signaling	5	10.4	2.17E-04
MMP11	1.18002052	0.1008	phosphatidylinositol phosphorylation	5	10.4	1.37E-04
PDGFA	1.15497199	0.0193	wound healing	5	10.4	7.29E-05
FLRT2	1.1399933	0.3171	regulation of phosphatidylinositol 3-kinase signaling	5	10.4	6.60E-05
RAPGEF2*	1.12498977	0.1685	regulation of cell adhesion	5	10.4	6.14E-06
SEMA3A	1.11002909	0.2403	skin development	4	8.3	1.16E-04
TACSTD2	1.09998842	0.2952				
TGFB3	1.07498489	0.2649				
ADAM17	1.00498873	0.2048				
COL4A6	0.85996955	0.0664				
LAMA2	0.84502832	0.0713				
DKK1	0.84004018	0.2317				
JUP	0.81500152	0.2873				
MDM2	0.81500152	0.2072				
SATB1	0.80999058	0.0704				
MMP14	0.79501881	0.0599				
HEG1	0.77997406	0.3686				
LRRC6	0.76502547	0.2697				
OFD1	0.76502547	0.0787				
TAC1	0.70999637	0.0803				
SNAIL	0.69001474	0.3345				
COL3A1	0.67500292	0.1166				
FBN1	0.67002451	0.085				
RAPGEF2*	0.639973	0.0792				
NRG1	0.6200233	0.3425				
B9D1	0.60502015	0.0681				
ARHGAP7	0.59998423	0.3036				
DSP	0.59502618	0.2578				
ANG	0.56003012	0.2184				
GJA1	0.54497988	0.1546				
EPS15	0.54002727	0.1942				
PDGFRA	0.52004502	0.156				
COL5A1	0.51005046	0.0746				
LAMA4	0.51005046	0.1471				
ARHGAP24	0.49998616	0.076				

Fig. 5. List of 49 upregulated genes, including doublet RAPGEF2, and top 20 biological process GO terms of 48 upregulated genes

Assessment of transcriptional and protein expression of upregulated genes in ECM1-knockdown fibroblasts

To check the reproducibility of our microarray results, we conducted real-time RT-PCR using fibroblasts transfected with two siRNAs (#1 and #2 siECM1s). Among 48 upregulated genes (Fig. 5), representative concerning diverse fibroblast biology, such as CTGF, SMAD7, FBN1, KIT, S1PR1, F11R, TGFB3, COL4A1, and LAMA3, were increased significantly in transfectants with both siECM1s, whereas PDGFA and CCDC103 showed a significant increase only in those with #1 siECM1 (Fig. 6). The transcription of COL7A1 was unchanged, consistent with our microarray.

The expression of the corresponding protein was validated by a time-course analysis for two major molecules, COL-4a1 (COL4A1) and laminin-a3 (LAMA3), responsible for the structural integrity of skin basement membrane and vasculature via direct binding with ECM1 (Sercu et al. *Matrix Biol* 2009;28:160-9). On antigen-specific ELISA, increased expression of COL-4a1 protein was found by 48h after transfection with #1 siECM1 and maintained at least up to 96h (Fig. 7, *lt.*). In contrast, the expression of laminin-a3 protein started to increase at ~96h after transfection (Fig. 7, *rt.*).

Fig. 6. Validation of cDNA microarray data using ECM1-knockdown fibroblasts on real-time RT-PCR

Fig. 7. Validation of cDNA microarray data using ECM1-knockdown fibroblasts on ELISA

LS lesional skin harbors molecule-dependent regulation of major antigens expressed in the dermal-epidermal junction

Considering the histological features of LS, we examined the expression profile of major BMZ and vessel wall antigens, which may contribute to the establishment of LS pathology, by immunohistochemistry using a panel of antibodies on a CLSM. Compared with age-/sex-matched control skin, the LS lesional skin (n=23) revealed unchanged expression of hemidesmosomal antigens including BP230, BP180 NC16a (as visualized by BP180 ELISA-positive BP patients' serum)(data not shown), BP180-COOH terminus, integrin b4 (Figs. 8a-c, arrow heads), but instead unique expression pattern of laminin-332 (6/23, 26.1%) and two major collagens, type IV (20/23, 87.0%) and VII collagens (19/23, 82.6%) (Figs. 8d-f).

The immunosignals for COL-4 and laminin-332 showed periodical thickness, reduplication and abrupt disappearance, and multifocal notches downward along with BMZ (Figs. 8d, e, *lt. upper*). These features were more intensely with immunostaining of COL-7 (Fig. 8f, *upper*). The vasculature visualized by anti-COL-4 antibody showed pronounced thickness and reduplication of the dermal vessel walls (23/23, 100%) (Fig. 8e, *rt. upper*).

These results suggest that the LS lesional skin harbors microscopic disruption of laminin-332 (lamina densa) and hemidesmosomal collagens; COL-4 (lamina densa/dermal vessel wall) and COL-7 (sublamina densa).

Fig. 8. Comparison of immunoreactivity to BMZ and vascular antigens between LS and control skin

Evidence for direct binding of recombinant ECM1a and COL-7

Our previous evidence that ECM1 binds to laminin-332 and COL-4 may aid to understand the uncovering finding between the lack of COL-7 gene dysregulation in our microarray and its abnormal expression pattern in the LS lesional skin (Sercu et al, *J Invest Dermatol* 2008;128:1397-408). We therefore performed in vitro binding assay using bacterially expressed recombinant human full-length ECM1a (Oyama et al, *J Clin Invest* 2004;113:1550-9). As detected by anti-ECM1 antibodies raised against the COOH-terminal end of ECM1 (Utsunomiya et al, *J Invest Dermatol* 2018;138 abstr), the recombinant ECM1a bound dose-dependently to COL-7 immobilized onto a microtiter plate (Fig. 9). COL-7, which did not satisfy the ECM1-silencing upregulation but showed abnormal expression pattern in the LS skin, bound to ECM1 recombinant protein. Impaired ECM1 function may thus cause increased expression and selective disassembly of basement membrane, vascular, and extracellular matrix molecules, as well as growth factors and its receptors facilitating fibroblast proliferation, contributing to the LS skin pathology.

Fig. 9. Binding of recombinant ECM1a to immobilized COL-7 protein

CONCLUSION

1. Indirect IF-CLSM can be a reliable approach for detection of circulating IgG autoantibodies specific for ECM1 in LS sera.
2. Our results demonstrate the possible involvement of microstructural disorganization of the skin basement membrane and vascular molecules in ECM1-knockout dermal fibroblasts. We also highlight that ECM1 binds to COL-7.
3. Functional impairment of in vivo ECM1 may cause altered expression of major structural proteins, COL-4, COL-7, and laminin-332, in dermo-epidermal junction and dermal blood vessels.
4. Impaired ECM1 function may thus cause increased expression and selective disassembly of basement membrane, vascular, and extracellular matrix molecules, as well as growth factors and its receptors facilitating fibroblast proliferation, contributing to the LS skin pathology.

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