INVESTIGATIVE REPORT

Decreased Tissue and Serum Expression of Galectin-7 in Patients with Hypertrophic Scars

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Hypertrophic scars (HS) result from an imbalance between collagen biosynthesis and matrix degradation during wound healing. In this study a proteomics approach was used to compare the protein profiles of skin tissue obtained from patients with HS and healthy controls. One of the epidermal proteins, galectin-7 was markedly down-regulated in HS. Serum levels of galectin-7 in 27 patients with HS were less than 1/3 of those in 15 healthy controls. Tissue protein expression was subsequently evaluated using immunohistochemical staining on HS tissue and on serially-obtained control tissue during wound healing. Weaker galectin-7 immunoreactivity was detected along the cytoplasmic membrane of basal and suprabasal cells in samples from HS. In addition, galectin-7 was stained in the extracellular space of the upper papillary dermis in HS tissue. Ablative laser treatment, used to induce wound healing of healthy control tissue, demonstrated marked galectin-7 expression at the cytoplasmic membrane on days 3, 5, 14 and 21. Pronounced galectin-7 staining at the upper papillary dermis was detected on days 1, 3 and 10. These results suggest that the differences in galectin-7 expression and subcellular and extracellular distribution may be crucially involved in the pathogenic process of HS. *Key words: hypertrophic scar; galectin-7; proteomics; wound healing.*

Hypertrophic scars (HS) and keloids are benign fibrous growths characterized by excessive deposition of extracellular matrix at or beyond sites of prior dermal injury and wound repair, and they are thought to occur due to an imbalance between collagen biosynthesis and matrix degradation (1–3). Both increased basal levels of connective tissue growth factor (CTGF) and exaggerated production of CTGF stimulated by the transforming growth factor-β have been suggested as major contributing factors to the pathogenesis of HS and keloid scars (4–6). Activation of neuregulin-1 and its receptor have been shown to stimulate PI3K- or Src-mediated CTGF expression in cultured fibroblasts obtained from HS (6). In addition, increased release of monocyte chemoattractant protein-1 by CD14+ cells in keloid tissues is thought to enhance fibroblast proliferation and initiate keloid formation (7).

Previous comparative proteomic analyses of keloid scar and normal skin extracts have identified various proteins that are upregulated or uniquely expressed in keloid scars, including inflammatory/differentiated keratinocyte markers, wound healing proteins, fibrogenetic proteins, anti-fibrotic proteins, tumour suppressor proteins, and anti-angiogenic proteins (8). In particular, and of note, the protein expression levels of asporin, stratifin, galectin-1, and macrophage migration inhibitory factor are significantly increased in keloid scar tissues (8). Galctin-7 is a marker of keratinocyte differentiation, which appears in the epidermis, but has not been studied in HS before.

The present study took a proteomics approach to compare the protein profiles of scar tissue from HS patients with those of healthy control skin.

MATERIALS AND METHODS

Patients

Twenty-seven subjects (18 males and 9 females; mean age 26.9 years; age range 6–55 years) with biopsy-confirmed HS and 5 healthy volunteers (4 males and 1 female; mean age 26.8 years; age range 16–36 years) with no history of HS or keloid scar formation were enrolled in this study. Patients were excluded for receiving treatment for HS within 3 months of the study, including intralesional steroid injections, silicone gel sheeting, topical silicone gels, pressure dressing, systemic tranilast, radiotherapy, cryotherapy and laser or light therapies. No participant had an undiagnosed inflammatory condition, and all presented with normal ranges of full blood count, renal and liver function, blood glucose level, C-reactive protein, erythrocyte sedimentation rate, anti-streptolysin O titre, antinuclear antibodies, rheumatoid factor, and venereal disease serum levels.

Tissue samples were obtained from 5 HS patients for 2-DE and immunohistochemical staining by 4.0-mm punch biopsy. Serum was collected from 27 HS patients for use in ELISA. Normal skin tissues obtained from 5 healthy volunteers were used as controls, and sera from 15 healthy donors were purchased (Innovative Research Inc., Novi, MI, USA). All blood samples were collected in serum-separating tubes with clot activator and

were allowed to clot at 4ºC for 24 h. The sera was separated, with the supernatant divided into 100 µl aliquots and stored at –70ºC until analysis. The study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Ethics approval included laser wounding and 6 serial biopsies in a healthy volunteer.

Two-dimensional electrophoresis and proteomic analysis

Two-dimensional electrophoresis (2-DE) followed by amino acid sequencing by liquid chromatography-matrix assisted laser desorption/ionization-tandem time-of-flight analysis (LC-MALDI-TOF/TOF) was used to assess qualitative and quantitative differences in protein profiles from patients with HS and healthy controls.

2-DE analysis was performed as described previously (8). Briefly, frozen tissue samples obtained from HS patients ($n = 5$) or healthy controls ($n = 5$) were homogenized in lysis buffer and the supernatants were collected. Mixed tissue extracts in the strips were then electrophoresed using an 8–18% gradient polyacrylamide gel in the presence of sodium dodecyl sulphate. All samples were analysed in duplicate. The 2-DE gels were then developed with silver staining (9). For mass spectrometric analyses, gels were stained with Coomassie blue using a modified method described previously (10). Quantitative analyses of the spot intensities were performed using PDQuest system (Bio-Rad Laboratories, Hercules, CA, USA) after scanning stained 2-DE gels according to the protocols.

LC-MALDI-TOF/TOF was then performed with excised gel pieces using a capillary LC equipped Q-TOF Ultima mass spectrometer (Waters, Milford, MA, USA), where the data-dependent analysis mode with an mass spectrometry (MS) precursor scan (200–1,800 atomic mass unit (amu)) was used for peptide ion detection and followed by 3 data-dependent MS scans. The DNA sequence of identified protein was defined at the National Center for Biotechnology Information (NCBI) (11). The data from all collected raw MS/MS spectra were used by the Mascot Search program (available at http://www.matrixscience.com/) to search Swiss-Prot and NCBI human databases (11). A mass tolerance of 1.0 amu was used to accept molar masses of both the precursor peptide and peptide fragment ions. For search data screening, only peptides yielding a Mascot score greater than 30 were considered as notable homologues.

Measurement of galectin-7 serum concentration

The serum concentration of galectin-7 was assessed in samples from 15 subjects with HS and 15 healthy controls using a galectin-7 human ELISA kit (Abcam, Cambridge, UK). The manufacturer-provided range for normal serum galectin-7 levels (derived from healthy volunteers) was 13.72–10,000 pg/ml.

Laser treatment and immunohistochemical staining

For the evaluation of galectin-7 expression during wound healing, one participant with no history of HS or keloid scar formation was treated with a single session of 10,600-nm ablative carbon dioxide fractional laser (CO_2 FS) using a Mosaic eCO$_2$™ laser (Lutronic Corporation, Goyang, Korea) on the lower back. Laser fluences were initially delivered on the back with settings of pulse energy of 50 mJ, density of 200 spots/cm^2, single pass, and static operating mode (coverage 12.7%). Full-thickness skin samples were obtained at baseline (control), 6 h (day 1), 3, 5, 7, 10, 14 and 21 days after the laser treatment. Samples were formalin-fixed and embedded in paraffin for haematoxylin-eosin staining and immunohistochemistry analysis.

Sections (4 µm thick) from HS lesion biopsies ($n = 5$), CO_2 FS-treated skin ($n = 1$; 8 samples), and healthy control skin ($n = 5$) were incubated with diluted primary anti-sera at room temperature for 1 h. The rabbit anti-human galectin-7 polyclonal antibody (Abcam) was diluted at 1:400. After washing with phosphate-buffered saline (PBS), the sections were incubated in horse-radish peroxidase (HRP)-conjugated secondary anti-sera at a dilution of 1:100 for 30 min. Sections were then lightly counterstained with haematoxylin. Negative controls were obtained by omitting the primary antibody. The experiments were performed in triplicate. Stain intensities were analysed using Metamorph software ver. 7.7.1.0 (Molecular Devices Inc., Sunnyvale, CA, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

In order to evaluate *in situ* apoptosis, an ApopTag apoptosis detection kit (Serologicals, Norcross, GA, USA) was used according to the manufacturer's protocol. Briefly, the paraffin sections were incubated with 20 µg/ml proteinase K, and endogenous peroxidase activity was blocked with 3% hydrogen
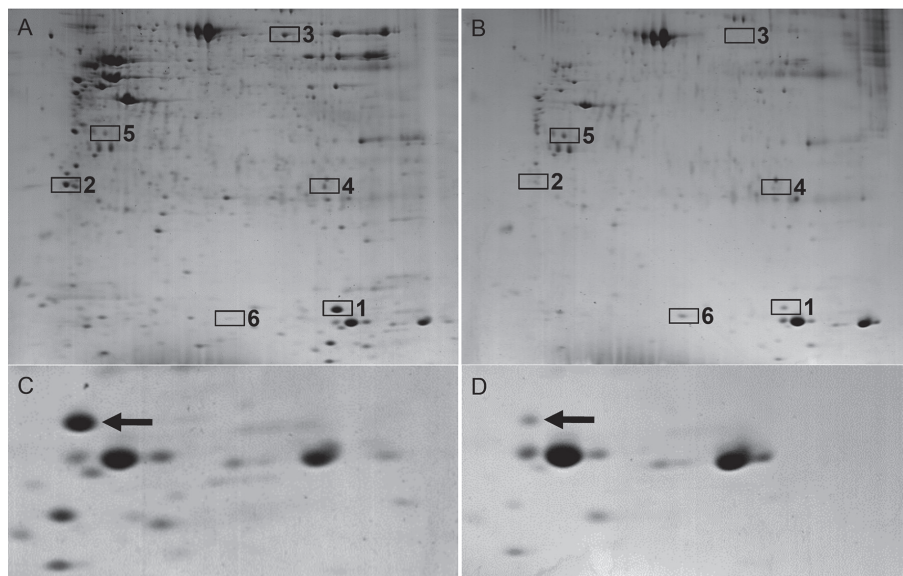

Fig. 1. Comparative analysis of 2-dimensional electrophoresis (2-DE) gel maps. 2-DE performed with mixed skin tissue samples from: (A) adult healthy controls and (B) subjects with hypertrophic scar (HS). Four downregulated protein spots (numbers 1–4) and 2 upregulated protein spots (numbers 5 and 6) were identified in tissue protein profiles of patients with HS. Galectin-7 was identified by proteomic analysis of the downregulated protein spot (number 1) in the tissue samples from (C) healthy controls and (D) subjects with HS.
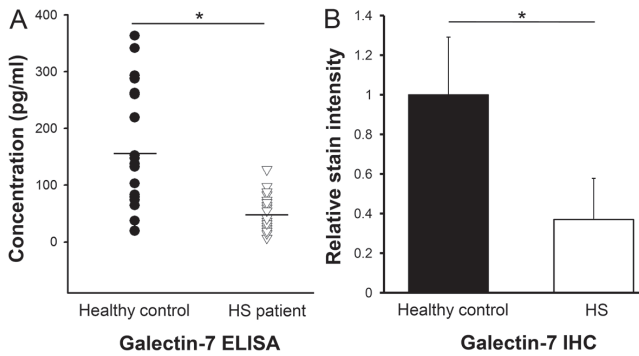
peroxide. The sections were applied with equilibrium buffer and then treated with TdT enzyme at 37ºC for 1 h. The slides were applied with anti-digoxigenin peroxidase for 30 min, then developed in 3,3'-diaminobenzidine (DAB) substrate solution, and counterstained with haematoxylin. The results were quantified by counting the number of TUNEL-positive cells in the epidermis.

Statistical analysis

Relative galectin-7 serum concentrations and galectin-7 stain intensities were compared between HS and healthy subjects, using non-parametric Mann-Whitney U tests. Values are expressed as means ± SD. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 18.0 (SPSS Inc., Chicago, IL, USA). p-values less than 0.05 were considered statistically significant.

RESULTS

Comparative analysis of 2-DE gel proteomic maps

Mixed tissue samples from HS patients and healthy controls were subjected to 2-DE and silver staining,

which generated typical, highly-resolved profiles for highly abundant proteins. Compared with the control profiles (Fig. 1A), 4 protein spots were downregulated in the tissue protein profiles of patients with HS, while 2 protein spots were upregulated (Fig. 1B).

After amino acid sequencing by LC-MALDI-TOF/TOF, spots of altered expression were identified using a similar protein. The NCBI database was then searched for the corresponding DNA sequence of this altered protein, and data containing immunoglobulins and false positives were excluded. Specifically, the following proteins were found to be downregulated in HS patients' tissue samples: galectin-7 (Fig. 1C and D), 14-3-3 protein sigma (also known as epithelial cell marker protein 1 or stratifin), keratin 1, and RBM44 protein (Table SI; available from http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1583). In contrast, tissue proteins including RIB43A-like with coiled-coils protein 1 isoform 1 and tubulin tyrosine ligase-like family member 11 isoform CRA_b, were found to be upregulated in HS tissue samples compared with normal.

Serum concentrations of galectin-7

Galectin-7 ELISA was performed on sera obtained from the 27 HS patients and 15 healthy donors, based on a previously-described relationship between galectin-7 and epidermal homeostasis during wound repair (12). The serum levels of galectin-7 were significantly lower among the patients with HS (mean 48.8 ± 30.2, range 7.1–127.7 pg/ml) compared with controls (mean 160.3 ± 106.7, range 19.5–341.8 pg/ml, $p < 0.0001$) (Fig. 2A).

Immunohistochemical staining and TUNEL staining

Tissue samples obtained from healthy controls demonstrated strong immunoreactivity against anti-galectin-7 antibody in the epidermis (Fig. 2B and 3A). In particular, uniformly intensive cytoplasmic staining was found in
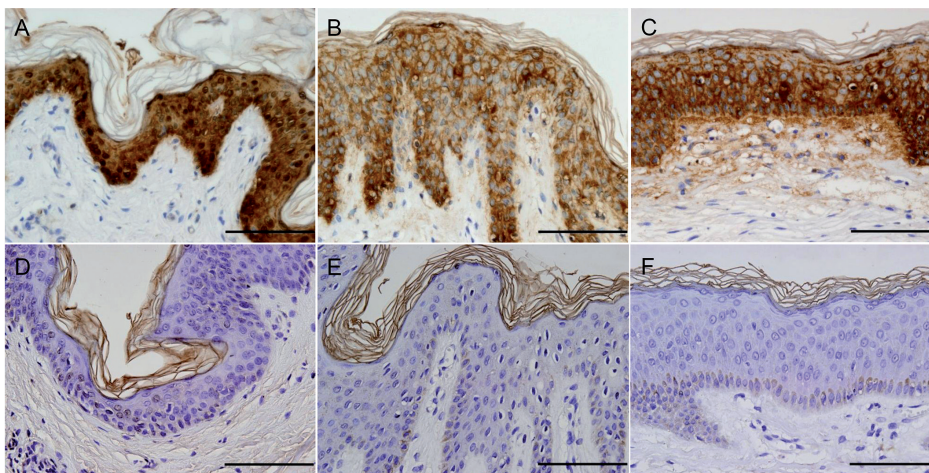
basal and suprabasal keratinocytes. In contrast, tissue samples from patients with HS demonstrated weaker galectin-7 immunoreactivity (Fig. 3B, C). In addition, galectin-7 staining in these tissues was localized to the cytoplasmic membrane rather than in the cytoplasm of the basal and suprabasal cells. In addition, galectin-7 was stained in the extracellular space of the upper papillary dermis in HS tissues, but not in control tissues. TUNEL staining revealed that 1.3% of TUNEL-positive cells in the epidermis were observed in tissue samples obtained from healthy controls (Fig. 3D), whereas 0.2% of TUNEL-positive cells were present in HS (Fig. 3E, F).

In order to evaluate galectin-7 expression during wound healing, we used ablative laser treatment on the lower back of a healthy patient. This consisted of performing a single session of CO_2 FS and subsequently serially-obtaining skin tissues at baseline (control), 6 h (day 1), 3, 5, 7, 10, 14 and 21 days after the laser treatment. As described above, control tissue revealed immunoreactivity against anti-galectin-7 antibody on the epidermis and follicular outer root sheath with uniformly intensive cytoplasmic staining pattern (Fig. 4). During the wound healing process following laser treatment, there was marked galectin-7 expression on the cytoplasmic membrane seen at days 3, 5, 14 and 21. In addition, there was pronounced galectin-7 staining on the upper papillary dermis detected at days 1, 3 and 10.

DISCUSSION

Galectin-7 is a prototype galectin that appears in the epidermis, coinciding with epidermal stratification as a marker of keratinocyte differentiation (12, 13). The gene encoding for galectin-7 was first described for its responsiveness to retinoic acid and for its down-regu-lated expression in actively proliferating keratinocytes (12, 14, 15). Galectin-7 is thought to play a role during wound healing, functioning as a regulator of keratinocyte proliferation and migration that is central in maintaining and restoring epidermal homeostasis (12, 13).

Furthermore, galectin-7 has been proposed a regulator of apoptosis, based on its proapoptotic effects noted in epidermal responses to a variety of stimuli (13, 16). Indeed, galectin-7 has been considered as the product of tumour suppressor gene p53-induced gene 1 (17). However, Gendronneau et al. (12) demonstrated that galectin-7$^{-/-}$ mice have apoptotic responses against ultraviolet B (UVB) stimulation that begin earlier and last longer than those of wild-type mice. We propose that the decreased tissue and serum expression of galectin-7 in patients with HS in the present study might have resulted in the dysregulation of apoptosis and excessive deposition of scar tissues.

Galectins can be found in the nucleus, cytoplasm, cytoplasmic membrane, and extracelluar space (13). In C57Bl/6 mouse skin, galectin-7 is present in epidermal basal cells, distributed uniformly in the cytoplasm, whereas in suprabasal keratinocytes it is found to be strongly, but irregularly, distributed along plasma membranes (12). In healthy adult human skin, galectin-7 is evenly distributed in the basal and suprabasal layers of the epidermis and can be detected in the interfollicular epidermis, oral mucosal epithelia, follicular outer root sheath, oesophagus, thymus, and cornea (18). It has been proposed that the various subcellular or extracellular localization of galectin-7 may correlate with the different functions of this protein (13).

In the present study, we noted differences between control skin and HS tissue, not only in the intensity of immunoreactivity against anti-galectin-7 antibody, but
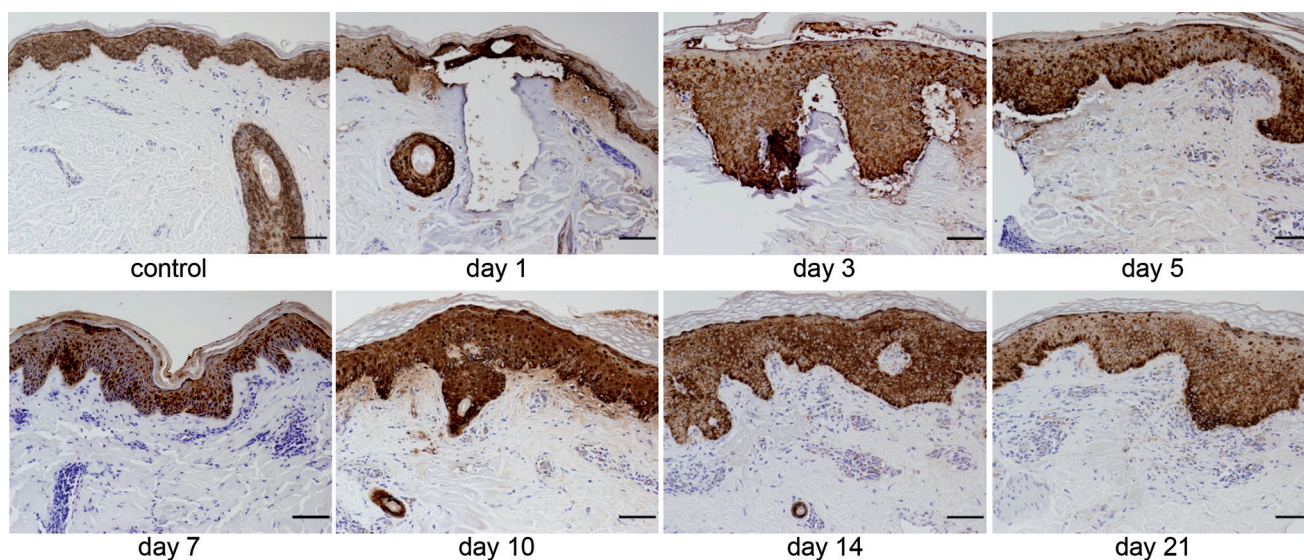

Fig. 4. Galectin-7 expression during the wound healing process in healthy control skin. Marked galectin-7 expression at the cytoplasmic membrane is visible on days 3, 5, 14 and 21 after ablative 10,600-nm carbon dioxide fractional laser treatment, whereas pronounced galectin-7 staining at the upper papillary dermis is noted on days 1, 3 and 10. Original magnification ×200. Bars = 100 μm.

also in the subcellular distribution of galectin-7 protein. The epidermis of HS tissues had a characteristic net-like distribution of galectin-7 along the cytoplasmic membrane of the basal and suprabasal keratinocytes with relatively weak immunoreactivity. Moreover, galectin-7 was found in the extracellular space of the upper papillary dermis in HS tissues, but not in control tissues. We also showed that the pattern of subcellular and extracellular galectin-7 expression was markedly changed during wound healing after laser treatment in healthy control tissue. In a previous study, galectin-7 was upregulated during wound healing after corneal injury, and exogenous galectin-3 and galectin-7 promoted the re-epithelialization of wounded cornea in animal models (19). We propose that galectin-7 localized to the plasma membrane in the epidermis and extracellular galectin-7 in the upper papillary dermis may contribute to the unbalanced scar formation in HS patients.

A previous comparative proteomic study revealed that the protein expression of stratifin and galectin-1 was upregulated in keloid scar (8), whereas our present study found that stratifin expression was downregulated in HS. Inactivation of stratifin, which is a known direct transcriptional target of p53, results in continuous keratinocyte proliferation (8, 20). In addition, galectin-1, which is expressed in human mesenchymal cells, negatively regulates the growth of fibroblasts and has proapoptotic effects and anti-inflammatory activities (8, 21). We posit that this contradictory finding might stem from the pathogenetic differences between keloid scar and HS, as well as racial and genetic differences in participants.

In conclusion, our data demonstrate that HS is associated with decreased tissue and serum levels of galectin-7. These results suggest that the differences in galectin-7 expression and subcellular and extracellular distribution may be crucially involved in the pathogenic process of HS. However, the specific clinical values and the exact aetiological role of tissue and serum galectin-7 require further clarification.

ACKNOWLEDGEMENTS

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