The Peripheral Lymphatic System Is Impaired by the Loss of Neuronal Control Associated with Chronic Spinal Cord Injury

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Spinal cord injury (SCI) is associated with venous vascular dysfunction below the level of injury, resulting in dysregulation of tissue fluid homeostasis in affected skin. The purpose of this study was to determine whether loss of neuronal control in chronic SCI also affects the skin lymphatic system. Morphology of lymphatics was characterized by immunohistochemistry and lymphatic gene expression profiles determined by DNA microarray analysis. In SCI, skin lymphatic function appeared to be impaired, because the ratio of functionally dilated versus collapsed lymphatic vessels was 10-fold decreased compared with control. Consequently, the average lumen area of lymphatic vessels was almost halved, possibly due to the known impaired connective tissue integrity of SCI skin. In fact, collagenases were found to be overexpressed in SCI skin, and dermal collagen structure was impaired. Molecular profiling also suggested an SCI-specific phenotype of increased connective tissue turnover and decreased lymphatic contractility. The total number of lymphatic vessels in SCI skin, however, was doubled, pointing to enhanced lymphangiogenesis. In conclusion, these data show, for the first time, that lymphatic function and development in human skin are under neuronal control. Because peripheral venous and lymphatic vascular defects are associated with disturbed fluid homeostasis, inappropriate wound-healing reactions, and impaired skin immunity, they might contribute to the predisposition of afflicted individuals to pressure ulcer formation and wound-healing disorders. (Am J Pathol 2022, 1–10; https://doi.org/10.1016/j.ajpath.2022.06.012)

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G.B. and M.S.R. contributed equally to this work.
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edema formation. Skin fluid homeostasis, however, is maintained by the peripheral microvascular system of both blood vessels and lymphatics, with the latter draining excess fluid, particulates, and cells from the tissue. Whether aberrant nerve activity, in addition to impairing blood vessel function, may also affect skin lymphatic function; and how this potentially contributes, in addition to increased venous vascular permeability, to the disturbed tissue fluid homeostasis in chronic SCI is not known.

To address this question, a histologic analysis of the lymphatic system in chronic SCI skin was performed, and the molecular effects of aberrant nerve activity were examined by defining SCI-specific molecular profiles for lymphatic function, connective tissue integrity, and lymphangiogenesis.

Materials and Methods

To identify SCI-specific alterations in the morphology of the lymphatic system, we quantified, using immunohistochemistry combined with image analysis, number and functionality of lymph vessels in SCI skin from areas affected by loss of neuronal control in comparison to skin from healthy individuals. In addition, we identified, at the molecular level, SCI-specific lymphatic gene regulation by whole-genome gene expression analysis. This was confirmed, by (immuno)histochemistry specific for collagenases and collagen.

Tissue Samples

Following informed consent, tissue samples of intact skin or of the wound edge of pressure ulcers, both from skin areas affected by loss of neuronal control, were collected during routine surgery of chronic SCI patients at the Werner Wicker Klinik (Bad Wildungen, Germany). Patients (n = 31; paraplegia n = 15; tetraplegia n = 16) were classified on the basis of the impairment scale (grades A through E) developed by the American Spinal Injury Association (Table 1). All patients were classified to American Spinal Injury Association grade A or B [ie, were afflicted by a complex of both motor and sensory function (grade A) or only motor function (grade B) below the level of injury]. Tissue samples of pressure ulcers were taken from the ischium (70%), the coccyx (13%), or the sacrum (9%) (8% from undefined body locations). During debridement and coverage of pressure ulcers, intact SCI skin samples were collected from areas approximately 10 cm distant to the wound and to high-risk weight-bearing skin areas, respectively.

Following informed consent, control tissue samples of normal skin were collected, during routine surgical procedures, from various body locations of able-bodied (AB) patients (n = 22) at the Fachklinik Hornheide (Münster, Germany).

Tissue samples were stored fresh-frozen at −80°C or formalin fixed and paraffin embedded at room temperature.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
<td>SCI patients (n = 31)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27 (87)</td>
</tr>
<tr>
<td>Female</td>
<td>4 (13)</td>
</tr>
<tr>
<td>Age, years</td>
<td>50 (14.4)</td>
</tr>
<tr>
<td>SCI level</td>
<td></td>
</tr>
<tr>
<td>Paraplegia</td>
<td>15 (48)</td>
</tr>
<tr>
<td>Tetraplegia</td>
<td>16 (52)</td>
</tr>
<tr>
<td>ASIA Impairment Scale</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>21 (68)</td>
</tr>
<tr>
<td>B</td>
<td>10 (32)</td>
</tr>
<tr>
<td>SCI duration, years</td>
<td>14.7 (11.5)</td>
</tr>
</tbody>
</table>

The patient cohort is identical to the cohort analyzed by Brunner et al. Data are given as number (percentage) or mean (SD). ASIA, American Spinal Injury Association; SCI, spinal cord injury.

Procedures of tissue sample collection were approved by the local ethical committee (Ärztekammer Westfalen-Lippe, Münster, Germany).

Gene Expression Analysis

Gene expression analysis was performed as described previously. Briefly, total RNA was prepared from fresh-frozen tissue (intact SCI skin, n = 17; pressure ulcers, n = 15; and AB control skin, n = 16) using RNeasy Fibrous Tissue Mini Kits (Qiagen, Hilden, Germany) and cyanine-3 labeled by RT-IVT. Whole human genome gene expression profiles were obtained for each sample group in blinded triplicates using G3 Human Gene Expression 8 × 60K Microarrays (Agilent, Waldbronn, Germany). Gene expression data were normalized to the mean expression of the housekeeping genes, PUM1, GUSB, and HPRT1.

Immunohistochemistry of Lymphatic Vessels

Formalin-fixed, paraffin-embedded tissue sections (5 μm thick) were dewaxed in xylene and rehydrated in decreasing ethanol concentrations. Following proteinase K treatment (Qiagen; 20 μg/mL) for 20 minutes at room temperature, endogenous peroxidase was inactivated using NOVADetect Peroxid—Block (Dianova, Hamburg, Germany) for 15 minutes at room temperature, and free protein binding sites were blocked with 5% human serum for 30 minutes at room temperature. Tissue sections were incubated overnight at 4°C with primary antibodies to the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1; polyclonal goat IgG at 5 μg/mL; AF2089; R&D Systems, Abingdon, UK) or nonimmune goat IgG as negative control. Antibody binding was detected by incubation for 45 minutes at room temperature with secondary anti–goat IgG horseradish peroxidase. Diaminobenzidine (Dianova) was used as a chromogenic substrate for peroxidase. Tissue sections were counterstained with Papanicolaou (Merck, Darmstadt, Germany).
Image Analysis of Lymphatic Vessels

Microphotographs of LYVE-1–immunostained skin tissue sections were taken using a Diaplan microscope (Leitz, Wetzlar, Germany), equipped with a DFC320 digital camera and FireCam 3.4 software (Leica Microsystems, Wetzlar, Germany), and imported into Photoshop software (Adobe, San Jose, CA). Lymph vessels were identified on the basis of LYVE-1 staining and counted, and the lumen area of the vessels as well as the length of the epidermis in the tissue section were quantified in Photoshop using the respective selection tools.

Statistical Analysis

All graphs show mean values and SEM. Statistical significance was determined using the Kruskal-Wallis test or the t-test, with Bonferroni correction for multiple comparisons.

Results

Characterization of the Lymphatic Vessel System in SCI Skin

To analyze the skin for potential morphologic and functional alterations caused by the loss of neuronal control in chronic SCI, dermal lymphatic vessels were identified and characterized by immunostaining for the lymphatic marker, LYVE-1 (Figure 1A).12 The shape of the lumen of lymphatic vessels varied, and three categories of vessels were defined on the basis of the appearance of their lumen (open, intermediate, or collapsed) (Figure 1C).

To quantify lymphatic vessel number and lumen area, we developed an image analysis procedure, depicted in Figure 2. Microphotographs covering the entire area of a skin tissue section, immunostained for LYVE-1, were imported into Photoshop software, rearranged to cover and visualize the entire area of the section; immunostaining was digitally enhanced to facilitate subsequent image analysis. Lymphatic vessels were numbered and categorized into the three vessel types, defined in Figure 1C (open, green; intermediate, yellow; and collapsed, red). Following conversion of LYVE-1 immunostaining to black and white, the lumen areas of the lymphatic vessels in the section were determined. Following conversion of the Papanicolaou staining to black and white, the length of the epidermis in the section was measured.
Loss of Neuronal Control in Chronic SCI Skin Induces Collagenase Expression and Impairs Collagen Fibril Structure as Well as Functional Lymphatic Morphology

Lymphatic function requires opening of the lumen as well as contractile properties of lymphatic vessels. In chronic SCI skin, the proportion of open lymphatic vessels was drastically reduced (by 4.9-fold compared with AB control skin) (Figure 3A). Correspondingly, the proportion of collapsed vessels was almost doubled. Thus, the ratio of functionally dilated versus collapsed lymphatic vessels decreased by almost 10-fold compared with AB control skin. These alterations were reflected in a 1.7-fold reduction in the average lumen area of individual lymphatic vessels in SCI skin compared with control (Figure 3B). These findings suggested impairment of lymphatic function.

To support the above morphologic observations, we performed differential gene expression analysis of functional lymphatic markers in SCI (n = 17) versus AB (n = 16) skin. As a reference to wound healing, the analysis also comprised the wound edge of pressure ulcers (n = 15).

We identified an SCI-specific molecular signature of eight known functional lymphatic markers [CACNG1, ACTA1, LYVE1, ESR, PF4, MYLK2, TLR4, and transforming growth factor (TGF)-β1], SCI-specific differential regulation of which may result in impaired lymphatic contractility and immune cell trafficking (Table 2),13–22 supporting the above morphologic findings. Differential regulation of four of the eight signature markers (ACTA1, LYVE1, ESR, and TGF-β1) was further enhanced (P < 0.001) in pressure ulcers of SCI individuals.

Opening of lymphatic vessels and proper skin lymphatic function are critically dependent on the mechanical integrity of the dermal connective tissue.23 Previous findings indicating increased collagen degradation in skin and bone of SCI patients23–25 prompted us to investigate connective tissue weakening as a potential cause contributing to skin lymphatic dysfunction. Differential gene expression profiling of tissue degradation and production identified an SCI-specific 13-gene signature of proteinases (MMP1, MMP13, KLK12, and MMP8), enzyme inhibitors (SPINK2 and HPSE2), connective tissue constituents (FNDC1, THBS4, TNN, DPT, and TNXB), and modifying enzymes (HS3ST2 and LOXL4). Differential gene expression of this signature might result in a shift of SCI connective tissue homeostasis toward enhanced degradation (in particular, of collagen) and reduced production (Table 3).26–30 Differential expression of 6 of the 13 signature genes was further enhanced (P < 0.001) in pressure ulcers (MMP1, MMP13, KLK12, HPSE2, TNXB, and LOXL4) (Table 3).

To provide further support for increased collagen degradation in SCI skin, as suggested previously,22,24 and by our gene expression profiling (see above), we analyzed collagenase protein expression and dermal collagen structure by (immuno)histochemistry. Collagenase-3 [matrix metalloproteinase (MMP)-13] was strongly and collagenase-2...
Table 2  SCI-Specific Molecular Signature of Lymphatic Function in the Skin

<table>
<thead>
<tr>
<th>Gene/protein symbol</th>
<th>Gene/protein name</th>
<th>SCI versus AB</th>
<th>P value</th>
<th>Pressure ulcer versus AB</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACNG1</td>
<td>Calcium channel γ subunit</td>
<td>&gt;34 ▲ ▲</td>
<td>&lt;0.001</td>
<td>21.7 ▲ ▲</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACTA1</td>
<td>Actin α 1</td>
<td>8.7 ▼ ▲</td>
<td>&lt;0.001</td>
<td>78.7 ▼ ▲</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LYVE1</td>
<td>Lymphatic vessel endothelial hyaluronan receptor-1</td>
<td>5.4 ▼ ▲</td>
<td>&lt;0.001</td>
<td>8.5 ▼ ▲</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ESR</td>
<td>Estrogen receptor</td>
<td>4.3 ▼ ▲</td>
<td>&lt;0.001</td>
<td>7.7 ▼ ▲</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PF4</td>
<td>Platelet factor 4</td>
<td>4.1 ▲ ▲</td>
<td>&lt;0.001</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CCL21</td>
<td>Chemokine (C-C motif) ligand 21</td>
<td>3.7 ▼ ▲</td>
<td>&lt;0.001</td>
<td>3.6 ▼ ▲</td>
<td>0.016</td>
</tr>
<tr>
<td>MYLK2</td>
<td>Myosin light chain kinase 2</td>
<td>3.6 ▼ ▲</td>
<td>&lt;0.001</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
<td>3.0 ▼ ▲</td>
<td>0.002</td>
<td>3.2 ▼ ▲</td>
<td>—</td>
</tr>
<tr>
<td>TGF-B1</td>
<td>Transforming growth factor-β22</td>
<td>2.2 ▲ ▲</td>
<td>&lt;0.001</td>
<td>3.3 ▲ ▲</td>
<td>—</td>
</tr>
</tbody>
</table>

* Determined by DNA microarray analysis (1 to 10 probes/gene, assayed on triplicate microarrays).

Table 3  SCI-Specific Molecular Signature of Skin Connective Tissue

<table>
<thead>
<tr>
<th>Gene/protein symbol</th>
<th>Gene/protein name</th>
<th>SCI versus AB</th>
<th>P value</th>
<th>Pressure ulcer versus AB</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Matrix metalloproteinase-1 (collagenase-1)</td>
<td>16.3 ▲ ▲</td>
<td>&lt;0.001</td>
<td>130.2 ▲ ▲</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Matrix metalloproteinase-13 (collagenase-3)</td>
<td>&gt;11 ▲ ▲</td>
<td>&lt;0.001</td>
<td>21.7 ▲ ▲</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>KLK12</td>
<td>Kallikrein-related peptidase 12</td>
<td>4.0 ▲ ▲</td>
<td>&lt;0.001</td>
<td>24.3 ▲ ▲</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SPINK2</td>
<td>Serine proteinase inhibitor Kazal type 2</td>
<td>4.0 ▲ ▲</td>
<td>&lt;0.001</td>
<td>5.2 ▲ ▲</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP8</td>
<td>Matrix metalloproteinase-8 (collagenase-2)</td>
<td>&gt;3.8 ▲ ▲</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HPSE2</td>
<td>Heparanase 2 (heparanase 1 inhibitor)</td>
<td>3.5 ▲ ▲</td>
<td>&lt;0.001</td>
<td>10.1 ▲ ▲</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Determined by plasminogen activator inhibitor-1/ucerfase bioassay (data taken from Brunner et al.).

Taken together, loss of neuronal control in chronic SCI appears to impair skin lymphatic function, as documented by a decrease in functionally dilated lymphatic vessels, by molecular signatures of functional inhibitors and connective tissue degradation, and by histopathologic evidence of impaired collagen structure.

Enhanced Lymphangiogenesis in Chronic SCI Skin

Immunohistochemical analysis of skin lymphatics revealed that the total number of lymphatic vessels (open, intermediate, and collapsed) was almost doubled in SCI skin compared with AB skin (Figure 6), indicating enhanced lymphangiogenesis.
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Figure 4  Induction of collagenase protein expression in spinal cord injury (SCI) skin. A–D: Expression was analyzed in SCI skin (A and C) and able-bodied control skin (B and D) by immunohistochemistry. A and B: Collagenase-3 [monoclonal mouse anti–matrix metalloproteinase (MMP)-13; 16 μg/mL]. C and D: Collagenase-2 (monoclonal mouse anti–MMP-8; 10 μg/mL). D: Arrows indicate single cells stained for MMP-8, most likely representing neutrophils. Staining with corresponding antibody isotype controls was negative (data not shown). Scale bar = 100 μm (A–D). Original magnification, ×10 (A–D).

To verify the above morphologic observation at the molecular level, lymphangiogenesis was analyzed by differential gene expression in SCI versus AB control skin. We identified an SCI-specific molecular signature of seven lymphangiogenic factors. Differential regulation of four of the seven factors (LYVE1, ESR, TLR4, and TGF-β1) results in impairment of both lymphatic function (Table 2) as well as lymphangiogenesis (Table 3). Differential expression of the remaining three signature genes (MMP13, HPSE2, and GJB2) stimulates specifically lymphangiogenesis, with MMP13 being most prominently up-regulated in SCI skin (13.3-fold compared with AB skin). Differential regulation of six of the seven signature markers was further enhanced (P < 0.001) in pressure ulcers of SCI individuals (LYVE1, ESR, TGF-β1, MMP13, HPSE2, and GJB2) (Table 4).

Taken together, increased lymphatic vessel numbers and strong activation of lymphangiogenic stimulators, such as MMP-13, documented enhanced lymphangiogenesis in chronic SCI skin, possibly in response to impaired lymphatic functionality.

Discussion

Chronic SCI is associated with prolonged dysfunction of the cardiovascular system below the level of injury, due to interruption of spinal motor pathways and impairment of the sympathetic nervous system. The consequences in affected body areas are loss of vascular tone, vasodilation, and venous vascular dysfunction. We have previously reported that this results in increased peripheral extravasation of plasma components from the microvasculature into the dermis, followed by activation of platelets and cytokines as well as wound-healing type cellular and molecular reactions, inappropriate to noninjured skin. Whether loss of neuronal control in chronic SCI also affects the skin lymphatic system has not yet been studied so far.

The lymphatic system regulates fluid homeostasis, absorption of gastrointestinal lipids, and trafficking of immune cells. Skin lymphatic vascular function critically depends on the composition, geometry, and integrity of the connective tissue. In a functional state, the lumina of lymphatic microvessels is dilated by the increased tissue fluid pressure stretching the connective tissue fibers to which the lymphatic endothelial cells are firmly attached. In SCI skin, however, the ratio of functionally dilated/collapsed lymphatic vessels was 10-fold reduced, and the average vessel lumen area was almost halved, compared with AB control skin. This suggested that lymphatic function in SCI skin was impaired, which was unexpected...
considering the presumably high tissue fluid pressure due to increased microvascular permeability, plasma leakage, and predisposition to edema formation.

One plausible explanation for the collapse of lymphatic vessels in SCI skin might be reduced mechanical integrity of the dermis. Structural impairment of SCI connective tissue has been suggested previously, based on increased collagen degradation in SCI skin and bone, enhanced excretion of collagen and glycosaminoglycan fragments in SCI individuals, and diminished collagen cross-linking by lysyl hydroxylation, altogether apparently diminishing the quality of the collagen structure. Increased turnover and impaired structure of SCI dermal collagen is, at least in part, consistent with our observations that gene and protein expression of two major collagen-degrading enzymes, MMP-13 (collagenase-3) and MMP-13 (collagenase-3) and MMP-13 (collagenase-3), was significantly induced de novo in SCI skin. Gene expression of the collagen cross-linking enzyme, lysyl oxidase-like 4 (LOXL4), was down-regulated, potentially further affecting structural integrity of collagen. In addition, heparanase-2 (HPSE2), an inhibitor of the glycosaminoglycan-degrading enzyme, heparanase-1, was down-regulated, which is consistent with an increased turnover of glycosaminoglycans. Finally, the ratio of thicker collagen type I fibrils/thinner type III fibrils has been found to be decreased in SCI dermis, also reducing collagen integrity and possibly explaining the less dense and less fibrillar appearance of the collagen network observed herein. Thus, connective tissue in SCI dermis might be affected by a shift of homeostasis toward enhanced turnover and reduced integrity, potentially leading to the observed collapse of lymphatic microvessels.

The functional state of lymphatics, however, cannot be determined solely based on their morphology. For example, proper fluid transport, particularly through the larger lymphatics in the deeper layer of the dermis, also critically depends on the contractile properties of lymphatic muscle cells. We defined an SCI-specific signature of eight molecular markers, which are inhibitory with regard to lymphatic function (CACNG1, ACTAI, LYVE1, ESR, PF4, MYLK2, TLR4, and TGF-β). Three of these markers (CACNG1, ACTAI, and MYLK2) are critical for smooth muscle cell contraction. The most striking lymphatic inhibitory feature of SCI skin was a >34-fold down-regulation of the voltage-dependent calcium channel γ subunit 1 (CACNG1). Because lymphatic contractility requires calcium influx into smooth muscle cells, the complete abolishment of muscle cell–specific CACNG1 expression in SCI skin (<1.4-fold background level) most likely has a significant impact on lymphatic function.

Although lymphatic function appeared to be impaired in SCI skin, lymphangiogenesis was almost doubled, as determined morphologically by analyzing total vessel numbers. Enhanced lymphangiogenesis was supported, at the molecular level, by SCI-specific differential expression of a seven-marker signature of lymphangiogenesis. Although the anti-lymphangiogenic part of the signature (LYVE1, ESR, TLR4, and TGF-β) overlaps with the inhibitory functional lymphatic signature, the prolymphangiogenic part (MMP13, HPSE2, and GJB2) partially overlaps with the connective tissue signature. In particular, the dominant de novo expression of the lymphangiogenic factor, MMP13, is consistent with a stimulation of lymphangiogenesis in SCI skin. One possible interpretation of
these data is that enhanced lymphangiogenesis might represent a skin response to counteract functional lymphatic defects and restore tissue fluid homeostasis. However, because the proportion of fully dilated vessels was fivefold decreased in SCI skin, a doubling in total vessel number is most likely insufficient to compensate for the impairment of lymphatic function.

In this study, we have extended our SCI-skin–specific gene expression profiling. Together with our previous study on venous dysfunction and inadequate wound-healing reactions in SCI skin, we have defined four distinct but partially overlapping molecular signatures (relating to wound-healing reactions, lymphatic function, connective tissue integrity, and lymphangiogenesis) comprising 26 differentially regulated molecular markers. Intriguingly, differential regulation of 15 of these markers was further enhanced in pressure ulcers, corroborating our previous hypothesis that intact, noninjured SCI skin is pre-activated and has already acquired part of the molecular properties of a chronic wound. However, in addition to the above 26 molecular markers, several additional genes were found to be differentially regulated in SCI skin. It must be assumed, therefore, that other skin functions and properties might be affected in a similar manner by the loss of neuronal control, requiring future, more comprehensive molecular and functional analyses.

In conclusion, venous and lymphatic skin vasculature and, consequently, tissue fluid homeostasis as well as connective tissue integrity and wound healing appear to be affected, directly or indirectly, by the loss of neuronal control in chronic SCI. The latter comprises, apart from motor and sensory dysfunctions, also the impairment of autonomic functions of the nervous system. Although the sympathetic neuronal control of blood flow in human skin has been extensively studied, control of lymphatic function in vivo by the autonomic nervous system has only recently been shown using an animal model. To our knowledge, our studies provide first evidence that, in human skin, the lymphatic vasculature is under neuronal control. However, because our study comprised a limited number of patients, results require confirmation in an independent patient cohort. Nevertheless, our findings may help to explain the frequently observed disturbance of tissue fluid homeostasis observed in several tissues of SCI individuals, including skin.

Table 4  SCI-Specific Molecular Signature of Lymphangiogenesis in the Skin

<table>
<thead>
<tr>
<th>Gene/protein symbol</th>
<th>Gene/protein name</th>
<th>Differential gene expression*/growth factor activity$^{x}$-fold</th>
<th>SCI versus AB</th>
<th>P value</th>
<th>Pressure ulcer versus AB</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of Lymphangiogenesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LYVE1</td>
<td>Lymphatic vessel endothelial hyaluronan receptor-1</td>
<td>$^{37,37}$$^{5.4}$</td>
<td>$^{&lt;0.001}$</td>
<td>$^{8.5}$</td>
<td>$^{&lt;0.001}$</td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>Estrogen receptor</td>
<td>$^{17}$</td>
<td>$^{&lt;0.001}$</td>
<td>$^{7.7}$</td>
<td>$^{&lt;0.001}$</td>
<td></td>
</tr>
<tr>
<td>CCL21</td>
<td>Chemokine (C-C motif) ligand 21</td>
<td>$^{38}$</td>
<td>$^{&lt;0.001}$</td>
<td>$^{3.6}$</td>
<td>$^{&lt;0.001}$</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
<td>$^{40,40}$</td>
<td>$^{&lt;0.001}$</td>
<td>$^{3.2}$</td>
<td>$^{0.016}$</td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor-β</td>
<td>$^{41,42}$</td>
<td>$^{&lt;0.001}$</td>
<td>$^{3.3}$</td>
<td>$^{&lt;0.001}$</td>
<td></td>
</tr>
<tr>
<td>Stimulation of Lymphangiogenesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP13</td>
<td>Matrix metalloproteinase-13</td>
<td>$^{43}$$^{&gt;11}$</td>
<td>$^{&lt;0.001}$</td>
<td>$^{47}$</td>
<td>$^{&lt;0.001}$</td>
<td></td>
</tr>
<tr>
<td>HPSE2</td>
<td>Heparanase 2 (heparanase 1 inhibitor)</td>
<td>$^{30,44}$</td>
<td>$^{&lt;0.001}$</td>
<td>$^{10.1}$</td>
<td>$^{&lt;0.001}$</td>
<td></td>
</tr>
<tr>
<td>GJB2</td>
<td>Gap junction protein β 2 (connexin-26)</td>
<td>$^{45}$</td>
<td>$^{&lt;0.001}$</td>
<td>$^{11.3}$</td>
<td>$^{&lt;0.001}$</td>
<td></td>
</tr>
</tbody>
</table>

$^{*}$Determined by DNA microarray analysis (one to nine probes/gene, assayed on triplicate microarrays).
$^{1}$Determined by plasminogen activator inhibitor-1/luciferase bioassay (data taken from Brunner et al.$^{9}$).
$^{2}$Data are x-fold differences of mean gene expression in the respective groups (SCI, $n=17$; AB control, $n=16$; and pressure ulcer, $n=15$).

$^{+}$Differentially down-regulated (threefold or greater); $^{\dagger}$, differentially up-regulated (threefold or greater); AB, able bodied; SCI, spinal cord injury.
Because the skin lymphatic system is not only involved in the regulation of tissue fluid homeostasis but is also essential for pathogen recognition and cellular responses in skin immunity, our results may have important implications with regard to the predisposition to and the treatment of wound-healing disorders of SCI individuals. Thus, molecular signatures defining the homeostatic balance of connective tissue turnover and lymphatic dysfunctionality in SCI skin, as identified herein, may have translational potential for diagnostic stratification and/or prognosis of SCI individuals with regard to the risk of pressure ulcer development. Furthermore, stimulation of contractile lymphatic function by specific receptor agonists or inhibition of inappropriate connective tissue degradation by specific MMP inhibitors might be evaluated, in future clinical studies, for its efficacy in preventing and/or treating pressure ulcers of individuals affected by SCI.

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Author Contributions

G.B. and T.M.: study concept and design; T.M. and V.B.: tissue samples and clinical data; M.S.R. and G.B.: development of method, experiments, statistical analysis, and writing of the article; M.S.R., T.F., N.B.-S., and M.B.: (immuno)histochemistry; G.B., T.M., and M.S.R.: analysis and interpretation of the data. All authors read and approved the final article. G.B. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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