Cellular and molecular changes that predispose skin in chronic spinal cord injury to pressure ulcer formation

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Abstract
Patients with spinal cord injury have a predisposition to develop pressure ulcers. Specific characteristics of the patients' skin potentially involved have not yet been identified. The purpose of this investigation was to determine whether loss of neuronal control affects cellular and molecular homeostasis in the skin. Intact afflicted skin, wound edge of pressure ulcers, and control skin were analysed. Platelets, transforming growth factor-β1, and activin A were identified by immunohistochemistry. Transforming growth factor-β-like activity was determined by bioassay, and gene expression by DNA microarray analysis or RT-PCR. In afflicted skin, enhanced platelet extravasation was detected. Transforming growth factor-β1 and activin A accumulated in the dermal-epidermal junction zone. Transforming growth factor-β-like activity and activin A expression were increased in intact afflicted skin (compared to control skin) and were further enhanced in pressure ulcers. In vitro, activity was generated by fibroblast-epithelial cell interactions, which also induced activin A. Thus, loss of neuronal control in spinal cord injury appears to trigger inappropriate wound healing processes in the patients' skin. Plasma leakage and increased transforming growth factor-β-like activity combined with shear forces potentially enhance the risk for pressure ulcer formation.

KEYWORDS
activins, pressure ulcer, spinal cord injury, transforming growth factor beta, wound healing

1 INTRODUCTION

There is increasing evidence that neuronal activity regulates skin homeostasis as well as physiological processes such as inflammation and wound repair.1,2 This is supported by the findings that in animal models, experimental denervation impairs regeneration and important wound healing responses such as re-epithelialisation.3,4 Conversely, in intact unaffected skin, cutaneous wound healing causes hyperinnervation of the skin,5 pointing to the release of neurotrophic factors such as nerve growth factor. The latter, in turn, stimulates cutaneous inflammation6 and promotes pressure ulcer (PU) healing.7 These observations illustrate that neuronal activity and wound healing responses are closely interlinked.2,3

List of abbreviations: FF, fresh-frozen; FFPE, formalin-fixed paraffin-embedded; PAI/L, plasminogen activator inhibitor-1/luciferase; PU, pressure ulcer; SCI, spinal cord injury; TGF, transforming growth factor.
However, the molecular and cellular mechanisms underlying these interactions are largely unknown.

Patients afflicted with peripheral neuropathies, for example, in diabetes or venous insufficiency, often suffer from chronic wounds. This is also true for patients with spinal cord injury (SCI) who have a striking predisposition to develop PUs in skin areas afflicted by impaired neuronal activity. SCI patients have a life-time risk of 85% for developing PUs, and the prevalence of PUs in chronic SCI ranges between 15 and 30%, causing annual health care costs of $1.4 billion in the United States and substantial impact on the quality of life of these patients. Several physiological abnormalities have been noted in SCI skin such as vascular abnormalities and abnormal connective tissue catabolism, both potentially impairing the healing of existing PUs. Most of the risk factors described for the predisposition to develop PUs, however, relate to clinical and sociodemographic characteristics. The aim of this study was to define, for the first time, cellular and molecular risk factors in the skin of SCI patients, potentially predisposing to PU development.

2 | METHODS

To identify SCI-specific risk factors for PU development, we compared non-injured SCI skin from areas afflicted by impaired neuronal activity to control skin from healthy individuals. We focused on cellular and molecular indicators of early-wound related processes inappropriately triggered in intact SCI skin, such as platelet extravasation and growth factor activation. Activation of molecular risk factors was verified in PU tissue.

2.1 | Tissue samples

Following informed consent tissue samples of intact skin or of the wound edge of PUs, both from areas afflicted 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 based on the impairment scale (grades A-E), developed by The American Spinal Injury Association (ASIA) (Table 1). PU tissue samples were taken from the ischium (70%), the coccyx (13%), or the sacrum (9%) (8% from undefined body locations). During PU debridement and coverage, intact SCI skin samples were taken from areas approximately 10 cm distant of PUs and high-risk weight-bearing skin areas, respectively. Following informed consent, tissue samples of normal skin were collected, during routine surgical procedures, from various body locations from able-bodied (AB) patients (n = 22) at the Fachklinik Hornheide (Münster, Germany). Tissue samples were stored fresh-frozen (FF) at −80°C or formalin-fixed and paraffin-embedded (FFPE) at room temperature. Procedures of tissue sample collection were approved by the local ethical committee (Ärztekammer Westfalen-Lippe, Münster, Germany).

2.2 | Cell culture

Primary adult human dermal fibroblasts and epidermal keratinocytes were obtained from PromoCell (Heidelberg, Germany). Fibroblasts were cultured in Medium 106 with low-serum growth supplement (Life Technologies, Darmstadt, Germany), and keratinocytes in EpiLife medium with defined growth supplement (Life Technologies). Cells were used between passages 2 and 5. Co-cultures of fibroblasts and keratinocytes were plated in 24-well plates (50 000 cells of each cell type in 300 μL of medium) and TGF-β activity determined in 24-hour conditioned media. In some experiments, fibroblasts were incubated with 1 ng/mL of recombinant human TGF-β1 (R&D Systems, Wiesbaden, Germany) and analysed for INHBA mRNA expression. Mink lung epithelial cells (MLEC) transfected with the plasminogen activator inhibitor-1 (PAI-1)/luciferase (PAI/L) reporter construct were a gift from Dr. D.B. Rifkin (New York University Medical Centre, New York, New York). MLEC...
were cultured in DMEM with 10% FCS, L-glutamine (2 mmol/L), Hepes (10 mmol/L), and geneticin (250 μg/mL). Cells were used between passages 8 and 35.

2.3 DNA microarray analysis

Total RNA was prepared from 5-μm tissue cryosections using RNeasy Fibrous Tissue Mini Kits (Qiagen, Hilden, Germany) following tissue homogenisation with a syringe in RLT buffer. Aliquots of 100 ng RNA were cyanine-3-labelled by RT-IVT. Whole human genome gene expression profiles of sample groups (AB controls, n = 16; SCI skin, n = 17, PU wound edge, n = 15) were obtained by hybridising 0.6-μg aliquots of labelled cRNA onto G3 Human Gene Expression 8x60K Microarrays (Agilent, Waldbronn, Germany) and signal detection using an Agilent DNA Microarray Scanner and Feature Extraction 10.5.1.1 software. Gene expression data were normalised to the mean expression of the housekeeping genes, PUM1, GUSB, and HPRT1.

2.4 Real-time RT-PCR

To analyse activin A expression, mRNA levels of the βA subunit of activin A, INHBA, were determined using real-time RT-PCR. Total RNA was prepared from cultured cells using QIAamp RNA Blood Mini Kits (Qiagen). RNA aliquots (1 μg/20 μL) were reverse-transcribed for 1 hour at 37°C using random hexamers (Promega, Mannheim, Germany) and High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, California), in the presence of 1 u/μL of RNase inhibitor (Promega). cDNA quality was verified by amplifying the housekeeping gene, GAPDH. Gene expression (50 ng cDNA/20 μL) was quantified by real-time PCR (Applied Biosystems 7500 Real-Time PCR system).

2.5 Immunohistochemistry

FFPE tissue sections (5 μm) were dewaxed in xylene and rehydrated in decreasing ethanol concentrations. Following proteinase K treatment (Qiagen, 20 μg/mL, 20 minutes at RT), endogenous peroxidase was inactivated using NOVADetect Peroxid-Block (Dianova, Hamburg, Germany; 15 minutes at RT) and free protein binding sites blocked with 5% human serum (30 minutes at RT). Tissue sections were incubated (overnight at 4°C) with the primary antibodies, goat anti-glycoprotein IIb (GPIIb, anti-CD41, 5 μg/mL), rabbit anti-TGF-β1 (10 μg/mL; both from Santa Cruz Biotechnology, Santa Cruz, California), or rabbit anti-activin A (5 μg/mL; Agrenvec, Madrid, Spain). Anti-CD41 blocking peptide (5 μg/mL; Santa Cruz Biotechnology) or non-immune rabbit IgG were used as negative controls. Antibody binding was detected by incubation (45 minutes at RT) with anti-goat horseradish peroxidase or anti-rabbit alkaline phosphatase (0.8 μg/mL; both from Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). Chromogenic substrates used were DAB for peroxidase and Permanent Red for phosphatase (Dianova). Tissue sections were counterstained with Papanicolaou.

2.6 PAI/L bioassay for TGF-β

TGF-β is secreted as a latent protein complex, which requires extracellular activation for bioactivity. TGF-β-like activity in FF skin tissue was determined ex vivo using the PAI/L bioassay. Briefly, coverslips carrying 20-μm tissue cryosections were placed upside down onto MLEC plated in 24-well plates. Tissue sections and recombinant human TGF-β1 standards (R&D Systems, 0 to 250 pg/mL) were incubated with MLEC overnight at 37°C. Luciferase activity in the cell lysates was determined using an MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, Virginia). The tissue volume was calculated from the thickness (20 μm) and the area of the cryosections, determined by image analysis (ImageJ software; NIH, Bethesda, Maryland). The ratio of TGF-β and activin A activity in the tissue was determined by neutralisation with 5 μg/mL of rabbit pan-specific anti-TGF-β1,2,3 IgG or monoclonal mouse anti-activin A IgG (R&D Systems). Species-specific non-immune IgGs were used as controls. In order to mimic dermal-epidermal interactions in vitro, primary human cells were cultured in DMEM with 10% FCS, L-glutamine (2 mmol/L), Hepes (10 mmol/L), and geneticin (250 μg/mL). Cells were used between passages 8 and 35.
fibroblasts and keratinocytes were co-cultured, with direct cell-to-cell contact, and analysed for TGF-β like activity. Activity in skin-cell conditioned media was determined using the PAI/L bioassay.12,14

2.7 | Data analysis

All graphs show mean values and SEM. Statistical significance was determined using the Kruskal-Wallis test with
Bonferroni correction for multiple comparisons or the Student's *t*-test.

### 3 | RESULTS

#### 3.1 | Platelet extravasation

One of the most prominent early wound healing processes is plasma leakage and the release and activation of platelets in the injured tissue. We, therefore, examined intact SCI skin for the presence of extravasated platelets using immunostaining for the platelet marker, GPllb. Platelets were indeed identified outside of blood vessels (Figure 1), scattered throughout the dermis or clustered in small groups (B, D), in some cases adjacent to blood vessels (F). In contrast, in the skin of AB controls, platelets were not seen outside (A, C) and only occasionally inside of blood vessels (E). The observed staining was platelet-specific, since it was abolished by anti-GPllb antibody blocking peptide (data not shown). Platelet extravasation in SCI skin was not due to an increase in circulating platelets, since platelet counts of SCI patients without PUs were decreased rather than increased when compared with AB controls (SCI: $2.7 \pm 0.2 \times 10^5/\mu L$, $n = 10$; AB: $3.0 \pm 0.2 \times 10^5/\mu L$, $n = 11$). Thus, chronic SCI appears to result in peripheral leakage of plasma components such as platelets into the dermis (see also Reference 15).

#### 3.2 | Platelet TGF-β1 release

Activated platelets release a variety of growth factors, the most prominent one being latent TGF-β1.$^{16,17}$ Indeed, increased TGF-β1 protein levels were detected by immunostaining of SCI skin when compared with AB controls (Figure 2B compared to A). TGF-β1 was observed throughout the dermis and appeared to accumulate in the sub-epidermal junction zone (B). This indicated that TGF-β1 was released into the dermis, at least in part, by activated platelets.

**FIGURE 2** Accumulation of transforming growth factor (TGF)-β1 and activin A in the dermis of SCI patients. Immunostaining for TGF-β1, A,B or activin A, D,E of intact SCI skin, B,C,E,F or AB control skin, A,D; C,F, Control staining with non-immune IgG. TGF-β1 (B, arrow) and activin A (E, arrow) accumulated in the dermal-epidermal junction zone of SCI skin. TGF-β1 was also found throughout SCI dermis, and activin A in sweat glands of both SCI and AB skin (D,E; arrowheads)
3.3 | TGF-β-like activity in vivo

We determined TGF-β-like activity in SCI skin vs AB control skin using the plasminogen activator inhibitor-1 (PAI/L) bioassay. Activity stepwise increased from AB control skin (76 sections analysed from 21 individuals; 76/21) to intact SCI skin (58/20), and further to wound edge of pressure ulcers (PUs) (66/23). Data are mean values + SEM and differed significantly from each other (p < 0.00001). B. TGF-β-like activity in intact SCI skin (36/5) or wound edge of PUs (26/6), in the presence of neutralising IgG or non-immune IgG as a control. Data are mean values + SEM of TGF-β activity (in the presence of anti-activin A IgG) and activin A activity (in the presence of pan-specific anti-TGF-β IgG), expressed as percentage of total TGF-β-like activity (in the presence of non-immune IgG).
activin A contributed to the elevated TGF-β-like activity in SCI skin and PUs.

### 3.4 | TGF-β-like activity in vitro

Our previous studies indicated that, apart from activated platelets, dermal-epidermal interactions may be involved in TGF-β activation during wound repair in vivo and in vitro. Fibroblast-keratinocyte interactions in co-cultures strongly induced TGF-β-like activity (6.4-fold compared to fibroblasts alone; Figure 4B). Similar to SCI skin in vivo, TGF-β-like activity in skin cell cultures in vitro was a mixture of TGF-β and activin A (Figure 4C).

### 3.5 | Activin A expression

In SCI skin INHBA mRNA expression was significantly elevated (1.3 fold) compared with AB controls (Figure 5A). Expression was further increased at the wound edge of PUs (1.9 fold, 2.5-fold compared to AB controls), which is consistent with the involvement of activin in wound repair. While platelets do not release activin A, dermal-epidermal interactions induced activin A protein expression and bioactivity (Figures 2E and 4C). In vitro, fibroblast-keratinocyte interactions strongly induced INHBA expression (7.7-fold compared to fibroblasts alone; Figure 5B). This co-culture effect could in part be substituted for by exogenous addition of TGF-β1 to fibroblasts. Enhanced bioactivity of activin A in SCI skin was also documented by the induction of known activin target genes (Table 2). The activin target gene signature in SCI skin (induced 4.1-16.3 fold) comprised four genes, MMP1, S100A9, S100A8, and HBB. Target gene expression was further increased in PUs.

### 4 | DISCUSSION

The striking predisposition of SCI patients to develop PUs is not fully understood. Whereas behavioural and physiological risk factors such as decreased mobility, lack of sensation, or shear forces have been documented, a potential contribution of abnormalities in SCI skin at the cellular or molecular level has not yet been analysed. Our hypothesis was that the loss of neuronal control in chronic SCI alters the cellular and molecular homeostasis in the skin of these patients, thereby promoting PU formation. In support of this hypothesis, we found that tissue fluid homeostasis is dysregulated in SCI skin towards enhanced microvascular permeability and plasma leakage, as documented by extravascular platelets in the dermis. Microvessels are under neuronal control, and our results are consistent with the increased capillary

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**FIGURE 5** Increased INHBA gene expression in SCI skin ex vivo and in skin cells in vitro. INHBA mRNA expression was measured in tissue samples ex vivo, A using DNA microarray analysis or in skin cells in vitro, B using real-time RT-PCR. A, Expression stepwise and significantly (*p* < 0.00001) increased from AB control skin to intact SCI skin, and further to wound edge of PUs. Data are mean fluorescence + SEM of 10 INHBA-specific probes on duplicate (AB) or triplicate arrays (SCI, PU). B, INHBA mRNA expression was increased by co-culture of primary human adult skin fibroblasts and keratinocytes (*p* < 0.002, co-culture vs homotypic cultures) or by stimulation of fibroblasts with 1 ng/mL of TGF-β1 (*p* = 0.05). Data are mean mRNA copy numbers + SEM.

**TABLE 2** Activin A target gene induction in intact spinal cord injury (SCI) skin and pressure ulcers (PUs)

| Gene   | SCI skin     | PUs          |
|--------|--------------|--------------|
| MMP1   | 16.3 (1.6)a  | 130.2 (13.6) |
| S100A9 | 6.9 (0.2)    | 37.2 (2.7)   |
| S100A8 | 4.7 (0.1)    | 19.2 (1.2)   |
| HBB    | 4.1 (0.1)    | 9.1 (0.2)    |

*a*Data are mean (SD) differential gene expression (x-fold) vs able bodied control skin, determined with *n* = 2 (*S100A9, S100A8*) or *n* = 10 (MMP1, HBB) gene-specific probes on triplicate DNA microarrays. Differences were significant (*p* < 0.005) for all comparisons.
permeability observed in the skin of diabetic patients, caused by the loss of sympathetic tone in diabetic neuropathy. Clinically, this is illustrated in SCI skin by oedema formation and skin thickening.

Following contact with connective tissue, extravascular platelets become activated and release growth factors such as TGF-β. Probably as a result, TGF-β levels in SCI skin were increased. TGF-β accumulated in the dermal-epidermal junction zone, most likely due to binding of latent TGF-β to specific components enriched in this region. Latent TGF-β sequestration into extracellular matrix is an essential step regulating growth factor activity, candidate latent TGF-β binding sites in the dermal-epidermal junction zone are fibronectin or fibrillin microfibrils. Platelets contain 100-fold more TGF-β than any other cell type. Thus, platelet degranulation may provide a rich extracellular matrix reservoir of latent TGF-β in SCI skin, accessible to extracellular activation upon suitable physiological signals. Indeed, TGF-β protein levels were paralleled by activity levels, which were elevated in SCI skin and further increased in PUs. To our knowledge, this is the first demonstration of the active form of TGF-β in human tissue ex vivo and is consistent with its role in wound repair. However, significant TGF-β activity levels in SCI skin, amounting to two-thirds of those in PUs, are inappropriate for non-injured skin and might indicate a “pre-activated state” of intact SCI skin with regard to wound-related factors and processes. Since TGF-β has important functions in early wound healing, including initiation of inflammatory responses and activation of skin cells, inappropriate TGF-β activation in non-injured, resting SCI skin might facilitate PU formation, for example, upon further signals given by shear-stress induced tissue damage.

TGF-β is secreted as a latent protein complex, which requires extracellular activation by cell-type specific mechanisms. Thrombin-stimulated platelets, for instance, activate part of their latent TGF-β by corelease of a furin-like enzyme. However, although platelet TGF-β activity levels were biologically significant (corresponding to half-maximal signalling in the PAI/L bioassay), the majority remained in latent form, representing a growth factor source available to activation by other cell types and mechanisms. Intriguingly, the latent TGF-β complex is receptive to shear forces generated by connective tissue mediating latent TGF-β activation by interaction with integrin receptors. Frequently changing shear forces appear to be most effective in activating latent TGF-β. Whether locally occurring shear forces in SCI skin, in conjunction with platelet latent TGF-β deposition and integrin binding in the dermis, may contribute to the elevated TGF-β activity levels observed in these patients, remains to be investigated.

Several members of the TGF-β superfamily are involved in wound repair and might, therefore, contribute to the TGF-β-like activity observed in SCI skin. TGF-β-like activity in SCI skin and PUs, as measured by the PAI/L bioassay, was composed of TGF-β and the TGF-β superfamily member, activin A. This is the first time that activin A has been shown to contribute to the activity signal measured in the PAI/L bioassay. Elevated activin A levels in SCI skin and PUs were indirectly confirmed by increased expression of a signature of four known activin target genes, comprising the regulator of inflammation and wound healing, matrix metalloproteinase (MMP)-1, the Ca²⁺-binding protein dimer, S100A8/A9, induced by activin A in acute wounds and ulcers, and the haemoglobin subunit beta. Similar to TGF-β, activin A regulates early-wound related processes such as cell activation, inflammation, and connective tissue turnover. Increased activin A levels in intact SCI skin are, therefore, inappropriate and confirm a “pre-activated state” of SCI skin with regard to wound signals, potentially promoting PU formation. Apart from regulating inflammation and wound repair, activin A induces epidermal thickening due to augmented keratinocyte proliferation, thus potentially contributing, in conjunction with oedema formation, to the skin thickening observed in SCI patients. In contrast, at weight-bearing sites SCI skin thinning has been observed. Additional alterations of the biomechanical properties of SCI skin are skin stiffening and increased collagen degradation, the latter occurring several months prior to PU development. This is consistent with our findings that expression of MMP-1 (Table 2) and MMP-13 (data not shown), which degrade skin collagens type I and II, was more than 10-fold enhanced in intact, non-injured SCI skin. These altered biomechanical and molecular properties of SCI skin increase shear stress and are considered risk factors for PU development. Increased shear stress, particularly in weight-bearing skin areas, might further enhance, in a positive feedback loop, stress-induced TGF-β activation and TGF-β-induced expression of activin A and MMPs. While activin A has been reported not to be among the growth factors released from activated platelets, activin A expression was induced, similarly to latent TGF-β activation, by dermal-epidermal interactions in vitro as demonstrated by co-culture of primary fibroblasts and keratinocytes. This extends the previous observation that activin A is induced in fibroblasts, co-cultured with immortalised HaCaT keratinocytes, to co-cultures of primary skin cells. The requirement of
dermal-epidermal interactions could be partially substituted for by exogenous addition of TGF-β, which induced activin A in fibroblasts in vitro (Figure 5B), suggesting a potential sequential activation of TGF-β and activin A. Activin A induction by dermal-epidermal interactions or as a consequence of platelet TGF-β activation might explain activin accumulation, similarly to TGF-β, in the dermal-epidermal junction zone of SCI skin. It is intriguing that, apart from regulating wound repair, activin A also acts as a neuroprotective factor and is induced by acute neuronal damage, for example, SCI. These findings raise the possibility that activin A might have been induced in chronic SCI skin as a response to impaired neuronal activity.

PU formation is a frequent medical complication in chronic SCI, has a major impact on the patient's quality of life, and constitutes a significant financial burden for the health care systems. We provide evidence to support our hypothesis that loss of neuronal control in chronic SCI, in conjunction with biomechanical risk factors at weight-bearing body sites, may trigger a series of early-wound related processes inappropriate for non-injured skin. This might confer to intact SCI skin a “pre-activated,” potentially wound-promoting phenotype characterised by increased levels of TGF-β, activin A, and MMPs equalling two-thirds of those in PUs, that is, are “more than halfway there” regarding PU formation. However, PU formation is a complex process and PU predisposition may, therefore, involve a number of different molecular factors. This is illustrated by the fact that several hundred genes were differentially expressed in SCI skin (unpublished data). Thus, a more complete characterisation of PU predisposition of SCI skin will require comparative analyses of global gene expression profiles as well as integration of altered biomechanical properties.

In summary, loss of neuronal control was associated with peripheral platelet leakage and activation in SCI skin. In addition to this vascular abnormality local growth factor imbalance was observed in SCI skin, predominantly in the dermal-epidermal junction zone, possibly due to sequential induction and activation of TGF-β and activin A. These wound-related processes are inappropriate for non-injured, intact skin and potentially predispose SCI skin to PU development.

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CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT
Data available on request from the authors

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