Advanced wound care:
from genetics to skin cell therapy

Summary of Ph.D. thesis

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1. INTRODUCTION

1.1. Chronic wounds and leg ulcers

The number of chronic wound patients is increasing world wide, despite the fact that the last twenty years have seen major innovations in wound healing. Unfortunately this also means a steep rise in the number of lower limb amputations due to previous tissue loss. Chronic wounds are a growing challenge due to demographic changes and increased risks of atherosclerosis and diabetes. In developing countries the prevalence of diabetes is about 5-7%, and it is estimated that 15% of all diabetic foot ulcers will result in amputations. Both tissue loss and amputations have an enormous impact on one's everyday life resulting in social isolation, loss of independence, psychological stress and increased health costs for the patient and the society as well. These health costs already present a significant economic problem for health care providers, and with the patient numbers increasing they forecast a boom in expenses in the near future. The abundance of wound care methods and products resulted in an information boom regarding wound healing, making every day decision making harder. Treating a wound properly now equals to establishing a complex care strategy for the patient with the involvement of a multidisciplinary wound care team.

1.2. Overview of advanced wound care

The basic goals of chronic wound care: first - treat the cause, second - apply adequate local wound care. In the case of acute wounds normal wound healing is described as a cascade of physiological events resulting in full epithelisation. In chronic wounds these events often take place not one by one, but at the same time. A chronic wound is defined as a break in the skin of long duration (more than 6 weeks). Analyzing a chronic wound, one must attempt to determine which aspect of the healing process gone wrong and why. It should be carefully assessed whether it is the result of local or systemic microenvironmental factors. With this approach, chronic wound can be considered as a symptom of an underlying disease. For most cases successful wound healing is based on two pillars (goals): *treating the underlying cause or disease* behind the chronic wound and adequate *local wound care* resulting in a wound bed ready for epithelisation. The basic goal of local wound care is to

prepare a wound bed ready for epithelisation in the shortest, safest, pain free manner in the most cost-effective way possible. The main goal of these wound dressings is to maintain and protect a moist wound environment with the possible capability to absorb the excess exudates to prevent maceration. Besides the use of modern moist wound dressings, a wide array of wound care methods have been developed to solve common wound bed disorders. Evidently, accomplishment of only one of these two goals will never result in long term success.

1.3. The non-healing wound: could leg ulcers be hereditary?

The ulceration of the leg is a multifactorial disorder with several known and yet unknown environmental and genetic factors contribute to its etiology. To study the genetic factors involved in the mechanism of leg ulcer development and to minimize statistical bias, we have chosen one of the major homogenous subgroup of leg ulcers; therefore our genetic studies were carried out on patients with venous leg ulcer. The complexity of venous leg ulcer pathogenesis is increased by the fact that in the majority of the cases its development has polyetiological origin. Besides environmental factors, several predisposing genetic factors have already been shown to be associated with venous leg ulcer susceptibility such as the presence of certain gene polymorphisms and gene expression abnormalities. The known genetic factors predisposing to venous leg ulcer development are the followings factor V Leiden mutation, prothrombin gene G20210A mutation, antithrombin deficiency, activated protein C resistance, protein S deficiency, hyperhomocysteinaemia and abnormal TGF-β type 2 receptor expression (Munkvad et al., 1996; Peus et al., 1997). These genetic factors impair different processes and phases of wound healing, which might suggest that multiple molecular mechanisms of venous leg ulcer development exist or similarly to carcinogenesis the co-existence of different precipitating factors leads to the development of the disease. To develop causative treatment opportunities for those who have genetic deficiencies in the pathogenesis, there is an emerging importance to characterize the genetic abnormalities of leg ulcer development.

1.4. Aims

The aims of our genetic studies were to identify common single nucleotide polymorphisms (SNP), which are associated with leg ulcer

development and gene expression abnormalities related to pathologic wound healing.
We also aimed to develop a new autologous skin cell transplantation method that makes use of hairy scalps as a donor site, with an artificial mitogen free culturing method containing melanocytes, fibroblasts and keratinocytes.

2. MATERIALS AND METHODS

2.1. Human skin biopsy samples for genetic studies

Two-by-two cm shave biopsies were collected under local anesthesia from venous leg ulcer patients (n=15) and from age and sex-matched leg ulcer-free control individuals (n=15). The average duration of the ulcers was 5.8±5.1 years and 24.2% of the leg ulcer patients had suffered from deep vein thrombosis previously. Leg ulcer patients of other causes were excluded from the study. Venous leg ulcer patients were also screened a series of other diseases, for example trauma, erysipelas, cardiac diseases, atherosclerosis and autoimmune disorders. The study was approved by the Internal Review Board. Written informed consent was collected from all the donors. The study was conducted according to the Principles of the Declaration of Helsinki.

2.2. SNP detection

Blood samples were collected from patients with leg ulcer (n=82, average age: 67.0±24.8 years) and from the members of an age-matched control group (n=82, average age: 53.0±29.2 years). DNA was isolated by standard proteinase K digestion (DNA Isolation Kit, Eppendorf). PCR reactions were carried out on all of the samples with TaqMan mutation detection probes (Assays-on-Demand SNP Genotyping Kit, Applied Biosystems). The Assays-On-Demand SNP Analysing Mix contains two main components: specific primers for the amplification of a short DNA fragment involving the examined SNP and oligonucleotides labelled with fluorescent dyes (FAM and VIC). The sequences of the labelled oligonucleotides differ from each other only in one nucleotide, which is in the position of the polymorphism. During the PCR reaction, one or both of the oligonucleotides bind to the DNA amplicon, depending on the genotype of the examined individual, and is cut into fragments by the exonuclease activity of the Taq DNA

polymerase. Thus, at the end of the PCR reaction, the fluorescent signal of one or both of the cut fragments can be detect After the PCR reaction. End-point detection was performed with an ABI Prism 7000HT Sequence Detection System. The statistical significance of the differences between data distributions was determined by using the Chi^2 probe and a probability level $P<0.05$ was considered statistically significant.

2.3. Gene expression at mRNA level – real-time RT-PCR

Total RNA was isolated from samples through the use of the TRIzol reagent, following the instructions of the manual. cDNAs werw generated with oligo(dT) and random hexamer primers from 1 μg of RNA, using the iScriptTM cDNA Synthesis Kit of Bio-Rad Laboratories (Hercules, CA, USA) in a final volume of 20 μl. Then real-time RT-PCR was performed to quantify the abundance of the mRNA levels of the studied genes. RNA expression data were normalized to the 18S ribosomal RNA expression data of each of the examined samples. TaqMan probes were purchased from IDT Probes (Chicago, IL, USA). The real-time RT-PCR reactions were conducted by using iQ Supermix (Bio-Rad Laboratories) in an iCycler (Bio-Rad Laboratories).

2.4. Gene expression at protein level – Western blot analysis

Total protein extracts were prepared from the skin biopsy samples using TRIzol reagent according to the instruction of the manual. In order to verify the equivalent loading of proteins in the wells, the following procedure was carried out: based on the measured density OD_{280}, the protein concentration of each sample was calculated and the samples were then run on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie brilliant blue (CBB; Sigma-Aldrich), dried and scanned. The lanes were analyzed by densitometry. The amounts of the loaded protein samples were corrected according to the densitometry data and checked again on SDS-PAGE.

2.5. Statistical analysis

Statistical analysis, on the groups of leg ulcer patients and leg ulcer-free controls, was carried out according to the rules of case-control allelic association study design. The statistical significance of the association between the mRNA or protein expression profiles of the different genes

and venous leg ulcer development was assessed by one-way ANOVA for two independent samples. The statistical significance of the association between the studied SNPs of FGFR2 and venous leg ulcer was determined with the Chi^2 probe. The Bonferroni correction for the multiple hypothesis of the analysed different SNPs (n=5) was also defined. The statistical significance of the association between the -308 TNF-α SNP and venous leg ulcer was calculated by means of the Fisher Exact Probability Test. To allow comparison with the findings of Wallace et al. (2006), logistic regression analysis was also carried out. For all SNPs the odds ratios (ORs) with 95% confidence intervals (CIs) were also determined.

2.6. Human skin biopsy samples for culturing

We took biopsies from six patients after approval of the study protocol by the Ethical Commitee of the University of Szeged. All patients were informed of the procedures and all have signed the consent form. The samples were taken from three different sites under local anesthesia: split skin graft of the hairy scalp, full thickness graft of the groin and split thickness graft of the upper leg. All samples were 9 cm^2 in size. Each individual (ages 61-74) had chronic leg ulcers for over 10 years.

2.7. Cell culturing and autolgous keratinocyte transplantation

Adult epidermal keatinocytes and melanocytes were isolated and cultured from the groin, thigh and hairy scalp of healthy Caucasian donors. Skin specimens were first washed in Salsol A solution (Human Rt, Gödöllő, Hungary) supplemented with 2% antibiotic, antimycotic solution (Sigma). The subcutis and part of the dermis was removed and the tissue was cut into small strips. Overnight incubation in Dispase solution (Grade II, Roche Diagnostics, Mannheim, Germany) was carried out at 4 °C to separate the dermis from the epidermis (Kitano et al, 1983). Next day the epidermis was peeled of the dermis. The epidermis was put into 0.25% trypsin (Gibco) for 30 minutes at 37 °C. Following trypsinization, the epidermis was mechanically torn apart and vigorously washed to release epidermal cells. The epidermal cell suspension was filtered through a 100 μm nylon mesh (BioDesign, Saco, Maine, USA) and centrifuged at 200*g* for 10 minutes at 4 °C. Epidermal cells were then placed into 75 cm^2 tissue culture dishes

(Corning Incorporated, Corning, NY, USA) at a cell density of $4x10^5$ cells/cm^2. Cells were washed with fresh culture medium 12-18 hours after seeding to remove floating cells from the culture. Fresh culture medium was put on cells three times weekly. Primary cultures reached ~90% confluence in 7–9 days. Confluent primary cultures were treated with 0.05% EDTA in PBS and cells were harvested by a short, 1-2 minute trypsinization with 0.01 % trypsin (Gibco). Harvested cells were divided into two aliquots at passages. The cultures were grown at 37 °C in humidified atmosphere containing 5% CO_2. Keratinocyte and melanocyte co-cultures have been established and expanded from the different donor sites selectively *in vitro*. Patients were transplanted with autologous keratinocytes using the Tissucol® system from Baxter.

3. RESULTS

3.1. SNP of the 3' untranslated region of the *FGFR2* gene (2451A/G)

Comparison of the SNP data on venous leg ulcer patients (n=82) with those on healthy individuals (n=82) we identified an SNP located in the 3' untranslated region (UTR) of FGFR2 (2451A→G; 900 bp downstream from the open reading frame) which displayed a significant difference in the allelic distribution between the leg ulcer patients and the healthy individuals (P=0.0103). Our results allow to conclude that expression of the FGFR2-IIIb isoform is related to the proliferation states of the keratinocytes. The fact that FGFR2-IIIb exhibits a lower expression level in venous leg ulcer patients suggests a receptor dysfunction which, through impairment of the proliferation phase of re-epithelization, leads to pathologic wound healing in these patients. A possible underlying mechanism for the abnormal expression of FGFR2-IIIb seen in venous leg ulcer patients may be the presence of the 2451 A/G 3'UTR SNP, which could alter the stability of the mRNA and results in decreased amounts of FGFR2 protein and receptor dysfunction. In this case, both the re-epithelization through FGFR2-IIIb and the angiogenesis through FGFR2-IIIc may be impaired.

3.2. Gene expression abnormalities

Syndecan 4 (SDC4), a heparan sulfate proteoglycan, and neuropilin-1 (NRP1), a transmembrane receptor, are both involved in normal wound healing, but little is known about their possible role in venous leg ulcer

pathogenesis. We aimed to study whether there are expression abnormalities and/or gene polymorphisms of SDC4 and NRP1 associated with venous leg ulcer. SDC4 showed significantly lower mRNA and protein expression levels in the uninvolved dermis of venous leg ulcer patients (n=15) as compared to the control (n=15; P=0.0136), while NRP1 showed no expression abnormalities. We hypothesize that SDC4 may play an essential role not only in the inflammation and tissue formation phases of normal wound healing, but its expression abnormalities observed in the uninvolved dermis of venous leg ulcer patients may contribute to venous leg ulcer development.

3.3. Artificial chemical mitogen free method to culture human adult melanocytes and keratinocytes

In Mel-mix medium (AIM-V and Keratinocyte Basal Medium (KBM) (v:v) supplemented with 2.5% fetal bovine serum, 2.5 ng/ml epidermal growth factor, 25 μg/ml bovine pituitary extract) normal human adult keratinocytes and melanocytes grew *in vitro* without chemical mitogens. To compare the cultured cells to cells grown in conventional TPA containing medium melanocytes from the same skin specimen were cultured simultaneously with 10 ng/ml TPA in the complete Mel-mix medium. Melanocytes cultured in the presence of TPA had a highly dendritic appearance showing multiple dendritic processes, well developed ramifications and an overall delicate architecture compared to their TPA-free counterparts. Multiple contacts were present between the cells grown with TPA. Melanocytes cultured in the TPA-free medium also showed the typical dendritic appearance, however the dendritic projections were notably more voluminous and cell contacts were notably less. As both culture types matured and grew toward confluency, cells started to show a tripolar or bipolar spindle-shaped morphology. Cells typically became polygonal and flat in senescence in both cultures.

3.4. Hairy scalp the ideal donor site for split thickness and autologous keratinocyte-melanocyte transplantation

Analyses of freshly isolated keratinocytes from hairy scalp skin showed that the percentage of p63 positive cells was significantly higher (39.8±12.0%) as compared to upper leg (31.7±8.3%, P=0.007) or groin

(27.8±16.0, P=0.03). Percentage of the K1/K10 negative cells was also significantly higher (52.6±10.2%) than in the case of keratinocytes separated from groin (35.4±4.5%, P=0.018) or from the upper leg (36.1±3.2%, P=0.028). Moreover hairy scalp yielded a higher number of epidermal cells from the same size of skin specimen

4. DISCUSSION

4.1. Genetic studies

Leg ulceration is a multifactorial disorder with several known and yet unknown environmental and genetic factors contribute to its etiology. Our genetic studies concerned a homogenic subgroup of leg ulcers, to venous leg ulcers. However the complexity of venous leg ulcer pathogenesis is increased by the fact that in the majority of the cases its development has polyetiological origin. Besides environmental factors, several predisposing genetic factors have already been shown to be associated with venous leg ulcer susceptibility such as the presence of certain gene polymorphisms and gene expression abnormalities. These genetic factors impair different processes and phases of wound healing, which might suggest that multiple molecular mechanisms of venous leg ulcer development exist or similarly to carcinogenesis the co-existence of different precipitating factors leads to the development of the disease. To develop causative treatment opportunities for those who have genetic deficiencies in the pathogenesis, there is an emerging importance to characterize the genetic abnormalities of leg ulcer development.

The difficulties of the studies regarding the molecular mechanisms of pathologic wound healing stem from the fact that these inherited factors form a complex multifactorial genetic background, which do not follow the Mendelian rules. Moreover each genetic component contributes differently to the pathogenesis of leg ulcers and the assessment of its importance in the development of leg ulcers is difficult and variable. To determine the putative genetic factors and to minimize statistical bias, the currently most widely accepted approach is the formation of highly homogenous subgroups of leg ulcer patients and assessment of the genetic factors within the subgroups. Therefore, a homogenous venous leg ulcer patient group has been selected with 157 individuals with non-healing ulcers despite of proper therapy. The duration of the presence of

the leg ulcer exceeded 6 weeks. The female (48.4%) male (51.6%) ratio was nearly 1:1. The average duration of the ulcers was 5.8±5.1 years. The three most frequent and clinically relevant parameters in the history of the patients were: deep vein thrombosis, 29.9% of the leg ulcer patients had deep vein thrombosis previously. Leg fracture, 22.9% of the patients had leg fracture previously. Soft tissue infection of the affected lower leg, 47.1% of the patients had erysipelas or cellulites in their history. These clinical parameters strongly affect the circulation of the leg and contribute to the pathogenesis of venous leg ulcer development. Three subgroups of the patients have been created to further reduce statistical fluctuations Group A contained patients without thrombosis, fracture or soft tissue infection in the history. Patients with fracture, but without thrombosis and soft tissue infection has formed group B. While patients with thrombosis or erysipelas, but without fractures has been placed to group C. Other combinations of fracture, thrombosis and soft tissue infection were represented in such a small number that those patients as all put together in group D. Leg ulcer patients with diabetes of either type 1 or type 2 or with arterial disease were excluded from the study. Venous leg ulcer patients were also screened for cardiac diseases (incidence was 49.1%), atherosclerosis (incidence was 20.4%) and autoimmune disorders (incidence was 5.1%). The study was approved by the Internal Review Board. Written informed consent was obtained from all donors, and the study was conducted according to the Principles of the Declaration of Helsinki.

The importance of several putative genetic factors has been assessed in the homogenous group of venous leg ulcer patients such as the G1691A factor V Leiden mutation, G20210A prothrombin gene mutation, 2451 A/G polymorphism (SNP) of the *FGFR2* gene and the -308 G/A SNP of the promoter region of the *TNFα* gene (Jebeleanu *et al.,* 2001, Hafner *et al.,* 2001; Nagy *et al.,* 2005 and 2007). The investigations were carried out using RT-PCR with TaqMan probes and RFLP techniques described in details by previous studies (Jebeleanu *et al.,* 2001, Hafner *et al.,* 2001; Nagy *et al.,* 2005 and 2007). The Leiden mutation was detected in a heterozygous form in 11 patients with the overall frequency of 7.9%, showing higher presentation in the group A and group C than in group B (data not shown). The prothrombin mutation occurred also only in 3

patients with heterozygous form, every other patients carried only the wild type allele (data not shown).

The distribution of the mutant genotypes of the studied FGFR2 gene polymorphism (2451A/G SNP at the 3'UTR) was the highest in group A (ratio of homozygous mutants 18.8%, mutant allele frequency MAF=0.46), while the lowest in group B (ratio of homozygous mutants 8.8%, MAF=0.37, Fisher Exact Probability Test P=0.12, Odds ratio 1.49, CI 0.88 – 1.81;. However it has been proven previously, that the 2451A/G polymorphism in the 3'UTR of the FGFR2 gene is associated with venous leg ulcer development, its distribution did not show significant difference between the subgroups of venous leg ulcer patients defined on the basis of their medical history.

The single nucleotide change of the TNFα promoter also showed the highest frequency in group A (ratio of homozygous mutants 5.8%, MAF=0.22), while in group B and C homozygous mutant form was not detected and only the heterozygous form was present (group B MAF=0.18, group C MAF=0.11; group A vs. group B P=0.27, Odds ratio 1.35, CI 0.70 – 2.32; group A vs. group C P=0.017, Odds ratio 2.38, CI 1.07 – 4.01). It has been demonstrated previously that the -308A/G SNP of the promoter region of the *TNFα* gene is a predisposing factor to venous leg ulcer development (Wallace *et al.,* 2006; Nagy *et al.,* 2007). Our data demonstrate that the homozygous mutant form of the -308 TNFα SNP was represented in a significantly higher ratio among venous leg ulcer patients without other predisposing factor in their history (group A: no thrombosis, fracture or soft tissue infection) than among patients with other known etiological event in their history (group C: patients with previous thrombosis or soft tissue infection; group A vs. group C Fisher Exact Probability Test P=0.017). Our data further emphasize that the putative etiologic factors investigated in our study has a key importance in the development of therapy-resistant leg ulcer cases, and they might have a tremendous significance in the development of future causative treatment strategies.

Several recent studies characterized in details putative genetic factors contributing to the development of leg ulcer. However these discoveries have no impact yet on the current guidelines of leg ulcer management and thus they have no clinical relevance yet. Further larger studies in different populations are needed to characterize the underlying genetic abnormalities in the mechanism of leg ulcer development and to work

out cost-effective investigation techniques for the routine diagnostic assessments of the putative genetic factors and causative treatment options for these patients.

4.2. Local wound care studies

Several methods and several media formulations have been published over the years for the separation and subsequent culturing of human epidermal melanocytes and keratinocytes (Eisinger at al, 1982; Gilchrest et al, 1984; Hu at al, 1957). With few exceptions, all methods used chemical mitogens to enhance cell growth. Co-cultures of melanocytes with keratinocytes in a melanocyte growth medium (MGM) supplemented with EGF, BPE, and FBS resulted in sufficient *in vitro* melanocyte growth without the need for chemical mitogens (Donatien et al, 1993). The use of basic fibroblast growth factor (bFGF), endothelin-1 (ET-1) and α-melanocyte stimulating hormone (α-MSH) have also been reported to be able to replace the need for TPA and BPE in already established *in vitro* cultures of human neonatal melanocytes (Swope et al, 1995). The culture system that we describe here is unique, in that relatively long term, pure melanocyte cultures, with cells derived from adult epidermis, that are never exposed to chemical mitogens, can be established easily. Although additional growth factor supplementation (e.g. EGF, BPE) increases cell growth, the use of serum is sufficient to establish and maintain the *in vitro* culture of these cells in our medium. We found autologous human serum (AHS) to be somewhat superior to FBS in supporting the *in vitro* melanocyte growth. Cells expanded *in vitro* in Mel-mix supplemented with autologous human serum allow autologous transplantation of cultured skin cells in chronic wounds and in vitiligo (Lontz et al, 1994; Olsson et al, 1993; Olsson et al, 1995). We offer a novel culturing technique to isolate and culture human epidermal melanocytes and keratinocytes in a chemical mitogen-free environment. With this method it is possible to easily establish pure human epidermal skin cell cultures in which melanocytes propagate sufficiently already in the early passages, when their pigmentation is not lost, therefore they can be transplanted to cover vitiligo lesions. Further work will show whether the *in vitro* dedifferentiation of the cells that occurs in older cultures can be reversed with agents that allow autologous transplantations. Cells grown in our cultures regain their *in vitro* growth

after freezing and storage allowing repeated transplantation from the same harvest in chronic wound and vitiligo patients.

In vitro culturing technique involves subcultivations to expand the number of keratinocytes. Transplantation techniques of cultured keratinocytes are based on the self-renewing capacity of the epidermis. This characteristic of the epidermis is the result of the presence of multi-potent stem cells which reside in the bulge region of the hair follicle as well as in the interfollicular epidermis. Epidermal stem cells undergo asymmetric cell division and give rise to daughter stem cells and to transit amplifying cells. After a few rounds of cell division the transit amplifying cells exit from the cell cycle and undergo terminal differentiation. Differentiating keratinocytes constitute a significant percentage of the cultured keratinocytes separated from skin biopsies and in consequence have a finite replicative capacity and lifespan. The increased number of cell divisions *in vitro* leads to telomere shortening, which in turn reduces lifespan of the autograph, hence it is of outmost importance to use undifferentiated keratinocytes for autologous transplantation purposes (Counter et al, 2003).

Stem cells and transit amplifying cells are characterized by expression of the transcription factor p63, a member of the p53 gene family. Recent data suggest that p63 has a role in initiating epithelial stratification during development and a role in maintaining the proliferative potential of basal keratinocytes in mature epidermis (Koster et al, 2004). As soon as the transit amplifying cells cease proliferation, the expression of p63 is down-regulated and the cells start to produce K1 and K10 keratins. Production of K1 and K10 keratins mirrors the commitment to differentiation and is among the earliest events in the program of cellular terminal differentiation (Eichner et al, 1990). The proportion of p63 positive and K1/K10 negative cells in transplanted keratinocytes is important both for the short term and long term clinical outcome.

We have shown that the epidermal cell suspension prepared from hairy scalp contains an abundance of undifferentiated keratinocytes as compared to conventional donor sites (groin, upper leg). Considering that stem cells are more abundant in hair follicles, the use of hairy scalp as a donor site provides a keratinocyte culture rich in undifferentiated keratinocytes (Taylor et al, 2000). Therefore, cell cultures prepared from hairy scalp skin specimens can proliferate to yield the required cell number for transplantation in a shorter period as compared to

conventional donor sites. Furthermore, the hairy-scalp derived cells have longer lifespan and longer regenerative capacities. The results reported here suggest that keratinocytes and melanocytes isolated and cultured from hairy scalp donor sites are more suitable for autologous transplantation of chronic leg ulcers and burnt skin areas than grafts established from conventional donor sites. Like other epidermal keratinocytes, the cultured cells can be successfully frozen and stored for possible future transplantations, an advantage that should prove useful both for patient and clinician.

5. SUMMARY

In our genetic studies we compared the SNP data of leg ulcer patients with healthy individuals for FGFR2-IIIb isoforms. Our results showed that FGFR2-IIIb exhibits a lower expression level in venous leg ulcer patients which results in the impairment of re-epithelisation, thus pathologic wound healing. The SNP data for the promoter region of the TNF-α gene suggested a primary association with obesity and only a secondary association with leg ulceration. We also set out to determine the expression abnormalities and/or gene polymorphisms of SDC4 and NRP1. We found out that SDC4 showed a significantly lower mRNA and protein expression levels in the uninvolved dermis of venous leg ulcer patients, compared with normal controls. In the case of NRP1 no expression abnormalities were detected.

In our experiments regarding cell culturing we established a chemical and artificial mitogen free autologous keratinocyte/melanocyte culturing technique. We developed a special culture media to suit the needs of melanocytes as well as keratinocytes. This method enabled us to prepare autologous skin cell cultures suitable for the grafting of chronic wounds. Further, with the use of the hairy scalp as a donor site, we could establish cultures that consisted primarily of keratinocytes stem cells. This autologous skin cell culture was used for the grafting of chronic leg ulcers with the Tissucol® system.

With the knowledge of our culturing results, we developed a split skin grafting technique for the transplantation of chronic leg ulcers. The split skin grafts taken from the hairy scalp were used for grafting with V.A.C. fixation of the grafts on the wound bed. With the combination of the hairy scalp and the V.A.C. technique all the grafts attached and vitalized on the wound bed.

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I dedicate this work especially to my children, Emma and Vendel.

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