Dexpanthenol Modulates Gene Expression in Skin Wound Healing in vivo

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Introduction

Dexpanthenol, the stable alcohol form of pantothenic acid (vitamin B\textsubscript{5}), is well absorbed through the skin where it is rapidly converted enzymatically to pantothenic acid, a component of coenzyme A (CoA), which is important in cellular skin metabolism \cite{1, 2}. The topical application of dexpanthenol is widely used in skin care and in treating various dermatologic diseases because it stimulates skin regeneration and promotes wound healing. Many clinical in vivo studies provide experimental evidence of the beneficial effects of topically applied dexpanthenol. In a randomized, double-blind, placebo-controlled study topical treatment with dexpanthenol on the epidermal barrier function was studied in vivo. Treatment with dexpanthenol improved stratum corneum hydration and reduced transepidermal water loss \cite{3}. A clinical study by Camargo et al. \cite{4} also evaluated the skin-moisturizing effects of dexpanthenol-containing formulations. Additional clinical trials by Pugliese et al. \cite{5} and Presto et al. \cite{6} documented the efficacy of dexpanthenol in experimental in vivo models of skin injuries where improved epidermal regeneration and signif-

Key Words
Pantothenate \cdot Pantothenic acid \cdot S100 calcium-binding protein A7A \cdot Keratin-associated protein 4–12 \cdot Cytokines \cdot Chemokines

Abstract
Topical application of dexpanthenol is widely used in clinical practice for the improvement of wound healing. Previous in vitro experiments identified a stimulatory effect of pantothenate on migration, proliferation and gene regulation in cultured human dermal fibroblasts. To correlate these in vitro findings with the more complex in vivo situation of wound healing, a clinical trial was performed in which the dexpanthenol-induced gene expression profile in punch biopsies of previously injured and dexpanthenol-treated skin in comparison to placebo-treated skin was analyzed at the molecular level by Affymetrix\textsuperscript{\textregistered} GeneChip analysis. Upregulation of IL-6, IL-1\textsubscript{\beta}, CYP1B1, CXCL1, CCL18 and KAP 4–2 gene expression and downregulation of psorasin mRNA and protein expression were identified in samples treated topically with dexpanthenol. This in vivo study might provide new insight into the molecular mechanisms responsible for the effect of dexpanthenol in wound healing and shows strong correlations to previous in vitro data using cultured dermal fibroblasts.
Significant accelerated wound healing was observed by showing more elastic and solid tissue regeneration and a reduction of erythema. Furthermore, dexpanthenol was shown to stimulate epithelialization and granulation and had an antipruritic and anti-inflammatory effect on experimental ultraviolet-induced erythema [7, 8]. A prospective, randomized, double-blind study showed that the prophylactic continued use of topically applied dexpanthenol within radiation therapy for breast cancer reduced the clinical signs of acute radiation dermatitis [9]. Other clinical trials proved that topical application of dexpanthenol is effective in the treatment and protection against skin irritation and inflammation [2, 10]. In a multicenter study, 483 patients with atopic dermatitis, ichthyosis, psoriasis or contact dermatitis requiring adjuvant skin care received dexpanthenol in topical formulations. Skin irritation symptoms such as xerosis, erythema, pruritus and roughness improved considerably [11]. A pilot study of Udompataikul et al. [12] demonstrated that the effectiveness of dexpanthenol is equal to that of hydrocortisone in the treatment of mild-to-moderate childhood atopic dermatitis. The positive influence of dexpanthenol on fibroblast proliferation, which is an important factor in wound healing, is well documented in several in vitro studies [1, 13, 14]. Wiederholt et al. [1] analyzed the molecular mechanisms resulting in the proliferative effect of pantothenate by in vitro examination of the effect of pantothenate on the gene expression profile in human dermal fibroblasts. Microarray analysis revealed an upregulation of IL-6, IL-8, CCL2 and CXCL1 expression by pantothenate which may contribute to the wound-healing properties of this compound [1]. To correlate these in vitro findings to the more complex in vivo situation of wound healing, a clinical trial was performed in which the dexpanthenol-induced gene expression profile in punch biopsies of previously injured and dexpanthenol-treated skin in comparison to placebo-treated skin was analyzed at the molecular level by Affymetrix® GeneChip analysis.

**Material and Methods**

**Generation of Probes**

Skin of 12 healthy human volunteers (8 female and 4 male, 32–46 years, 100% Caucasian) was injured by two 4-mm punch biopsies. One skin wound was subsequently treated topically with biopsies. One skin wound was subsequently treated topically with.

**RNA Extraction**

8-mm punch biopsies of placebo- or dexpanthenol-treated skin were homogenized in a Tissue Lyser II (Qiagen, Hilden, Germany) and subsequently total RNA was isolated using the Nucleo Spin II kit, (Macherey and Nagel, Düren, Germany) according to the instructions of the manufacturer, respectively. The RNA was quantified by means of photometric measurement using the Nanodrop®-ND-1000 spectrophotometer (Nanodrop, Wilmington, Del., USA), and the integrity was proved by using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif., USA).

**Reverse Transcription and Quantitative Real-Time PCR**

Purified RNA was reverse-transcribed utilizing the SuperScriptTM III Platinum®Two Step qRT-PCR Kit (Invitrogen-Gibco, Paisley, UK). TaqMan experiments were carried out on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Carlsbad, Calif., USA) using Assay-on-Demand gene expression products (Applied Biosystems) for human CCLI8 (Hs00268113), CCR1 (Hs00174097), CXCL1 (Hs0039726857), CYP1B1 (Hs00174298), CXCL1 (Hs0036937), CYP1B1 (Hs00164383), IL-1β (Hs00174097), IL-6 (Hs00985642), S100A7 (Hs00161488) and KRTAP4–12 (Hs00258949) according to the manufacturer’s recommendations. An Assay-on-Demand product for HPRT rRNA (Hs99999909_m1) was used as an internal reference to normalize the target transcripts. For CCLI8, CCR1, CXCL1, CYP1B1, IL-1β, IL-6, S100A7, KRTAP4–12 and HPRT all measurements were performed in triplicates in separate reaction wells.

**Analysis of Gene Expression Using Exon Expression Arrays**

RNA samples of punch biopsies of placebo- and dexpanthenol-treated skin were collected. The Ambion® WT Expression Kit (Ambion, Kauflung, Germany) was used to generate purified sense-strand cDNA with incorporated dUTP according to the technical manual. Fragmentation and labeling was done using the Affymetrix GeneChip WT Terminal Labeling kit (Affymetrix, Santa Clara, Calif., USA) according to the manufac-
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Results

Dexpanthenol Affects Gene Expression in Skin

In a randomized, double-blind, single-center, placebo-controlled study, skin of 9 healthy human donors was injured by two 4-mm punch biopsies. One skin wound was subsequently treated topically with ointment containing dexpanthenol every 12 h, the other skin wound was treated with placebo every 12 h. Participants of the clinical trial in group I received one application of the test materials, probands in group II received 5 applications and in group III they received 11 applications. Eight-millimeter punch biopsies of placebo- or dexpanthenol-treated areas were taken after 24 (group I), 72 (group II) and 144 h (group III). Tissue samples of punch biopsies of placebo- or dexpanthenol-treated skin were collected, homogenized and subsequently RNA was isolated. RNA samples of punch biopsies of placebo- or dexpanthenol-treated skin were used to generate purified sense-strand cDNA. After fragmentation and labeling, each sample was hybridized to an Affymetrix Gene Chip Human Exon 1.0 ST array. Expression values of each probe set were determined and placebo samples were compared to dexpanthenol-treated probes using the GeneSpring software.
Effects of 5 Applications of Dexpansenol on Gene Expression in Human Skin

Significant effects of dexpansenol on gene regulation in comparison to placebo treatment could be revealed in all samples from all three time points analyzed but especially in group II (5 applications of dexpansenol), we could detect an upregulation of 95 genes after dexpansenol treatment by gene chip analysis (fig. 1, 2). We focused on the expression patterns of the most highly regulated genes and confirmed these genes by quantitative real-time PCR. Therefore, total RNAs were isolated from punch biopsies of placebo- or dexpansenol-treated skin 12 h after the last of a total of 5 applications and subjected to quantitative real-time PCR analysis using specific probes to detect human IL-6, IL-1β, CYP1B1, CXCL1, CCR1 and CCL18 mRNA and HPRT mRNA as internal references (fig. 3). In biopsies of dexpansenol-treated skin up to 7-fold higher IL-6 mRNA levels could be observed in comparison to IL-6 mRNA levels detected in biopsies of placebo-treated control skin. As shown in figure 3, dexpansenol treatment upregulates IL-1β and CYP1B1 expression nearly 6-fold in comparison to placebo treatment. Treatment of skin with dexpansenol leads to a 3.5-fold induction of CCR1 expression. As demonstrated in figure 3, a 2.5-fold upregulation of CCL18 and CXCL1 expression after dexpansenol treatment in comparison to placebo treatment could be shown.

Expression of Psoriasin is downregulated by Dexpansenol

Our findings from the Affymetrix GeneChip analysis suggested that dexpansenol downregulates the expression of psoriasin (S100 calcium-binding protein A7A) in...
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**Fig 4.** Expression of psoriasin is downregulated by dexpanthenol. 
**a** The expression of psoriasin was analyzed by qRT-PCR 12 h after the last of a total of 11 applications of dexpanthenol. Therefore, purified RNA of placebo- or dexpanthenol-treated skin was reverse-transcribed and subsequently the TaqMan experiment was carried out using the Assay-on-Demand gene expression product for human S100A7 (psoriasin). Displayed are relative expression levels from one patient of group III comparing placebo-treated skin with dexpanthenol-treated skin, normalized to HPRT expression. The measurements were performed in triplicates and the mean values and SD of the three measurements are presented. Statistical significance of technical triplicates was evaluated using the one-way ANOVA with Tukey’s post test (**p < 0.001) [34]. **b** The downregulation of psoriasin expression on the protein level was analyzed in histological sections of 8-mm punch biopsies of placebo- or dexpanthenol-treated areas from one patient of group III by immunohistochemistry. Immunohistochemistry was performed with 4-μm paraffin-embedded sections of formalin-fixed biopsy using a primary monoclonal mouse anti-human S100A7 (psoriasin) antibody. Visualization of psoriasin expression is based on the alkaline phosphatase fast-red reaction.

**Fig 5.** Expression of keratin-associated protein 4–12 is upregulated by dexpanthenol. The expression of keratin-associated protein 4–12 was analyzed by qRT-PCR 12 h after dexpanthenol treatment (**a**) and 12 h after the last of a total of 11 applications (**b**) of dexpanthenol. Therefore, purified RNA of placebo- or dexpanthenol-treated skin was reverse-transcribed and subsequently the TaqMan experiments were carried out using the Assay-on-Demand gene expression product for human KRTAP 4–12. Displayed are relative expression levels from one patient of group I (**a**) and from one patient of group III (**b**) comparing placebo-treated skin with dexpanthenol-treated skin normalized to HPRT expression. The measurements were performed in triplicates and the mean values and SD of the three measurements are presented. Statistical significance of technical triplicates was evaluated using the one-way ANOVA with Tukey’s post-test (**p < 0.001) [34].

Expression of Keratin-Associated Protein 4–12 Is Upregulated by Dexpanthenol

The analysis of the expression patterns detected by Affymetrix GeneChip analysis revealed an upregulation of keratin-associated protein 4–12 (KAP 4–12) by dexpanthenol in tissue material from patients of group I (one application of dexpanthenol) and group III (11 applications of group III were analyzed for psoriasin expression by immunohistochemistry. Figure 4b clearly demonstrates the downregulation of psoriasin in biopsies of dexpanthenol-treated skin in comparison to placebo-treated skin.

tissue material of patients treated 11 times within 144 h with the test material (data not shown). To confirm this result at the RNA level, total RNA was isolated from punch biopsies of placebo- or dexpanthenol-treated skin 12 h after the last of a total of 11 applications and subjected to quantitative real-time PCR analysis using a specific probe to detect human psoriasin and HPRT mRNA as the internal reference. As shown in figure 4a, dexpanthenol treatment downregulates psoriasin expression by 36%. To confirm the downregulation of psoriasin at the protein level, histological sections of punch biopsies of placebo- or dexpanthenol-treated areas from one patient
of dexpanthenol). This finding could be confirmed by quantitative real-time PCR analysis using a specific probe to detect KAP 4–12 and HPRT mRNA as the internal reference. As shown in figure 5, two exemplified experiments of a skin sample from one donor of group I treated with dexpanthenol once within 24 h and one donor of group III treated with dexpanthenol 11 times within 144 h in comparison to placebo revealed a 3.6-fold (group I, A) and 8.1-fold (group III, B) induction of KAP 4–12 expression.

**Discussion**

Although the positive influence of panthenol and pantothenic acid on skin irritation [2, 4] and wound healing [5, 6, 17] was well known and documented in several in vivo and in vitro studies, the accompanying molecular effects of these compounds on skin cells remained unclear. In terms of the effects of pantothenic acid on dermal fibroblasts, Weimann and Hermann [13] revealed that Ca D-pantothenic acid promoted fibroblast proliferation and migration. Wiederholt et al. [1] confirmed the stimulatory effect of pantothenate at a concentration of 20 µg/ml on the proliferation of cultured dermal fibroblasts and in the same experiment revealed by microarray analysis the simultaneous regulation of various genes. To correlate these in vitro findings to the more complex in vivo situation of wound healing employing various cell types and three to four sequential, yet overlapping phases of wound healing (hemostasis, inflammatory, proliferative and remodeling), a randomized, double-blind, single center, placebo-controlled study was performed. In this study, skin of healthy human donors was injured by two 4-mm punch biopsies and skin wounds were subsequently treated in vivo topically with dexpanthenol-containing ointment and placebo every 12 h (fig. 1). Three groups received different numbers of treatments in order to monitor the effect of dexpanthenol on gene expression in different phases of wound healing (24, 72 and 144 h after initial wounding). Noticeable effects of dexpanthenol on gene regulation in comparison to placebo treatment could be revealed in all samples from all three time points analyzed (fig. 1), but especially in group II (72 h after wounding, receiving 5 applications of dexpanthenol) we could detect an upregulation of 95 genes after dexpanthenol treatment by gene chip analysis (fig. 2). These findings correlate with results retrieved from animal studies [18] analyzing the effect of vacuum-assisted closure therapy, moist wound healing or gauze on gene expression during wound healing in a Zucker diabetic fatty (fa/fa) male inbred rat model. In these studies major differences in gene expression between the three different wound treatments could be detected in tissues retrieved 2 days after wound creation.

The alteration of gene expression by topical dexpanthenol treatment for 3 days included upregulation of IL-6, IL-1β, CYP1B1, CXCL1, CCL2, CCR1 and CCL18 mRNA expression (fig. 2, 3). These findings correlate to previous in vitro studies [1] revealing an upregulation of IL-6, CYP1B1, CCL2 and CXCL1 in proliferating dermal fibroblasts by pantothenate. Therefore, it is tempting to speculate that proliferating dermal fibroblasts are at least in part the source of the enhanced mRNA levels in wounded skin tissue treated with dexpanthenol. Weimann and Hermann [13] suggested that the turnover of CoA in damaged skin is relatively high, so that the requirement for pantothenic acid increases during wound healing. Therefore, it is likely that additional quantities of pantothenate are locally needed to optimize the many cellular processes involved in wound healing. This might explain why the highest amount of gene regulations are detected in the dexpanthenol-treated skin samples derived during the proliferative phase of wound healing (fig. 1).

It is well known from the analysis of IL-6-deficient (IL-6KO) mice, which display significantly delayed cutaneous wound healing characterized by decreased re-epithelialization, granulation tissue and wound closure [19], that IL-6 plays an important role in wound healing. In addition, recent studies have shown the secretion of this wound-healing mediator from stimulated fibroblasts in dermal substitutes [20]. Upregulation of this cytokine has been shown in previous in vitro [1] and in this in vivo study in wound healing models treated with dexpanthenol (fig. 2, 3) or pantothenate, supporting the thesis that dermal fibroblasts are a source of this cytokine.

The chemokine receptor CCR1, which binds several chemokines present at the wound site, has been shown to be barely detectable in nonwounded murine skin, but a strong upregulation was observed after injury in wild-type mice. In addition, the healing abnormalities observed in glucocorticoid-treated mice and activin-overexpressing transgenic mice correlated with an altered expression of CCR1. CCR1-positive cells were identified as macrophages and neutrophils within the wounded area [21] which correlates with our findings that upregulation of CCR1 was only detected in the more complex in vivo wound-healing model including various inflammatory cells (fig. 2, 3) but not in the in vitro ‘scratch’ model, containing only dermal fibroblasts [1].

In contrast, the chemokine (C-X-C motif) ligand 1 (CXCL1) – signaling through the chemokine receptor
CXCR2 – has been shown to be expressed not only in inflammatory but also in epithelial cells and fibroblasts [22]. In addition, in vitro wounding experiments with cultures of keratinocytes established from CXCR2−/− and wild-type mice revealed a retardation in wound closure in CXCR2−/− keratinocytes, suggesting a role for this receptor on keratinocytes in epithelial resurfacing that is independent of neutrophil recruitment [23]. Upregulation of CXCL1 has been detected in both the previously reported fibroblast in vitro model [1] and in human skin wounds treated with dexpanthenol for 3 days (fig. 2, 3), suggesting that fibroblasts are the source for this enhanced mRNA expression.

These findings suggest that in vitro models are capable of analyzing aspects of the complex processes and that they can help us determine the individual role of cell types such as the role of dermal fibroblasts in wound healing. But clinical studies implying skin samples as presented in this study are still a prerequisite to understand the complete effects of compounds such as dexpanthenol on different cell types in human tissues.

Array analysis of skin samples taken during the early and late phases of wound healing revealed an enhanced expression of keratin-associated protein 4–12 (KAP 4–12) by dexpanthenol treatment. Hair keratin-associated proteins are a major component of the hair fiber, and play crucial roles in forming a strong hair shaft through a cross-linked network with keratin intermediate filaments, which are produced from hair keratins [24]. Recently, it has been shown that aging processes influence the gene expression of KAP4 family members in human hair follicles which are expressed predominantly in the highly differentiated portions of the middle and upper cortex [25]. Previously, changes in epithelial keratin expression during healing of rabbit corneal wounds and delayed wound healing in keratin 6a knockout mice have been reported [26, 27]. Since it is known that hair follicle stem cells contribute to wound healing and even minor wounding resulted in mobilization of follicle stem cells to generate daughter cells that quickly move into the wound area [28, 29], further studies would be of interest to define the putative role of keratin-associated proteins in wound healing.

Affymetrix GeneChip analysis (fig. 1) suggested that dexpanthenol-treated wound tissue (11 treatments within 144 h) revealed a considerably lower expression of psoriasin (S100 calcium-binding protein A7A) in comparison to placebo-treated skin wounds. This effect could be confirmed on the protein level using immunohistochemistry. Psoriasin is known to function as a transglutaminase substrate/cornified envelope precursor, signal transduction protein, chemokine, and antibacterial protein in normal epidermis [30]. S100A7 has been shown to be markedly increased in epidermal hyperproliferative disorders and was detected in wound exudates and in parts of the epidermis surrounding acute wounds and in the margins of nonhealing chronic leg ulcers, but not in unwounded epidermis [31]. The downregulation of psoriasin expression in the maturation and remodeling phase of wound healing might be correlated to the improved wound healing described previously in clinical studies. It might also – at least in part – be due to the additional antimicrobial/bactericidal property of pantothenol mediated by interfering with the bacterial CoA pathway as demonstrated by Kumar et al. [32]. This also correlates with recent findings studying bacteria recolonization in a suction blister wound model. In these studies, the authors could show that treatment of wounds with dexpanthenol cream once daily completely suppressed recolonization over the test period of 7 days [17]. Decreased colonization of wounds with bacteria might lead to the downregulation of antibacterial proteins such as psoriasin.

In a previous study, healthy young (18–55 years of age) and elderly (more than 65 years of age) human volunteers received a 2 × 2 cm, superficial, split-thickness wound on the anterior aspect of the thigh, and the rate of epithelialization was measured [33]. Holt et al. [33] could show that the elderly volunteers had a significant delay of 1.9 days in epithelialization. No effect of age on collagen synthesis was noted, although accumulation of wound noncollagenous protein was decreased. The average age of the volunteers enrolled in our present study was 35 ± 7.4 years which is similar to the median age of the first group studied by Holt et al. [33] revealing a normal wound healing.

In conclusion, this in vivo study revealed considerable effects of dexpanthenol on gene regulation in comparison to placebo treatment in all samples from all three time points of wound healing but especially in those samples retrieved 3 days after wounding. These investigations which could be confirmed by qRT-PCR and immunohistochemistry might provide new insight into the molecular mechanisms responsible for the effect of dexpanthenol in wound healing and showed strong correlations to previous in vitro data using cultured dermal fibroblasts.

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References


