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rAAV2-mediated restoration of LEKTI in LEKTI-deficient cells from Netherton patients

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ABSTRACT

Background: Netherton syndrome (NS, MIM 256500) is a potential life threatening autosomal-recessive skin disorder clinically characterized by the trias of congenital erythroderma, hair shaft anomalies and atopic diathesis. It is caused by mutations in the gene *SPINK5* resulting in a deficiency of its processed protein named lympho-epithelial Kazal-type related inhibitor (LEKTI). LEKTI controls the activity of several serine proteases in the skin that are involved in terminal differentiation. Loss of LEKTI results in protease hyperactivity, increased degradation of intercellular junctions, reduced stratum corneum adhesion and impaired skin barrier function. Today NS can only be treated symptomatically.

Objective: Does gene transfer offer a therapeutic option for NS in the future?

Methods: A recombinant adeno-associated virus type 2 vector was constructed containing the full length cDNA (rAAV2/C-*SPINK5*) of functional human LEKTI. Infectious virus particles were used for transfection of LEKTI-deficient-keratinocytes of NS patients *in vitro*.

Results: Gene transfer of *SPINK5* in NS-keratinocytes led to a five-fold increase in mRNA expression of *SPINK5* reaching almost 75% of normal value. The functionality of the expressed LEKTI was proven in a hydrolytic activity assay demonstrating that the activity of LEKTI after gene transfer increased closely to the level seen in keratinocytes of healthy individuals.

Conclusion: The results provide first evidence that gene transfer of *SPINK5* results in increased LEKTI activity in NS-keratinocytes, thus offering a rationale to further pursue such a gene therapy approach for NS.

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1. Introduction

Netherton syndrome (NS, MIM 256500) is an autosomal-recessive skin disorder clinically characterized by congenital erythroderma, trichorrhexis invaginata (bamboo hair), and atopic diathesis. It accounts for up to 18% of congenital erythrodermas [1], which can be life-threatening in the neonatal period due to severe complications such as hypernatremic dehydration, hypothermia, extreme weight loss, bronchopneumonia, recurrent bacterial infections and sepsis [2].

NS is caused by genetic defects in the gene *SPINK5* (serine protease inhibitor kazal-type 5) [3–6]. *SPINK5* is located on chromosome 5q32 and encodes serine protease inhibitor LEKTI (lympho-epithelial Kazal-type related inhibitor). The protein LEKTI is a 15-domain Kazal-type related serine protease inhibitor [7,8]. In the epidermis, LEKTI is confined to the granular layer and

stratum corneum [6,8] where it co-localizes with kallikreins (KLK) 5 and 7 [9]. Several domains of LEKTI have shown to inhibit KLK 5 and 7 [10] that are involved in the degradation of corneodesmosomes such as desmoglein 1 (Dsg1) and desmocollin 1 (Dsc1) [11]. Normally, the interplay between the inhibitory pressure of LEKTI and the proteolytic activity of KLK 5 and 7 is well-balanced leading to a controlled desquamation process and an intact skin barrier.

In NS, mutations result in a complete or severe loss of LEKTI and subsequently a reduced inhibition of serine proteases. The enhanced activity of KLK 5 and 7 induce premature degradation of corneodesmosomal cadherins as demonstrated by a dramatically reduction of Dsg1 and Dsc1 [11,12]. This mechanism has been suggested to result in loss of stratum corneum integrity and severe skin barrier defect.

Various therapeutic options have been used in NS to reduce clinical symptoms, among them topical steroids, calcipotriene, calcineurin-inhibitors, topical and systemic retinoids, PUVA, and intravenous immunoglobulin all with variable and only temporary success [13,14]. Today, no causal and permanent treatment for NS

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does exist, although the severity and continuity of the diseases are demanding new developments. Herein, we describe the first developmental steps towards a genetic treatment approach for NS.

2. Materials and methods

2.1. Cell culture

Human primary NS-keratinocytes were isolated from punch biopsies derived from 4 NS patients after informed consent had been given; normal human keratinocytes (NHK) came from circumcised foreskin (provided by Dr. Thomas A. Angerpointer, Pediatric Surgery Practice, Munich, Germany). The specimens were processed according to a standard protocol described previously [15–17]. Briefly, skin was cleaned of fat, cut into small piece (if necessary) and incubated overnight at 4 °C in thermolysin buffer containing 0.5 mg/ml protease X (Sigma, Munich, Germany). Epidermal sheets were peeled off the dermis, washed in phosphate-buffered saline (PBS, Invitrogen, Carlsbad, USA) and incubated in 0.25% (w/v) trypsin and 0.02% (w/v) EDTA (Biochrom, Berlin, Germany) for 20 min at 37 °C in order to achieve single-cell suspension. After trypsin action was stopped by fetal calf serum (FCS) containing medium, cells were washed and resuspended in culture medium. NS-keratinocytes were cultured onto mitomycin C-treated NIH3T3 cells (purchased from German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) using keratinocyte growth medium KGM consisting of a 3:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 supplemented with 10% FCS, 10 ng/ml epidermal growth factor (EGF), 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 500 ng/ml hydrocortisone, 5 µg/ml insulin, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all purchased from Biochrom). Medium was renewed every 2–3 days and cells were usually passaged at 80% confluence. NHK were cultured without feeder layer cells in serum-free KSFM (Invitrogen) supplemented with 50 µg/ml bovine pituitary gland extract, 10 ng/ml epidermal growth factor (EGF), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

2.2. rAAV plasmid construction

pTR-UF/C represents the rAAV2 gene transfer vector pTR-UF containing the inverted terminal repeats (ITRs) of AAV2 as originally described and kindly provided by Dr. R.J. Samulski (University of North Carolina, USA) [18]. In addition, it contains an expression cassette with a cytomegalovirus immediate-early promoter, a multiple cloning site, and an SV40 late polyadenylation signal as previously constructed [16,19]. Within the multiple cloning site of pTR-UF/C, the 3252 bp *KpnI*-*NotI* fragment of pTRE2hyg-LEKTI was inserted by sticky end ligation containing the open reading frame of the functional *SPINK5* cDNA (GenBank NM_006846, kind gift of Professor G.L. Clayman, University of Texas, Houston, USA). This vector construct was named pTR-UF/C-SPINK5 (Fig. 1), and used for the generation of rAAV2/C-SPINK5 particles. pTR-UF/C-GFP contained the low-toxic humanized green fluorescent protein (Stratagene, La Jolla, CA, USA) instead of *SPINK5* and was used for the generation of rAAV2/C-GFP particles. As in previous studies, rAAV2/C-GFP served as a control for specific transgene transductions [16,17,19].

2.3. rAAV2 vector production

rAAV2 vector particles were generated according to a standard protocol described by Xiao et al. by triple calcium-phosphate transduction of (1) transgene containing plasmids pTR-UF/C-SPINK5 or pTR-UF/C-GFP, (2) packaging plasmid pRC containing the replication (REP) and capsid (CAP)-gene of AAV2, and (3) adenovirus helper plasmid pXX6 of human 293 cells over a 3-day period [18]. Viral particles were purified by ammonium sulphate precipitation and iodixanol gradient centrifugation [20]. The physical titres of each preparation was measured by a commercially available AAV2 titration ELISA (Progen, Heidelberg, Germany), and averaged around 7×10^{11} particles/ml.

2.4. rAAV2 transduction of primary keratinocytes

Keratinocytes were grown for one passage in the absence of feeder cells and transduced at 70–80% confluence. To enhance transduction efficiency by promoting viral second strand synthesis, cells were cultured in KSFM without EGF 2 days before transduction, and 2 h before transduction, they were incubated with 100 µM AG1478 (4-(3-chlorophenylamino)-6,7-dimethoxyquinazoline, Calbiochem, EMD Biosciences, Darmstadt, Germany) as described previously [19]. Thereafter, cells were transduced at an MOI of 10,000 in EGF-free KSFM medium containing the equivalent amount of vector lysate for 12 h. The next day cells were passaged and expanded on fresh culture dishes as usual.

2.5. RNA extraction, reverse transcriptase-PCR and realtime (RT)-PCR

Total RNA was isolated from normal and NS-keratinocytes using peqGOLD RNAPure kit (peqLab, Erlangen, Germany) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA by First Strand cDNA Synthesis Kit for reverse transcriptase-PCR (AMV) (Roche, Penzberg, Germany). The resulting cDNA was used as template for PCR amplification of the first 268 bps of *SPINK5* with sense-primer 5'-CGCTAGCAA-CATGAAGATAG-3' and anti-sense-primer 5'-CTAGAAGCATTTGCG GTGGACGATGGAGGG-3'. Control PCR was performed for β -actin using commercially available primers (Invitrogen). PCR conditions for *SPINK5* were 5 min 95 °C, 1 cycle, followed by 30 cycles of 30 s 95 °C, 45 s 54 °C and 40 s 72 °C, followed by 1 cycle end extension, 10 min 72 °C. PCR products were visualized by running 1.5% agarose gel electrophoresis.

For real time-PCR, cDNA was amplified by SYBR Green ERTM qPCR SuperMix for ABI PRISM[®] (Invitrogen) using the same primers as mentioned above. Values were quantified using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) and normalized to the housekeeping gene β -actin. Each reaction was performed in duplicates.

2.6. Indirect LEKTI activity assay (hydrolytic activity assay)

The inhibitory activity of LEKTI was determined indirectly by measuring the trypsin-like hydrolytic activity in homogenates of keratinocytes according to a modified protocol by Komatsu et al. [21]. In short, a synthetic peptide containing 7-amino-4-methylcoumarin (Boc-Phe-Ser-Arg-AMC; MP Biomedicals, Santa Ana, USA) was used as substrate in a reaction mixture, containing 1 mg

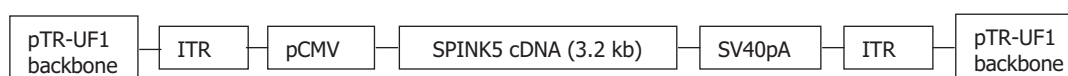


Fig. 1. rAAV2/C-SPINK5 vector construct containing the open reading frame of human *SPINK5* encoding LEKTI, driven by a cytomegalovirus immediate-early promoter (CMV), terminated by an SV40 late polyadenylation signal (SV40pA) and flanked by inverted terminal repeats (ITR) of AAV2.

cell homogenate, 20 ml of N,N-dimethylformamide, 0.48 ml of 0.1% Triton X-100 (w/w), 0.35 ml of 0.2 mol per l Tris–HCl buffer (pH 8.0). The reaction mixtures were incubated at 37 °C for 2 h, and released AMC was measured using a fluorescence spectrophotometer (BMG LABTECH, Offenburg, Germany) at excitation/emission spectra of 355/460 nm. The activity was calculated in relation to a standard reference AMC (Sigma, Munich, Germany) curve as nmol released AMC per mg protein and per minute using the linear part of a trend line from the resulting curves. Each assay was performed in duplicates.

2.7. Statistics

Values were expressed by their mean and standard error of the mean. The significance of the difference was calculated by a two-tailed Student's *t*-test with values of $P < 0.05$ being considered to be significant.

3. Results

3.1. Netherton patients

Keratinocytes of four patients with typical clinical presentation of NS were available for the study. The underlying genetic defects are summarized in Table 1. Mutation c.316delAG and c.1432-13 G > A had been published previously [6,9]. All mutations resulted in premature termination of *SPINK5*. In addition all demonstrated reduced mRNA expression of *SPINK5*, and increase hydrolytic activity consistent with a deficiency of LEKTI.

3.2. Increased expression of *SPINK5* in Netherton keratinocytes following gene transfer of *SPINK5*

In the skin, *SPINK5* expression is known to be restricted to the upper most layers of the epidermis, which is entirely composed of differentiated keratinocytes. Since most keratinocytes during monolayer culture are at their proliferative, non-differentiated stage, any analyses of *SPINK5* require induction of differentiation. In order to induce differentiation and subsequently *SPINK5* expression in a physiological way, keratinocytes were cultured in differentiation medium containing 1.2 mM CaCl_2 over a 3-day period prior to any *SPINK5* analyses. In accordance with results by Bitoun et al. [8], *SPINK5* mRNA as measured by reverse-transcribed PCR was low in proliferating NHK cultured in low-calcium containing medium (NHK-prol), and increased substantially after the induction of differentiation (NHK-dif) (Fig. 2). *SPINK5* expression was not detectable in keratinocytes derived from NS patients in proliferation medium, and remained almost completely reduced in differentiation medium (Fig. 2). Following the gene transfer of *SPINK5*, *SPINK5* mRNA increased substantially in NS-keratinocytes.

In order to provide a more quantitative analyses of the enhancement of *SPINK5* after *SPINK5* transduction, real time PCR

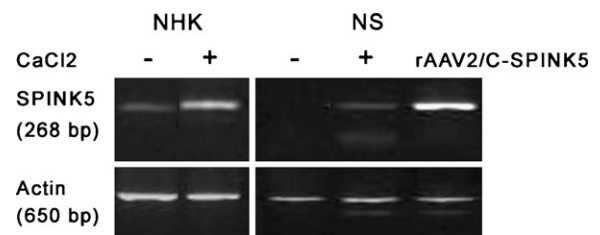


Fig. 2. Reverse transcribed PCR analyses of *SPINK5* mRNA expression in normal human keratinocytes (NHK) and Netherton (NS) keratinocytes (patient 1). *SPINK5* expression was low in NHK cultured in proliferation medium (low calcium) and increased in differentiation medium (1.2 mM CaCl_2), whereas in NS-keratinocytes, *SPINK5* remained decreased even in differentiation medium. Following gene transfer, *SPINK5* demonstrated increased expression in NS. (Shown is one representative out of 4 independent experiments.)

was performed. As shown in Fig. 3, *SPINK5* expression was 6.7 times higher in NHK-dif than in NS-keratinocytes ($p = 0.009$). Following gene transfer of *SPINK5*, the its expression in NS-keratinocytes increased fivefold ($p = 0.014$) and reach a level of up to 75% of that seen in NHK-dif. GFP transduced NS increased as well ($p = 0.027$), most likely artificially, but the difference between GFP and *SPINK5* transduced NS was still significant ($p = 0.0381$) pointing to a specific increase due to gene transfer.

3.3. Enhancement of the activity of LEKTI in transduced NS-keratinocytes

In order to investigate whether the increased expression of transduced *SPINK5* results in functional protein assembly, the activity of LEKTI was measured indirectly in a trypsin-like hydrolytic activity assay. In this assay, enhancement of LEKTI results in downregulation of the hydrolytic activity of trypsin. All experiments were performed after cells have been cultured in differentiation medium for 3 days. NHK-dif revealed a low hydrolytic activity due to high LEKTI. In contrary, NS-keratinocytes demonstrated a high hydrolytic activity due to deficiency in LEKTI ($p < 0.01$). The same was shown for GFP-transduced NS-keratino-

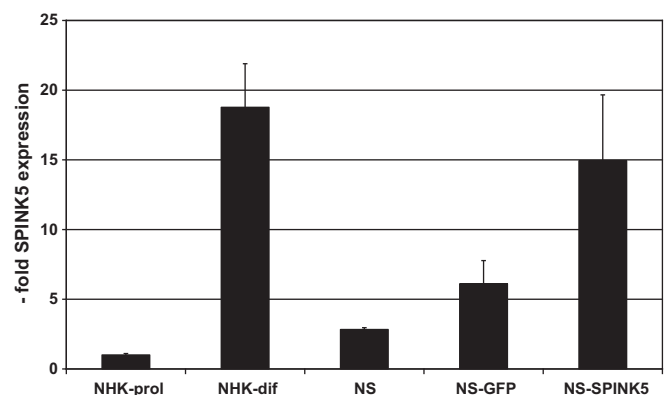


Fig. 3. *SPINK5*-transduction of keratinocytes from patients with Netherton syndrome (NS) results in increased *SPINK5*-mRNA expression. *SPINK5* expression was demonstrated by real time PCR analysis in normal human keratinocytes (NHK) in proliferation (NHK-prol) and differentiation medium (NHK-dif) (each $n = 5$), and in keratinocytes from NS-patients which were untransduced (NS), or transduced with GFP (NS-GFP) or *SPINK5* (NS-SPINK5) (each $n = 3$), each cultured in differentiation medium. *SPINK5* mRNA was 6.7-fold higher in NHK than in NS. The expression of *SPINK5* increased almost fivefold to a level of 75% of normal in NS-keratinocytes after gene transfer of *SPINK5* ($p = 0.0114$). GFP transduced NS increased as well ($p = 0.027$), most likely artificially, and the difference between GFP and *SPINK5* transduced NS was still significant ($p = 0.0381$) pointing to a specific effect of the gene transfer (shown are mean and SD of the indicated n , each performed in duplicates).

Table 1

Gene defects of patients with Netherton syndrome.

Patient	Sex	Age	Mutation	Location	Consequence
1	f	12	c.316delAG ?	exon 5 (domain 2)	PTC
2	f	1 1/2	c.354delTTGT c.1432-13G > A	exon 5 (domain2) exon 16 (domain7)	PTC PTC
3	m	15	c.1432-13G > A c.1432-13G > A	exon 16 (domain7) exon 16 (domain7)	PTC PTC
4	f	1	c.900A > G c.1913delT	exon 11 (domain 5) exon 21 (domain 10)	PTC PTC

Abbreviations: age is given in years; PTC=premature termination codon.

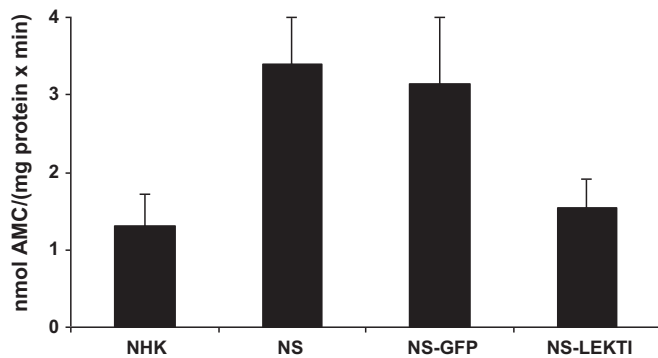


Fig. 4. Hydrolytic activity assay measuring indirectly the activity of LEKTI. Normal human keratinocytes (NHK) grown in differentiation medium displayed a low hydrolytic activity due to high amount of LEKTI, whereas Netherton keratinocytes (NS) and NS-keratinocytes transduced with GFP (NS-GFP) showed high hydrolytic activity due to lack of LEKTI ($p < 0.01$). NS-keratinocytes transduced with SPINK5 (NS-LEKTI) demonstrated restoration of LEKTI deficiency ($p < 0.01$). (Shown are means and SD of three independent transduction experiments each performed in cells from different NS patients measured in duplicates).

cytes (Fig. 4). Following gene transfer of *SPINK5*, LEKTI activity enhanced in NS-keratinocytes resulting in a downregulation of the hydrolytic activity to a level almost equivalent to the level seen in healthy keratinocytes ($p = 0.008$).

4. Discussion

Netherton syndrome is a genetic disorder with severe and chronic skin manifestations that can currently only be treated in a symptomatic way. There are two promising molecular concepts at the horizon that might offer causal therapeutic benefit – protein replacement therapy and gene therapy. In theory, protein replacement by means of topical application of recombinant LEKTI might allow LEKTI to permeate the impaired skin barrier of NS patients and become active at the granular layer and thus ameliorate LEKTI deficiency. Since recombinant LEKTI is yet not available, alpha1-antitrypsin has been tried topically instead, but had failed to be beneficial in NS [22]. Gene therapy is by far the more complex and complicated development for treating NS, but recent results from the first human skin gene therapy trial for hereditary epidermolysis bullosa were very encouraging and demonstrated that skin gene therapy is feasible and can lead to a “cure” of the underlying genodermatosis [23]. Herein, we present the first developmental steps of such a gene therapy concept for NS. We constructed a recombinant viral vector based on adeno-associated virus type 2 which expressed the functional cDNA of human *SPINK5* and generated infectious vector particles. With this vector we transduced human keratinocytes from 4 unrelated patients with NS and found that the gene transfer of *SPINK5* led to a fivefold increase in mRNA expression of *SPINK5* reaching almost 75% of normal (Figs. 2 and 3). In order to proof whether this increase results in an enhancement of the activity of LEKTI, we performed a hydrolytic activity assay, which indeed demonstrated that the inhibitory capacity of the transduced *SPINK5* increased closely to the level seen in keratinocytes of healthy individuals (Fig. 4).

These results demonstrate that the gene transfer of *SPINK5* into LEKTI-deficient NS-keratinocytes might be an appropriate approach for future treatment of NS. Although the next consecutive developmental step would be an *in vivo* study, this is hampered by the early death (few hours after birth) of the mice in the existing knock-out models. Therefore, highly complicated nude mice transplantation models are required for analyzing long term epidermal effects of *SPINK5* gene transfer such as activity of KLK 5

and 7, degradation of Dsg1 and Dsc1, or IgE-level [12,24]. One advantage of NS as a candidate disease for gene therapy is that the phenotype of NS seems to depend on the residual activity of LEKTI [9], thus a partial increase in LEKTI activity already might lead to a phenotypical benefit. Besides *ex vivo* gene transfer with subsequent epithelial sheet transplantation, NS might also be suitable for direct *in vivo* gene transfer due to the impaired skin barrier which may facilitate transcorneal permeation of viral vector particles. Herein, we used a conventional rAAV2 viral vector that is capable for both, *ex* and *in vivo* gene transfer to the skin [25]. In NS, there might be a chance to reduce the full length cDNA of *SPINK5* to a shortened version without loss of protein function due to an optimized combination of a selection of some of the 15 LEKTI-domains. Recently, Deraison et al. identified certain biologically active fragments of LEKTI in particular domains D5, D6, D8–D11, and D9–15 that showed specific inhibition of KLK 5, 7 [10]. Such shorter transgenes could possibly being transduced at higher efficacy and the corresponding protein fragments might be sufficient for treating at least some NS patients, but further research is necessary to find out which combination of domains works best for which mutations. A small transgene would also be suitable for non viral topical gene transfer approaches in the sense of a gene cream for instance by using transposon elements forcing stable integration of the transgene and a liposomal formulation that facilitate transcorneal permeation [26].

In conclusion, the data presented here provide the initial basis and rational that gene therapy might be a promising future therapeutic approach for NS.

Conflict of interest

The authors declare no conflict of interest.

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