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ORIGINAL ARTICLE

Age-associated decrease in GDNF and its cognate receptor GFRα-1 protein expression in human skin

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SUMMARY

Glial cell line-derived neurotrophic factor (GDNF) and its cognate receptor (GFRα-1) are expressed in normal human skin. They are involved in murine hair follicle morphogenesis and cycling control. We hypothesize that 'GDNF and GFRα-1 protein expression in human skin undergoes age-associated alterations. To test our hypothesis, the expression of these proteins was examined in human skin specimens obtained from 30 healthy individuals representing three age groups: children (5–18 years), adults (19–60 years) and the elderly (61–81 years). Immunofluorescent and light microscopic immunohistologic analyses were performed using tyramide signal amplification and avidin–biotin complex staining methods respectively. GDNF mRNA expression was examined by RT-PCR analysis. GDNF mRNA and protein as well as GFRα-1 protein expressions were detected in normal human skin. We found significantly reduced epidermal expression of these proteins with ageing. In the epidermis, the expression was strong in the skin of children and declined gradually with ageing, being moderate in adults and weak in the elderly. In children and adults, the expression of both GDNF and GFRα-1 proteins was strongest in the stratum basale and decreased gradually towards the surface layers where it was completely absent in the stratum corneum. In the elderly, GDNF and GFRα-1 protein expression was confined to the stratum basale. In the dermis, both GDNF and GFRα-1 proteins had strong expressions in the fibroblasts, sweat glands, sebaceous glands, hair follicles and blood vessels regardless of the age. Thus there is a decrease in epidermal GDNF and GFRα-1 protein expression in normal human skin with ageing. Our findings suggest that the consequences of this is that GFRα-1-mediated signalling is altered during the ageing process. The clinical and therapeutic ramifications of these observations mandate further investigations.

Keywords
ageing, GDNF, GFRα-1, healthy skin

Introduction

Glial cell line-derived neurotrophic factor (GDNF) belongs to a novel family of neurotrophic factors, the GDNF family, which is closely related to the TGF-β superfamily. GDNF family includes three other ligands: neurturin, artemin and persephin. The GDNF family of ligands signals through a receptor complex consisting of a transmembrane tyrosine kinase signalling subunit and a ligand binding subunit, GFRα. Four different GFRα family members, GFRα-1, GFRα-2, GFRα-3 and GFRα-4, operate as cognate receptors for GDNF, neurturin, artemin and persephin respectively (Adly *et al.* 2006a,b,c,d; Adly *et al.* 2008).

GDNF family ligands (GFLs) were originally discovered in the nervous system as potent survival factors for embryonic and adult central dopaminergic, noradrenergic and motor neurons (Nosrat *et al.* 1996) as well as a variety of peripheral neurons including sympathetic, parasympathetic and enteric neurons (Worley *et al.* 2000). They play a central role in promoting neurite and axon outgrowths from these neurons (Yan *et al.* 2003). In addition, these neurotrophic factors have protective function for degenerating,

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axotomy-injured and chemically damaged neurons (Ugarte *et al.* 2003).

Outside the nervous system, GDNF ligands and receptors are also expressed in the developing retina, inner ear, skeletal muscles, adrenal chromaffin cells and in the embryonic and adult thymocytes of mice (Kondo *et al.* 2003). GDNF ligands and their cognate receptors are also essential for the development of organs that are formed on the basis of complex epithelial–mesenchymal interaction, such as kidney, gut, lung, teeth, murine and human skin and hair follicles (Adly *et al.* 2006b; Adly *et al.* 2008).

Adly *et al.* reported the expression pattern of GDNF and GFRalpha-1 in human scalp skin using immunohistological methods. The expression of GDNF protein was strong in the epidermis and sebaceous and sweat glands. The results of RT-PCR analysis revealed that GDNF protein is synthesized in the epidermis. The expression of GFRalpha-1 was strong in the papillary dermis and sebaceous and sweat glands. The authors concluded that the expression of these proteins in the skin suggests their possible roles in skin homeostasis (Adly *et al.* 2006a,b,c,d). The authors also examined the expression of these proteins (GDNF and GFRalpha-1) in the different stages of the hair follicle cycle using immunofluorescence, immunoalkaline phosphatase staining methods and reverse transcription-polymerase chain reaction. GDNF and GFRalpha-1 proteins were weakly expressed in catagen and telogen hair follicles. In contrast, they were strongly expressed in the epithelial and mesenchymal compartments of the anagen hair follicles. The GDNF gene was transcribed, both in the human scalp skin and in the isolated anagen hair follicles. In hair follicles organ culture, GDNF increased the number of the proliferating HF keratinocytes (Adly *et al.* 2008).

To date, reports that bear directly on the expression of GDNF and GFRα-1 in different age groups are lacking. Here, we hypothesize that 'GDNF and GFRα-1 protein expression in human skin undergoes age-associated alterations'. We have examined the expression pattern of the GDNF and its high-affinity receptor, GFRα-1, proteins in healthy human skin of different ages using immunohistochemical staining methods.

Materials and methods

Ethical approval

The study and the study design were approved by the Institutional Review Board at the Faculty of Science, Sohag University.

Skin specimens

Thirty normal human skin biopsy specimens were obtained from 30 donors after obtaining informed consent. None of the donors had a skin disorder. The specimens were obtained from sun-protected areas (back skin) so that changes in GDNF and GFRα-1 would reflect true ageing rather than photoageing due to sun exposure. The individuals were divided into three age groups: children and adolescents (5–18 years; $n = 10$), adults (19–60 years; $n = 10$) and the elderly (61–80 years; $n = 10$). After excision, skin specimens were frozen immediately in liquid nitrogen and stored at −80°C until be used; 7-μm-thick cryosections were prepared and dried at room temperature for about 30 min. Sections were then fixed in cold acetone (−20°C) for 10 min, re-dried at RT for 60 min and preserved at −20°C. In nine cases a second skin biopsy specimen was obtained (skin of the back) and was used for real-time PCR analysis. These specimens represented the three age groups (three cases for each age group). The specimens were collected in dimethyl sulphoxide (DMSO) and processed for RNA purification.

RNA extraction. Total RNA was extracted from the examined samples using QIAGen RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA were stored at −80°C until used.

Real-time polymerase chain reaction. To determine GDNF and GFRα-1 RNA transcripts, real-time PCR was carried out on the R Corbett (Corbett Life Science, Sydney, Australia) Real-time PCR detection system (Bio Rad) using gene-specific primers: for GDNF, sense: 5′-AAACATCCGAGGACAAGG TG -3′, antisense: 5′- ACATCCACACCTTTTAGCGG -3′; for GFRα-1, sense: 5′- AGGGAAATGATCTGCTGGAGGA -3′, antisense: 5′- CTCTGGCTGGCAGTTGGTAAAA -3′; and for GAPDH, sense: 5′ GAAGGTGAAGGTCGGAGTC-3′, antisense: 5′-GAAGATGGTGATGGGATTTC-3′. The specificity of the primers was tested under normal PCR conditions. Reverse transcription was carried out using 10 μg of total RNA in 20 μl of the reaction mixture. Three microlitres of the cDNA product was used as a template in real-time PCR in 25 μl of the reaction mixture. The PCR cycle conditions were carried out as follows: (i) 95°C for 3 min, one cycle, 95°C for 30 s followed by 60°C for 30 s, 40 cycles.

Immunohistochemistry. Cryosections of normal back human skin were immunostained using specific polyclonal primary antibodies against the human GDNF and GFRα-1. The primary antibodies applied and their source, dilution and host species are listed in Table 1. Two labelling techniques were performed to visualize antigen–antibody complexes: the avidin–biotin complex (ABC) labelling (Vector Labs, Burlingame, CA, USA) and the highly sensitive immunofluorescent tyramide signal amplification (TSA) labelling (PerkinElmer Life Science, Boston, MA, USA).

For ABC labelling method, the cryosections of normal human back skin were washed in Tris-buffered saline (TBS, 0.05 M, pH 7.6) and preincubated with avidin–biotin blocking kit solution (Vector Laboratories) followed by incubation with protein-blocking agent (Immunotech; Krefeld, Germany) to prevent non-specific binding. The sections were then incubated with the primary antibodies diluted in TBS (for dilution, see Table 1) containing 2% goat serum (for GDNF) or 2% rabbit serum (for GFRα-1), for 1 h at room temperature or overnight at 4°C. Thereafter, the sections were incubated with biotinylated secondary antibodies goat anti-rabbit IgG and rabbit anti-goat IgG (Jackson Immu-

Table 1 Primary antibodies

Antigen	Species	Dilution (ABC)	Dilution (TSA)	Source	Positive control
Human GDNF	Rabbit	1:100	1:1000	Santa Cruz Biotechnology, Santa Cruz, CA	Mouse brain
Human GFRα-1	Goat	1:50	1:1000	Santa Cruz Biotechnology, Santa Cruz, CA	Mouse brain

noResearch Laboratories, West Grove, PA, USA) diluted as 1:200 in TBS containing 2% goat or rabbit serum, respectively, for 30 min at room temperature. Next, the sections were incubated with avidin–biotin–alkaline phosphatase complex (Vecta-Stain Kits; Vector Laboratories Inc.) diluted in TBS (1:100) for 30 min at room temperature. The alkaline phosphatase colour reaction was developed by applying a staining protocols described before (Eichmuller *et al.* 1996; Eichmuller *et al.* 1998), using fast red tablets (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Finally, the sections were counterstained with Mayer's hemalum, covered with Kaiser glycerol (Dako Deutschland GmbH, Hamburg, Germany) and stored at 4°C for microscopic examination and analysis.

For TSA labelling technique (Shindler *et al.* 1996), the cryosections were washed in Tris–acid–Tween buffer (TNT, pH 7.5), followed by washing in 3% hydrogen peroxide (H_2O_2). The sections were then incubated with lower concentrations of primary antibodies diluted in Tris–acid-blocking buffer (TNB, pH 7.2; for dilution, see Table 1) overnight at 4°C. Next, the sections were washed in TNT and incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated F(ab)2 fragments of goat anti-rabbit IgG and rabbit anti-goat IgG secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in TNB (1:200) containing 2% goat or rabbit serum, respectively, for 30 min at room temperature. Thereafter, the sections were incubated with streptavidin–horseradish peroxidase (1:50 in TNB) for 30 min at room temperature. Finally, tetramethylrhodamine isothiocyanate–tyramide amplification reagent was administrated (1:50 in amplification diluent provided with the kit) for 30 min at room temperature, followed by counterstaining with 4′,6′-diamidino-2-phenylindole (DAPI) and mounting in levamisole (DAKO Corporation; Carpinteria, CA, USA). The TSA signals were visualized under a Zeiss fluorescence microscope (Zeiss, Jena, Germany) (Table 1).

Positive and negative controls. Negative controls were obtained by the omission of primary antibodies, whereas positive controls were obtained by the incubation of mouse brain cryosections with primary antibodies specific for GDNF and GFRα-1. The GDNF immunostaining results were verified by the positive control from mouse cerebellum and the negative control from human back skin non-incubated with anti-GDNF antibody. Similarly, GFRα-1 immunostaining results were contrasted by the positive control from mouse cerebellum and the negative control from human back skin non-incubated with anti-GFRα-1 antibody.

Semiquantitation of GDNF and GFRα-1 protein expressions. To assess the GDNF and GFRα-1 immunoreactivity (IR) and alteration in staining intensity (SI) among different ages, semiquantitation of the immunostaining was carried out by calculating the immunoreactivity score (IR score) following Adly *et al.* (2006a,b,c,d, 2008). The IR score was evaluated by multiplying the percentage of positive cells (PP %) and the staining intensity (SI). First, the PP% was scored as 0 for <5%, 1 for 5–25%, 2 for 25–50%, 3 for 50–75% and 4 for >75%. Second, the SI was scored as 1 for weak, 2 for medium and 3 for intense staining.

Statistical analysis. Statistical analysis was carried out using Kruskal–Wallis one-way analysis of variance (Armitage *et al.* 2002). The rationale behind using this nonparametric is that it represents a simple technique appropriate to compare one variable across more than two groups of data that has assumptions more easily met. The results are presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

The positive and negative controls were positive and negative respectively (Figure 1).

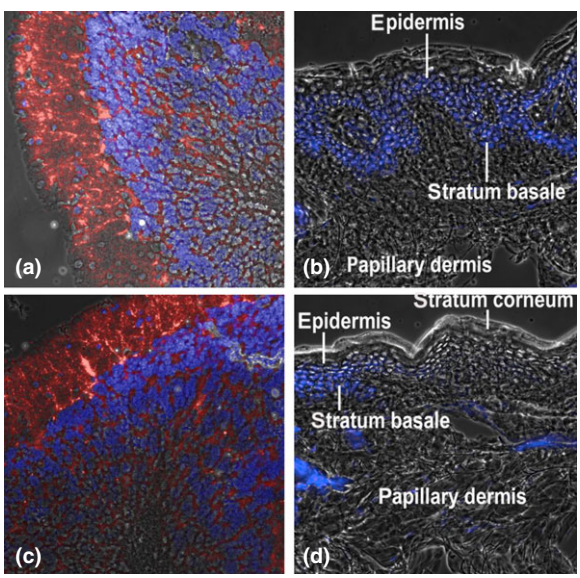


Figure 1 GDNF and GFRα-1 immunoreactivity (tyramide signal amplification) in the positive and negative controls. The colour of a positive signal is red. (a) GDNF-positive control from mouse cerebellum. (b) GDNF-negative control from human skin. (c) GFRα-1-positive control from mouse cerebellum. (d) GFRα-1-negative control from human skin.

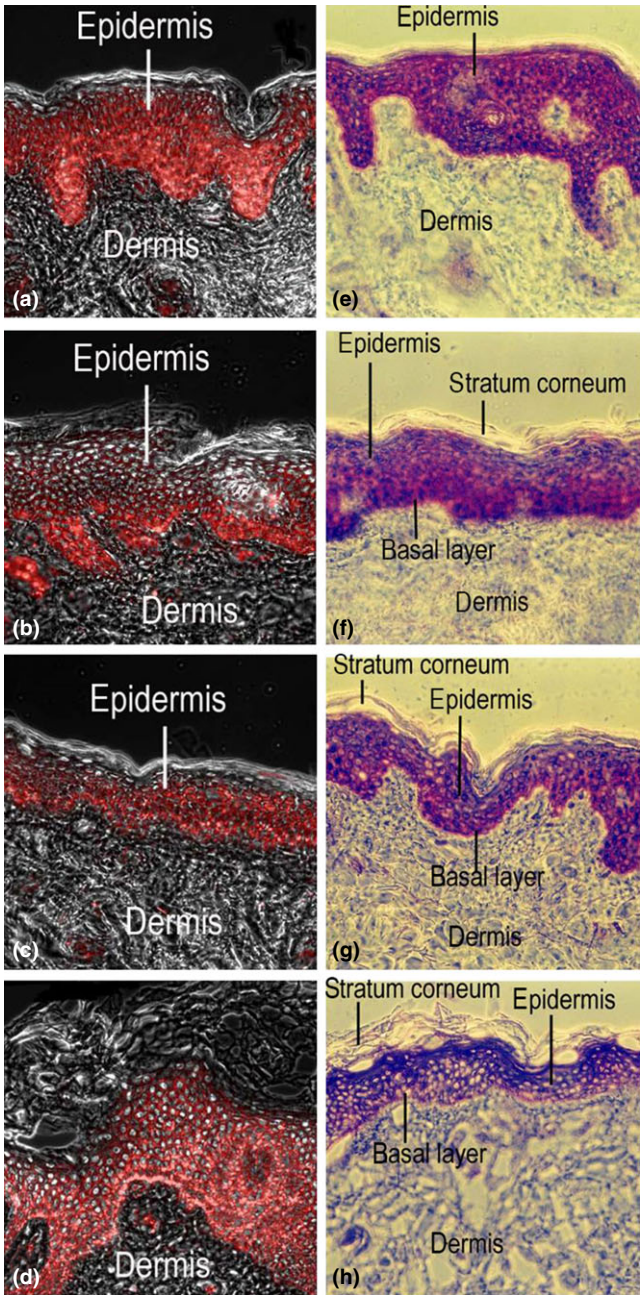


Figure 2 GDNF immunoreactivity shown with tyramide signal amplification and avidin–biotin complex staining in young and mid-ages. The colour of a positive signal is red. (a and e) 6 years old, (b and f) 18 years old, (c and g) 33 years old, (d and h) 39 years old.

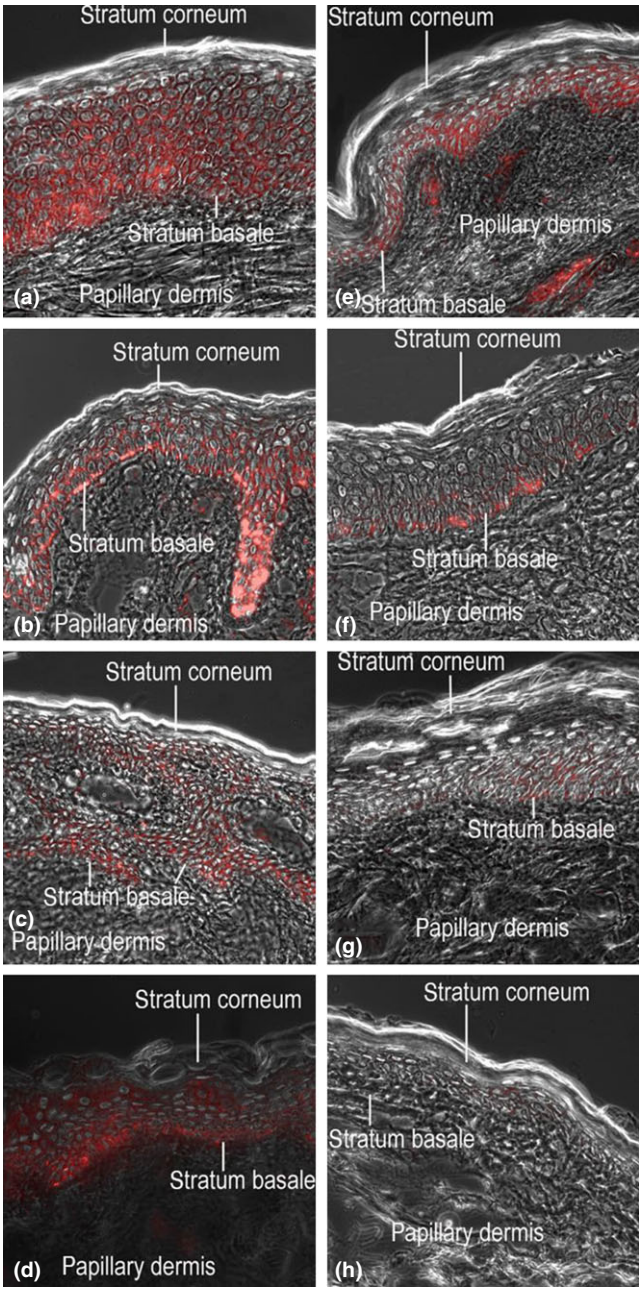


Figure 3 GFR1 immunoreactivity shown with tyramide signal amplification staining. The colour of a positive signal is red. (A) 6 years old, (B) 18 years old, (C) 34 years old, (D) 39 years old, (E) 60 years old, (F) 68 years old, (G) 71 years old, (H) 81 years old.

Gradual decrease in the expression of both GDNF and GFRα-1 proteins in the human skin epidermis with ageing

In children, we found the expression of GDNF and GFRα-1 proteins in the human back skin. GDNF and GFRα-1 expression was strong in the basal layer and decreased gradually towards the granular layer (Figures 2 and 3a-d). A prominent GDNF and GFRα-1 expression was noted in the sweat and sebaceous glands, in which the immunopositivity was strong in the peripheral sebocytes (Figure 4).

In the back skin, a strong GFRα-1 protein expression was detected in the epidermis, myofibroblasts, sweat and sebaceous glands. The expression was strong in the basal and spinous layers and few rows of the granular layer, sweat and sebaceous glands (especially in the peripheral sebocytes) (Figure 3), but was completely absent in the stratum corneum.

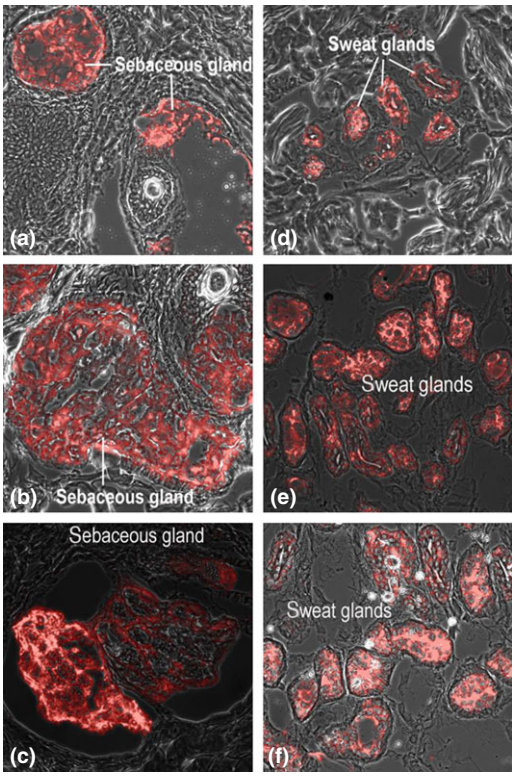


Figure 4 GDNF immunoreactivity shown with TSA staining. The colour of a positive signal is red. (A) 6Y (sebaceous glands), (B) 33Y (sebaceous glands), (C) 71Y (sebaceous glands), (D) 6 years old (sweat glands), (E) 53 years old (sweat glands), (F) 71 years old (sweat glands).

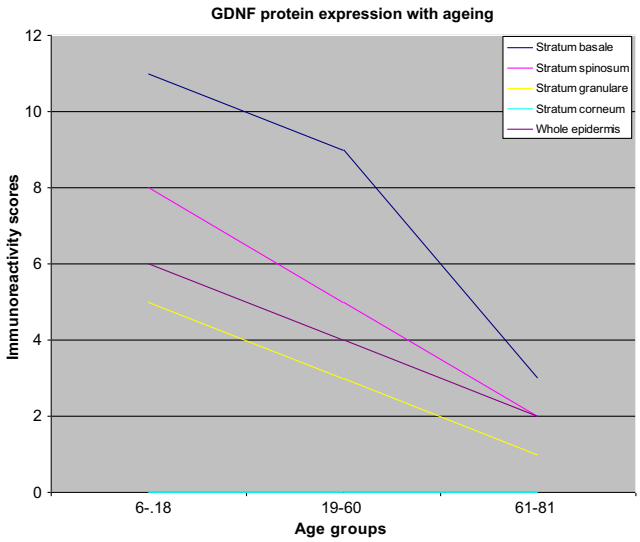


Figure 6 GDNF expression values.

In the epidermis, GDNF and GFRα-1 protein expression was strong in the skin of children and then declined gradually with ageing, wherein they became moderate in the skin of adults and weak in elderly subjects (Figures 5 and 3e–h). Compared with children, both GDNF and GFRα-1 protein expressions were statistically significantly reduced in the stratum basale, stratum spinosum and stratum granulare of elderly subjects ($p < 0.05$). Both GDNF and GFRα-1 protein productions were absent in the stratum corneum of all subjects. In contrast to GDNF, which had

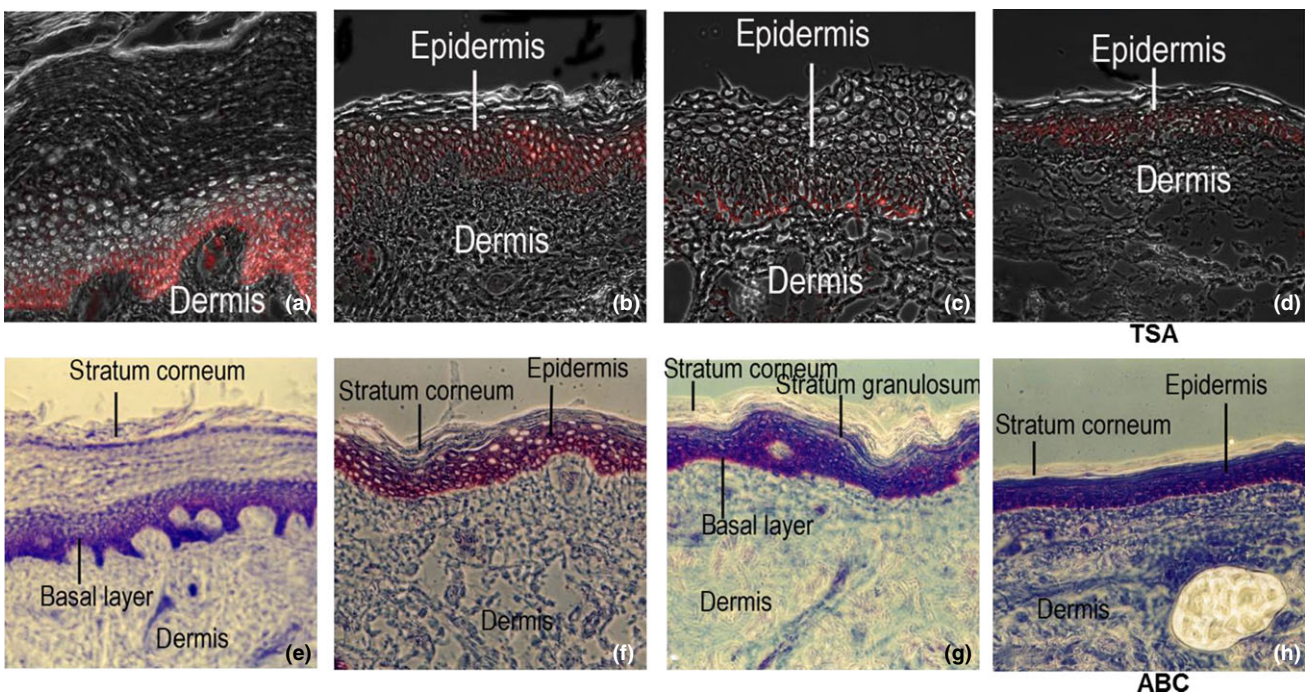


Figure 5 GDNF immunoreactivity shown with tyramide signal amplification and avidin–biotin complex staining in old ages. The colour of a positive signal is red. (A) 64 years old (B) 71 years old (C) 78 years old (D) 81 years old.

Table 2 Expression values (average immunoreactivity scores) of Glial cell line-derived neurotrophic factor protein in normal back human skin of different-aged donors (mean ± SEM)

Age group (years)	Stratum basale	Stratum spinosum	Stratum granulare	Stratum corneum	Whole epidermis
6–18	10.9 ± 0.2	7.8 ± 0.2	5.1 ± 0.1	Absent	6.1 ± 0.5
19–60	8.5 ± 0.3	5.4 ± 0.1	3.1 ± 0.2	Absent	4.2 ± 0.4
61–81	3.4 ± 0.4	1.6 ± 0.3	1.3 ± 0.3	Absent	1.2 ± 0.3

The average immunoreactivity score (IRSc) was evaluated by multiplying the percentage of positive cells (PP%) and the staining intensity (SI). The PP% was scored as 0 for <5%, 1 for 5–25%, 2 for 25–50%, 3 for 50–75% and 4 for >75%. The SI was scored as 0 for absent, 1 for weak, 2 for medium and 3 for intense staining.

Table 3 Expression values (average immunoreactivity scores) of GFRα-1 protein in normal back human skin of different-aged donors (mean ± SEM)

Age group (years)	Stratum basale	Stratum spinosum	Stratum granulare	Stratum corneum	Whole epidermis
6–18	8.1 ± 0.4	5.4 ± 0.3	2.7 ± 0.3	Absent	4.2 ± 0.6
19–60	6.1 ± 0.5	3.1 ± 0.4	1.1 ± 0.2	Absent	2.6 ± 0.3
61–81	2.3 ± 0.3	0.8 ± 0.2	0.2 ± 0.1	Absent	0.7 ± 0.2

The average immunoreactivity score (IRSc) was evaluated by multiplying the percentage of positive cells (PP%) and the staining intensity (SI). The PP% was scored as 0 for <5%, 1 for 5–25%, 2 for 25–50%, 3 for 50–75% and 4 for >75%. The SI was scored as 0 for absent, 1 for weak, 2 for medium and 3 for intense staining.

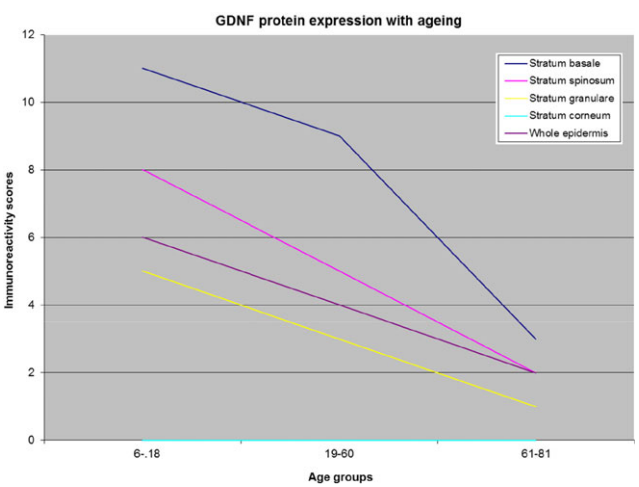
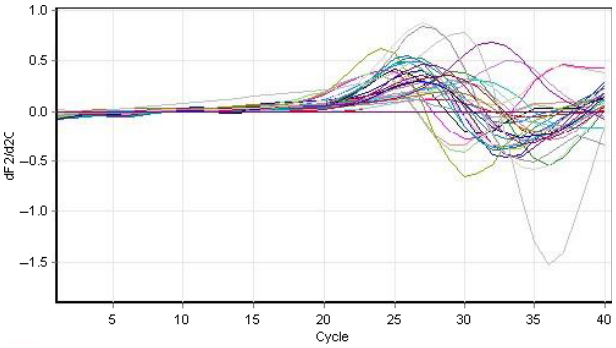


Figure 7 GFRa-1 expression values.

preserved expression at least in the stratum basale of elderly subjects, GFRα-1 protein production was completely absent in all epidermal layers in the elderly. A summary of the expression values is shown in Tables 2 and 3 and Figures 6 and 7.



No.	Colour	Name	Calculated con. (Ullml)
1		GDNF in 6 Y	0.12
2		GDNF in 18 Y	0.15
3		GDNF in 33 Y	0.17
4		GDNF in 39 Y	0.19
5		GDNF in 45 Y	0.14
6		GDNF in 64 Y	0.09
7		GDNF in 71 Y	0.06
8		GDNF in 78 Y	0.05
9		GDNF in 81 Y	0.03
		GAPDH	0.05

Figure 8 Real -Time PCR analysis of GDNF mRNA expression in human skin of different ages.

Absence of alterations of both GDNF and GFRα-1 protein productions in the adnexal structures with ageing

Both GDNF and GFRα-1 protein expressions were strong in fibroblasts, sweat glands, sebaceous glands, blood vessels and hair follicles. There were no statistically significant differences in GDNF and GFRα-1 protein expression in these structures with ageing. Both GDNF and GFRα-1 protein expressions in the dermis (glands) were similar in children, adults and elderly subjects (Figure 4).

GDNF and GFRα-1 genes were transcribed in the human skin of different ages

Real-time PCR analysis revealed gene expression of both GDNF and GFRα-1 in the human skin of different ages.

The absolute concentrations of GDNF and GFRα-1 transcripts in the skin samples of different ages are summarized in Figure 8. They ranged from 0.03 Ul/ml in very old ages to 0.19 Ul/ml in middle-age groups, while the relative concentrations were 0.6 Ul/ml and 3.8 Ul/ml respectively. In the nine cases, the staining scores (GDNF and GFRα-1) and the real-time PCR results (when matched for the same subjects) were directly correlated (Figure 8).

Discussion

In this investigation, we hypothesized that GDNF and GFRα-1 protein production, in normal human skin, undergoes age-associated alterations. To test our hypothesis and to fill this gap in the literature, we carried out the present investigation. We report (i) the production of GDNF and GFRα-1 proteins in the human skin at different ages and (ii) an age-associated decrease in the production of these proteins in the human epidermis.

The production of GDNF and GFRα-1 proteins in the human skin at different ages

Consistent with our previous studies on the hair follicles (Adly *et al.* 2008), we found strong expression of GDNF and GFRα-1 proteins in normal human epidermal keratinocytes of the children and adults. Our findings indicate possible roles for GDNF and GFRα-1 proteins as neuronal signalling molecules that regulate skin functions and the growth kinetics of the keratinocytes. The possible sources of GDNF and GFRα-1 proteins include cutaneous (nerve endings, fibroblasts and Merkel cells) and extracutaneous sites of synthesis (Suvanto *et al.* 1996; Nosrat *et al.* 1997; Honda *et al.* 1999). The strong cellular expression and localization of GDNF and GFRα-1 proteins in both basal and spinous layers indicates their roles in the regeneration of these layers through its ability to support angiogenesis (McLeod *et al.* 2006; Shvartsman *et al.* 2014) and antagonizing apoptosis (McAlhany *et al.* 2000). Several observations support the angiogenic roles of GDNF. Blais *et al.* showed that the neurotrophic factors (such as NGF and GDNF) can modulate the cutaneous microvascular network. Using a human tissue-engineered angiogenesis model, the authors reported that these peptides are highly potent angiogenic factors. A 40–80% increase in the number of capillary-like tubes was observed after the addition of 10 ng/ml of NGF and 50 ng/ml of GDNF. This angiogenic effect was mediated directly through binding with the neurotrophic factor receptors tropomyosin-receptor kinase A (TrkA), TrkB, GFRalpha-1 and c-ret that were all expressed by human endothelial cells. The authors concluded that as NGF and GDNF can moderately regulate the microvascular network in normal skin, they might have the ability to increase angiogenesis in pathological situations (Blais *et al.* 2013). Using microarray profiling of Neuro2A-20 cells, Piltonen *et al.* demonstrated up-regulation of vascular endothelial growth factor-C (VEGF) by GDNF (Piltonen *et al.* 2011). Moreover, in mice, the expression of GDNF and NGF was induced by VEGF presentation. Using both *in vitro* and *in vivo* modelling approaches, the authors indicated that the activity of NGF and GDNF regulates VEGF-driven angiogenesis, controlling endothelial cell sprouting and blood vessel maturation (Shvartsman *et al.* 2014). Similarly, in keeping with previous studies (Adly *et al.* 2008), strong expression of these proteins was found in the sweat and sebaceous glands. This strong reactivity suggests that GDNF- and GFRα-1-mediated signalling is critical for their homeostasis.

Age-associated decrease in GDNF and its cognate receptor GFRα-1 protein expression in the human skin

Here, we report a decreased GDNF and GFRα-1 protein production in the human skin with ageing. Our findings of age-related decrease in GDNF protein expression in the human epidermis agree with those of previous studies (Yurek & Fletcher-Turner 2001) (Adly *et al.* 2006a) (Adly *et al.* 2006a,b,c,d) (Li *et al.* 2013).

Yurek *et al.* measured GDNF in the striatum and ventral midbrain of young and aged Brown Norway hybrid rats following a unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway. At 2 weeks postlesion, protein levels of GDNF were higher in the denervated striatum when compared to the intact striatum for young (4- to 5-month-old), but not old (31- to 33-month-old) rats. Young rats showed higher GDNF protein levels in both the striatum (lesioned or intact) and ventral midbrain (lesioned or intact) than old rats. The endogenous GDNF was differentially affected by a 6-OHDA lesion in the ageing nigrostriatal system with young brain showing a significantly compensatory increase in these two factors in the denervated striatum, while no compensatory increase is observed in aged brain (Yurek & Fletcher-Turner 2001).

Li *et al.* examined the effects of chronic stress on the spatial learning memory and the role of GDNF of prefrontal cortex (PFC) and hippocampus (HP) in different-aged mice. The chronic stress model mice in 21 days with multiple chronic unpredictable stressors were applied. The expression of GDNF in HP and PFC was detected by immunohistochemical method. Compared with young mice, the GDNF expression in the CA3 (*Cornu Ammonis* area 3), DG (dentate gyrus) of HP and PFC was significantly reduced in aged mice. The expression of GDNF in HP and PFC was remarkably reduced in stress group mice. The aged stress mice had more serious changes after chronic stress (Li *et al.* 2013).

Skin is a major source of secretion of the neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin-3 and GDNF (Blais *et al.* 2013). Adly *et al.* examined NGF protein expression in the human epidermis and adnexal structure. The authors reported prominent age-related alterations in the former, but not in the latter. The intense NGF protein expression values were seen in the epidermis of young individuals.

Alternatively, weak NGF protein expression values were seen in the epidermis of old individuals (Adly *et al.* 2006a). CD1d belongs to a family of antigen-presenting molecules that are structurally related to the classic major histocompatibility complex class I (MHC I) proteins. However, unlike MHC I molecules, which bind protein antigens, CD1d binds to lipid and glycolipid antigens. CD1d is expressed by cells of lymphoid origin and by cells outside of the lymphoid lineages, such as human keratinocytes of psoriatic skin. We previously examined the expression pattern of CD1d protein in normal human skin with ageing (children, adults and the elderly) using immunofluorescent and immunoalkaline phosphatase staining methods. In the epidermis, CD1d protein production was strong in the skin of the children and declined gradually with age, being moderate in adults and weak in the elderly. As compared with values in children, there was a statistically significant decrease in CD1d protein production in the elderly. In the dermis, CD1d protein production was strong in the fibroblasts, sweat glands, sebaceous glands, blood vessels and hair follicles regardless of the age (Adly *et al.* 2005, 2006b; Adly & Abdelwahed Hussein 2011).

Mechanisms of age-associated decrease in GDNF and its cognate receptor GFRα-1 protein expression in the human skin of the elderly

The decreased GDNF and GFRα-1 protein production in the epidermal keratinocytes in the elderly may reflect senescence of epidermal keratinocytes, increased sensitivity of GDNF- and GFRα-1-positive cells for apoptosis with ageing or an altered expression of certain cytokines that influence the production of these proteins (Unsicker 1996). It is also possible that the down-regulation of GDNF and GFRα-1 protein expression in aged skin may be due to the reduction in the number of high-affinity GDNF binding sites. The down-regulation of these molecules with ageing may have a critical permissive role in the development of cutaneous infections and neoplasms. Interestingly, GDNF protein has a reparative action during the healing of the wounds of corneal epithelial cell and ischaemic skeletal muscles (You *et al.* 2001; Shvartsman *et al.* 2014). Alternatively, the strong expression of GDNF and GFRα-1 proteins in the skin of young individuals may reflect an increased receptor-mediated internalization of these proteins (Hase *et al.* 1999; Schober *et al.* 2007).

Here, we report a decreased GDNF and GFRα-1 protein production in the human skin with ageing. GDNF and GFRα-1 protein production on keratinocytes may be a potential marker for ageing. The possible clinical ramifications of these findings (such as the potential reparative actions of GDNF in skin wound healing) are open for further investigations.

Conflicts of interest

None to declare.

Disclosure of financial and other funding sources

None to declare.

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