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Vitamin B_{12} Activates the *Wnt*-Pathway in Human Hair Follicle Cells by Induction of β-Catenin and Inhibition of Glycogensynthase Kinase-3 Transcription

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ABSTRACT

***Background and Objectives*:** *Micrograft transplantation is accompanied by a transient induction of telogen in transplanted hair follicles (HF), which might be avoided by supporting the metabolic pathways of the micrograft during the ex vivo period. Vitamin B_{12} (cobalamin) has been suggested to influence HF growth and cycling in humans, but the mechanisms are unclear.* ***Method*:** *HFs were obtained from patients undergoing routine micrograft transplantation and were cultured for 5 days in Dulbecco's modified Eagles Medium, supplemented with different amounts of vitamin B_{12}. Hair shaft elongation (HSE) of the isolated HFs as well as quantitative changes of mRNA for β-catenin, glykogensynthase kinase-3 (GSK-3) and TCF/Lef-1 in HF cells were determined.* ***Results*:** *In vitro HSE demonstrated a dose dependent induction of HSE after stimulation with 2.5 μg/ml and 25 μg/ml vitamin B_{12} (6.2 ± 2.1% and 15.4 ± 3.8% respectively). A dose dependent induction of β-catenin-mRNA could be demonstrated in cultured HFs after stimulation with 2.5 μg/ml and 25 μg/ml vitamin B_{12} (fold change compared to DMEM: 9.5 ± 2.7, $p < 0.05$ and 23.1 ± 7.4, $p < 0.01$; respectively). Concomitantly the amounts of GSK-3 were significantly reduced after stimulation with 25 μg/ml vitamin B_{12} (fold change compared to DMEM: 0.76 ± 0.12, $p < 0.05$).* ***Conclusions*:** *Our data demonstrate a hair growth promoting effect of vitamin B_{12} in vitro. This effect is accompanied by the modulation of intracellular signal transduction molecules of the wnt-pathway and might promote hair growth after micrograft transplantation.*

***Keywords*:** *Hair Follicle, Growth Factors, Cell Signaling*

1. Introduction

During hair restoration surgery, transplanted hair follicles (HF) undergo cycling after transplantation, resulting in a period of reduced hair growth immediately after transplantation. Recent progress in studying the biology of isolated micrografts during hair restoration procedures identified key factors for high viability of the micrografts, which has opened new ways of treating the isolated micrografts for better grafting efficiency. However, the posttransplantational effluvium is not completely prevented by improved storage conditions and other factors has to be taken into account.

Vitamin B_{12} (cobalamin) is an essential cofactor for two enzymes: methionin synthase (MS) and methylmalonylCoA mutase. MS provides methionine for protein synthesis, but also interacts with the folate cycle, which provides methyl groups for the conversion of dUMP to dTMP during nucleotide synthesis and therefore influences DNA replication. Both actions of vitamin B_{12} are essential for cell proliferation [1,2]. Deficiency of vitamin B_{12} is therefore associated with severe changes and abnormalities in rapidly dividing tissues [3]. The mechanisms involved in vitamin B_{12} associated diseases are not clearly understood in many of the pathologic conditions, but rely on disturbed DNA synthesis in megaloblastic anemia [2,4] and/or on altered methylation of genomic DNA and intracellular proteins in demyelinating diseases [4].

The association of vitamin B_{12} with diseases of the skin and its appendages is much less characterized. It has been shown, that vitamin B_{12} deficiency is accompanied by hyperpigmentation of skin [5] and hair [6,7]. This

effect of vitamin B_{12} seems to be due to altered melatonin production. It has been demonstrated in the agouti mouse model, that dietary supplementation favor the production of eumelanin and decrease the production of pheomelanin [7]. In addition, inhibition of hair growth was repeatedly reported, but experimental data are rare. Most recently, it has been demonstrated that topical administration of cyanocobalamin suppressed the effect of the potassium channel inhibitor tolbutamide and induced anagen phase in hair follicles in a mouse model [8].

The *wnt*-pathway controls both morphogenesis and cycling of different compartments of the hair follicle [9]. It has been shown, that forced expression of Wnt-5a in epithelial wounds induced formation of rudimentary hair follicles and sebaceous gland in an β-catenin dependent manner [10]. The action of Wnt signaling in hair follicle development seems further is closely associated to Notch signaling, and stimulation of Notch signaling is induced by β-catenin dependent Jag1 transcription [11]. Wnt/β-catenin signaling serves also as a crucial proximal signal for the telogen-anagen transition [9,12]. Chronic activation of β-catenin in telogen hair follicles resulted in changes consistent with induction of an exaggerated, aberrant growth phase (anagen), and transient activation of β-catenin produced normal anagen hairs [12].

In this study, we investigated the postulated growth promoting effect of vitamin B_{12} in an in vitro hair shaft elongation model. In addition, the influence of vitamin B_{12} stimulation of hair follicles on transcription of molecules of the *wnt*-pathway was investigated.

2. Materials and Methods

Sample preparation:

Human hair follicles (HF) were prepared by standard surgical techniques used in hair restoration surgery. Micrografts were taken from the occipital region of patients undergoing hair restoration surgery and had given informed consent for the usage of micrografts for this in vitro study.

Stimulation of HFs:

To evaluate the specific effect of vitamin B_{12} on hair shaft elongation (HSE) and gene expression, 5 HFs were cultured for 5 days in 1.5 ml of Dulbecco's modified Eagle's medium (Sigma-Aldrich Co, StLouis, MO), supplemented with 10% fetal bovine serum (Sigma) and antibiotics (Sigma).

Stimulation with vitamin B_{12} (Sigma) was performed at concentrations of 2.5 μg/ml and 25 μg/ml vitamin B_{12}.

Evaluation of in vitro hair shaft elongation (*HSE*):

To evaluate the effect of vitamin B_{12} on in vitro HSE, hair shaft length was measured at day 0 and day 5 of culture using an invert microscope. Data are expressed as percent change compared to HFs cultured in DMEM.

Real time RT—polymerase chain reaction:

After 5 days of HF culture, mRNA was prepared using the Quiagen RNeasy kit (Quiagen, Hilden, Germany), followed by reversed transcription into cDNA with oligo-dT primers (Clontech, Palo Alto, CA). 5 μl of the cDNA was amplified with specific primer for GADPH, β-catenin, glykogensynthase kinase-3 (GSK-3) or Lef1/TCF (**Table 1**). PCR reactions containing SYBR-green were amplified on a Corbett Real Time PCR machine (Rotor gene 2000, Corbett research). For each sample, Ct (cycle threshold)-values obtained for each target gene was normalized to the internal control. Standard curves were constructed from standard reactions for each target gene and internal control by plotting Ct-values vs. log cDNA dilution. Because the amplification efficiencies of target genes and internal control were equal, the relative change of target gene expression compared to the unstimulated control could be calculated using the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct(\text{stimulated}) - \Delta Ct(\text{control})$. The ΔCt values were determined by subtracting the average GADPH Ct-value from the average target gene Ct-value.

After each real time RT PCR, a melting profile as well as agarose gel electrophoresis of each sample was performed to rule out non-specific PCR products and primer dimers.

Statistics:

Data are expressed as mean ± SD of at least five independent experiments. Data were analyzed using standard statistical software and tests.

3. Results

HSE of vitamin B_{12} stimulated HFs:

To evaluate the influence of vitamin B_{12} on hair shaft

Table 1. Primer pairs used for real time RT-PCR.

	Forward primer (5'-3')	Reverse primer (5'-3')	PCR product length (bp)
G3DPH	CCCATCAGGATCTTCCAG	CCTGCTTCACCACCTTCT	590
β-catenin	TGCGGACTCAGAAGGAACTCAT	ACTAGTCGTGGAATGGCACC	162
GSK-3	AACTGCCCGACTAACAACAC	ATTGGTCTGTCCACGGTCTC	253
Lef1/TCF	AATCATCCCGGCCAGCA	TGTCGTGGTAGGGCTCCTC	234

growth in vitro, HF were cultured in DMEM and vitro hair shaft elongation HSE was measured on day 0 and day 5. In these experiments HSE reveals increased elongation in HFs containing vitamin B_{12} as compared to HFs cultured in DMEM (**Figure 1**). Supplementation of DMEM with 2.5 μg/ml vitamin B_{12} showed a significant increase of HSE (6.2% ± 2.1%, $p < 0.05$). Furthermore, a dose dependent increase could be observed, reaching a maximum at 25 μg/ml vitamin B_{12} (15.4% ± 3.8%, $p < 0.01$). No signs of induction of catagen (as determined by thinning of the hair shaft at the dermal papilla/hair shaft border) were observed in any of the cultures.

Modulation of β-catenin, GSK-3 and Lef1/TCF by vitamin B_{12}:

Analysis of mRNA transcription in cultured HFs by real time-RT-PCR revealed a dose dependent induction of β-catenin transcription after vitamin B_{12} stimulation. We found, that addition of 2.5 μg/ml vitamin B_{12} lead to a significant induction of β-catenin transcription (9.5 ± 2.7fold; $p < 0.05$; **Figure 2**) as compared to HFs cultured in DMEM only. Higher doses of vitamin B_{12} further enhanced the transcription of β-catenin. showing a 23.1 ± 7.4 fold increase at 25 μg/ml vitamin B_{12} (compared to DMEM; $p < 0.01$; **Figure 2**).

Transcription of GSK-3 showed a slight but significant decrease after stimulation with 25 μg/ml of vitamin B_{12} (fold change compared to DMEM: 0.96 ± 0.06, p = n.s. and 0.76 ± 0.12, $p < 0.05$; respectively; **Figure 2**). With lower concentrations, no significant changes in GSK-3 transcription could be observed.

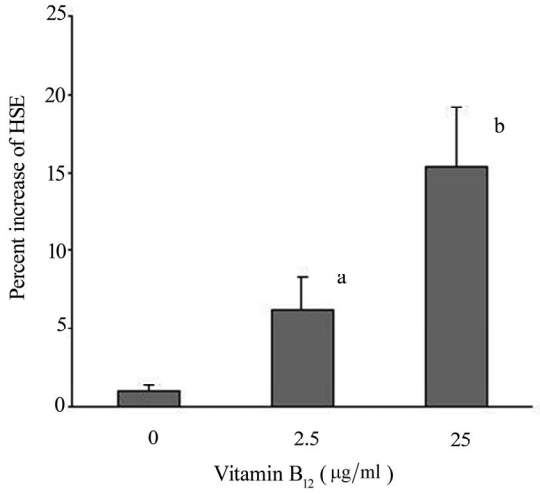

Figure 1. HF of 4 different patients were cultured in quadruplicate for 5 days at 37˚C in DMEM, 10% FBS supplemented with different concentrations of vitamin B_{12}. Hair shaft length was measured at day 0, and day 5 and HSE was given as percent increase compared to unstimulated HF. a: $p < 0,05$, b: $p < 0,01$.

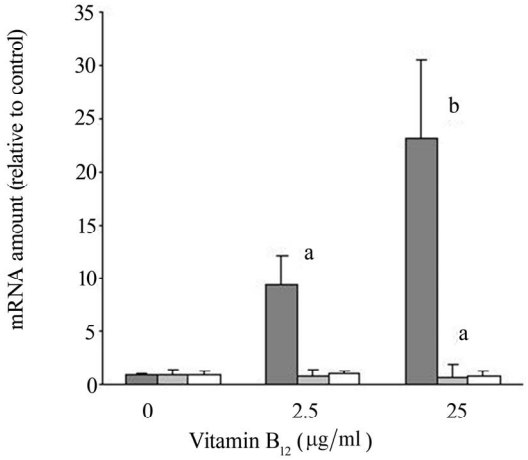

Figure 2. HF of 4 different patients were cultured in quadruplicate for 5 days at 37˚C in DMEM, 10% FBS supplemented with different concentrations of vitamin B_{12}. After culture, specific mRNA for β-catenin (■), GSK-3 (▒) and Lef1/TCF (□) was quantified by rT-PCR. Data represent fold change ± SD of specific mRNA levels. a: p=0,05, b: p<0.01.

No changes in the transcription of Lef1/TCF were found in HFs stimulated with 2.5 μg/ml vitamin B_{12} or 25 μg/ml vitamin B_{12} (**Figure 2**).

4. Discussion

Cells of the HF are rapidly dividing cells, and proliferation of the cells is dependent upon synthesis of DNA and therefore on sufficient supply with vitamin B_{12} and folic acid [3]. It has been shown, that many growth factors are able to induce cell proliferation and DNA synthesis in various compartments of the HF [13,14]. In addition, disturbed DNA synthesis, induced by cytokines or growth factors, lead to transition of the HF from anagen into telogen [15]. On the other hand, transition of the HF from telogen into anagen is also accompanied by cell proliferation and DNA synthesis and is therefore dependent on vitamin B_{12} and folic acid.

A key pathway in hair follicle morphogenesis and hair follicle cycling is the *wnt*-pathway. This pathway is regulated by a variety of soluble factors, which are known to influence hair growth and hair follicle cycling. Signaling via the *wnt*-pathway results in release of membrane bound β-catenin in activation of a down stream cascade which, at least activates the transcription factor Lef1/TCF. As a result of *wnt*-pathway activation in hair follicle cells, cell proliferation is increased in different compartments of the hair follicle leading organogenesis in hair follicle placodes or the induction of anagen in mature hair follicles (reviewed in [9]). The importance of

wnt-pathway in hair follicle biology was further supported by the finding that activation of this pathway results in the formation of ectopic follicles from existing follicles, interfollicular epidermis and sebaceous glands [16].

In this study, we have evaluated the effect of different concentrations of vitamin B_{12} in an in vitro HSE model. In this model, elongation of the hair shaft occurs under standard tissue culture conditions [17].

We found, that supplementation of medium with vitamin B_{12} resulted in enhanced HSE in this system. This finding demonstrates the hair shaft growth promoting effect of vitamin B_{12}. The cellular actions of vitamin B_{12} are dependent on vitamin B_{12} uptake into the cell either by internalization of free cobalamin or, more important, on receptor mediated uptake of the transcobalamin II (TCII)/vitamin B_{12} complex into the cells via the TCII-receptor [18]. It has been shown, that cultured cells produce endogenous TCII and the amount of internalized cobalamin correlates directly to the capacity of the cells to produce TCII [19]. Our data suggests that in the in vitro model of HSE cellular expression of TCII and TCII-receptor occurs due to the lack of vitamin B_{12} in the tissue culture medium. A similar decrease of vitamin B_{12} can be postulated for the storage period of micrografts during hair restoration surgery, and this might be causative for the observed posttransplantational effluvium.

It has been demonstrated recently, that topical administration of vitamin B_{12} results in induction of anagen in the mouse model [8]. Similarly, our data suggest that vitamin B_{12} supports the transition of the HF into anagen by increased transcription of β-catenin and reduced transcription of GSK-3. Although clinical trials have to confirm the role of vitamin B_{12}, the present data support the hypothesis that vitamin B_{12} stabilize and/or initiate the anagen phase of the HF and might reduce posttransplantational effluvium in hair restoration surgery.

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