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Growth Factor mRNA Levels in Alopecia Areata Before and After Treatment with the Contact Allergen Diphenylcyclopropenone

ROLF HOFFMANN, ELKE WENZEL, ANDREA HUTH, PIETER VAN DER STEEN, MONIKA SCHÄUFELE, ARNE KÖNIG and RUDOLF HAPPLE

Department of Dermatology, Philipp University, Marburg, Germany

The early immune response in alopecia areata is characterized by a Th1 T helper cell cytokine pattern and an aberrant expression of ICAM-1 and HLA-DR molecules on lesional hair bulbs. A counteracting cytokine pattern induced by a therapeutic contact dermatitis is supposed to mediate the hair regrowth. In addition to cytokines, growth factors have been shown to influence immune responses, and we therefore investigated the expression levels for a panel of growth factors in untreated versus alopecia areata after treatment with the contact sensitizer diphenylcyclopropenone. Using semiquantitative reverse-transcriptase polymerase chain reaction we detected a striking overexpression of transforming growth factor β1 mRNA in successfully treated patients. This cytokine has been shown to be a potent immune response modifier, which can suppress Th1 immune responses. The way in which topical immunotherapy induces hair regrowth in alopecia areata is unknown, but a lesional increased expression of transforming growth factor β1 may be a possible mechanism. ***Key words: hair growth; PCR; HPLC.***

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R. Hoffmann, MD, Zentrum für Hautkrankheiten, Philipps-Universität, Deutschhausstraße 9, 35033 Marburg, Germany.

There is increasing evidence that immune/inflammatory cells, such as T-cells, participate in the pathophysiology of several clinically distinct alopecias (1). In alopecia areata (AA) the mechanisms by which the hair loss is mediated are not understood, but it is well known that AA is associated with a peri- and intrafollicular lymphocytic infiltrate, consisting primarily of $CD4^+$ T-lymphocytes (2, 3) and with an aberrant expression of ICAM-1 and HLA-DR molecules on affected hair follicle keratinocytes and papilla cells (4–8). The nature of the noxious signal, however, and its anatomical target remain elusive. It has been assumed that an autoimmune process is involved (9, 10). Apparently the infiltrating T-lymphocytes do not destroy the hair follicle but rather initiate a "switch-off" mechanism of the hair cycle, which is present as long as the lymphocytic infiltrate persists.

Recently we have hypothesized that during the AA-specific immune response T-lymphocytes might mediate their effects through soluble mediators, which arrest the hair cycle in stage IV anagen (10), and we were able to demonstrate a Th1-type T helper cell cytokine pattern in active, untreated AA (11). Other soluble mediators, such as growth factors, have been shown to mediate a rather broad spectrum of biological functions. Interestingly, treatment with TGF-α or EGF in newborn mice was shown to decrease hair growth (12), and mice with a null mutation of the TGF-α gene have abnormal skin architecture with wavy hair (13). *In situ* hybridization experiments have localized high expression levels for TGF-α mRNA within the inner root sheath of anagen hair follicles, indicating a pivotal role of this polypeptide during hair growth (14). Moreover, EGF and its receptor have been demonstrated to be differentially expressed during early hair follicle development (15–18). Furthermore, growth factors have been shown to influence active hair growth *in vitro*. In this regard EGF and TGF-α were shown to induce catagen-like changes, whereas TGF-β1 inhibited the hair growth (19–23). Following this line of thought, one may postulate growth factor-dependent mechanisms which play an essential role during hair growth and cycling. In hair diseases such as AA they may affect the differentiation and proliferation of hair follicle cells. Therefore, the aim of this study was to investigate the expression of a variety of growth factors in scalp biopsies from untreated AA versus healthy controls and AA after successful and unsuccessful treatment with the contact allergen diphenylcyclopropenone (DCP).

MATERIALS AND METHODS

Patients

With informed consent, deep excisional biopsies were taken from the temporal aspect of the scalp. All patients had had stable alopecia areata totalis for at least 2 years. No patient described a history of spontaneous hair regrowth. As controls, scalp biopsies obtained from 5 patients (31–65 years, mean 41 years) undergoing elective neurosurgery were used. They had no inflammatory scalp disease, no systemic disease and no immunosuppressive therapy. Biopsies were taken from 13 patients (25–55 years, mean 36 years) with alopecia areata of the totalis type. After skin biopsy, sensitization was induced by painting 25 cm^2 of the patient scalp with 2% DCP. A contact dermatitis was maintained by weekly unilateral application of DCP concentrations, varying from 1% to 0.0000001%. After a response in the form of unilateral hair regrowth (approximately 8–18 weeks after the initiation of the contact dermatitis), additional scalp biopsies were taken 24–36 h after the last DCP application.

Chemicals

Guanidinium thiocyanate, Tris-borate, glycerol, bromophenol blue, Tris-HCl, $NaClNa_2H_2$ ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (Deisenhofen, Germany), and phenol, isopropanol and chloroform from Merck (Darmstadt, Germany). The taq polymerase and the kit for reverse transcription (RT kit) came from Pharmacia (Uppsala, Sweden). Ethidium bromide and the DNA-VIII molecular weight marker came from Boehringer Mannheim (Mannheim, Germany). Primers for PCR amplification of β-actin were bought from Clonetech (Palo Alto, CA). Primers for EGF, TGF-α, TGF-β1;β2;β3 and PDGFβ were designed according to published sequences (24) and manufactured with the aid of an automatic oligonucleotide synthesizer. These primer pairs are all intron-spanning to avoid amplification of any contaminating genomic DNA. The sequences, annealing temperatures and cycle numbers are given in Table I.

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Table I. *List of primers used for amplification of growth factor transcripts*

The primers are designed as described in *Methods* and the annealing temperatures, cycle numbers ultimatively used and sequences are provided.

Primer	°C	bp	Cycles	Sequences
β-actin	60	838	20	5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′
TGF-α	65	671	28	5′-CGCTCTGGGTATTGTGTTGG-3′ 5′-TCGTGGTCCGCTGATTTCTT-3′
TGF-β1	65	365	30	5′GCCCTGGACACCAACTATTGC-3′ 5′-GCTGCACTTGCAGGAGCGCAC-3′
TGF-β2	65	573	32	5′-GCTCTGTGGGTACCTTGATGCCATCC-3′ 5′-TTCTTCCGCCGGTTGGTCTGTTGTG-3′
TGF-β3	65	538	30	5′-ACTGCCGAGTGGCTGTCCTTTGATG-3′ 5′-AGGCAGATGCTTCAGGGTTCAGAGTG-3′
EGF	65	522	33	5′-ACTGCTTGGTGTTCGTGTCG-3′ 5′-GCCTCCCTCTGTATTTGTGC-3′
PDGF-β	65	497	30	5′-TGCACGGAGACCCCGGAGAGGAAGATGG-3′ 5′-GCACCGTCCGAATGGTCACCCGAGTTTG-3′

Extraction of total RNA from excisional scalp biopsies

Total RNA from scalp skin was isolated according to Chomczynski & Sacchi (25). Twenty milligrams wet weight scalp skin was frozen in liquid nitrogen, minced and resuspended in guanidinium thiocyanate, followed by addition of acidified phenol/chloroform. After centrifugation, the RNA was precipitated from the aqueous phase with isopropanol. Individual RNA samples from different patients were equalized with regard to their RNA concentration by ultraviolet (UV) spectroscopy. Approximately 10 μg total RNA was isolated from each preparation.

RT-PCR analysis of growth factor mRNA and β-actin mRNA

Analysis of growth factor mRNA expressions was performed as previously described (21). In brief, 1 μg total RNA from individual biopsies was reverse-transcribed with random hexamer primers and murine moloney leukemia virus reverse transcriptase. Each primer pair was tested on total RNA and subsequent PCR amplification without prior reverse-transcription. No PCR products were obtained in controls. For each primer pair a three-temperature step PCR cycle program was carried out (1 min annealing, 2 min extension at 72°C and 1 min denaturation at 94°C). This PCR set-up was optimized to obtain only one PCR product of the expected length. Linear amplification conditions were determined by (1) identical amounts of cDNA subjected to increasing PCR cycles, and (2) increasing amounts of cDNA subjected to a defined PCR cycle number. PCR cycle numbers used are given in Table I. Semiquantitative analysis of the PCR amplification products was achieved by high-performance liquid chromatography (HPLC) (26). The specific PCR products were recognized by their elution time in comparison to a DNA standard. The peak areas of the corresponding PCR products are proportional to the amount of DNA loaded onto the analytical column, allowing precise quantitation. Before calculation of the growth factor mRNA expressions, the probes were normalized for β-actin mRNA as the house-keeping gene. This was achieved by using one third of the cDNA and amplification of the β-actin mRNA to each growth factor mRNA followed by HPLC analysis. Similar experiments were performed by using GAPDH as the house-keeping gene. Those experiments showed identical results (data not shown). The mean ±SD peak area for each growth factor in 13 patients/5 controls was calculated. All experiments were performed at least in triplicate (Fig.1).

HPLC operating conditions

A Perkin–Elmer TSK DEAE-NPR (35 × 4.6 mm i.d.) analytical column packed with 2.5 μm particles of hydrophilic resin bonded with DEAE groups was used. The samples were injected into the column equilibrated for 2 min with 70% buffer A (25 mM Tris-HCl, pH 9.0) + 30% buffer B (25 mM Tris-HCl, pH 9.0 containing 1.0 mol/l NaCl). The eluent was then brought to 50% B within 1 min, followed by a 15-min linear gradient from 50% B to 65% B using an eluent flow rate of 1 ml/min. The temperature of the analytical column was maintained at 40°C.

RESULTS

Using our experimental approach we detected steady state mRNA levels for all growth factors tested in intact scalp from healthy donors, as well as in scalp from patients with AA before and after DCP treatment. The expression of growth factor transcripts appeared to be reduced in active AA compared to controls (Fig. 1). This was especially evident for TGF-β1 mRNA expression, where mRNA levels was reduced by nearly 60% in comparison to the controls.

After successful (11 patients) and unsuccessful (2 patients) DCP-treatment (2 patients) additional scalp biopsies were taken 24–36 h after the last DCP application. The experimental design and semiquantitative analysis of the different growth factors were performed in the same way as described above. At this point in time, when only the first vellus-like hair was

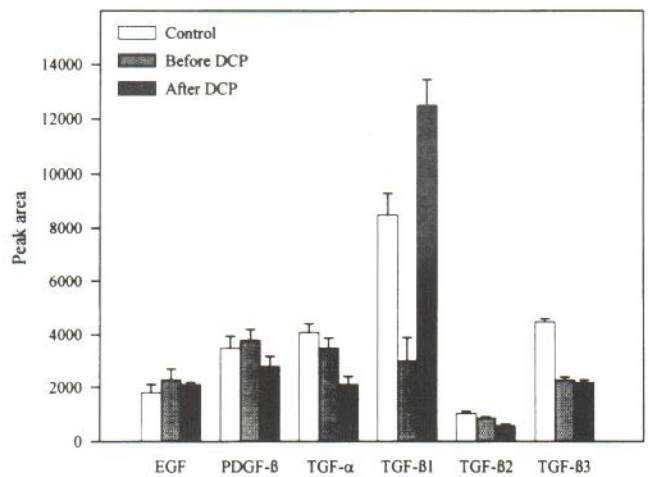

Fig. 1. Growth factor mRNA expression in untreated versus treated alopecia areata as compared to healthy controls.

visible, most of the growth factor expressions remained unchanged. However, compared to controls and untreated AA, overexpression of TGF-β1 mRNA was noted (Fig. 1). Compared to the untreated situation the TGF-β1 mRNA showed a nearly threefold induction after DCP-treatment. In 2 patients who were non-responsive to DCP-treatment the growth factor expression pattern was nearly similar to that of successfully treated patients (Fig. 2).

DISCUSSION

In AA affected hair roots are rather "switched off" than destroyed, and recently we have provided experimental evidence that an aberrant Th1 cytokine profile plus expression of IL-1β might trigger this "switch off" mechanism (11). Scalp areas that were treated with DCP revealed a different cytokine profile. In particular, we hypothesized that IL-10, representing a potent immune response modifier, mediates the induction of hair regrowth after DCP treatment.

Apparently the induction of contact eczema induces a rather broad spectrum of immune responses within the scalp (28–30). For this reason, a more complex network of mediators may be actively involved in the therapeutic mechanism. Therefore, we investigated the expression patterns of a panel of growth factors before and after treatment in affected scalp areas from patients with AA. We were able to detect all growth factors tested. The noted decrease of growth factor expression in AA versus controls may reflect a lower growth activity, as in AA the hair cycle is arrested in early anagen and only small hair follicles are present. After DCP-treatment we noted an up-regulation of TGF-β1 mRNA expression, above the control levels.

TGF-β1 has been described to promote a wide spectrum of biological responses, and there is increasing evidence that this polypeptide acts as a potent immune response modifier. Although TGF-β1 can promote cell recruitment and inflammation at sites of tissue damage, the effects of TGF-β1 are generally of a negative nature, suppressing immune functions (34). The absence of TGF-β1 has been demonstrated to potentially contribute to autoimmune activation. In this regard

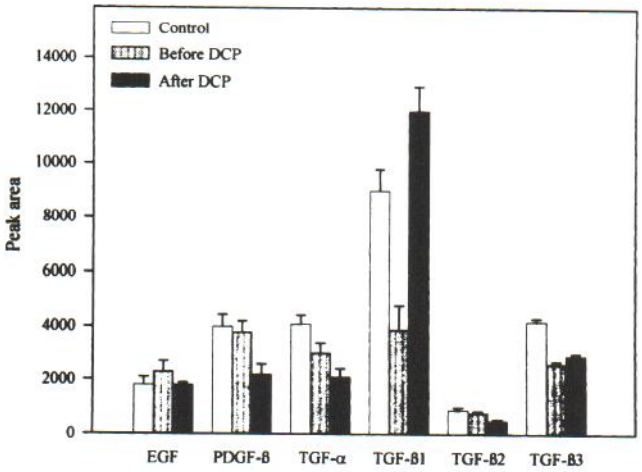

Fig. 2. Growth factor mRNA expression in untreated versus DCP non-responsive patients (2) with alopecia areata. The overall expression pattern of the various growth factors is nearly similar to the pattern detected in successfully treated patients.

TGF-β1 has been suggested to be a pivotal regulator of both classes of MHC molecules, with implications for antigen presentation (35).

In AA an early event is the aberrant expression of ICAM-1 and HLA-DR molecules on affected hair follicle cells. After successful treatment with potent contact sensitizers, this aberrant expression was shown to be reduced (4). In our view, ICAM-1 and HLA-DR expression is induced by IFN-γ, which is present in active AA. TGF-β1 has the ability to inhibit IFN-γ synthesis (36, 37), IFN-γ-induced ICAM-1 expression and HLA-DR expression (38). In treated AA locally secreted TGF-β1 may promote a suppressive effect on the immune response responsible for the disease. Possibly in concert with other mediators of the contact allergy, in particular with IL-10 (11 for ref.), TGF-β1 might promote a local down-modulation of essential molecules such as HLA-DR and ICAM-1. This immunomodulation would diminish the severity of the lymphocytic infiltration. A diminished immune response would allow to induce a "switch on" signal, of the interrupted hair cycle and hair regrowth would be the result.

Since we detected increased TGF-β1 transcripts in DCP non-responsive patients, TGF-β1 expression could not reflect an epiphenomenon due to the beginning hair growth. We rather suppose an essential mechanism during the late phase of the contact dermatitis. To our knowledge this proposed action of TGF-β1 has not been reported yet. However, because of the small numbers of unresponsive patients, we cannot draw a definite conclusion. It remains to be shown whether our hypothesis is true. It has been shown that patients resistant to DCP-treatment tend to have a severe, chronic and stable form of the disease. In routine scalp biopsies a tendency of decreased hair follicle counts and slight but consistent fibrosis were noted (39). Those cases were discussed as "end stage" AA with only a small chance of hair regrowth. The non-responders in our study had had AA for years, and when we assessed the scalp biopsies histologically, only limited numbers of hair follicles were seen with only little or none lymphocytic inflammation. Obviously DCP-therapy induces regulatory cytokines such as IL-10 or TGF-β1. These mediators, however, can only be effective in inducing hair regrowth if the target, the inflamed hair follicle, is present. In long-standing AA this is not necessarily the case, which explains why DCP-therapy may be ineffective.

In conclusion, we have determined the expression levels for a panel of growth factors in the scalp from patients with untreated AA versus healthy controls and versus DCP-treated AA. We provide experimental evidence that TGF-β1 expression is up-regulated after DCP-treatment in the scalp above control levels. This finding may be an important factor limiting the immune-mediated response in AA. TGF-β1 may be responsible for the inhibition of an AA-specific cytokine profile and subsequent ICAM-1 and MHC-molecule expression on affected hair follicle papilla cells. The vanishing AA-specific immune response would give way for the induction of a "switch on" signal of the interrupted hair cycle.

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