The Role of the VEGF, KGF, EGF, and TGF-β1 Growth Factors in the Pathogenesis of Telogen Effluvium in Women

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ABSTRACT

In the recent time, several growth factors were revealed that appear to control the development and the cycle of a hair follicle. However, the contribution of these factors into the pathogenesis of alopecia keeps being poorly studied. So far, all the studies have experimental nature, most of them conducted in vitro or on animal models. Thus, the role of the growth factors in the pathogenesis of different types of alopecia and their impact of the severity of this pathology remains unclear. The purpose of this study was to investigate the role of the VEGF, KGF, EGF, and TGF-β1 growth factors in the development of telogen effluvium in women. The study involved 30 female patients with telogen effluvium and 8 healthy volunteers. All the patients were examined, medical history was collected, trichoscopy and phototrichogram tests were performed. From every patient, a sample of scalp was taken by punch biopsy (4mm), which was analyzed by the standard immunohistochemical study. We discovered changes in the expression of the growth factors in women with telogen effluvium in comparison with healthy volunteers. Our results showed that all the studied growth factors (VEGF, KGF, EGF, TGF-β1) contribute to the development of telogen effluvium; VEGF has the biggest impact and EGF has the lowest impact on hair loss.

Keywords: Growth factors, Hair loss, Telogen effluvium, Hair follicle, Hair cycle, Immunohistochemical research, VEGF, KGF, EGF, TGF-β1.

INTRODUCTION

The term *telogen effluvium* was first proposed by A.M. Kligman in 1961. The condition is characterized by a diffuse loss of scalp hair in the telogen phase, which usually occurs 2-3 months after a triggering impact[1,2]. The list of trigger factors includes: hypo- and hyperthyroidism, chronic systemic diseases, autoimmune and infectious diseases, microcirculation disorders of the skin of the scalp, lack of a number of microelements, some medications[3-6]. Telogen effluvium can be acute (the disease lasts up to 6 months), chronic (more than 6 months), or chronically recurrent[6,7].

The trigger factor affects the process of keratinocytes division and differentiation, and induces changes in metabolic processes in the growth plate of a hair bulb, which leads to premature termination of the anagen phase. The hair follicle gradually passes into the catagen phase, and then into the telogen phase, which incurs a change in the ratio between telogen and anagen hair. To diagnose telogen effluvium, the crucial factor is the ratio of the number of hairs that are in the anagen to the number of those in the telogen phases[8,9].

Today it is known that there is a large number of signal molecules involved in regulation

of a hair cycle and regeneration of a follicle. Those include certain genes, some kinds of growth factors, nuclear receptors, cytokines, subcellular signaling pathways[10-13]. Each stage of a follicle morphological development is characterized by a unique pattern formed by the expression of growth factors, their receptors and antagonists, adhesion molecules and components of subcellular signaling[14-17].

Contemporary studies have made it possible to detect the growth factors that can control the development and the cycle of a hair follicle; those include: epidermal growth factor (EGF), transforming growth factor (TGF), keratinocyte growth factor (KGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), etc[18-19].

The studies on experimental animals revealed that in the anagen phase such growth factors as IGF-1, HGF, FGF-7, VEGF get activated in the dermal papilla cells, affecting the follicular keratinocytes and melanocytes by paracrine mechanism. In the transition from the anagen phase to the catagen phase, FGF-5, TGF-â1, and EGF get activated in follicular keratinocytes. These growth factors affect the hair follicle in an autocrine or paracrine manner.

However, as of today, the numerous studies have purely experimental nature, and there are still unsolved problems concerning the role of growth factors in the pathogenesis of various types of alopecia and their influence on the severity of the pathological process.

The purpose of this study was to investigate the role of VEGF, KGF, EGF, and TGF-â1 growth factors in the development of telogen effluvium in women.

MATERIALS AND METHODS

The study involved 30 women diagnosed with telogen effluvium, their average age being 37.1 ± 9.29. The control group was formed by 8 healthy women in the average age of 38.5 ± 7.4.

The criteria for inclusion in the study listed the informed consent of patients to participate in the study, the age between 18 and 60, and the confirmed diagnosis of telogen effluvium.

The exclusion criteria included the thyroid gland disease, skin diseases in the acute stage, infectious diseases, hyperandrogenism, concurrent somatic pathologies, and other types of alopecia.

Clinical examination of patients with hair loss

The patients were examined after signing the informed consent with the requirements of Good Clinical Practice (GCP), and the ethical principles stated in the Helsinki declaration with amendments of 2008.

The clinical study was conducted in accordance with the specially developed individual cards of patients, which listed the data on age, sex, anamnesis of the disease, specific characteristics of hair loss, hereditable predisposition, earlier and concomitant diseases, precipitating factors, earlier treatment and its effectiveness, etc.

The clinical research of the scalp included the assessment of visible thinning, thinning of the hair shaft, intensity of sebaceous excretions, and the presence of pathological rash on the scalp.

Assessment of the hair and scalp condition

The assessment of density and the diameter of hair in the androgen dependent and androgen independent zones of the scalp was carried out in all patients, followed by calculations of the percentage of vellus and terminal hair. The percentage of hair in follicular units within the studied zones and its distribution was calculated and assessed. Likewise, calculations were performed with respect to the ratio of anagen and telogen hair, and the ratio of vellus hair among the anagen and telogen hair. Automatic calculation of the total amount of hair on a patient's head was performed as well as the calculation of the hair growth rate, which was implemented with use of the special Aramo SG micro camera (manufactured by Aram HUVIS Co., Ltd., Republic of Korea) and the two lenses (magnification 60x was used to study the external skin surface and the hairs, and

magnification 200x, to investigate the state of the hair follicles and the scalp). The camera was used in conjunction with the specialized computer diagnostic program "Program for professional diagnosis in Trichology/Trichoscience v. 1.7 (RUS)" (Russia), a fixed area being marked with indelible Tribal black ink (Manufactured by Startbrite Color, USA).

Conditions for obtaining biological material

Biopsy scalp samples served as research materials in the course of the study. The skin samples were taken from women with telogen effluvium by punch biopsy with the use of EPITHEASY, a disposable device for skin biopsy, diameter 4 mm (Manufactured by Medax, Italy), under local anesthesia, the voluntary consent of the patient necessarily acquired. For local anesthesia, lidocaine (manufactured by Egis Pharmaceuticals PLC, Hungary) was administered subcutaneously around the site of biopsy. The biopsy samples were taken from the parietal area.

As control, 8 scalp samples were obtained from patients with no reports of hair loss or clinical signs of alopecia, during surgery or routine operations on nonmalignant lesions, the voluntary consent of the patients necessarily acquired (8 women, aged 21 to 52 years).

Immunohistochemical research of growth factors expression in hair follicle

The expression of VEGF, KGF, EGF, and TGF-â1 growth factors was examined in scalp samples of 30 patients with telogen effluvium and 8 healthy individuals. Immunohistochemical research was conducted by immunofluorescent method in line with the standard procedure: after deparaffination and rehydratation in a series of

liquids (for 3-10 min.) in the sequence: xylene I, xylene II, 100% ethanol, 96% ethanol I, 96% ethanol II, 75% ethanol, the glasses were washed with distilled water. The procedure of antigen retrieval was performed: the container with glasses was incubated in citrate buffer (PH 6.08-6.10) for 20 minutes at high pressure and the temperature of 95-98° C. After cooling, the glasses were washed with Wash buffer (Dako). Blocking of nonspecific binding with Protein Block (Abcam) was performed. After washing in Wash buffer (Dako), incubation was carried out with the primary specific antibodies. The glasses were transferred to a moist chamber and incubated (see Table 1).

After washing with Wash buffer (Dako), glasses were incubated with secondary antibodies conjugated with fluorochrome Alexa 488 (1:1000, Abcam) and Alexa 647 (1:1000, Abam) for 30 minutes, at room temperature, in the dark. Washed with Wash-buffer (Dako), nucleuses were counterstained with Hoechst 33258 (Sigma, USA). The preparations were placed under cover glasses into the Dako Fluorescent Mounting Medium (Dako, USA).

To assess the results of immunofluorescent staining, morphometric research was conducted using the system of computer analysis of microscopic images, which involved an Olympus FlueView1000 confocal microscope, a personal computer on the basis of IntelPentium 4, and Videotest Morphology 5.0 (Russia) software. In each case, 5 fields of vision were analyzed at 200x magnification.

Measurements of optical density, the average brightness and the area of expression were conducted. The area of expression was calculated

Table 1: Antibodies used in the immunofluorescence method

Antibodies	Manufacturer	Dilution	Incubation conditions
Anti-VEGF antibody (mouse)	Abcam (UK)	1:50	30 min at room temperature
Anti-TGF beta 1 antibody (rabbit)	Novus Biologicals (UK)	1:100	1 hour at room temperature
Anti-KGF antibody (mouse)	Abcam (UK)	1:200	1 hour at room temperature
Anti-EGF antibody (mouse)	Abcam (UK)	1:100	30 min at room temperature

as the ratio of the area occupied by immunopositive cells to the total area of cells in the field of vision expressed as a percentage.

Statistical calculations were performed using the statistical programming language R version v3.2.0. The distribution of patients according to the values of indicators was carried out by means of the Shapiro-Wilk test (W). Descriptive statistics were calculated according to generally accepted methods. For quantitative characteristics, if the distribution was recognized as normal, average and standard deviation was calculated. If the distribution was different from the normal, the median and the 25% -75% quartiles were calculated. For qualitative characteristics, mode and the 25% -75% quartiles were calculated. For comparison of patients in terms of indicators between groups, Mann-Whitney's test was used. Analysis of correlations in pairs of indicators was performed using the Spearman criterion. Analysis of the effect of the growth factors on the development of alopecia was performed using unifactor and multifactorial linear regression;

with $p < 0.05$, the null hypothesis of no linear relationship between the growth factors and the fact of alopecia development was rejected. Analysis of the power of influence of the growth factors on alopecia development was performed using univariate and multivariate dispersion analysis of the previously obtained linear models. The strength of the effect of the growth factors on the development of alopecia was determined by the value of the partial eta-squared ($ç^2$), showing the percentage of the dispersion of the dependent variable explained by the independent variable.

RESULTS

The study involved 30 women with diagnosis of telogen effluvium, in the average age of 37.1 ± 9.29, the average age of the disease onset being 26 (22; 33; 25), and the average disease duration being 5 (0,75; 16) years.

The anamnestic data revealed that the women with telogen effluvium associated the onset

Table 2: Indicators of the relative area of growth factor expression in scalp of women with telogen effluvium and healthy individuals

Growth factor Research groups	VEGF (relative area of expression, %)	KGF (relative area of expression, %)	EGF (relative area of expression, %)	TGF-β1 (relative area of expression, %)
Women with telogen effluvium (n=15)	25.79 ± 1.81*	35.83 ± 0.7*	19.17 (18.93; 19.79)	63.14 (60.84; 64.7)*
Healthy women (n=8)	68.53 ± 1.08	47.68 ± 0.93	19.96 ± 3	51.72 ± 2.21

*- statistically significant differences between patients with the TE condition and healthy individuals; at $p < 0.05$;

Table 3: Strength of impact of independent variables (growth factors) on the probability of development of telogen effluvium in women

Indicator	η^2	Sum of Squares (SS)	Fisher test (F)	p
VEGF	0.836328	93.05723	209.5008	0.000
KGF	0.197145	4.471948	10.06775	0.003
EGF	0.130318	2.728923	6.143657	0.017
TGF-β1	0.255143	6.238207	14.04415	0.001

of their disease with childbirth (n=6; 20%) and stress (n=12; 40%), while one patient (n=1; 3.33%) attributed it to her diet violation. 11 women (36.67%) could not associate the onset of their disease with a specific precipitating factor. From the anamnesis, it was found that 11 (30%) women with telogen effluvium had a burdened heredity: 10 patients (33.33%), by paternal line and 4 (13.33%), by maternal line including 3 patients with a hereditary burden in both paternal and maternal line, the burdening factor present in the first line of parentage in all patients.

Chronic-relapsing nature of hair loss (n=18, 60%) prevailed in patients, the condition reported as not seasonal in 17 (56.67%) women.

Most patients practiced self-treatment using hair care remedies for external care (13; 43.33%) and ingesting drugs (10; 33.33%) . Various cosmetic lotions, shampoos and popular remedies were used externally while complex vitamins and mineral agents were ingested. 7 (23.33%) women with telogen effluvium received a cycle of mesotherapy. 4 (21%) patients had presented with a temporary effect of the therapy they had received while 11 (57.89%) women reported absence of positive dynamics resulting from treatment.

Among the patients involved in the study, 11 (36.67%) women had not received any treatment.

Data of trichoscopy and phototrichogram

Trichoscopy and phototrichogram revealed that the patients in the study presented with an abnormal percentage ratio of anagen to telogen hair, with the increased proportion of telogen hair (37.02 ± 11.26 in the parietal region, 24.49 ± 6.52 in the occipital region). At the same time, in the occipital and parietal regions, the total number of hair per square centimeter (242.4 (231.9; 289.6) and 242.75 ± 34.81, accordingly), as well as the percentage ratio of terminal and vellus hair (12 (6; 16) in the parietal region, 12.6 ± 7.31 in the occipital region) proved to correspond to the norm. Also, in healthy volunteers the results of trichoscopy and phototrichogram were within the normal parameters.

Expression of growth factors (VEGF, KGF, EGF, TGF-â1) in scalp specimens

The analysis of the received results revealed that in women with telogen effluvium, indicators of the relative area of VEGF expression (25.79 ± 1.81) and KGF expression (35.83 ± 0.7) proved to be significantly lower than those in healthy women. The level of TGF-â1 expression tended to increase in comparison with that of healthy people reaching 63.14 (60.84; 64.7) (p<0.05). At the same time, the baseline EGF (19.17 (18.93; 19.79)) in women with telogen effluvium did not differ from that of healthy women (p = 0.830) (Table 2).

The strength of the impact of growth factors (VEGF, KGF, EGF, TGF-â1) in scalp on the development of telogen effluvium

The growth factors that influence the development of telogen effluvium were detected by unifactor linear regression. For each statistically significant case, a linear model was constructed. To determine the strength of the effect of the growth factors on the development of telogen effluvium (partial eta-squared ($ç^2$), the linear models were evaluated by single-factor analysis of variance

The analysis of the influence of the VEGF, KGF, EGF, and TGF-â1 growth factors on the development of telogen effluvium in women with the help of unifactor linear regression showed that each of the growth factors (VEGF, KGF, EGF, TGF-â1) had statistically important influence on the development of telogen effluvium. Particularly, a decrease in the values of VEGF ($ç^2$ = 99.3%), KGF ($ç^2$ = 97.2%), and EGF ($ç^2$ = 22.8%), and the rise of TGF-â1 ($ç^2$ = 84.5%) proved to increase the possibility of development of telogen effluvium in women (ð>0.05).

A model taking account of the simultaneous influence of the listed growth factors (VEGF, KGF, EGF, TGF-â1) on the probability of development of telogen effluvium in women was received with the use of multifactorial linear regression. When analyzing the multifactorial model, it was established that all the indicators appear to be predictors of the disease. The growth factor VEGF ($ç^2$ = 0.836328, ð<0.05) affects the development of telogen effluvium in women in a

greater degree compared to the impact of TGF-â1 ($ç^2$ = 0.255143, ð<0.05) KGF ($ç^2$ = 0.197145, ð<0.05) and EGF ($ç^2$ = 0.130318, ð<0.05), which is smaller (summary in Table 3).

DISCUSSION

According to the data obtained from the research, patients with telogen effluvium present with chronic and recurrent course of the disease. Telogen effluvium prevails in young women, the most common causes of hair loss being psychological stress and childbirth. The previously conducted complex treatment of the patients in the study did not result in a significant therapeutic effect, which once again speaks for the importance of search of new highly effective remedies that would inhibit the catagen phase or induce the anagen phase.

Trichoscopy and phototrichogram revealed an altered ratio of anagen to telogen hair, the proportion of hair in the telogen phase increased in the parietal and occipital areas. The total number of hair per square centimeter, and the ratio of terminal to vellus hair in the parietal and occipital areas proved to correspond to the norm.

The immunofluorescence analysis revealed a change in the expression of the growth factors in women with telogen effluvium in comparison with that of healthy individuals: a decrease in the expression of VEGF and KGF factors, and an increase in the value of TGF-â1 proved to be statistically significant. The difference in the indicators of the EGF expression level is not statistically reliable.

The analysis of variance showed that the development of telogen effluvium is influenced by all the studied growth factors (VEGF, KGF, EGF, TGF-â1). An decrease in the value of VEGF, KGF, and EGF, and an increase in TGF-â1 rises the probability of development of telogen effluvium. When analyzing the multivariate model, it was established that VEGF exerts the greatest influence on the development of this type of alopecia, while the impact of EGF is the least.

Thus, the data obtained in our study are consistent with the results obtained by many authors who conducted their studies on an animal models and assumed an important role of EGF and KGF in the development and differentiation of a hair follicle and hair growth[20-23].

In vivo studies revealed that VEGF has a great importance in the development and the life of hair. It promotes growth, determines the differentiation, the structure and the duration of growth of the hair follicle and the hair shaft[19, 24]. VEGF has a huge impact on vascularization and angiogenesis, thus stimulating hair growth[25-27]. The conducted research also shows the importance of the decrease in the level of the VEGF expression in the hair follicle for the development of telogen effluvium.

The study of the mechanisms of apoptosis in the hair follicle, identified the important role of TGF-â factor[28-32]. Back in 1994, M.P. Philpott and coauthors indicated that TGF-â is a negative regulator of a hair follicle growth[33]. An increased level of expression of TGF-â1 in scalp of patients with telogen effluvium confirms the role of this factor in the pathogenesis of alopecia as a factor involved in suppression of the anagen phase and maintenance of the catagen phase.

CONCLUSION

Our results showed that all the studied growth factors (VEGF, KGF, EGF, TGF-â1) contribute to the development of telogen effluvium, the biggest impact exerted by VEGF, and the smallest by EGF. It should be noted that all the growth factors are closely interrelated and affect each other in both regulating and controlling manner. Further research in this area should be aimed at finding other regulatory substances and mechanisms of initiation of catagen and telogen, and inhibition of anagen in the hair follicle.

ACKNOWLEDGEMENTS

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