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Transforming Growth Factor β1 (TGFβ1) and Vascular Endothelial Growth Factor (VEGF) in the blood of healthy people and patients with Graves' orbitopathy — a new mechanism of glucocorticoids action?

Transformujący czynnik wzrostu β1 (TGFβ1) i naczyniowo-śródbłonkowy czynnik wzrostu (VEGF) we krwi ludzi zdrowych i chorych z orbitopatią Gravesa — nowy mechanizm działania glikokortykosteroidów?

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Abstract

Introduction: The first part of this paper is related to healthy people and presents concentrations of TGFβ1 and VEGF in blood (with and without dividing data with respect to sex), their single measurement values (at 8am), Mean Daily Concentrations (MDC), Area Under the Curves (AUC; total daily secretion), and circadian rhythm. The second part of the work is related to Graves' orbitopathy (GO). The aim of the study were: 1) to determine the physiological pattern of TGFβ1 and VEGF secretion; 2) to compare the serum TGFβ1 and VEGF circadian profile in newly diagnosed thyreotoxic patients with active GO and healthy controls (H); and 3) to estimate the influence of high-dose intravenous methylprednisolone pulse therapy (MP) on TGFβ1 and VEGF blood levels in GO.

Material and methods: Twenty-two healthy (H) subjects and 16 hyperthyroid GO patients were treated with MP (6 g/14 days) and followed up by ophthalmological assessment. Blood was collected before and after 2 weeks MP-therapy. TGFβ1 and VEGF levels were determined by the ELISA method.

Results: No difference was observed in the concentrations of TGFβ1 and VEGF in the blood of healthy women and men — in further analysis, a combined healthy male and female cohort was used (H). While the absence of circadian rhythms in the concentrations of TGFβ1 and VEGF allows the application of a single measurement approach, MDC and AUC measurements were found to be more precise. There were no differences in TGFβ1 MDC/AUC between GO and H. VEGF MDC/AUC in GO were higher than in H. MP-therapy increased TGFβ1 MDC/AUC, thus in GO after MP, the TGFβ1 MDC/AUC were higher than in H. There were no differences in VEGF MDC/AUC during MP-therapy. MP-therapy was effective in 15/16 patients.

Conclusions:

1. MP-therapy increases MDC and AUC of TGFβ1. The effectiveness of MP-therapy in patients with active GO may be related to its influence on TGFβ1 concentrations in blood. The results suggest the existence of a new mechanism of glucocorticoids action, consisting of an increase in the secretion of TGFβ1.
2. The elevated serum VEGF in thyreotoxic patients with active GO may reflect long-standing autoimmune processes in orbital and thyroid tissues and intensified angiogenesis in the thyroid gland. **(Endokrynol Pol 2014; 65 (5): 348–356)**

Key words: *TGFbeta1; VEGF; transforming growth factor beta 1; vascular-endothelial growth factor; healthy; thyroid; Graves; orbitopathy; fibrosis; angiogenesis; methylprednisolone; glucocorticoids*

Streszczenie

Wstęp: Pierwsza część pracy dotyczy ludzi zdrowych — dostarcza dane o stężeniach TGFβ1 i VEGF we krwi (z podziałem i bez podziału na płeć), ich wartości punktowe (godz. 8), średniodobowe stężenie (MDC), pole pod krzywą (AUC; całkowite dobowe wydzielanie), rytm okołodobowy. Druga część pracy dotyczy orbitopatii Gravesa (GO). Celem pracy było: 1) określenie fizjologicznego wzoru wydzielania TGFβ1 i VEGF; 2) porównanie okołodobowych surowiczych stężeń TGFβ1 i VEGF u chorych z nowo rozpoznaną nadczynnością tarczycy i aktywną fazą GO oraz u ludzi zdrowych (H); 3) ocena wpływu wysokodawkowej dożylnej pulsacyjnej terapii metyloprednizolonem (MP) na stężenia TGFβ1 i VEGF we krwi chorych z GO.

Materiał i metody: Przebadano 22 zdrowych (H); 16 pacjentów z nadczynnością tarczycy i GO leczonych MP (6 g/14 dni) obserwowanych okulistycznie. Krew pobierano przed i po 2 tygodniach terapii MP. Stężenia TGFβ1 i VEGF oznaczono metodą ELISA.

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Wyniki: Nie stwierdzono różnic w stężeniu TGFβ1 i VEGF we krwi między zdrowymi kobietami i zdrowymi mężczyznami — dalszym analizom poddawano więc grupę zdrowych (H) składającą się z kobiet i mężczyzn. Krew pobierano przed i po 2 tygodniach terapii MP. Choć wykazanie braku rytmów okołodobowych stężeń TGFβ1 i VEGF we krwi umożliwia zastosowanie ich oceny punktowej to jednak stwierdzono, że bardziej precyzyjną jest ocena MDC i AUC. Nie wykazano różnicy w TGFβ1 MDC/AUC między GO i H. VEGF MDC/AUC były wyższe u GO niż u H. MP zwiększył TGFβ1 MDC/AUC, tym samym u GO po MP, TGFβ1 MDC/AUC były też wyższe niż u H. Nie stwierdzono różnicy w VEGF MDC/AUC podczas MP. MP był skuteczny u 15/16 chorych.

Wnioski:

1. Terapia MP zwiększa MDC i AUC TGFβ1. Skuteczność MP u chorych z aktywną fazą GO może być związana z jego wpływem na stężenia TGFβ1 we krwi, co sugeruje istnienie nieznanego dotąd mechanizmu działania glikokortykosteroidów polegającego na zwiększaniu wydzielania TGFβ1.
2. Podwyższone stężenia VEGF we krwi u chorych z nadczynnością tarczycy i aktywną fazą GO mogą być odzwierciedleniem długotrwałego procesu autoimmunologicznego w tkankach oczodołu i tarczycy oraz nasilonej angiogenezy w gruczole tarczowym.
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Słowa kluczowe: *TGFbeta1; VEGF; transformujący czynnik wzrostu beta 1; naczyniowo-śródbłonkowy czynnik wzrostu; zdrowi; tarczyca; Graves; orbitopatia; włóknienie; angiogeneza; metyloprednizolon; glikokortykosteroidy*

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Introduction

TGFβ1 is a multifunctional cytokine. Organ and tissue distinctiveness hinder the unambiguous characterisation of the cytokine. However, there are constant functions of TGFβ1 inducing no controversy: it participates in the control of cell growth and differentiation, induces fibrosis and scar formation (the process of 'wound healing'), causes the suppression of immune response, and is involved in angiogenesis, the development of tumours, and inflammatory processes. Thus, there are three fundamental directions of its activities: I. TGFβ1 regulates cell proliferation, growth, differentiation and cells movement; II. It has immunomodulatory effects; III. It has profibrogenic effects [1–5]. TGFβ1 is involved in the suppression of the immune response. It inhibits the proliferation, differentiation and activity of cells involved in humoral and cellular responses, reduces the expression of MHC molecules, the cellular toxicity, the production of antibodies, and inhibits the secretion of cytokines [2, 3, 5–7]. On the surface of B cells are located receptors for TGFβ1 — their stimulation leads to the inhibition of B cell proliferation [8–10]. TGFβ1 inhibits T cell proliferation [10] in response to polyclonal mitogens [11]. On the other hand, in the inflamed tissues, an increase of activation of T helper cells CD4+ which produce TGFβ1 is seen [9, 12]. This cytokine inhibits macrophages' maturation and activity [6, 10, 11]. The immunosuppressive and anti-inflammatory effects of TGFβ1 [3] have been confirmed *in vitro* [1, 6, 13] and *in vivo* [1, 13]. Mouse model studies have demonstrated that TGFβ1 is involved in the pathogenesis of autoimmune diseases — systemic administration of TGFβ1 suppresses autoimmune disease while the anti-TGFβ1 antibodies cause its progression. It has been demonstrated that mutations in the TGFβ1 gene result in the development of a phenotype with characteristics typical of autoimmune diseases [5, 14]. Furthermore, the relationship between TGFβ1 and fibrosis of various tissues and organs is increasingly being described. Although TGFβ1 plays a critical role in tissue repair, overproduction of the cytokine can lead to an excessive, uncontrolled deposition of fibrous tissue [5, 15].

Angiogenesis is an important component of many physiological and pathological processes [16–27]. Disease processes which have been shown to increase vascularity include, in particular, endocrine glands illness [24, 25], acromegaly [19], pituitary adenomas [26, 27], autoimmune thyroid disease, goitre, neoplasms [18, 22] and eye disorders — retinopathy occurring in premature infants [18, 28], diabetic retinopathy [18, 20, 21, 29–31], uveitis and other ocular illnesses. Angiogenesis commonly occurs in inflammatory diseases [18–20, 22, 24–27]. Activators of angiogenesis include VEGF (induces vascular permeability, endothelial cells proliferation, migration and adhesion of leukocytes) and TGFβ1 (promotes maturation of blood vessels, deposition of extracellular matrix components, induces endothelial cells proliferation, differentiation of mesenchymal cells to pericytes) [5, 24, 25]. VEGF is produced by the endothelial cells, fibroblasts and the majority of inflammatory cells (macrophages, lymphocytes, neutrophils and eosinophils) [24, 32]. Starting the process of angiogenesis is usually dependent on several factors. These include the immune/inflammatory response (e.g. immune/tissue infiltrating inflammatory cells) [18, 24] and hormonal changes (e.g. increased levels of IGF-I, TSH stimuli) and VEGF, which plays a role in all the situations in which angiogenesis occurs [24, 25, 33] Among the mechanical factors that trigger angiogenesis, an important role is played by increased blood flow in the capillary vascular (e.g. hyperthyroidism) [24].

The first part of this paper is related to healthy people — it presents concentrations of TGFβ1 and VEGF in

blood (with and without dividing the data with respect to sex), their single measurement values (at 8 am), Mean Daily Concentrations (MDC), Area Under the Curves (AUC; total daily secretion), and circadian rhythm.

The second part of this work is related to Graves' orbitopathy (GO). There are two clinical phases of GO: active (oedematous-infiltrative orbitopathy) and non active (fibrosis). GO is thought to be an inflammatory disorder of autoimmune background. The thyroid-stimulating hormone (TSH) receptor (TSHR) is the autoantigen responsible for the hyperthyroidism of Graves' disease. Its role in the development of GO is unclear [34–38]. Expression of TSHR mRNA is augmented in orbital tissues from GO patients, and in newly differentiated adipocytes derived from precursor cells within the orbit. TGFβ1 inhibits TSHR expression and adipogenesis by orbital fibroblasts *in vitro*, effects that would seem to favour disease remission [34, 39]. TGFβ mRNA levels have also been found to be present at higher levels in orbital tissue from GO patients compared to controls [40]. VEGF is produced by thyroid follicles in response to chronic activation of TSHR. VEGF stimulates its receptors on thyroid endothelial cells, leading to hypervascularity of the thyroid gland [24, 25]. It is possible that glucocorticoids modulate TGFβ1 and VEGF production.

The objective of this study was to evaluate the role of selected growth factors related to the processes of fibrosis, immunosuppression and angiogenesis in the GO pathogenesis. The aims of the study were: 1) to determine the physiological pattern of TGFβ1 and VEGF secretion (assessment of their concentrations in the blood of healthy people with and without stratification with respect to sex, circadian rhythm, mean daily concentrations, and total daily secretion); 2) to compare the serum TGFβ1 and VEGF circadian profile in newly diagnosed thyreotoxic patients with active GO and healthy controls; and 3) to estimate the influence of high-dose intravenous methylprednisolone pulse therapy on TGFβ1 and VEGF blood levels in patients with active GO and to assess their potential as a guide to immunosuppresive therapy.

Material and methods

The fundamental part of this study was conducted in 38 individuals: 16 newly diagnosed thyreotoxic patients with active Graves' orbitopathy (oedematous-infiltrative phase of the disease) (group GO) (12 women, 4 men; mean age 37 ± 9 years) were treated with high-dose intravenous methylprednisolone pulse therapy (MP) (6 g/14 days — 1 g per day repeated within two weeks) (groups: GO before MP *vs.* GO after MP) and followed up by an ophthalmological assessment (two and four weeks after the beginning of MP therapy); the control group (group H) consisted of 22 healthy volunteers age- and sex-matched to group GO. The research was approved by the Ethical Committee of the Medical University of Silesia, and was carried out in 2002–2007.

In all those examined, the current endocrinological status was defined on the basis of thyroid gland ultrasound, blood levels of TSH, fT4 (eventually +fT3), thyrotropin receptor antibodies (TRAb), and anti-thyroid peroxidase antibodies (TPOAb). An ophthalmological assessment relating to the status of cornea, extraocular muscles, eyelids, proptosis and optic nerve function was carried out during the study. This provided a measurement of visual acuity, intraocular pressure, proptosis, fundoscopy, the assessment of ocular motility, visual fields, colour vision, lagophthalmos and corneal changes. To evaluate eye disease, a clinical activity score (CAS) and an ophthalmopathy index (OI) were used. The active phase of GO was confirmed before MP treatment with magnetic resonance imaging (MRI) of the orbits.

In GO and H groups, blood was collected at four points during the day (at 7 am, 1 pm, 7 pm, and 1 am) to determine the presence of a possible circadian rhythm of TGFβ1 and VEGF, the calculation of their Mean Daily Concentrations (MDC) in blood and total daily secretion (Area Under the Curves; AUC) to blood. In a similar manner, blood was collected two weeks after MP therapy. Whole blood and serum and plasma derived from its parts were stored at –80°C until testing. The creation of a control group of healthy people (H) composed of men and women (for estimation of MDC/AUC) was preceded by a comparative analysis of the concentrations of TGFβ1 and VEGF in the blood of women (n = 31) and the concentrations of TGFβ1 and VEGF in the blood of men (n = 28). For the purposes of this analysis, blood was taken once at 8 am.

Serum levels of TGFβ1 and VEGF were measured by an enzyme-linked immunosorbent assay (ELISA) method using Quantikine Immunoassay, (R&D Systems, USA) kits. Tests were performed in the Department of Pathophysiology and Endocrinology with a Universal Microplate Spectrophotometer – μ QUANT (BIOTEK INC). The sensitivity of the method was: for TGFβ1 — < 7 pg/mL, and for VEGF — < 5 pg/mL. Intra-assay error and inter-assay error were respectively: for TGFβ1 — 4.9% to 10.3%, and for VEGF — 5.1% to 6.2%. TRAb and TPOAb were estimated, respectively, by a radioimmune assay (RIA) method and the ELISA method. Serum levels of TSH and free thyroid hormones were assayed immunoenzymatically (MEIA), by routine laboratory techniques.

For each quantitive parameter, basic statistical characteristics were made — mean, SD (standard deviation), SEM (standard error of the mean), minimal and maximal

Table Ia. *Comparison of concentrations of TGFβ1 and VEGF in blood of healthy women and healthy men (single measurement at 8 am)*

Tabela Ia. *Porównanie stężeń TGFβ1 i VEGF we krwi zdrowych kobiet i zdrowych mężczyzn (oznaczenie punktowe o godz. 8)*

	♀ (n = 31)	♂ (n = 28)	
	Mean ± SD	**Mean ± SD**	**p**
TGFβ1 [ng/mL]	37.89 ± 10.48	41.98 ± 12.16	0.171
VEGF [ng/mL]	298.65 ± 179.85	214.24 ± 210.56	0.1023

value, median, quartiles 25% and 75%, 95% confidence interval. In the statistical analysis, $p < 0.05$ was regarded as statistically significant. Shapiro-Wilk test performed for evaluation of normal distribution of parameter. Homogeneity of variance was checked by Fischer test. Student's *t*-test was used for comparison of unpaired groups of parameters that follow normal distribution. Pre-treatment and post-treatment data within the same group were compared with Student's paired t-test. For the assessment of intergroup correlations, a Spearman Rank Correlation test was used. Chronobiological parameter estimation was performed using the cosinor method [Model: M+A*cos((Pi/12)*T+fi)].

Results

The first part of this paper is related to healthy people. Table I (a, b, c) shows the concentrations of TGFβ1 and VEGF in the blood of healthy people. These Tables (Ia, Ib, Ic) illustrate the way how we finally formed a control group of healthy people (H) who took part in the second, fundamental, part of the experiment concerning Graves' orbitopathy. No difference was observed in the concentrations of TGFβ1 and VEGF in the blood of healthy women and healthy men (single measurement at 8 am) (Table Ia) (Table Ib presents the same material without stratification with respect to sex). In view of this, it was concluded that in further analysis we could use a combined male and female cohort. Despite the fact that the absence of circadian rhythms in the concentrations of TGFβ1 and VEGF (Table III) allowed for an application of a single measurement approach, we found it was more precise to evaluate the Mean Daily Concentrations (MDC) and Area Under the Curves (AUC) of the TGFβ1 and VEGF. So we finally formed a control group of healthy people (H) (Table Ic), of whom the MDC and AUC of TGFβ1 and VEGF were then compared to the study group (GO) (Table II, IV, V, VI).

There were no significant (NS) differences in mean daily serum concentrations (MDC) and area under the curve (AUC) of TGFβ1 between patients with GO and healthy controls (H). In GO patients, the MDC of VEGF was significantly higher than in healthy people (H) and the AUC of VEGF was also significantly higher than in H (Table IV).

High-dose intravenous methylprednisolone pulse therapy (MP) significantly increased MDC and AUC of TGFβ1 ($TGF\beta1_{MDC}$ and $TGF\beta1_{AUC}$ increased during MP therapy in 15/16 patients). In this way, in patients

Table Ib. *Concentrations of TGFβ1 and VEGF in blood of healthy people (single measurement at 8 am)*

Tabela Ib. *Stężenia TGFβ1 i VEGF we krwi ludzi zdrowych (oznaczenie punktowe o godz. 8)*

Healthy ♀♂ (n = 59)	**Mean**	**SD**	**SEM**	Confidence interval –95%	Confidence interval +95%	Median	Minimum	Maximum	Lower quartile	Upper quartile
TGFβ1 [ng/mL]	39.83	11.4	1.48	36.86	42.8	37.68	16.24	67.1	33.47	44.92
VEGF [ng/mL]	258.59	197.93	25.77	207.01	310.17	172.32	37.38	987.87	116.3	371.31

Table Ic. *Mean Daily Concentrations (MDC) and Area Under the Curves (AUC; total daily secretion) of TGFβ1 and VEGF in blood of healthy people (group H)*

Tabela Ic. *Średnie dobowych stężeń (MDC) i całkowitego dobowego wydzielania (pól pod krzywą; AUC) dla TGFβ1 i VEGF we krwi ludzi zdrowych (grupa H)*

Healthy (gr. H) ♀♂ (n = 22)	**Mean**	**SD**	**SEM**	Confidence interval –95%	Confidence interval +95%	Median	Minimum	Maximum	Lower quartile	Upper quartile
$TGF\beta1_{MDC}$ [ng/mL]	34.57	6.66	1.42	31.61	37.52	32.98	25.21	46.34	29.13	42.14
$TGF\beta1_{AUC}$	412.05	81.97	17.48	375.71	448.4	391.54	299.02	551.64	358.58	509.08
$VEGF_{MDC}$ [ng/mL]	236.11	106.68	22.74	188.81	283.41	224.44	61.78	415.11	150.81	326.87
$VEGF_{AUC}$	2,824.76	1,292.3	275.52	2,251.78	3,397.73	2,643.68	725.06	4,904.72	1,821.82	3,922.92

Table II. *Mean Daily Concentrations (MDC) and Area Under the Curves (AUC; total daily secretion) of TGFβ1 and VEGF in blood of patients with active Graves' Orbitopathy (group GO)*

Tabela II. *Średnie dobowych stężeń (MDC) i całkowitego dobowego wydzielania (pól pod krzywą; AUC) dla TGFβ1 i VEGF we krwi chorych z czynną orbitopatią Gravesa (grupa GO)*

Graves' Orbitopathy (gr. GO) (n = 16)	**Mean**	**SD**	**SEM**	Confidence interval –95%	Confidence interval +95%	Median	Minimum	Maximum	Lower quartile	Upper quartile
$TGF\beta1_{MDC}$ [ng/mL]	39.15	9.6	2.4	34.04	44.27	38.69	22.84	59.33	33.35	47.3
$TGF\beta1_{AUC}$	471.12	114.89	28.72	409.9	532.34	465.65	277.62	708.5	401.7	568.8
$VEGF_{MDC}$ [ng/mL]	463.12	330.45	82.61	287.03	639.21	356.9	99.71	1,366.02	270.4	572.7
$VEGF_{AUC}$	5,603.44	3,943.28	985.82	3,502.22	7,704.66	4,350.87	1,297.76	16,322.4	3,331.64	6,909.85

Table III. *Assessment of circadian rhythm of TGFβ1 and VEGF concentrations in blood of healthy people (group H) and patients with active Graves' Orbitopathy (group GO)*

Tabela III. *Ocena rytmu dobowego stężeń TGFβ1 i VEGF we krwi u ludzi zdrowych (grupa H) oraz pacjentów z czynną orbitopatią Gravesa (grupa GO)*

Circadian rhythm (cosinor method)	**Healthy (gr. H)**	**Graves' Orbitopathy (gr. GO)**
TGFβ1 [ng/mL]	No rhythm	No rhythm
VEGF [ng/mL]	No rhythm	No rhythm

Table IV. *Mean Daily Concentrations (MDC) and Area Under the Curves (AUC; total daily secretion) of TGFβ1 and VEGF in blood of patients with active Graves' Orbitopathy (GO) and in healthy controls (H)*

Tabela IV. *Porównanie średnich dobowych stężeń (MDC) i całkowitego dobowego wydzielania (pól pod krzywą; AUC) dla TGFβ1 i VEGF we krwi chorych z aktywną oftalmopatią Gravesa (GO) i w grupie kontrolnej zdrowych (H)*

Groups	**TGFβ1 [ng/mL]** **MDC** ± SD; ± SEM	**TGFβ1** **AUC** ± SD; ± SEM	**VEGF [ng/mL]** **MDC** ± SD; ± SEM	**VEGF** **AUC** ± SD; ± SEM
GO	39.15 ± 9.6; ± 2.4	471.12 ± 114.89; ± 28.72	463.12 ± 330.45; ± 82.61	5,603.44 ± 3,943.28; ± 985.82
H	34.57 ± 6.66; ± 1.42	412.05 ± 81.97; ± 17.48	236.11 ± 106.68; ± 22.74	2,824.76 ± 1,292.3; ± 275.52
GO *vs.* H	NS	NS	< 0.05	< 0.05

'GO after MP', the $TGF\beta1_{MDC}$ and $TGF\beta1_{AUC}$ were significantly higher than in healthy people (H) (Table V). There were no differences in MDC and AUC of VEGF during MP therapy. Also in GO patients before MP, as in 'GO after MP', the $VEGF_{MDC}$ and $VEGF_{AUC}$ were significantly higher than in healthy people (H) (Table V, VI). The treatment was efficient – all patients showed a significant improvement in the signs and symptoms of orbital inflammation, and most patients showed an improvement in proptosis and diplopia. CAS fell within two weeks of MP therapy in 15 of 16 patients (before treatment: 5.7 ± 1.9 and after: 3.6 ± 0.9) and remained stable during the follow-up period. Post-treatment OI was improved (mean values 3.5 ± 1.3 *vs.* 2.6 ± 1.02). Proptosis was reduced (mean values 23.04 mm ± 1.54 *vs.* 21.64 mm ± 1.6) in 15 of 16 patients. Visual acuity was improved in 15 of 16 patients. Ocular motility was normalised or improved in 15 of 16 patients. The clinical assessment of the effect of the glucocorticoids pulse therapy for Graves' orbitopathy showed a good response in eight patients, a fair response in seven (responders), and no response in one (non-responder). No significant correlation was found between fT4, fT3 and TGFβ1, VEGF blood levels. We also did not find any correlation between measured growth factors and parameters of autoimmune thyroid disease such as TRAb or TPOAb.

Discussion

GO is an autoimmune disease in which CD4+ and CD8+ T cells, B cells, plasma cells and macrophages infiltrate the orbital soft tissue/periorbital space leading to tissue remodelling. Enlargement of orbital tissue mass occurs through the accumulation of extracellular matrix (ECM), scar-forming myofibroblasts, and/or fat

Table V. *Mean Daily Concentrations (MDC) and Area Under the Curves (AUC; total daily secretion) of TGFβ1 in blood of patients with active Graves' Orbitopathy (GO) treated with high-dose intravenous methylprednisolone pulse therapy (MP) (groups: GO before vs. GO after MP) and in healthy people (H)*

Tabela V. *Porównanie średnich dobowych stężeń (MDC) i całkowitego dobowego wydzielania (pól pod krzywą; AUC) dla TGFβ1 we krwi chorych z aktywną orbitopatią Gravesa (GO) poddanych wysoko dawkowej pulsacyjnej dożylnej terapii metyloprednizolonem (MP) (grupy: GO przed/before MP, GO po/after MP) i u zdrowych (H)*

Groups	**TGFβ1 [ng/mL]** **MDC** ± SD; ± SEM	**TGFβ1** **AUC** ± SD; ± SEM
GO before MP	39.15 ± 9.6; ± 2.4	471.12 ± 114.89; ± 28.72
GO after MP	44.79 ± 9.41; ± 2.35	542.35 ± 111.73; ± 27.93
H	34.57 ± 6.66; ± 1.42	412.05 ± 81.97; ± 17.48
GO before MP vs. GO after MP	p = 0.0003	p = 0.0002
GO before MP *vs.* H	p = 0.0904	p = 0.0722
GO after MP *vs.* H	p = 0.0004	p = 0.0002

Table VI. *Mean Daily Concentrations (MDC) and Area Under the Curves (AUC; total daily secretion) of VEGF in blood of patients with active Graves' Orbitopathy (GO) treated with high-dose intravenous methylprednisolone pulse therapy (MP) (groups: GO before MP vs. GO after MP) and in healthy people (H)*

Tabela VI. *Porównanie średnich dobowych stężeń (MDC) i całkowitego dobowego wydzielania (pól pod krzywą; AUC) dla VEGF we krwi u chorych z aktywną orbitopatią Gravesa (GO) poddanych wysoko dawkowej pulsacyjnej dożylnej terapii metyloprednizolonem (MP) (grupy: GO przed/before MP vs. GO po/after MP) i u zdrowych (H)*

Groups	**VEGF [ng/mL]** **MDC** ± SD; ± SEM	**VEGF** **AUC** ± SD; ± SEM
GO before MP	463.12 ± 330.45; ± 82.61	5,603.44 ± 3,943.28; ± 985.82
GO after MP	428.12 ± 284.85; ± 71.21	5,217.06 ± 3,454.85; ± 863.71
H	236.11 ± 106.68; ± 22.74	2,824.76 ± 1,292.3; ± 275.52
GO before MP *vs.* GO after MP	p = 0.1137	p = 0.1247
GO before MP *vs.* H	p = 0.0167	p = 0.0145
GO after MP *vs.* H	p = 0.0193	p = 0.0166

[41–45]. The earliest stages of GO involve the infiltration of the orbital tissue by T cells [41, 42]. T cell-fibroblast interactions are mediated through cytokines, adhesion molecules, and other co-stimulatory molecules [42, 46]. These interactions stimulate fibroblasts to deposit ECM [42, 47, 48] and/or differentiate into myofibroblasts or adipocytes [34, 42, 46, 49]. TGFβ1 is synthesised primarily by platelets, macrophages/monocytes, lymphocytes, fibroblasts, and epithelial cells [50]. TGFβ1 is a potent stimulator of glycosaminoglycans accumulation by retroocular connective tissue. Stimulation of glycosaminoglycans production by the cytokine, released from lymphocytes or macrophages infiltrating the retroocular space, may play a role in the accumulation of glycosaminoglycans in the retroocular and perimysial connective tissues in GO [47, 51]. TGFβ increases hyaluronan (the major ECM glycosaminoglycan) secretion into the culture medium of orbital fibroblasts *in vitro* [40, 42, 51]. TGFβ1 is one of the key mediators of fibrogenesis. An increased TGFβ1 expression has been observed in patients with pulmonary, kidney, and liver fibrosis. In chronic hepatitis, the prolonged stimulation of hepatic stellate cells, being the result of chronic damage to hepatocytes, results in the release of profibrogenic abundant factors such as TGFβ1 and leads to the development of liver cirrhosis [5]. On the other hand, TGFβ1 inhibits TSHR expression and adipogenesis in the orbit that would seem to favour disease remission [34, 39]. Human recombinant TGFβ1 was immunosuppressive in patients with Graves' disease and in controls. In both groups, it inhibited the proliferation of peripheral blood mononuclear cells and of peripheral and thyroid derived T cell lines and clones in response to non-specific stimuli. It also decreased the number of serine esterases expressing cytotoxic T cells and suppressed the recognition of thyroid epithelial cells by thyroid autoantigen specific T cell clones. TGFβ1 may exert a variety of down-regulatory influences in Graves' disease. It may be of importance for the suppression of autoaggression in persons predisposed to autoimmunity; it may be quantitatively overrun by immunostimulatory influences in the acute phase of the disease; and it may be

important for the induction of remission in patients with Graves' disease [52]. TGFβ1's action can be local and systemic. TGFβ1, released locally as a result of injury or the immune response, has pro-inflammatory properties (e.g. it stimulates granulocyte macrophage chemotaxis and the release of pro-inflammatory cytokines (TNF, IL-1, IL-6) [5, 24, 25, 53] and profibrogenic properties [5, 53, 54]. Among the systemic properties of TGFβ1, an immunosuppressive effect seems to be the most important [5, 24, 25, 53]. Serum concentrations of TGFβ1 were higher in patients with Graves' disease compared to controls and decreased after treatment for hyperthyroidism. Positive correlations were observed between thyroid hormones and TGFβ1 in patients with thyroid dysfunction [55].

Endocrine glands are well vascularised and the structure of their vessels (fenestration of epithelium) facilitates the exchange of various substances, including hormones. The amount of VEGF in the thyroid gland is greater in the frequently occurring parenchymal and nodular goitres compared to healthy subjects, although a difference between the pathological tissues is not stated [25, 33, 56]. Increased mRNA and protein expression of VEGF and its receptors detected in nodular tissue of uninodular and recurrent goitre compared to the corresponding normal tissue might be crucial in the proliferation of thyrocytes, and therefore may contribute to the development of goitre and goitre recurrence [56]. Tissues of lymphocytic thyroiditis and differentiated thyroid carcinomas have a stronger expression of VEGF than healthy gland tissues [25, 57, 58]. Increased VEGF expression in malignant thyroid tumours has been shown not only compared to healthy tissue, but also in relation to benign tumours. TSH stimulation of the thyroid cells culture induces their proliferation [25, 33]. Stimulation of human thyrocytes by the TSH and TRAb leads to an increase in their mRNA VEGF expression and *in vivo* to an increased mRNA expression of VEGF, Flt-1 (fms-like tyrosine kinase-1 receptor; VEGFR1) and KDR (Flk-1; foetal liver kinase-1 receptor; VEGFR2) in the endothelial cells of thyroid rats. This points to the participation of VEGF in angiogenesis occurring in the thyroid gland, also in humans with Graves' disease [24, 25, 58–60] in whom the constant stimulation of thyroid tissue by TRAb not only increases the production of thyroid hormones, but also enhances angiogenesis, leading to an increase in thyroid vascularity which has long been widely known [25, 33]. In people with Graves' disease, VEGF mRNA has been found to be localised in the hyperplastic thyroid follicular cells, and mRNA and protein of Flt-1 in the endothelial cells of all thyroid tissues [61]. The highest expression of VEGF and its receptors has been found in tissue obtained from patients with Graves' disease [56]. It has been found that serum VEGF levels are elevated in patients with untreated Graves' disease and Hashimoto's disease, and correlate positively with the degree of thyroid vascularity assessed by colour Doppler ultrasound. VEGF C and Flt-4 have also been observed in human tissues of goitre and autoimmune thyroiditis [25, 33, 58]. There was an increase in serum VEGF levels and a correlation between the levels and intrathyroidal vascular in untreated patients with Graves' disease who had a goitre larger than or equal to 40 cm^3 when compared to healthy subjects. Serum VEGF levels decreased in these patients after treatment [62]. The concentrations of serum VEGF were higher in GO and Graves' disease patients than in controls. The serum levels of VEGF in patients with active GO were higher than those in patients with inactive GO and those in Graves' disease patients. Moreover, serum VEGF concentrations were correlated with CAS in GO patients [38].

Glucocorticoids (e.g. methylprednisolone) modulate TGFβ1 production [63]. Some researchers, evaluating a single measurement of TGFβ1 levels in the blood, have not demonstrated such a relationship [64]. Glucocorticoids and their receptors interact with signal transduction of TGFβ1 at the transcription and translation level [65]. TGFβ1 increases glucocorticoids binding and signalling in macrophages through a Smad2/3-mediated process. This may represent a new target for intervention to increase glucocorticoids responsiveness [66]. Glucocorticoids, including methylprednisolone *in vitro* potentiated TGFβ signalling by the Smad1/5/8 signalling and blunted signalling by the Smad2/3 in primary lung fibroblasts, smooth muscle cells, and endothelial cells. Dexamethasone acted synergistically with TGFβ to drive differentiation of primary lung fibroblasts to myofibroblasts, revealed by the acquisition of smooth muscle actin and smooth muscle myosin, which are exclusively Smad1-dependent processes in fibroblasts [67]. There are also interesting observations concerning the activity of glucocorticoids approved in the treatment of autoimmune diseases. For example, in hepatic stellate cells after glucocorticoids administration, the TGFβ1 receptor III (TGFβRIII) expression increases in a dose dependent manner and time of their administration, while TGFβ1 receptor I and II (TGFβRI and TGFβRII) expression does not change.

It has been found that the degree of induction of TGFβIII expression is dependent on the nature of the stimulating hormone - dexamethasone, hydrocortisone, aldosterone and their doses. Glucocorticoids, through modulation of mRNA TGFβRIII expression, can influence the final effect of TGFβ1. Since TGFβ1 and gluco-

corticoids have immunosuppressive activity, and since after glucocorticoids' administration tissue sensitivity to the TGFβ1 is increased, it can be assumed that they act synergistically. Thus, the beneficial effects of glucocorticoids may be the result of a synergistic mechanism of intensifying immunosuppression.

This observation is related to autoimmune hepatitis [5, 65], but could it be also connected to glucocorticoid therapy of GO? Undoubtedly, in GO, the immunosuppressive effects seem to be clinically important, but TGFβ1 has also a profibrogenic action. Is the simultaneous immunosuppressive and profibrogenic response induced by glucocorticoid therapy beneficial? Does it contribute to the conversion of the active (oedematous-infiltrative orbitopathy) phase to the non active (fibrosis) phase?

Glucocorticoids (e.g. methylprednisolone) modulate also VEGF production. Ye et al. in 2014 showed that VEGF levels in corticosteroid-responsive patients decreased after corticosteroid treatment, and these changes were accompanied by a decrease of CAS. In their opinion, the results could reflect the degree of ocular inflammatory activity [38].

Conclusions

1. Methylprednisolone therapy increases serum TGFβ1 daily concentrations (and total daily secretion). The effectiveness of methyloprednisolone therapy in patients with active Graves' orbitopathy may be related to its influence on TGFβ1 concentrations in blood. The results suggest the existence of a new mechanism of glucocorticoids action, consisting of an increase in the secretion of TGFβ1.
2. The elevated serum VEGF in thyreotoxic patients with active Graves' orbitopathy may reflect long-standing autoimmune processes in orbital and thyroid tissues and intensified angiogenesis in the thyroid gland.

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