Endokrynologia Polska
DOI: 10.5603/EP.a2018.0004
Tom/Volume 69; Numer/Number 1/2018
ISSN 0423–104X

A preliminary study on the expression and clinical value of platelet-derived growth factor BB, hypoxia inducible factor-1α and C-C motif chemokine receptor-2 in peripheral blood during the pathogenesis of Graves' disease

Badanie wstępne ekspresji i wartości klinicznej oznaczenia czynnika wzrostu pochodzenia płytkowego BB, czynnika indukowanego hipoksją-1α i receptora chemokiny C-C typu 2 we krwi obwodowej w patogenezie choroby Gravesa-Basedowa

Ying Liu, Jinglan Tang, Qiaohong Hu, Kefeng Lu, Chunjie Hou

Department of ultrasonography, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China

Abstract

Introduction: Platelet-derived growth factor BB (PDGF-BB) plays an important role in the development of GD (Graves' disease). However, it is still unknown whether PDGF-BB is expressed in peripheral blood and whether the expression of PDGF-BB contributes to GD. We aim to study the expression of PDGF-BB, hypoxia inducible factor (HIF)-1α and C-C motif chemokine receptor (CCR)-2 in peripheral blood of patients with GD and explore its effect and potential mechanism in pathogenesis.

Material and methods: 41 patients with GD (GD group) and forty-five healthy people (control group) were chosen. The concentration of PDGF-BB and HIF-1α in peripheral blood specimens were detected and compared between the two groups. The expression of CCR2 in macrophages in the peripheral blood specimens were examined using FCM (Flow Cytometry).

Results: Both PDGF-BB and HIF-1α were expressed in human peripheral blood from the two groups. Compared with specimens from healthy people, there were statistically increased concentrations of PDGF-BB and HIF-1α in the GD group ($P < 0.05$). The proportion of CCR2-positive macrophages in peripheral blood in the GD group was significantly higher than that in the control group ($P < 0.05$).

Conclusions: CCR2-positive macrophages may induce the expression of PDGF-BB through HIF-1α signal, and the high expression of PDGF-BB may be involved in the pathogenesis of GD. **(Endokrynol Pol 2018; 69 (1): 9–15)**

Key words: *PDGF-BB, Graves' disease, HIF-1α, macrophages, immunology*

Streszczenie

Wprowadzenie: Czynnik wzrostu pochodzenia płytkowego BB (*platelet-derived growth factor BB*, PDGF-BB) odgrywa ważną rolę w rozwoju choroby Gravesa-Basedowa (*Graves' disease*, GD). Jednak wciąż nie wiadomo, czy PDGF-BB ulega ekspresji we krwi obwodowej i czy ekspresja PDGF-BB przyczynia się do GD. Badanie przeprowadzono w celu zbadania ekspresji PDGF-BB, czynnika indukowanego hipoksją-1α (*hypoxia inducible factor-1α*, HIF-1α) i receptora chemokiny C-C typu 2 (*C-C motif chemokine receptor-2*, CCR-2) we krwi obwodowej pacjentów z GD i zbadania wpływu tych cząsteczek i potencjalnego mechanizmu ich działania w patogenezie choroby.

Materiał i metody: Do badania włączono 41 pacjentów z GD (grupa GD) i 45 osób zdrowych (grupa kontrolna). Stężenie PDGF-BB i HIF-1α w próbkach krwi obwodowej oznaczono i porównano między grupami. Do pomiaru ekspresji CCR2 w makrofagach krwi obwodowej zastosowano metodę cytometrii przepływowej (*flow cytometry*, FCM).

Wyniki: W obu grupach badanych stwierdzono ekspresję PDGF-BB i HIF-1α we krwi obwodowej. W grupie GD odnotowano istotne statystycznie wyższe stężenia PDGF-BB i HIF-1α niż u osób zdrowych ($p < 0,05$). Odsetek makrofagów CCR2-dodatnich we krwi obwodowej w grupie GD był istotnie wyższy niż w grupie kontrolnej ($p < 0,05$).

Wnioski: Makrofagi CCR2-dodatnie mogą indukować ekspresję PDGF-BB za pośrednictwem sygnału HIF-1α, a wysoka ekspresja PDGF-BB może odgrywać rolę w patogenezie GD. **(Endokrynol Pol 2018; 69 (1): 9–15)**

Słowa kluczowe: *PDGF-BB, Choroba Gravesa i Basedowa, HIF-1α, makrofagi, immunologia*

Chunjie Hou, Master of Medicine, Department of ultrasonography, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, Zhejiang, 310014, P R China, China, tel: (86)13868167040, e-mail: houchunjie@hmc.edu.cn

Introduction

GD is an autoimmune disease, and the current research on it mainly focuses on its immunological pathogenesis. PDGF belong to the family of vascular endothelial growth factor (VEGF), and is mostly present as dimer [1, 2]. PDGF-BB, one member of the PDGF family [3, 4], has the ability to promote fibrosis, muscle growth and angiogenesis [5, 6].

HIF-1α is a transcription factor involved in homeostasis of oxygen concentration. Inflammatory cytokines play an important role in the regulation of HIF-1 alpha protein accumulation, DNA binding activity and the expression of downstream factors [7]. CCR2 is the main receptor of monocyte chemoattractant protein-1 (MCP-1), which is a member of the chemokine CC subfamily [8]. In chemotaxis, MCP-1 and CCR2 combine to activate intracellular signalling pathways and induce cell migration [9]. Some scholars believe that MCP-1 can regulate HIF-1α gene expression, and MCP-1 mainly exerts its biological effects by binding to the chemokine receptor CCR2 [10].

Several studies have shown that overexpression of PDGF-BB is closely related to cancer, atherosclerosis and fibrotic diseases [11, 12]. PDGF-BB may also be involved in the pathogenesis of GD by promoting fibrosis, oedema, inflammation, and non-specific immune response [13]. However, there have been few reports about the research on the expression of PDGF-BB in peripheral blood. In this study, we examined the expression of PDGF-BB, HIF-1α, and CCR2 in peripheral blood and analysed the correlation between PDGF-BB and GD.

Materials and methods

Subjects

A total of 41 patients (male: 24; female: 17; average age: 34 ± 12.6 years) with GD were initially diagnosed by ultrasound and confirmed in the Department of Endocrinology from October 2013 to June 2016. Healthy controls (n = 41; male: 14; female: 27; average age: 36 ± 10.3 years) from the medical examination centre of our hospital were included in this study. Inclusion criteria for GD [14] were: typical fatigue, heat intolerance, sweating, irritability, palpitations, tremor, hypermetabolic syndrome, goitre, exophthalmos and increased serum level of FT3 and FT4 and decreased level of TSH.

Main reagents and instruments

Calf serum and phosphate buffer solution were purchased from GIBCO (New York, USA). PDGF-BB and HIF-1α ELISA assay kits were purchased from R & D (Minnesota, USA). PE-labelled rabbit anti-human CCR2, FITC-labelled rabbit anti-human CD68 antibody and isotype control antibody IgG were purchased from BD Biosciences (New York, USA). Haemolytic agents were purchased from Invitrogen (California, USA). Instruments used in this study included Mylab90 ultrasonic device (Esaote, Shenzhen, China), a Beckman FC500 flow cytometer (Beckman, California, USA) and a microplate reader (New York, USA).

Ultrasound diagnosis of GD

Longitudinal and transverse sections of thyroids were examined by Mylab90 ultrasonic device using high-frequency linear array probe (8–13 MHz). Thyroid size, shape, echo characteristics, blood flow, and peripheral tissues were examined. Left and right lobe diameter, vertical diameter, thickness of the isthmus, thyroid artery peak systolic velocity (PSV), and the resistance index (RI) were measured at the standard sections.

Peripheral blood sample preparation

Peripheral blood (2 mL) from the subjects was collected into anticoagulant tubes. Plasma samples were obtained after centrifugation at 1800 rpm, 4°C for 5 min. The remaining red blood cells in the anticoagulant tube were lysed by adding 1 mL of distilled water. After centrifugation at 1800 rpm at room temperature for 5 min, the cell pellet containing mononuclear cells was harvested and used for subsequent flow cytometry analysis.

Detection of PDGF-BB and HIF-1α expression in peripheral blood by ELISA

PDGF-BB and HIF-1α plasma levels were determined by ELISA as described by the manufacturer (R&D). The concentration was determined by an ELISA reader at 450 nm. The percentage of PDGF-BB and HIF-1α results falling below the limit of sensitivity was stated for each study subgroup and calculations were based on observations above the detection limits. All ELISA readings of PDGF-BB and HIF-1α measurements were within the respective detection limits.

Flow cytometry detection of CCR2 positive macrophages in peripheral blood

The mononuclear cells were adjusted to the concentration of 2×10^5/mL and each reaction contained 5×10^4 cells. The resultant cells were stained with FITC-conjugated rabbit anti-human CD68 and PE-conjugated rabbit anti-human CCR2. Together with the samples stained with non-immunised rabbit IgG mAb as an isotype control.

Statistical analysis

The means and standard error of the mean (SEM) were calculated for all parameters determined in the study. Data were analysed statistically using one-way analysis

Table I. *The clinical conditions of the two groups*
Tabela I. *Dane kliniczne obu grup pacjentów*

Characteristics		Control group	GD group	*P*
Clinical manifestation	Participants [n]	41	41	
	Age [y]	36 ± 10.3	34 ± 12.6	0.25
	Infiltrative exophthalmos [n]	0	1	
	High metabolic syndrome [n]	0	41	< 0.01
	FT3 [pmol/L]	Normal	21.65 ± 7.13	< 0.01
	FT4 [pmol/L]	Normal	63.46 ± 25.71	< 0.01
	TSAb positive rate [%]	0	95 (39/41)	< 0.01

Data presented as n or mean ± SD.
P-value: t-testing (age); chi-square-testing (high metabolic syndrome)

of variance (ANOVA), two-tailed Student's t test, or chi square test. A value of $P < 0.05$ was considered statistically significant.

Results

General information of the subjects

All of the 41 subjects with GD had high metabolic syndrome, e.g. tachycardia, palpitations, bulimia, and significantly enlarged thyroid. There were 37 patients with exophthalmos (The exophthalmos was 17.27 ± 1.12 mm), and 1 case with infiltrative exophthalmos (The exophthalmos was 25 mm). The exophthalmus of infiltrative exophthalmos is 16–18 mm, and the exophthalmos of infiltrative exophthalmos is 19 mm [15]. Serum free T3, T4 concentration in the GD group was higher than in normal subjects; the concentrations were 21.65 ± 7.13 pmol/L (FT3) and 63.46 ± 25.71 pmol/L (FT4), respectively. The maximum value was 30.80 pmol/L (FT3) and 120.20 pmol/L (FT4). Thirty-nine patients' TSAbs were positive, and the TSAb positive rate in the Graves' disease group was 95% (Table I).

Ultrasound examination

Dimensional ultrasound showed that thyroid volume was significantly increased in patients with GD (where the isthmus had no significant thickening). The thyroid was encapsulated, with clear boundaries, and enhanced and evenly distributed echoes. Colour Doppler showed that the blood supply of the thyroid is abundant, significantly increased thyroid blood flow with flashing bright spot representative of a "sea of fire" sign (Figure 1A). Thyroid arteries were expanded and Doppler can detect the high-speed, low resistance turbulence spectrum (Figure 1B).

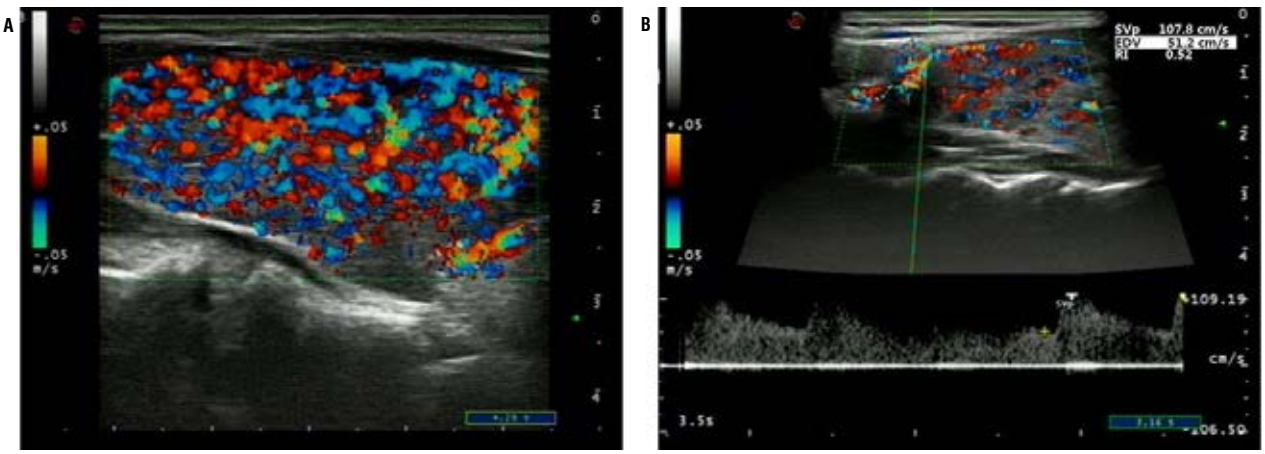

Figure 1. *The CDFI and PW-DTI of GD*
Rycina 1. *CDFI i PW-DTI choroby Gravesa-Basedowa*

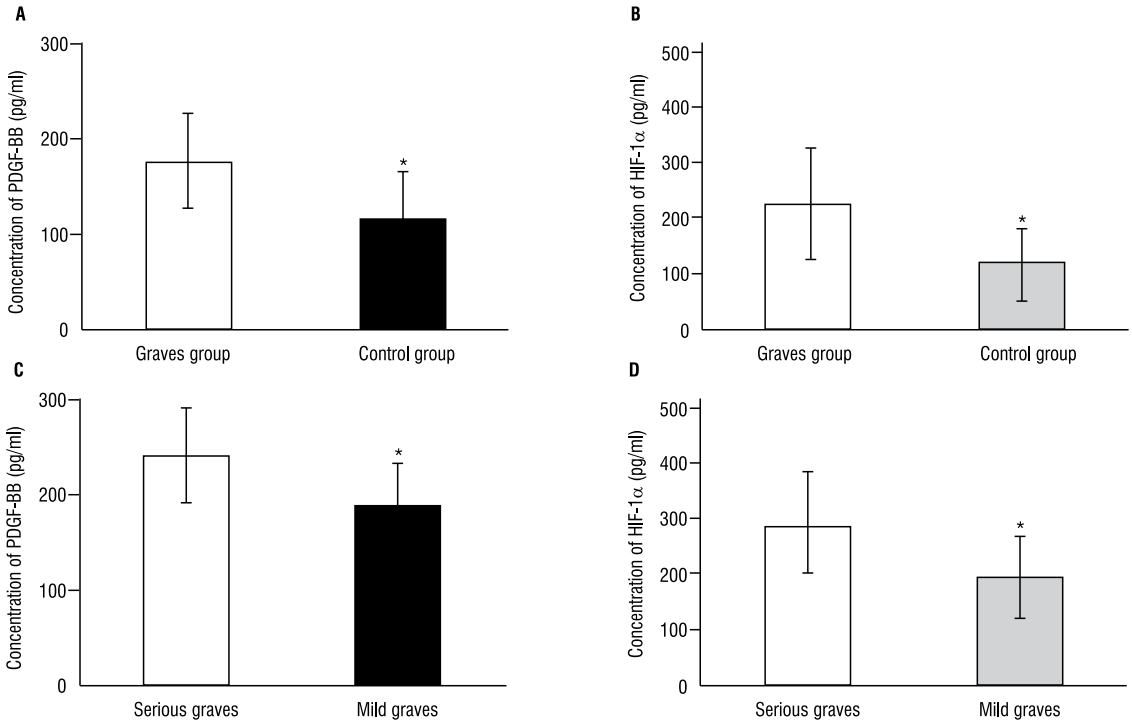

Figure 2. *The concentration of PDGF-BB and HIF-1α in peripheral blood. "*"means that the difference between the two groups was statistically significant (P < 0.05)*

Rycina 2. *Stężenie PDGF-BB i HIF-1α w krwi obwodowej. „*"różnica znamienna statystycznie pomiędzy dwoma grupami (P < 0,05)*

Expression of PDGF-BB and HIF-1α in the peripheral blood

The concentration of PDGF-BB and HIF-1α in the serum of patients with GD was 189.16 ± 22.37 pg/mL and 239.69 ± 32.97 pg/mL, respectively. The concentration of PDGF-BB and HIF-1α in the serum of control subjects was 121.12 ± 29.38 pg/mL and 106.45 ± 44.39 pg/mL, respectively. The concentration PDGF-BB and HIF-1α in the serum of patients with GD was significantly higher than that in the control subjects (P < 0.05) (Figure 2A and 2B). The concentration of PDGF-BB and HIF-1α in the seven patients with higher T3 concentration was 241.35 ± 51.22 pg/mL and 287.32 ± 37.46 pg/mL, respectively, which was significantly higher than the average concentration of PDGF-BB and HIF-1α in patients with GD (P < 0.05) (Figure 2C and 2D).

Expression of CCR2 positive macrophage

Flow cytometry showed that the positive rate of CD68 on the peripheral mononuclear cells was 13.19 ± 4.55% and these cells were macrophages. Subsequently, CCR2 expression on macrophage was measured. CCR2 was expressed on 55.25 ± 10.44% of macrophages in the GD group (Figure 3B), while in the control subjects, CCR2-positive rate was 41.21 ± 11.26% (Figure 3A). The CCR2-positive rate in GD group was significantly higher than that in the normal group (P < 0.05) (Figure 3D). Furthermore, The CCR2-positive rate in the seven patients with higher T3 concentration was 62.16 ± 9.85%, which was significantly higher than the average CCR2-positive rate in the patients with GD (P < 0.05) (Figure 3E).

Correlation between PDGF-BB and CCR2 positive macrophages

Statistical analysis showed that PDGF-BB concentration in the peripheral blood was positively correlated with the CCR2-positive rate on macrophage (R^2 = 0.5777) (Figure 4).

Discussion

GD is a kind of refractory disease [16]. The main manifestation of GD is hyperthyroidism. Hyperthyroidism can exacerbate immune disorders, which leads to increased anti-TR (anti-thyroid hormone receptors) antibody production, and thus increases thyrotoxicosis and creates a vicious cycle [17]. Foreign studies have found that PDGF-BB is involved in the pathogenesis of GD [1]. According to the data analysis, PDGF-BB can be regarded as an important regulatory factor in the pathophysiology of GD, especially Graves ophthalmopathy.

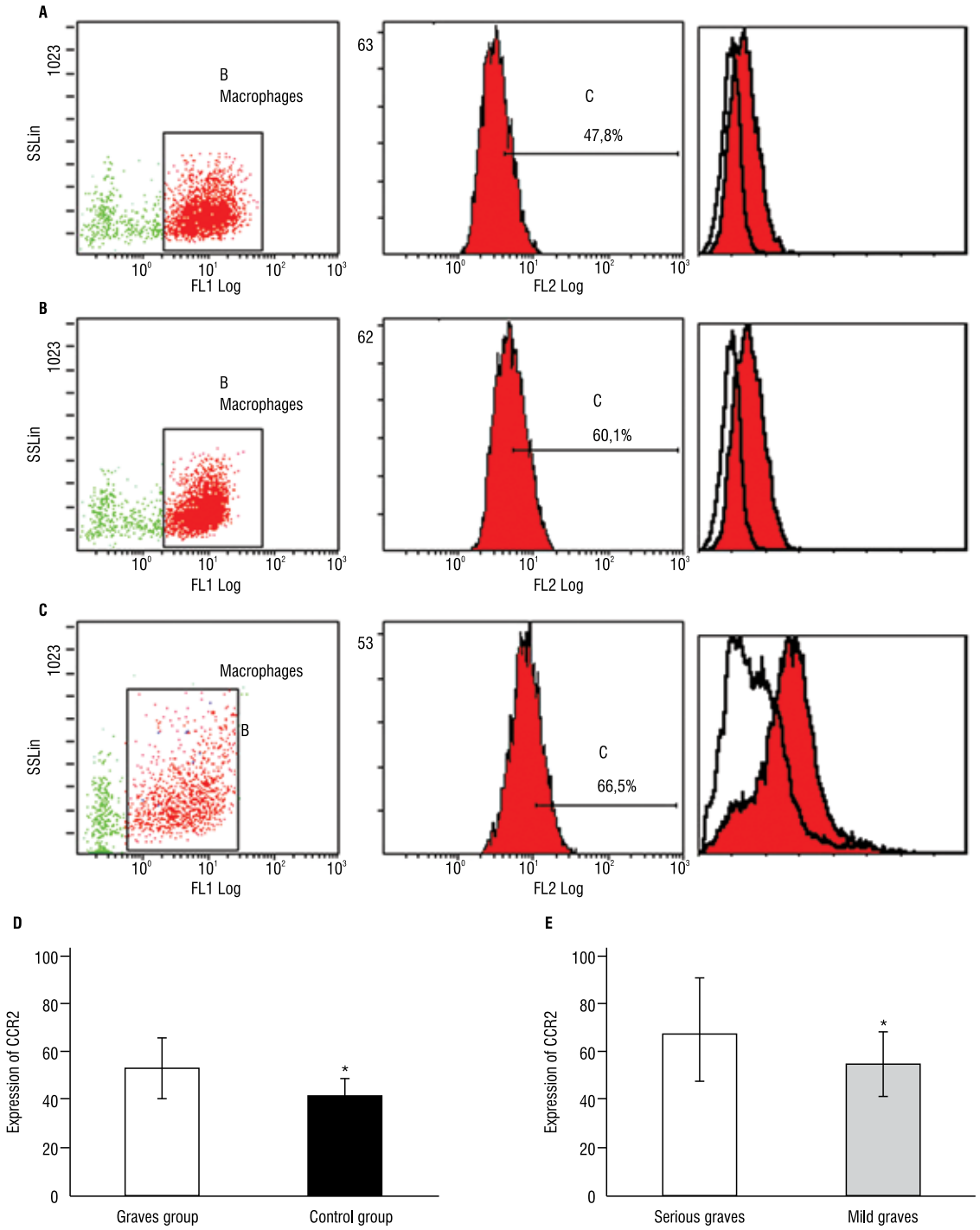

Figure 3. *The expression of CCR2 in peripheral blood of macrophages. Figure A represents the control group; Figure B represents GD; Figure C represents serious GD; "*" means that the difference between the two groups was statistically significant (P < 0.05). FL1 Log refers to fluorescein isothiocyanate (FITC) axis and FL2 Log refers to P-phycoerythrin (PE) axis. The cells in the gate were positive macrophages*

Rycina 3. *Ekspresja CCR2 w makrofagach krwi obwodowej. Rycina A przedstawia grupę kontrolną; Rycina B grupę pacjentów z GD; Rycina C przedstawia grupę pacjentów z chorobą GD w fazie zaawansowanej; „*"różnica znamienna statystycznie pomiędzy grupami (P < 0.05). FL1 Log — izotiocyjanian fluoresceiny (FITC), FL2 Log — fikoerytryna. Odnotowano makrofagi dodatnie*

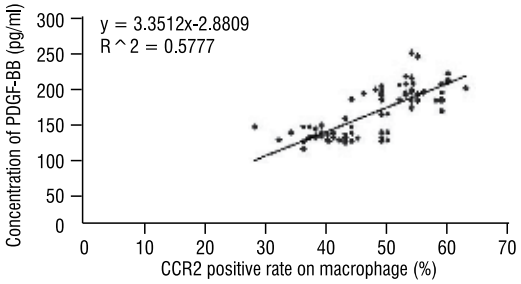

Figure 4. *The correlation of PDGF-BB expression and CCR2-positive macrophages*

Rycina 4. *Korelacja pomiędzy ekspresją PDGF-BB i makrofagami z dodatnim odczynem dla CCR2*

However, the expression of PDGF-BB in the peripheral blood of patients and its specific mechanisms need to be further studied.

Therefore, we detected the expression of PDGF-BB, HIF-1α, and CCR2 in the peripheral blood of patients. Compared with the control group, the expression of PDGF-BB, HIF-1α and CCR2 in peripheral blood of patients with GD increased significantly. Furthermore, the expression of PDGF-BB, HIF-1α and CCR2 in peripheral blood of patients with severe disease progression was higher than that in patients with mild symptoms. These results showed that the expression of PDGF-BB in peripheral blood may be related to the occurrence and development of GD.

This study demonstrated that the expression of PDGF-BB in peripheral blood of GD had an increasing trend, which may be caused by the increased HIF-1α. HIF-1α can cause CCR2-positive macrophage proliferation activity and then promote the secretion of VEGF and PDGF-BB. Currently, molecularly targeted therapy is an important means of clinical control of disease development. We may take PDGF-BB as a target and achieve effective control of Graves by blocking its molecular signalling pathway. The abnormal expression of PDGF-BB in peripheral blood of Graves patients provides a valuable experimental basis for clinical application.

The changes of PDGF-BB expression may be related to the immune regulation disorder of GD, and the heterogeneity of macrophages. We found that the expression of CCR2-positive macrophages in peripheral blood of patients with GD increased significantly, and the expression in patients with severe disease was also higher than that of patients with mild disease. The results indicate that the number of CCR2-positive macrophages in patients with GD was disordered. Macrophages are the major secretory cells of PDGF-BB. Furthermore, the correlation between PDGF-BB and positive macrophage CCR2 expression was analysed and the results showed that they were positively correlated. Thus, the expression of PDGF-BB may be related to the imbalance of CCR2-positive macrophages.

We also examined the expression of HIF-1α in peripheral blood and the results showed that HIF-1α expression had the similar trends with PDGF-BB expression. Previous studies have shown that HIF-1α is an important factor to stimulate macrophages [18, 19]. Upon HIF-1α stimulation, macrophages can upregulate the expression of VEGF and PDGF-BB, thus participating in the pathological process of GD.

Conclusions

Our present study investigated the expression of PDGF-BB in peripheral blood of patients with GD as well as the potential regulatory mechanisms, which laid the foundation for further exploration of the role of PDGF-BB in the pathological process of GD.

Funding information

General project supported by Zhejiang Provincial health and Family Planning Commission of China (2014KYA012).

Disclaimers

The views expressed in our submitted manuscript are our own and not an official position of the institution or funder.

Conflict of interest

We declare that the authors have no conflict of interest.

References

1. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev. 2008; 22(10): 1276–1312, doi: 10.1101/gad.1653708, indexed in Pubmed: 18483217.
2. Chen XS, Sun D, Yao X. Research Progress on PDGF/PDGFR in Cancer and Related Targeted Therapy. Chinese Journal of Clinical Oncology. 2012; 39: 1134–1137.
3. Li Q, Ma LK. Role and Possible Mechanism of PDGF Signaling Pathways During Process of Myocardial Fibrosis Induced by Aldosterone. Advances in Cardiovascular Diseases. 2012; 33: 85–88.
4. Liu HJ, Gao XZ, Jin Y, et al. Role of PDGFR- signal pathway in morphine tolerance. Journal of Medical Postgraduates. 2015; 28: 149–152.
5. Cheng P, Gao ZQ, Liu YH, et al. Platelet-derived growth factor BB promotes the migration of bone marrow-derived mesenchymal stem cells towards C6 glioma and up-regulates the expression of intracellular adhesion molecule-1. Neurosci Lett. 2009; 451(1): 52–56, doi: 10.1016/j.neulet.2008.12.044, indexed in Pubmed: 19133316.
6. Cheng J, Ye H, Liu Z, et al. Platelet-derived growth factor-BB accelerates prostate cancer growth by promoting the proliferation of mesenchymal stem cells. J Cell Biochem. 2013; 114(7): 1510–1518, doi: 10.1002/jcb.24492, indexed in Pubmed: 23297038.
7. Wang BL, Wang XR. Regulation of the hypoxia inducible factor-1α by the inflammatory mediators[J]. Inter J Pathol Clin Med. 2008; 90: 1673–1688.
8. Yoshimura T, Robinson EA, Tanaka S, et al. Purification and am ino acid analysis of two human glioma-derived monocyte chemoattractants[J] . J ExpMed. 1989; 169: 1449–1459.

9. Lee EY, Chung CH, Khoury CC, et al. The monocyte chemoattractant protein-1/CCR2 loop, inducible by TGF-beta, increases podocyte motility and albumin permeability. Am J Physiol Renal Physiol. 2009; 297(1): F85–F94, doi: 10.1152/ajprenal.90642.2008, indexed in Pubmed: 19420107.
10. Chen BY, Guan CN. The research progress of relationship between MCP-1 and HIF-1 and tumor angiogenesis. JMed Res. 2010; 39: 27–30.
11. Chen SJ, Xu XJ, Huo X. Research Development about Relationship of PDGFs and its Receptor with Related Diseases. Medical Recapitulate. 2008; 14: 1441–1444.
12. Qi L, Du J, Zhang Z, et al. Low differentiated microvascular density and low expression of platelet-derived growth factor-BB (PDGF-BB) predict distant metastasis and poor prognosis in clear cell renal cell carcinoma. BJU Int. 2013; 112(4): E415–E423, doi: 10.1111/bju.12191, indexed in Pubmed: 23879920.
13. van Steensel L, Hooijkaas H, Paridaens D, et al. PDGF enhances orbital fibroblast responses to TSHR stimulating autoantibodies in Graves' ophthalmopathy patients. J Clin Endocrinol Metab. 2012; 97(6): E944–E953, doi: 10.1210/jc.2012-1020, indexed in Pubmed: 22438231.
14. Compile Group of Endocrinology branch of Chinese medical association. Chinese guidelines of diagnosis and treatment of thyroid diseases - Hyperthyroidism. Chinese Journal of Internal Medicine. 2007; 46: 876–882.
15. Tang YG. Analysis of the clinical effect of methimazole combined with levothyroxine for treating hyperthyroid exophthalmos. Heilongjiang Medical Journal. 2016; 29: 279–280.
16. Fang WZ, Luo ZW. Progress in Treatment of Graves Ophthalmopathy. Medical Recapitulate. 2014; 20: 3534–3536.
17. Menconi F, Marcocci C, Marinò M. Diagnosis and classification of Graves' disease. Autoimmun Rev. 2014; 13(4-5): 398–402, doi: 10.1016/j.autrev.2014.01.013, indexed in Pubmed: 24424182.
18. Scholz CC, Taylor CT. Targeting the HIF pathway in inflammation and immunity. Curr Opin Pharmacol. 2013; 13(4): 646–653, doi: 10.1016/j.coph.2013.04.009, indexed in Pubmed: 23660374.
19. Chen W, Hill H, Christie A, et al. Targeting renal cell carcinoma with a HIF-2 antagonist. Nature. 2016; 539(7627): 112–117, doi: 10.1038/nature19796, indexed in Pubmed: 27595394.