Intravenous immunoglobulin does not increase FcγR II B expression levels on monocytes in children with immune thrombocytopenia

小児 ITP において免疫グロブリン大量療法は末梢血単球 FcγRⅡB 発現を増強しない

下村　麻衣子

山口大学大学院医学系研究科医学博士課程

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(1) 研究の背景

小児免疫性血小板減少性紫斑病（ITP)の病因は、血小板膜に反応する抗血小板抗体による血小板の破壊であると考えられている。血小板膜上の糖蛋白に抗体が結合した血小板は、早期に網内系の単核食細胞系（mononuclear phagocyte system：MPS）に貪食されて破壊される。血液を循環する単球や、脾臓や肝臓に存在するマクロファージは血小板に結合したIgGのFc部分にFcγRと呼ばれるFcレセプターを介して結合する。小児ITPに対する免疫グロブリン大量療法（IVIG)は迅速にかつ持続的に血小板を上昇させる治療法として確立されている。その機序はFcγRが介在する網内系の単核食細胞系による血小板のクリアランスを阻害することであると考えられている。しかしながら、ITP患者においてその機序は未だ不明である。

FcγRには、FcγRI、FcγRⅡ、FcγRIIIの3種類が存在し、免疫活性型と抑制型が知られている。活性型FcγRは細胞内領域に活性化のシグナル伝達に重要なアミノ酸モチーフITAM（immunoreceptor tyrosine-based activation motif）を有するサブユニットγ鎖(FcRγ)と会合し、リガンドとの結合によって細胞に活性化シグナルを伝達する。一方、抑制型FcγRであるFcγRIIB(CD32B)は、FcRγ鎖とは会合せず、細胞内領域にITIM（immunoreceptor tyrosine-based inhibitory motif）と呼ばれるアミノ酸配列を有し、活性化型FcγRとの共架橋刺激を介して、細胞に抑制性シグナルを伝達する。最近では、活性型FcγRと抑制性FcγRであるFcγRⅡBがバランスを取ってマクロファージなどのエフェクター細胞を調節し、B細胞上ではFcγRⅡBが抗体産生そのものを負に調節することもわかってきた。FcγRⅡBはB細胞、単球、マクロファージおよび形質細胞に発現しており、ITPにおいてIVIGの治療効果に関連する可能性が示唆されている。Samuelssonら(Science 2001; 291:484-6)は、抗血小板抗体が介在するITPのラットモデルにおいて、IVIGの治療効果にFcγRⅡBが関与していると報告した。さらに彼らは、IVIGによって脾臓マクロファージにおける抑制性レセプターの発現が増強することを報告し、ITPのモデルマウスにおいてそれを立証した。しかし、ヒトITP患者においてIVIGの効果にFcγRⅡBの存在が関与しているという同様の報告はない。逆に、FcγRⅢ(CD16)は活性型のFcレセプターであり、NK細胞、単球、マクロファージや形質細胞に発現している。

本研究では、小児ITP患者においてIVIGがCD14陽性末梢血単球とマクロファージ上のFcγRⅡBの発現を増加させるかどうかを検討し、さらに活性型FcγRⅢについても同様に検討した。

(2) 要旨

【背景】小児免疫性血小板減少性紫斑病 (ITP)は血小板膜に反応する抗血小板自己抗体が血小板を破壊し、血小板減少を来す自己免疫性疾患である。血小板膜上の糖蛋白に抗体が結合した血小板は、早期に網内系の単核食細胞系（mononuclear phagocyte system : MPS）に貪食後破壊される。免疫グロブリン大量療法 (IVIG) は小児ITP において迅速かつ持続的に血小板を上昇させる治療法として確立している。これまでにITPのラットモデルにおいてIVIGが脾臓マクロファージの抑制性Fcレセプターであ るFcγRⅡB（CD32B）の発現を増加させると報告されたが(Science 2001; 291:484–6)、ITP患者においてはこの機序は明らかにされていない。

【目的】小児ITP患者においてIVIG療法後の末梢血単球上FcγRⅡBの発現へ及ぼす影響ついてフローサイトメトリー法を用いて検討する。

【方法】対象は小児ITP症例20例 (男12例, 女8例, 中央値2歳11か月)である。患者の治療前（平均血小板数:9.5×10^9/L）と、IVIG投与後に血小板数上昇を確認した時点（IVIG後平均4.8日）の末梢血を用いた。フローサイトメーターを用いてIVIG投与前後で末梢血単球上のCD32Bの発現（FcγRIIB）とCD16の発現（FcγRⅢ）を比較した。全例が急性ITPであり、IVIG療法に対する反応性は良好であった。

【結果】小児ITP患者のIVIG前後におけるCD32B陽性単球の絶対数、単核細胞中および単球中におけるCD32B陽性細胞の比率にはいずれも有意差を認めなかった。また、CD16陽性単球についても同様にIVIG投与前後で絶対数、単核細胞中および単球中における比率のいずれも有意差を認めなかった。

【考察】IVIGが小児ITPにおいては末梢血単球上のFcγRⅡBの発現を増加させない可能性が示唆された。小児ITP患者のIVIG療法におけるFcγRⅡBを介した作用機序の関与についてはさらに検討が必要である。

Intravenous immunoglobulin does not increase FcγRIIB expression levels on monocytes in children with immune thrombocytopenic purpura

Short title: The effects of IVIG on FcγRIIB expression levels on monocytes in children with ITP

Maiko Shimomura M.D., Shunji Hasegawa M.D., Yumi Seki M.D., Reiji Fukano M.D., Noriko Hotta M.D., and Takashi Ichiyama M.D.

Department of Pediatrics, Yamaguchi University Graduate School of Medicine

Corresponding author:

Maiko Shimomura

Department of Pediatrics

Yamaguchi University Graduate School of Medicine

1-1-1 Minami-kogushi

Ube, Yamaguchi 755-8505

Japan

Tel.: +81-836-22-2258

Fax: +81-836-22-2257

E-mail: r004um@yamaguchi-u.ac.jp

Summary

Intravenous immunoglobulin (IVIG) produces a rapid and prolonged increase in the platelet counts of children with immune thrombocytopenic purpura (ITP). The mechanism of IVIG efficacy in a murine model of ITP has been reported to operate through an IVIG-mediated increase in the expression of the inhibitory Fc receptor FcγRIIB(CD32B) on splenic macrophages. This investigation examined whether IVIG administration results in a similar increase in FcγRIIB expression on peripheral blood $CD14^+$ monocytes in 20 children with ITP. FcγRIIB expression on peripheral blood monocytes was measured by flow cytometry in ITP patients, before and after IVIG therapy, as well as in control subjects. Peripheral blood monocytes were labeled with specific fluorescent specific antibodies. There were no significant differences in the percentages or numbers of $CD14^+CD32B^+$ monocytes, or in the percentage of $CD14^+CD32B^+$ monocytes present in children with ITP before and after IVIG therapy. We suggest that IVIG does not increase FcγRIIB expression in peripheral blood monocytes in children with ITP.

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Keywords: Monocytes, Immune thrombocytopenic purpura, FcγRIIB, Intravenous immunoglobulin

Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disease that causes abnormally increased destruction of platelets, which results in a reduced platelet count. Studies investigating the pathogenesis of this disease have revealed that platelet destruction is mediated by autoantibodies directed against platelet surface antigens [1,2]. Platelet clearance then occurs as a result of phagocytosis by the mononuclear phagocytic system (MPS) [3]. Circulating monocytes and resident macrophages in the spleen and liver bind to the exposed Fc portion of platelet-associated IgG molecules via the IgG Fc receptors, namely, FcγR [4]. Administration of intravenous immunoglobulin (IVIG) to children with ITP produces a rapid and prolonged increase in platelet counts [5], which is believed to be the result of the IVIG interfering with the FcγR-mediated platelet clearance [6]. However, the mechanism by which IVIG ameliorates human ITP remains poorly understood.

FcγRIIB (CD32B), the inhibitory, low-affinity receptor for IgG that is widely expressed on B cells, monocytes, macrophages, and mast cells, has attracted attention for its potential involvement in the IVIG induced amelioration of human ITP [7]. Samuelsson et al. reported that the protective effects of IVIG in an anti-platelet, antibody-mediated murine model of ITP depended on the presence of FcγRIIB; they also reported that IVIG administration increased the expression of this inhibitory receptor by splenic macrophages and confirmed this finding in a murine model of ITP [8]. However, reports regarding the involvement of FcγRIIB in human ITP are lacking. On the other hand, FcγRIII (CD16) is the low-affinity activating receptor for IgG and expressed on NKcells, monocytes, macrophages and mast cells[9].

In this study, we investigated whether IVIG increased the expression levels of FcγRIIB on peripheral $CD14^+$ monocytes and macrophages in children with ITP.

Patients and Methods

Immune thrombocytopenia purpura

Peripheral blood was obtained from 20 children with ITP (12 boys and 8 girls, age ranging from 2 months to 10 years 9 months; mean age, 2 years 11 months) on admission to Yamaguchi University Hospital between May 2003 and March 2011 (Table 1). The patients met the diagnostic and therapeutic criteria for ITP [10]. One patient received treatment with IVIG at 400 $mg \cdot kg \cdot^{-1} d^{-1}$ over a 5-day period, 12 patients received treatment with IVIG at 1 $g \cdot kg \cdot^{-1} d^{-1}$ over a 2-day period (1 of them also received prednisolone at 2 $mg \cdot kg \cdot^{-1} d^{-1}$ with the IVIG) and 7 patients received IVIG at 2 $g \cdot kg^{-1} \cdot d^{-1}$. All patients were acute type of ITP and almost all patients had good response to IVIG, increasing platelet count more than 30×10^9/L , within five-days after initiation of therapy. However four patients (No. 3, 4, 15, and 16) showed an incomplete response to IVIG therapy. They had response to IVIG at a slow pace and 2 patients of them needed additional IVIG therapy. None of the patients exhibited life-threatening bleeding complications. Blood samples were obtained on the day before treatment (platelet mean count = 9.5×10^9/L) and once after administration of IVIG (platelet counts were higher than the counts before the treatment).

Control subjects

The control subjects were 18 healthy children (8 boys and 10 girls; age ranging from 7 months to 6 years 2 months; mean age, 2 years 5 months).

Detection of FcγRIIB(CD 32B) expression on monocytes by flow cytometry

The FcγRIIB expression levels were measured using two-color flow cytometry. Peripheral blood cells labeled with phycoerythrin (PE)-conjugated anti-CD14 antibodies (BD Biosciences, San Jose, CA, USA) were incubated with anti-FcγRIIB(CD 32B) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibody was an affinity purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of CD32B of human origin. The cells were also labeled with a fluorescein isothiocyanate (FITC)-conjugated antibody. In addition, peripheral blood cells were labeled with FITC-conjugated anti-FcγRIII(CD 16) antibodies (BD Pharmingen, San Diego, CA, USA) and PE-conjugated anti-CD14 antibodies. Erythrocytes were lysed by adding 1.5 mL of lysing solution (Becton Dickinson, San Jose, CA, USA) for 10 min. The remaining cells were centrifuged, washed with phosphate-buffered saline (PBS), and analyzed using a FACScalibur flow cytometer with equipped with CellQuest software (Becton-Dickinson Bioscience, San Diego, CA, USA). In all, 5,000 $CD14^+$cells were analyzed for each subject.

Statistical analysis

The results are expressed as mean ± standard deviation (SD). Statistical analyses were performed using the Wilcoxon matched pairs test, the Mann-Whitney U-test, and Spearman's correlation; a *P* value of less than 0.05 was considered significant. Analyses were performed using SPSS ver. 12.0.(SPSS, Chicago, IL, USA).

Ethical considerations

Informed consent was obtained from the parents of the patients and control subjects enrolled in this study. The protocol was approved by the Institutional Review Board of Yamaguchi University Hospital (No.2011-34-4).

Results

Figure 1 shows the results of the two-color flow cytometry analyses of the expression of CD16(A) and CD32B(B) on peripheral $CD14^+$ monocytes and macrophages from Patient 2, before and after IVIG administration. The results from all of the flow cytometry analyses are presented in Tables 2 and 3. Both the percentage and absolute numbers of $CD14^+$ monocytes in peripheral blood mononuclear cells (PBMCs), from patients with ITP before the IVIG therapy, were significantly higher than those from the controls ($P = 0.004$ and $P = 0.008$, respectively). The percentage of $CD14^+$ monocytes, in patients with ITP, after the IVIG treatment was also significantly higher than that in the controls ($P = 0.008$) (Table 2). Significant differences were not observed in the percentage of $CD14^+CD16^+$ monocytes in ITP children before and after the IVIG therapy; further, significant differences were not observed between patients after the IVIG therapy and control patients ($P = 0.097$ and $P = 0.373$, respectively) (Table 2). There were no significant correlations between platelet counts in children with ITP on the day before the treatment and the percentage of $CD16^+$ monocytes among the recovered $CD14^+$ monocytes ($P = 0.44$).

Similarly, in the ITP patients, significant differences were not observed before and after the IVIG therapy with regard to the percentage of $CD14^+CD32B^+$ monocytes in the PBMC populations, the number of $CD14^+CD32B^+$ monocytes, or the percentage of $CD14^+CD32B^+$ monocytes in the $CD14^+$ monocyte populations ($P = 0.140$, $P = 0.145$,

and $P = 0.147$, respectively) (Table 3). Additionally, there were no significant differences in the percentages or absolute numbers of $CD14^+CD32B^+$ monocytes in PBMC samples obtained from ITP patients after the IVIG therapy and the controls ($P = 0.393$ and $P = 0.143$, respectively) (Table 3). The percentage of $CD14^+CD32B^+$ monocytes in the $CD14^+$ monocyte population in ITP patients, both before and after the IVIG treatment, were significantly lower than those in the controls ($P = 0.033$ and $P = 0.009$, respectively).

Discussion

FcγRII (CD32) represents a group of 3 closely related proteins (FcγRIIA, FcγRIIB, and FcγRIIC) that share a greater than 94% amino acid identity in their extracellular domains [11,12]. FcγRIIA and FcγRIIB are mainly expressed on human monocytes [13,14,15]; FcγRIIA is an active receptor and FcγRIIB is an inhibitory receptor. This is the first report of the analysis of $CD14^+$ monocytes recovered from peripheral blood samples from children with ITP. Interestingly, IVIG therapy did not increase the levels of FcγRIIB expression on the peripheral monocytes in children with ITP. These results differ from the results of FcγRIIB expression studies in monocytes in the murine model of ITP [8]. The difference may be attributed to the type of cells investigated. In this study, $CD14^+$ cells were analyzed as a subset of the PBMC population, whereas the murine ITP model investigation focused on splenic macrophages [8]. It is possible that IVIG does not increase the levels of FcγRIIB expression on monocytes in human ITP. Therefore, the contribution of FcγRIIB to IVIG effects may be species or cell subset dependent.

In Table 2, the percentages and absolute numbers of $CD14^+$ monocytes in the peripheral blood of ITP patients were significantly higher than those of the control

subjects. These results suggest the activation of the monocytes and may be indicative of them having a role in the pathogenesis of ITP. These findings do not correlate to those of Samuelsson et al. who reported that blocking the FcγRIII helped to prevent declining platelet counts in the murine ITP model [8]. The current study showed that the platelet counts in ITP patients, on the day before the treatment, did not correlate with the percentage of $CD16^+$ monocytes among the total $CD14^+$ monocyte population.

The function of the inhibitory receptor FcγRIIB requires recruitment of inositol phosphatase (SHIP1) to the immunoreceptor tyrosine-based inhibitory motif (ITIM) in both B cells and mast cells [16-19]. The FcγRIIB expressed on monocytes has been reported to lead to the down-regulation of activation in phagocytes [20]. However, it has not been established whether this pathway is involved in the mechanism of action of IVIG therapy in human ITP. Other authors have reported that the expression of SHIP1 is not required for IVIG action in ITP [21]. Therefore, the effect of IVIG on FcγRIIB in human ITP is still unclear. FcγRIIB-mediated inhibition of platelet phagocytosis cannot account for the therapeutic benefit provided by IVIG treatment [22]. Tovo et al. showed that an Fc-depleted IVIG preparation had some effect in ITP patients, although the magnitude of the effect was less than that observed with intact IVIG [23]. Recently, it has also been suggested that the interchain disulfide bonds of the gammaglobulins are important for the therapeutic amelioration of ITP and that the interaction of IVIG with the inhibitory receptor FcγRIIB is insufficient for this effect [24]. Thus, evidence is mounting that suggests that the FcγRIIB expression levels may not be sufficient to account for the mechanism through which IVIG is able to result in enhanced platelet counts in children with ITP.

Interestingly, the current study showed that the percentage of $CD14^+CD32B^+$ monocytes in the ITP patient $CD14^+$ monocyte population, both before and after the IVIG treatment, were significantly lower than that in the control subjects. These results suggest that the expression levels of FcγRIIB on peripheral monocytes may contribute the development of thrombocytopenia in children with ITP.

The primary limitations of this study were that the time of PBMC sampling (days) after IVIG therapy and the doses of IVIG administered were varied. Regardless, this study points to the need for further studies to clarify the mechanism of action of IVIG in human ITP. In conclusion, in this study, IVIG was not observed to increase FcγRIIB expression on monocytes in children with ITP.

The authors have no conflicts of interest to declare.

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Figure legend

Figure. 1

The expression of CD16(A) and CD32B(B) on peripheral $CD14^{+}$ monocytes and macrophages in Patient 2, as analyzed by two-color flow cytometry both before intravenous immunoglobulin (IVIG) and after IVIG treatment.

Table.1 Clinical characteristics of children with ITP

	Age,sex		PBMC counts on admission (/μl)	Plt count on admission ($\times 10^9$/l)	Administration method of IVIG	Sampling days after IVIG	Prognosis
1	10M	F	4,539	11	400mg/kg/day during 5days	2	acute
2	7Y6M	M	4,516	4	1g/kg/day during 2 days	5	acute
3	5Y6M	F	3,584	5	1g/kg/day during 2 days	4	acute
4	8Y3M	M	1,593	2	1g/kg/day during 2 days	2	acute
5	2Y0M	F	4,414	9	1g/kg/day during 2 days	2	acute
6	1Y7M	M	7,171	9	1g/kg/day during 2 days	3	acute
7	2Y1M	M	4,845	10	1g/kg/day during 2 days	4	acute
8	1Y1M	F	5,405	33	2g/kg/day	4	acute
9	8M	M	15,696	10	1g/kg/day during 2 days	7	acute
10	1Y2M	M	5,387	7	1g/kg/day during 2 days	7	acute
v11	1Y9M	F	6,242	18	1g/kg/day during 2 days	2	acute
12	10Y9M	M	1,744	6	1g/kg/day during 2 days	2	acute
13	2Y11M	F	3,383	1	1g/kg/day during 2 days	3	acute
14	6Y3M	F	3,357	10	2g/kg/day	6	acute
15	1Y8M	M	3,878	2	1g/kg/day during 2 days	3	acute
16	1Y10M	M	3,506	6	2g/kg/day	6	acute
17	1Y9M	F	6,699	14	2g/kg/day	4	acute
18	2M	M	4,863	7	2g/kg/day	8	acute
19	1Y11M	M	6,455	9	2g/kg/day	6	acute
20	4M	M	6,429	10	2g/kg/day	16	acute
median (range)	2Y11M (2M-10Y9M)		5,406 (1,593-15,696)	9.5 (1-33)		4.8 (2-16)	

Table.2 The comparison of CD14+ and CD14+CD16+ monocytes between patients with ITP and control subjects

	ITP (n=20)		Controls (n=18)	*P* value		
	Before IVIG Group A	After IVIG Group B	GroupC	A vs B	A vs C	B vs C
PBMCs(/μl)	5,046±3,045	4,599±1,834	5,870±1,789	0.218	0.096	0.050
CD14+cells in PBMCs(%)	11.3±6.7	11.1±7.2	6.2±2.7	0.341	0.004	0.008
CD14+cells(/μl)	482±224	454±221	344±153	0.247	0.008	0.111
CD14+CD16+ cells in CD14+ cells(%)	5.94±6.00	3.88±2.72	4.08±1.42	0.097	0.977	0.373

Table 3 The comparison of $CD14^{+}CD32B^{+}$ monocytes between patients with ITP and control subjects

	ITP (n=20)		Controls (n=18)	*P* value		
	Before IVIG Group A	After IVIG Group B	GroupC	A vs B	A vs C	B vs C
CD14+CD32B+ cells in PBMCs(%)	0.31±0.44	0.33±0.57	0.31±0.42	0.140	0.988	0.393
CD14+CD32B+ cells(/μl)	11.8±15.0	16.0±30.6	16.8±23.6	0.145	0.673	0.143
CD14+CD32B+ cells in CD14+ cells(%)	2.64±3.04	2.99±5.34	4.10±3.16	0.147	0.033	0.009

Figure.1