EDTA Inhibits the Binding of Clone 96.2C1, an Anti-CD41a Monoclonal Antibody, to the Platelets and Addition of Heparin and $CaCl_2$ to the Antibody Neutralizes the EDTA-induced Inhibitory Effect

Hyojin Chae M.D. and Hun Hee Park

Department of Laboratory Medicine, The Catholic University of Korea, Seoul, Korea

Background: The binding of some monoclonal antibodies platelet glycoprotein (GP) IIb/IIIa, which is frequently used for flow cytometric immnophenotyping, is known to be inhibited by EDTA. To select the ideal antibodies to be included in the 'Acute Leukemia Panel' for immunophenotyping of acute leukemia, we compared the inhibitory effect of EDTA on the binding of 5 different clones of monoclonal antibodies to platelet GP IIb/IIIa. We also discovered a simple method to neutralize this inhibitory effect.
Methods: Flow cytometric measurement of the number of platelet GP IIb/IIIa binding sites with different anticoagulants was performed using a panel of 5 clones of monoclonal antibodies against CD41 (clone PM6/248), CD41a (clone 96.2C1 & clone HIP8), CD41b (clone HIP2) and CD61 (clone VI-PL2), and the results are expressed as the mean equivalent soluble fluorochrome (MESF) values.
Results: The MESF value of the EDTA platelets stained with anti-CD41a, clone 96.2C1 antibody showed a significantly lower value than the MESF of platelets anticoagulated with heparin or citrate ($P<0.001$). The inhibitory effect of EDTA on the binding of anti-CD41a, clone 96.2C1 antibody to the platelets was neutralized by addition of heparin and $CaCl_2$. The mean MESF value of EDTA platelets stained with anti-CD41a, clone 96.2C1 antibody was significantly increased by the addition of heparin and $CaCl_2$ (P=0.0001).
Conclusion: The false-negative results of the binding of anti-CD41a, clone 96.2C1 antibody to the platelets seem to be due to the calcium chelating property of EDTA, and the addition of $CaCl_2$ and heparin could be used as an easy compensatory measure for the inhibitory effect of EDTA on other antibodies as well. ***(Korean J Hematol 2009;44:42-46.)***

Key Words: Anti-CD41a, Clone 96.2C1, Platelet glycoprotein IIb/IIIa, EDTA, $CaCl_2$

INTRODUCTION

Ethylenediamine tetra-acetic acid (EDTA) is the anticoagulant of choice for hematology cell counts and cell morphology. It is also one of the most commonly used anticoagulants for flow cytometric immunophenotyping.[1,2] CD41 and CD61 antigens, also known as glycoprotein (GP) IIb and IIIa, are the major cell surface glycoproteins in the platelet plasma membrane.[3] They are receptors for fibrinogen, von Willebrand's factor, fi-

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교신저자 : 박훈희, 서울시 서초구 반포동 505
㉾ 137-701, 가톨릭대학교 서울성모병원 진단검사의학과
Tel: 02-2258-1655, Fax: 02-2258-1719
E-mail: phh5240@hanmail.net

Correspondence to : Hun Hee Park, M.D.
Department of Laboratory Medicine, Seoul St. Mary's Hospital, The Catholic University of Korea
505, Banpo-dong, Seocho-gu, Seoul, Korea
Tel: +82-2-2258-1655, Fax: +82-2-2258-1719
E-mail: phh5240@hanmail.net

bronectin, and vitronectin and have been shown to be essential for platelet aggregation and adhesion.[4] Antibodies to one or both of these antigens are usually included in the 'Acute Leukemia Panel' for immunophenotyping in a typical hematology laboratory for the diagnosis of acute megakaryoblastic leukemia. It is also used for labeling platelets for immunoplatelet measurement.[5]

Binding of some of these antibodies is known to be inhibited by EDTA.[6] To select ideal antibodies to be included in the 'Acute Leukemia Panel' we compared the inhibitory effect of EDTA on binding of 5 different clones of monoclonal antibodies to platelet specific glycoproteins. In this article we present the results of these investigations.

MATERIALS AND METHODS

1. Platelet preparation

Peripheral blood samples from 8 healthy volunteers (including 6 authors) who had not taken aspirin for at least 10 days were collected in 3 tubes each containing different anticoagulants, K3-EDTA (Sekisui, Tokyo, Japan), sodium heparin (Becton Dickinson, San Jose, CA, USA), and sodium citrate (Greiner, Linz, Austria). Platelet rich plasma (PRP) was collected from peripheral blood by centrifugation.[7] The platelet count of PRP was measured using automatic hematology analyzer (XE-2100, Sysmex, Kobe, Japan) and the PRP was diluted to a platelet count of 10×10^9/L using phosphate buffered saline (PBS, pH 7.4) as diluent. Informed consents were obtained from all volunteers.

2. Comparative quantitative analysis of the number of platelet GP IIb/IIIa binding sites on the circulating platelets

Flow cytometric measurement of the number of platelet GP IIb/IIIa binding sites on the circulating platelets was performed using a panel of 5 clones of monoclonal antibodies against CD41 (clone PM6/248, Serotec, Oxford, UK), CD41a (clone 96.2C1, Ancell, Bayport, MN, USA & clone HIP8, Becton Dickinson, San Diego, CA, USA), CD41b (clone HIP2, BD Pharmingen, San Diego, CA, USA) and CD61 (clone VI-PL2, BD Pharmingen).

Briefly, 10uL each of fluorescein isothiocyanate (FITC) labeled anti-CD41, CD 41a, CD41b antibodies and phycoerythrin (PE)-conjugated anti-CD61 antibody were added to 100uL of diluted PRP and mixed. The mixture was then incubated in the dark for 15 minutes at room temperature. After washing with 3mL of PBS, the platelets were resuspended in 0.5mL of PBS and were analyzed using flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA, USA) and CellQuest program (Becton Dickinson). Quantum FITC and PE mean equivalent soluble fluorochrome (MESF) kits (Bangs Laboratories, Fishers, IN, USA) were analyzed together with the samples for comparative fluorescence analysis. Platelets and the control beads were gated using forward scattering (FSC) and side scattering (SSC) characteristics on a dot plot. The results were expressed as mean geometric fluorescence intensities, and the MESF was calculated using QuickCal v. 2.3 program (Bangs Laboratories).

3. Neutralization of the inhibitory effect of EDTA

To neutralize the inhibitory effect of EDTA on the binding of anti-CD41a, clone 96.2C1 antibody to the platelets, heparin and $CaCl_2$ were added to the antibody. One hundred units of sodium heparin and 10mg of $CaCl_2$ (Kanto chemical co. Inc, Japan) were dissolved in 1mL of distilled water, and 1 part of this mixture was added to 9 parts of antibody and kept at 4^oC until use. Flow cytometric measurement of the number of anti-CD41a binding sites on the circulating platelets from 8 normal healthy volunteers using this antibody mixture was performed in the same manner as described above.

4. Statistical analysis

Comparison of the numbers of binding sites of

the antibodies on the platelets among different anticoagulants was performed using one-way ANOVA test. *P* values less than 0.05 were considered statistically significant. When ANOVA test showed significant results, Student-Newman-Keuls test was performed for comparison of pair subgroups. Correlations of the number of binding sites of the antibodies on the platelets between different anticoagulants were estimated using Pearson's correlation coefficients and *P* value.

RESULTS

The results of comparative quantitative analysis of the number of binding sites of various clones of antibody on the platelets anticoagulated with different anticoagulants are shown as MESF in Table 1. The MESF value of EDTA platelets stained with anti-CD 41a, clone 96.2C1 antibody showed significantly lower value than MESF value of platelets anticoagulated with heparin or citrate with a F-ratio of 33.5 and a *P* value of <0.001 by one way ANOVA test. The MESF values of platelets stained with other antibody clones, anti-CD 41a (clone HIP 8), anti-CD41b (clone HIP 2), anti-CD41 (clone PM6/248), and anti-CD61 (clone VI-PL2) were not significantly different according to anticoagulants ($P>0.05$). Student-Newman-Keuls test for pairwise comparisons showed that only the MESF value of EDTA platelets stained with anti-CD 41a, clone 96.2C1 was different from that of heparinized and citrated platelets ($P<0.05$). When MESF data of anti-CD41a, clone 96.2C1 antibody were excluded, Pearson's correlation coefficients and *P* values between different anticoagulants showed good correlation (r=0.8652, $P=0.0119$ between EDTA and heparin, r=0.9814, $P=0.0001$ between EDTA and citrate, r=0.9758, $P<0.001$ between heparin and citrate).

Anti-CD41a, clone 96.2C1 antibody did not bind well to the platelets in EDTA-anticoagulated blood, and caused false negative results on the immunophenotyping (Fig. 1). The inhibitory effect of EDTA on binding of anti-CD41a, clone 96.2C1 antibody to the platelets was neutralized by addition of heparin and $CaCl_2$ in the antibody (Fig. 1). The mean MESF value of EDTA platelets stained with anti-CD 41a, clone 96.2C1 antibody was 2,707±3,681, and that stained with the same antibody mixed with heparin and $CaCl_2$ was significantly increased to 32,397±9,533 ($P=0.0001$).

DISCUSSION

Glycoprotein IIb/IIIa molecules are known to exist in a heterodimer complex in the intact platelet membrane, and when EDTA is added for anticoagulation, the lowered calcium level induces dissociation of the heterodimer complex into pure IIb and IIIa molecules.[8] As the heterodimer dissociates conformational changes of the molecule occur and this modifies, exposes or hide cer-

Table 1. MESF values of 5 clones of monoclonal antibodies against platelet glycoprotein IIb/IIIa with different anticoagulants

Antibody clone \ Anticoagulant		EDTA	Heparin	Citrate
		Mean±SD	Mean±SD	Mean±SD
CD41a	96.2C1*	2,092±2,182	77,529±21,110	91,531±21,830
CD41a	HIP8	45,993±3,397	48,655±4,517	49,487±4,216
CD41b	HIP2	2,367±129	2,210±205	2,364±469
CD41	PM6/248	40,908±10,948	35,155±6,118	38,595±7,941
CD61	VI-PL2	7,248±311	6,490±337	6,906±264
Negative control		307±5	304±5	307±7

*Clone 96.2C1 showed significant difference among anticoagulants (one way ANOVA test, $P<0.001$).
Abbreviations: MESF, mean equivalent soluble fluorochrome.

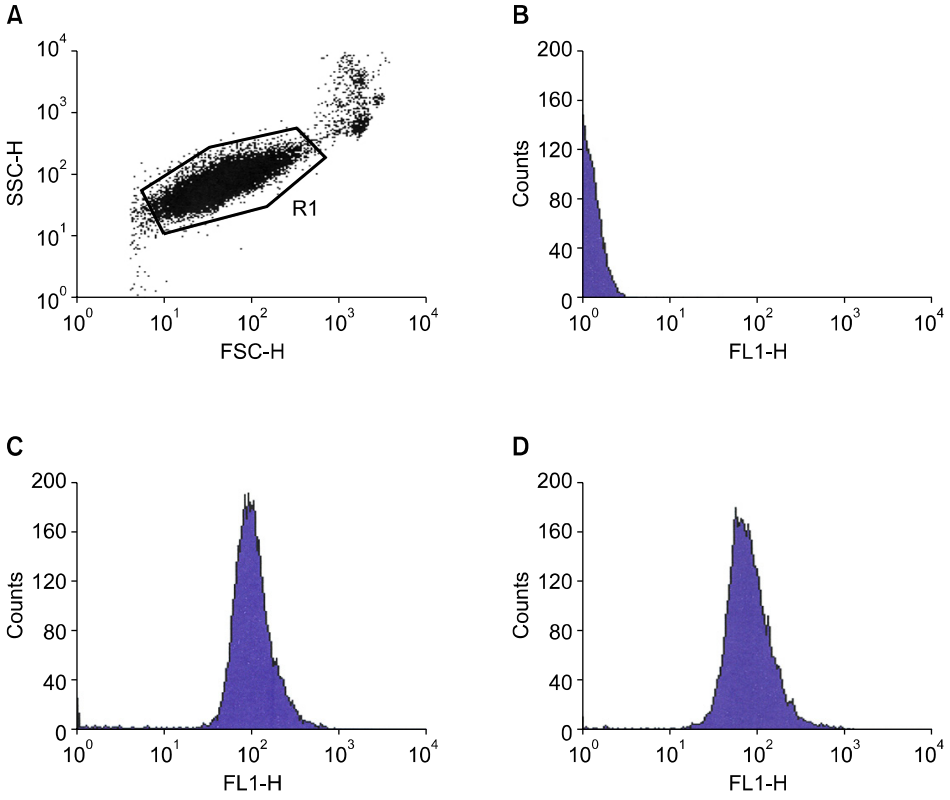

Fig. 1. Scattergram (A) and histograms of normal platelets after staining with FITC labeled anti-CD41a, clone 96.2C1 monoclonal antibody. EDTA-anticoagulated platelets (B) show low fluorescence consistent with inhibition of antibody binding, but citrated same platelets (C) and EDTA-anticoagulated platelets after staining with CD41a, clone 96.2C1 monoclonal antibody plus $CaCl_2$ and heparin show high fluorescence (D). It means the inhibitory effect of EDTA is completely neutralized by addition of heparin and $CaCl_2$.

tain epitopes.[9] The inhibitory effect of EDTA on the binding of antibody to the platelets was not seen with other antibodies including another clone of antibody against CD41a. Therefore, only the epitope of the anti-CD41a, clone 96.2C1 antibody is a EDTA-dependent antigen modified or hidden as the heterodimer dissociates when the calcium levels are lowered by EDTA. And since EDTA is known as a much stronger calcium chelating agent than citrate,[10] and is capable of removing calcium from the binding sites within the integrin molecules, this explains the fact that citrated platelets showed excellent binding with this same antibody even though it also is a calcium chelating agent.

The inhibitory effect is enough to cause false negative results on the immunophenotyping analysis. We had two cases of acute megakaryoblastic leukemia whose immunophenotyping results were misinterpreted as CD41a negative using anti-CD41a, clone 96.2C1 antibody and EDTA anticoagulated bone marrow samples (data not shown). Both cases were later proven to be CD41a positive using heparinized bone marrow samples with residual samples remained after chromosome analysis. The inhibitory effect of EDTA on the binding of anti-CD41a, clone 96.2C1 antibody to the platelets was neutralized by addition of heparin and $CaCl_2$. The specific concentration of heparin and $CaCl_2$ used for neutralization was deduced from a series of dilution experiments and the concentration that had maximal neutralization effect was chosen. The addition of heparin and $CaCl_2$ is a very simple and easy method for removing the inhibitory effect of EDTA, and although our data is limited to anti-CD41a, clone 96.2C1 antibody, this can be used as an easy compensatory measure for the inhibitory effect of EDTA on other antibodies as well. Since EDTA inhibited binding of some of the monoclonal antibodies against CD41a could lead to false negative results on immunophenotyping analysis of acute leukemia bone marrow samples, when selecting antibodies to be included in the 'Acute Leukemia Panel' one should verify the binding of the clone of anti-CD41a antibody

to EDTA anticoagulated platelets, and when this should show inhibition then $CaCl_2$ and heparin supplementation to the antibody should be incorporated as a routine measure to neutralize the inhibition.

요 약

배경: 면역표현형 검사에 자주 포함되는 혈소판 당단백 IIb/IIIa (CD41, CD61)에 대한 단일클론 항체는 EDTA에 의해 혈소판 항원과의 결합이 저해되는 것으로 알려져 있다. 그러므로 저자들은 급성백혈병 면역표현형검사패널에 포함시킬 혈소판 항체를 선별하기 위해 5개의 서로 다른 단일클론 항체에 대한 EDTA의 억제효과를 비교해 보았으며, EDTA에 의한 억제반응을 중화시킬 수 있는 간단한 방법을 발견하였다.

방법: 서로 다른 항응고제와 5개의 서로 다른 단일클론 항체를 사용하여 혈소판 당단백 IIb/IIIa 결합 부위의 수를 유세포 분석기를 이용하여 측정하였으며 이를 mean equivalent soluble fluorochrome (MESF) 수치로 환산하였다.

결과: 5개의 항체 중, 항-CD41a 항체(clone 96.2C1)의 경우에만, EDTA를 항응고제로 사용할 경우의 MESF 수치가 헤파린이나 구연산나트륨을 항응고제로 사용할 경우보다 통계적으로 유의하게 낮았다($P<0.001$). 이 같은 EDTA에 의한 항-CD41a 항체(clone 96.2C1) 결합 억제효과는 헤파린과 $CaCl_2$의 첨가에 의해 중화됨을 발견하였다. 즉, EDTA 항응고제 처리를 한 혈소판에 대한 항-CD41a 항체(clone 96.2C1)의 MESF 수치는 헤파린과 $CaCl_2$의 첨가에 의해 통계적으로 유의하게 증가하였다($P=0.0001$).

결론: 항-CD41a 항체(clone 96.2C1)의 혈소판 결합 억제효과는 EDTA에 의한 칼슘 농도 저하 때문에 일어나는 것으로 생각되며, 헤파린과 $CaCl_2$의 첨가에 의한 중화방법은 본 항체뿐만 아니라 EDTA에 의해 결합이 억제되는 다른 항체들에 대해서도 적용시킬 수 있는 편리하고 간단한 방법이다.

REFERENCES

1) Neumeier D, Pauls R, Wifling I, Fateh-Moghadam A, Knedel M. Immunophenotyping of lymphocyte subpopulations using monoclonal antibodies in an analytical flow cytometer: evaluation of the method and reference values. J Clin Chem Clin Biochem 1985; 23:765-75.
2) Behnken LJ, Heicke B, Löhr G, Maier-Lenz H, Musil J. Detection of lymphocyte subpopulations in EDTA whole blood samples using flow cytometry. Stability and normal values. Arzneimittelforschung 1985;35:1311-6.
3) Carrell NA, Fitzgerald LA, Steiner B, Erickson HP, Phillips DR. Structure of human platelet membrane glycoproteins IIb and IIIa as determined by electron microscopy. J Biol Chem 1985;260:1743-9.
4) Fitzgerald LA, Phillips DR. Calcium regulation of the platelet membrane glycoprotein IIb-IIIa complex. J Biol Chem 1985;260:11366-74.
5) Nishiyama M, Hayashi S, Futsukaichi Y, Suehisa E, Kurata Y. Usefulness of immunoplatelet measurement using the flow cytometric method for accurate platelet counting in hematological patients with severe thrombocytopenia. Rinsho Byori 2004;52:103-8.
6) Serotec. Mouse anti-human CD41 antibody datasheet. WWWdocument. URL. http://www.ab-direct.com/catalog/datasheet-MCA467A647T.html
7) Bauer JD. Methods in coagulation disorders. In: Bauer JD, Ackermann PG, Toro G, eds. Clinical Laboratory Methods. 9th ed. Mosby, USA: Mosby, 1982:256.
8) Nomura S, Nagata H, Oda K, Kokawa T, Yasunaga K. Effects of EDTA on the membrane glycoproteins IIb-IIIa complex--analysis using flow cytometry. Thromb Res 1987;47:47-58.
9) Fiorin F, Steffan A, Pradella P, Bizzaro N, Petenza R, De Angelis V. IgG platelet antibodies in EDTA-dependent pseudothrobocytopenia bind to platelet membrane glycoprotein IIb. Am J Clin Pathol 1998; 110:178-83.
10) Holt C. Inorganic constituents of milk. III. The colloidal calcium phosphate of cow's milk. J Dairy Res 1982;49:29-38.