[原著] Estimation of rabbit fibrinopeptide B using a monoclonal antibody

Estimation of rabbit fibrinopeptide B using a monoclonal antibody

Kiyohiko Kinjoh, Mariko Nakamura, Tetsuya Nejime,
Yoshihiro Tokeshi and Tadayoshi Kosugi

First Department of Physiology, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

(Received on August 4, 1998, accepted on November 24, 1998)

ABSTRACT

We produced a monoclonal antibody against rabbit fibrinopeptide B (FPB) and established an immunological assay system to estimate it. Rabbit FPB was purified from the supernatant of fibrin clot prepared with rabbit fibrinogen and bovine thrombin. The purified rabbit FPB was coupled to keyhole limpet hemocyanin (KLH), and BALB/c mice were immunized with the resultant FPB-KLH. The spleen cells of immunized mice were hybridized with P3-X63-Ag8-U1 myeloma cells. As a result, one hybridoma (IA12/4A2) was selected, which secreted an antibody against rabbit FPB. The monoclonal antibody obtained belonged to the IgM class and its light chain consisted of a κ-chain. The monoclonal antibody reacted not only with rabbit FPB but also rabbit fibrinogen, however it did not react with rabbit fibrinopeptide A. Utilizing this monoclonal antibody, we attempted to develop a competitive enzyme-linked immunoassay for estimating purified FPB. The working range of FPB varied between 5 and 50 μg/ml. *Ryukyu Med. J., 18(4)129~133, 1998*

Key words: rabbits, monoclonal antibody, rabbit fibrinopeptide B, competitive enzyme-linked immunoassay (CELIA).

INTRODUCTION

Thrombin releases fibrinopeptide A (FPA) initially and bring about the conversion of fibrinogen to fibrin I, then it releases fibrinopeptide B (FPB) consecutively and forms fibrin II[1-3]. Thus, FPA levels in plasma provide a direct index of thrombin action *in vivo*, and FPB levels in plasma reflect fibrin II formation *in vivo*, which may indicate the occurrence of occlusive thrombosis[4-6]. Consequently, for the investigation of the treatment of thrombotic disorders, radioimmunoassays for dog FPA[7], rat FPA[8] and guinea-pig FPA[9] have been developed. We have developed a competitive ELISA method (competitive enzyme-linked immunoassay, CELIA) for estimating rabbit FPA[10]. It has been reported that human FPB, which is an index of the formation of fibrin II, also possesses many biological effects such as the formation of atherosclerotic lesions[11,12]. Therefore, it is important to develop assay procedures for fibrinopeptide B using experimental animals in order to investigate thrombotic and atherosclerotic processes. However, no practical methods for the measurement of animal FPB have been developed. Therefore, we set out to develop an immunological method for estimating rabbit FPB. In the present study, we produced a monoclonal antibody against rabbit FPB, and attempted to develop a method for estimating rabbit FPB.

MATERIALS AND METHODS

Animals

BALB/c mice (six weeks old), and rabbits (Japanese white) weighing, 3 to 4 kg were purchased from Kyudo Co., Ltd. (Kumamoto, Japan).

Animal care and management was in compliance with the "Standard Relating to the Care and Management of Experimental Animals" (Notification No. 6, March 27, 1980, from the Prime Minister's Office, Tokyo, Japan) and the Guide for Animal Experiments of the University of the Ryukyus for the care and use of the animals. The animal experiment in this study was approved by the Committee on Animal Experiment of the University of the Ryukyus.

Reagents

Rabbit fibrinogen was purified according to the technique of Doolittle *et al.*[13] Bovine thrombin was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Rabbit FPB was prepared by column chromatography on Dowex 50W-X2 following the procedure of Blombäck *et*

al.[14,15], as described previously[10]. Sephacryl S-300 was purchased from Pharmacia Fine Chemicals Co., Ltd. (Uppsala, Sweden). Poly-L-lysine, keyhole limpet hemocyanin (KLH), and 2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dowex 50W-X2 (200-400 mesh; hydrogen form) and peroxidase (POD)-conjugated goat anti-mouse IgG and Mouse Typer® sub-isotyping kit were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). POD-conjugated anti-mouse IgM was purchased from The Binding Site (Birmingham, U.K.). Freund's complete adjuvant was purchased from Difco Laboratories (Detroit, MI, U.S.A.). Polyethylene glycol 4000 (for gas chromatography) was purchased from E. Merck AG (Darmastadt, F.R.G.). Glutaraldehyde was purchased from Nacalai tesque, Inc. (Kyoto, Japan). The mouse myeloma cell line P3-X63-Ag8-U1 (P3U1) was obtained from American Type Culture Collection (ATCC). Unless otherwise stated, only reagent-grade chemicals were used in all experiments.

Preparation of immunogen

Purified FPB was coupled to KLH using glutaraldehyde following the method of Ito *et al.*[16], as described previously[10].

Mouse immunization

A volume of 1.75 ml of FPB-KLH conjugate (520μg /ml) was mixed and emulsified with an equal volume of Freund's complete adjuvant, and 0.5 ml of this mixture was injected intraperitoneally into each of five male BAL B/c mice (seven weeks old) at an interval of 3 weeks for a total of 4 injections. At week 17, the last administration was performed by intravenous injection (0.1 ml of FPB-KLH conjugate) into the tail vein. Three days after the last injection, spleen cells of the immunized mice were subjected to cell fusion.

Cell fusion

The fusion procedure was based on the method of Köhler and Milstein[17], as described previously[10,18]. Spleen cells (4.1×10^8 cells) from an immunized mouse were mixed with 5.4×10^7 myeloma cells (P3U1), then hybridized by the addition of 50% (w/w) polyethylene glycol 4000. The procedure was similar to that described previously[18].

Determination of the mouse immunoglobulin class and subclass

The immunoglobulin class and subclass of the mouse monoclonal antibody were determined using the Mouse Typer® sub-isotyping kit, as described previously[18].

Purification of monoclonal antibody

Sephacryl S-300 was equilibrated in Dulbecco's PBS and packed into a column, 0.9×59 cm. The elution was carried out with Dulbecco's PBS. The flow rate was adjusted to 5 ml/hr, and eluates of 0.5 ml per tube were collected with a fraction collector (LKB 2112 Radirac Fraction Collector). Three milliliters of the culture medium was applied to this column. The eluted proteins were monitored by absorbance at 280 nm and the Folin-Lowry procedure[19]. The titer of anti-FPB antibody was determined by ELISA as described in the subsequent paragraph.

ELISA (enzyme-linked immunosorbent assay)

The coating of FPB onto polystyrene microtiter plate was carried out by coupling to polylysine coated plates with glutaraldehyde, as described previously[10]. After blocking with 1% gelatin, anti-FPB monoclonal antibody was added to each well. After incubation, POD-conjugated anti-mouse immunoglobulin was added to each well. Finally the substrate (ABTS) solution was added, and spectrophotometric readings (at 415 nm) were made, as described previously[10,18].

A competitive enzyme-linked immunoassay (CELIA) for the measurement of rabbit FPB

CELIA for the measurement of rabbit FPB was performed according to the method of Soria *et al.*[20], as described previously[10]. FPB (0-50 μg/ml) was mixed with equal volume of anti-FPB monoclonal antibody (10 μg/ml). After incubation (room temperature, 90 min), this FPB-anti-FPB mixtures were added to the FPB-coated wells containing 25 μg/ml of FPB. The following detection of anti-FPB was similar to that of ELISA.

RESULTS

Production of hybridoma

Among 21 wells in which cell growth was observed, four wells indicated the presence of antibodies to purified FPB, and hybridoma in three wells with the highest titer were used for limiting dilution. As a result, one hybridoma clone IA12/4A2, which showed the most rapid growth and the highest anti-FPB titers, was selected.

Immunoglobulin class and subclass

The monoclonal antibody produced by clone IA12/4A2 turned out to be IgM and its light chain consisted of a κ-chain.

Specificity of anti-FPB monoclonal antibody

On ELISA using the culture medium of clone IA12/4A2, the wells coated with rabbit FPB showed a positive reaction. However, FPA-coated wells did not reveal a positive reaction. The rabbit fibrinogen-coated well demonstrated a positive reaction only at concentrations greater than 100 μg/ml (Fig.1).

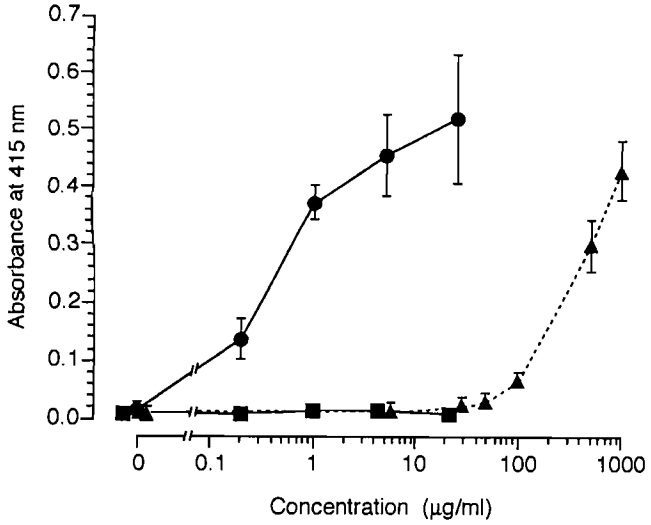

Fig. 1 Specificity of the monoclonal antibody using ELISA.
The response of monoclonal antibody in the culture media to wells which had been coated with FPB (●——●), FPA (■——■), and fibrinogen (▲----▲) is shown. Results are expressed as means ± S.D. (n = 4).

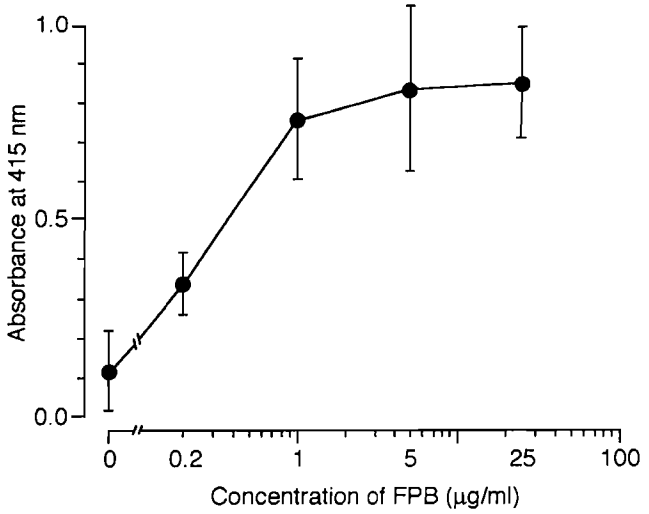

Fig. 3 Correlation between the concentration of FPB which was used for coating and the absorbance at 415 nm on ELISA.
Results are expressed as means ± S.D. (n = 4).

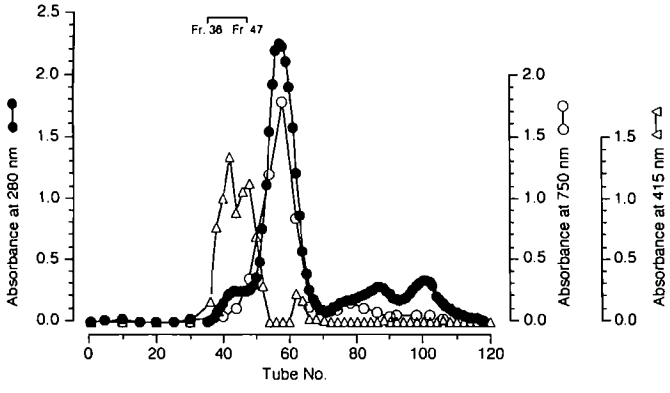

Fig. 2 The profile of gel filtration on Sephacryl S-300 to purify the monoclonal antibody.
The protein concentration was measured at 280 nm (●——●) and at 750 nm on the Folin-Lowry procedure (○——○). The anti-FPB activity of monoclonal antibody was determined by ELISA (△——△).

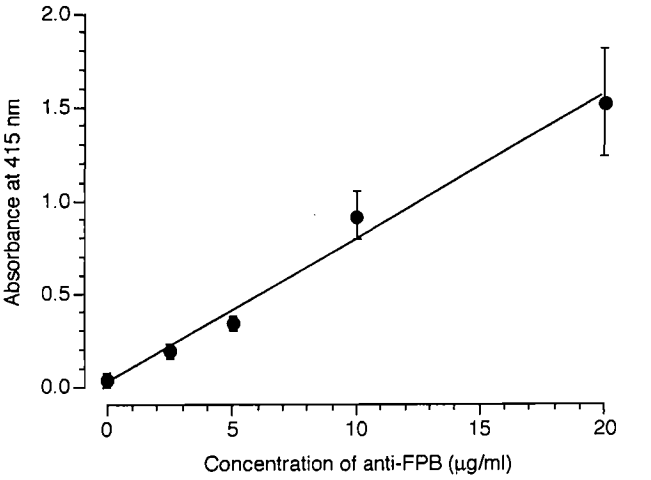

Fig. 4 Correlation between the concentration of anti-FPB monoclonal antibody and the absorbance at 415 nm on ELISA.
Results are expressed as means ± S.D. (n = 4).

Purification of anti-FPB antibody

The profile of gel filtration of the culture medium from the hybridoma using Sephacryl S-300 is shown in Fig. 2. The culture medium revealed several peaks of protein eluates, and the peak corresponding to the void volume demonstrated the anti-FPB activity. The eluates from tube No. 36 to No. 47 were pooled and employed in subsequent experiments.

Correlation between the ELISA value and the concentration of FPB coated onto microtiter plate

Various concentrations of rabbit FPB (0-25 μg/ml) were used to coat microtiterplate wells and then purified anti-FPB monoclonal antibodies (10 μg/ml) were added to each well. As shown in Fig. 3, the absorbance was increased in parallel with the concentration of FPB which was used for coating.

Correlation between the ELISA value and the concentration of anti-FPB monoclonal antibody

Twenty-five μg/ml of rabbit FPB was coated onto a microtiter plate and serially diluted anti-FPB monoclonal antibodies (0-20 μg/ml) was added to each well. As shown in Fig. 4, a good linear relationship between the ELISA value and the concentration of anti-FPB antibodies was observed.

Estimation of FPB using CELIA

The result of the CELIA with rabbit FPB and anti-

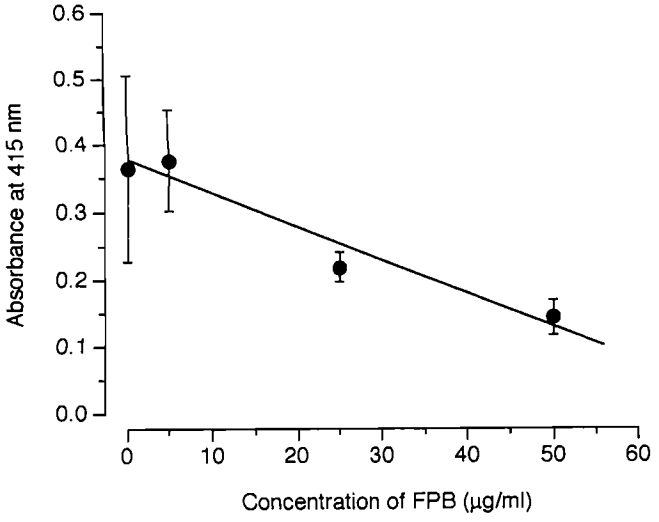

Fig. 5 Estimation of the FPB concentration using CELIA. Results are expressed as means ± S.D. (n = 3).

FPB monoclonal antibody is shown in Fig. 5. A linear relationship between the ELISA value and the concentration of FPB was observed, and the working range of FPB varied between 5.0 and 50 μg/ml. The equation of the regression line was $y=-0.0049x+0.374$, and the correlation coefficient, $r=0.947$.

DISCUSSION

Some problems have been encountered in the assay of human FPB in the circulating blood; that is, anti-human FPB antibodies have been demonstrated to cross-react not only with human fibrinogen but also with Bβ-chain fragments, Bβ 1-21 and Bβ 1-42, which were the products cleaved by plasmin[5,21,22]. Our anti-FPB antibody in the present study showed a cross-reaction with rabbit fibrinogen. Consequently, this antibody might also cross-react with Bβ-chain fragments of rabbit fibrinogen digested by plasmin. For the selective detection of rabbit FPB in plasma samples, it is necessary to remove cross-reactive fibrinogen and Bβ-chain fragments by alcohol precipitation and/or adsorption onto bentonite, as described by Eckfardt *et al.*[21]. In addition, human FPB was rapidly converted by plasma carboxypeptidase B to its desarginyl derivative which demonstrated a major decrease in immunoreactivity to anti-human FPB antibodies[23,24]. The amino acid sequence of rabbit FPB used in this experiment proved to be ADDYDDEVLPDAR, which was identical to that reported by Blombäck *et al.*[25] (data not shown). In plasma samples, therefore, the C-terminal residue (Arg) in rabbit FPB could be cleaved by carboxypeptidase B. The immunochemical properties of rabbit FPB and its derivatives or FPB-containing fragments have not been investigated. Our anti-rabbit FPB monoclonal antibody would be useful to clarify these problems. As mentioned previously, FPB possesses many biological effects such as a chemoattractant or stimulant for macrophages and smooth muscle cells in the presence of hyperchlesterolemia[11,12] as well as a constrictor of perfused aorta and isolated uterus[26-28]. However no amino acid residue has been identified to be responsible for these biological effects[27]. Therefore, a accurate measurement of native FPB in rabbit plasma is needed to clarify its physiological roles.

It has been reported that the range of plasma FPB of normal healthy persons is below 0.6 pmol/ml[21]. However, in this study, the working range of the CELIA procedure for measuring rabbit FPB was found to be between 5 and 50 μg/ml (3.5-34.5 nmol/ml), which is low in sensitivity to detect rabbit FPB in the circulating blood. There is therefore the need to improve the procedure described in this study for estimating rabbit FPB at submicrogram levels. Radioimmunoassay or techniques utilizing fluorochrome conjugates could provide an enhanced sensitivity for peptide detection.

REFERENCES

1) Blombäck B., Hessel B., Hogg D. and Therkildsen L.: A two-step fibrinogen-fibrin transition in blood coagulation. Nature 275: 501-505, 1978.
2) Scheraga H.A.: Chemical basis of thrombin interaction with fibrinogen. Ann. N.Y. Acad. Sci. 485: 124-133, 1986.
3) Weisel J.W., Velklich Y. and Gorkun O.: The sequence of cleavage of fibrinopeptides from fibrinogen is important for protofibril formation and enhancement of lateral aggregation in fibrin clots. J. Mol. Biol. 232: 285-297, 1993.
4) Blombäck B., Hessel B., Okada M. and Egberg N.: Mechanism of fibrin formation and its regulation. Ann. N.Y. Acad. Sci. 370: 536-544, 1981.
5) Bilezikian S.B., Nossel H.L., Butler V.P., Jr. and Canfield R.E.: Radioimmunoassay of human fibrinopeptide B and kinetics of fibrinopeptide cleavage by different enzymes. J. Clin. Invest. 56: 438-445, 1975.
6) Butler V.P. Jr., Nossel H.L. and Canfield R.E.: Immunoassay of human fibrinopeptides. Methods Enzymol. 84: 102-118, 1982.
7) Wilner G.D.: Measurement of fibrinopeptide A in canine blood - an interim report. Thromb. Res. 15: 601-610, 1979.
8) McLaughlin L.F., Drummond O. and MacGregor I.R.: A novel rat model of thrombogenicity: its use in evaluation of prothrombin complex concentrates and high purity factor IX concentrates. Thromb. Haemost. 68: 511-515, 1992.
9) Chen J.P., Marsh L.C. and Schroeder E.C.: Haemostatic derangements associated with arenavirus infection in the guinea-pig: radioimmunoassay of fibrinopeptide A to assess thrombin action in infected animals. Blood Coagul. Fibrinolysis 4: 165-172, 1993.

10) Kinjoh K., Nakamura M. and Kosugi T.: Application of a monoclonal antibody to estimate rabbit fibrinopeptide A released by habutobin. Toxicon 32: 1413-1423, 1994.
11) Singh T. M., Kadowaki M. H., Glasgov S. and Zarins C. K.: The role of fibrinopeptide B in early atherosclerotic lesion formation. Am. J. Surg. 160: 156-159, 1990.
12) Kadowaki M.K., Singh T.M., Zarins C. K., Glasgov S. and Meredith S.C.: The effects of hypercholesterolemia on early atherosclerotic lesions initiated by fibrinopeptide B. J. Surg. Res. 51: 524-528, 1991.
13) Doolittle R.F., Schubert D. and Schwartz S.A.: Amino acid sequence studies on artiodactyl fibrinopeptides. I. Dromedary camel, mule deer, and cape buffalo. Arch. Biochem. Biophys. 118: 456-467, 1967.
14) Blombäck B. and Vestermark A.: Isolation of fibrinopeptides by chromatography. Arkiv Kemi 12: 173-182, 1958.
15) Blombäck B., Boström H. and Vestermark A.: On the [^{35}S] sulphate incorporation in fibrinopeptide B from rabbit fibrinogen. Biochim. Biophys. Acta 38: 502-512, 1960.
16) Ito K., Iwata Y. and Kasama T.: Fibrinopeptide A. Rinsho Byori 81: 162-167, 1989 (in Japanese).
17) Köhler G. and Milstein C.: Continuous culture of fused cells secreting antibody of predefined specificity. Nature 256: 495-497, 1975.
18) Nakamura M., Kinjoh K. and Kosugi T.: Production of monoclonal antibody against the thrombin-like enzyme, habutobin, from *Trimeresurus flavoviridis* venom. Toxicon 30: 1177-1188, 1992.
19) Lowry O.H., Rosenbrough N.J., Farr A.L. and Randall R.L.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.
20) Soria J., Soria C. and Ryckwaert J.J.: A solid phase immuno enzymological assay for the measurement of human fibrinopeptide A. Thromb. Res. 20: 425-435, 1980.
21) Eckfardt T., Nossel H. L., Hurlet-Jensen A., La Gamma K. S., Owen J. and Auerbach M.: Measurement of desarginine fibrinopeptide B in human blood. J. Clin. Invest. 67: 809-816, 1981.
22) Plow E.F. and Edgington T.S.: Surface markers of fibrinogen and its physiologic derivatives revealed by antibody probes. Semin. Thromb. Hemost. 8: 36-56, 1982.
23) Tager-Nilsson A. C.: Degradation of human fibrinopeptide A and B in blood serum in vitro. Acta Chem. Scand. 22: 3171-3182, 1968.
24) La Gamma K.S. and Nossel H.L.: The stability of fibrinopeptide B immunoreactivity in blood. Thromb. Res. 12: 447-454, 1978.
25) Blombäck B., Blombäck M. and Gröndahl N.J.: Studies on fibrinopeptides from mammals. Acta Chem. Scand. 19: 1789-1791, 1965.
26) Gladner J. A., Murtauph P.A., Folk J.E. and Laki K.: Nature of peptides released by thrombin. Ann. N.Y. Acad. Sci. 104: 47-52, 1963.
27) Iwanaga S.: Structure and function of fibrinogen. Tannpakushitu Kakusan Koso 14: 175-195, 1969 (in Japanese).
28) Moore P. K., Hussaini I. and Bhardwaj R. Cardiovascular effect of fibrinopeptide B. J. Pharm. Pharmacol. 40: 558-561, 1988.