Vol. 124, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1984 Pages 290-295

ANTIBODIES AGAINST A 23Kd HEPARIN BINDING FRAGMENT OF THROMBOSPONDIN INHIBIT PLATELET AGGREGATION

T. Kent Gartner*, Daniel A. Walz, Martha Aiken,
Linda Starr-Spires* and Mary L. Ogilvie*

*Department of Biology
Memphis State University, Memphis, TN 38152

Department of Physiology
Wayne State University School of Medicine, Detroit, MI 48201

Received September 11, 1984

SUMMARY: Antiserum against a 23Kd heparin binding fragment of thrombospondin inhibits the aggregation of platelets in response to ADP, collagen or thrombin. The antiserum inhibits the secretion-dependent second phase, but not the primary phase of aggregation of platelets responding to ADP. Although immune serum added during the second phase of ADP-induced aggregation causes some inhibition of secretion, it also causes reversal of aggregation to the level produced during primary aggregation. Since thrombospondin is the endogenous lectin of human platelets, these results support the conclusion that the endogenous lectin mediates, at least in part, the secretion-dependent aggregation of platelets. Our data suggest that the region of thrombospondin which contains the heparin binding domain(s) present in the 23Kd fragment play(s) a critical role in secretion-dependent aggregation of platelets.

INTRODUCTION: The endogenous platelet lectin is a lectin-like agglutinin expressed by platelets in response to stimulation by a variety of agonists (1-3). The lectin, present in resting platelets in secretory vesicles called α-granules, is expressed as a consequence of secretion (2-4). The lectin is expressed exclusively as a membrane-bound activity in platelets stimulated with the Ca^{2+} ionophore A23187, γ-thrombin or thrombocytin (2). The aggregation of platelets in response to thrombin appears to be mediated, at least in part, by this agglutinin (1,3). The agglutinin is thrombospondin (5,6). Thrombospondin (TSP) is a trimeric, disulfide linked glycoprotein with an (7,8) apparent molecular weight of 450Kd which is secreted from α-granules (9) and can bind to the platelet surface (10-12). Here we report that antiserum against a 23Kd heparin binding fragment of TSP inhibits the secretion-dependent aggregation of platelets.

0006-291X/84 $1.50

MATERIALS AND METHODS: Thrombospondin (TSP) was isolated from 30 L of thrombin activated bovine platelets by the method of Lawler et al. (7). Platelet releasate was collected by centrifugation and applied to a column (1.6 x 10 cm) of heparin agarose (Bio-Rad Laboratories, Richmond, CA) pre-equilibrated with 15 mM Tris, 0.14 M NaCl, 2 mM $CaCl_2$, pH 7.6. The column was washed with 0.14, 0.20, 0.25, 0.55 and 1.0 M NaCl; thrombospondin was recovered predominately in the 0.55 M NaCl fraction. This fraction was pooled, concentrated and gel filtered through a column (5 x 95 cm) of agarose A-1.5m equilibrated in the heparin agarose elution buffer. The final product was concentrated to 1 mg/ml and stored frozen at -50 C. As judged by SDS electrophoresis, the reduced final product was >90% 180Kd form of thrombospondin. The amino-terminus of this sample was aspartic acid. Bovine TSP cross-reacts with rabbit antibodies against human TSP (unpublished observation).

PREPARATION 23Kd HEPARIN BINDING FRAGMENT: Bovine platelets were collected as above and stored frozen for 3 months at -70C. The platelets were subsequently freeze-thaw lysed and the resulting lysate fractionated by chromatography on dextran-sulfate Sepharose or heparin-Sepharose as described (13). The fraction eluted from these matrices with 0.5 M NaCl was desalted, freeze-dried, and gel filtered through G-75 equilibrated with 15 mM Tris, 0.75 M NaCl, pH 7.6. The fraction eluted last was homogenous by SDS electrophoresis and had aspartic acid as the single amino-terminus which is identical to that of TSP (14).

ANTIBODY GENERATION: Rabbits were immunized with the 23Kd heparin binding fragment or TSP as previously described (15). Sera were collected and utilized without further fractionation. Electrophoresis and subsequent immunoblotting verified the specificity of the antisera for the primary antigens as well as demonstrated that the 23Kd heparin binding fragment shared determinants with thrombospondin. Each antiserum formed lines of immunoidentity with platelet releasate products; neither antiserum reacted with the platelet released products platelet factor 4 and fibrinogen.

ADSORPTION OF ANTISERA: Sepharose 4B (Pharmacia Fine Chemicals) was derivatized (16) with fibrinogen (Kabi Sweden, L-grade fibrinogen at 30 mg/ml treated with phenylmethyl-sulfonylfluoride (Sigma Chemical Co.) at 10 mg/ml and dialyzed against 0.3 M NaCl). Anti-heparin binding fragment serum (AHBFS), 0.25 ml, was passed over the immobilized fibrinogen. The AHBFS was diluted 42X by passage over the column, nonetheless it still inhibited platelet aggregation. Anti-fibrinogen serum passed over the fibrinogen column lost its ability to precipitate fibrinogen (after adjustment to the original protein concentration). The anti-fibrinogen Ig was recovered from the immobilized fibrinogen in a functional form. The AHBFS that had been passed over the immobilized fibrinogen was adsorbed against the heparin-binding fragment (3 ml of Sepharose 4B derivatized with 3 mg heparin binding fragment). The flow-through from the column did not inhibit platelet aggregation even though the starting material diluted to the same extent did inhibit aggregation. The anti-23Kd immunoglobulin was recovered from the immobilized heparin binding fragments.

PLATELET AGGREGATION: Platelet-rich plasma was prepared and aggregation studied as described (4) except that 9 parts of blood were drawn into 1 part of 100mM Na citrate, 136mM glucose, pH 6.4. Materials used as platelet agonists were ADP (Sigma Chemical Co.) collagen (Chrono-Log Corp., Havertown, PA.) and γ-thrombin (John Fenton, II, New York State Department of Health, Albany, N.Y.). All other chemicals were reagent grade.

RESULTS: Antiserum against a 23Kd heparin binding fragment of TSP was tested for its ability to inhibit secretion-dependent aggregation of platelets. Under appropriate conditions, platelets aggregating in response to ADP undergo

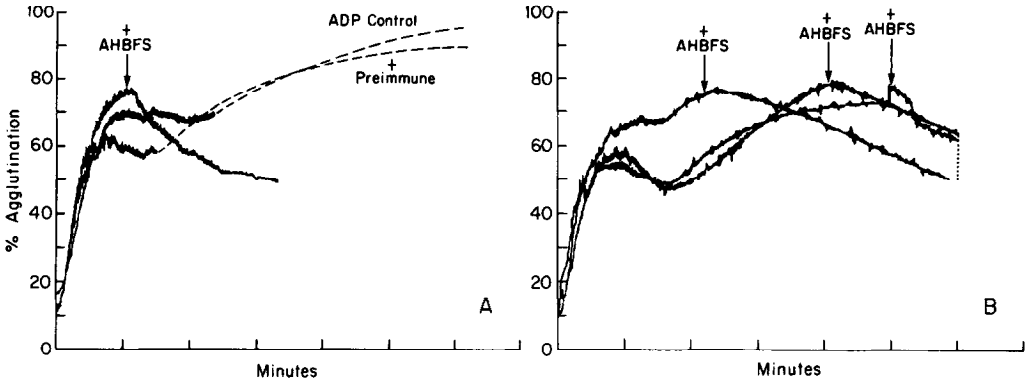

Fig. 1. Inhibition of aggregation of platelets stimulated with ADP. A. Platelets in platelet rich plasma (PRP) were stimulated with 2 μM ADP in the presence or absence of 1 μl of anti-heparin binding fragment serum (AHBFS) or preimmune serum. For the ADP and preimmune serum controls, the solid lines (——) approximate primary aggregation, the dashed lines (-----) approximate secretion-dependent aggregation. B. Platelets in PRP were stimulated with 2 μM ADP and exposed to 1 μl of AHBFS at approximately 2,4 and 5 minutes after addition of ADP. The dotted line (.....) depicts the extent of reversal of aggregation which occurred 10 minutes after the addition of AHBFS. The AHBFS had been added 5 minutes after the addition of ADP. Significant reversal of primary aggregation did not occur.

biphasic aggregation (17). The first phase of aggregation, called primary aggregation is not secretion-dependent. The second phase does not occur in the absence of secretion of the contents of platelet storage granules (dense bodies and α-granules) (17). The role of the secreted substances in secretion-dependent aggregation is not fully understood. Figure 1A reveals biphasic aggregation of platelets in response to ADP. Anti-heparin binding fragment serum (AHBFS) was added to platelet-rich plasma (PRP) at 1 minute after the addition of agonist. It is clear that this immune serum, but not the preimmune serum, inhibited secretion (Table 1) and the second wave, or secretion-dependent aggregation of platelets, without causing significant dissociation of the primary aggregates (Fig. 1A). The results in Fib. 1B reveal that AHBFS added during the second phase of aggregation caused reversal of aggregation to the level formed during primary aggregation. This effect on aggregation occurred without concomittant drastic inhibition of secretion of contents of the α-granules (Table 1). The effects of the AHBFS on secretion may mean that secretion and secretion-dependent aggregation are coupled via a feed-back mechanism.

TABLE 1

RELEASE OF β-TG FROM PLATELETS IN RESPONSE TO ADP IN THE PRESENCE OF ANTISERUM AGAINST THE 23Kd HEPARIN BINDING FRAGMENT OF THROMBOSPONDIN

Time*	β-TG (ng/ml)	% of control
1	2110	29.2
2	3655	50.5
4	3575	49.5
5	3725	51.5

*Minutes after the addition of ADP when the AHBFS was added to the platelet rich plasma. Samples for β-thromboglobulin (β-TG) determinations were obtained from the aggregation trials depicted in Fig. 1. Samples were taken at the end of each aggregation and the released β-TG determined as described (4,15). The control level of secreted β-TG (7220 ng/ml) is the amount released by the ADP control in Fig. 1A. Notice that the extent of primary aggregation in Fig. 1B is less than that in Fig. 1A and the maxima of aggregation and extent of secretion of all the second wave aggregations in Fig. 1B are about the same. In each instance the AHBFS reversed secondary, but not primary aggregation.

The ability of the AHBFS to inhibit platelet aggregation is not limited to platelets aggregating in response to ADP. The antiserum also inhibited the aggregation of platelets in response to collagen or thrombin (Fig. 2).

DISCUSSION: The data presented in Fig. 1 demonstrate that antibodies against the 23Kd heparin binding fragment of bovine TSP inhibit secretion-dependent

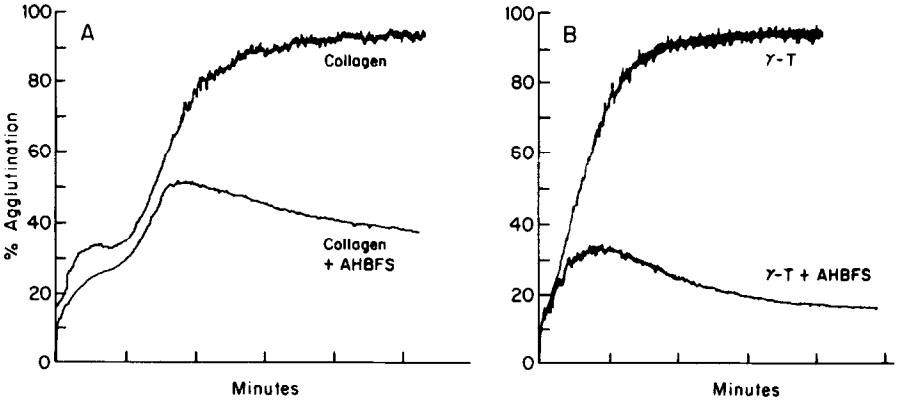

Fig. 2. Inhibition of aggregation of platelets stimulated with collagen or γ-thrombin. A. Platelets in PRP were stimulated with 2.5 μg/ml of equine tendon collagen (Chrono-Log Corporation) in the presence or absence of 1 μl of AHBFS. The AHBFS was added before the collagen. B. Platelets in PRP were treated with 7 μg/ml final concentration of γ-thrombin in the presence or absence of AHBFS. The AHBFS was added before the γ-thrombin.

aggregation of human platelets. The antiserum inhibited aggregation of platelets occurring in response to ADP, collagen or thrombin (Fig. 1 and 2). Thus, the inhibitory effect of the antiserum is not limited to platelets aggregating in response to weak agonists (18). These results confirm and extend the results reported by Nurden et al. (19) and Leung et al. (20).

The inhibition of aggregation caused by the AHBFS was due to the anti-TSP Ig in the serum. This is demonstrated by the facts that passage of the AHBFS over immobilized fibrinogen did not diminish the inhibitory potency of the serum, whereas adsorption of the serum against immobilized 23Kd heparin binding fragments of TSP eliminated its inhibitory activity (see Materials and Methods for details).

A variety of evidence indicates that TSP is the endogenous platelet lectin (5,6). Since TSP is the endogenous platelet lectin, the data presented here demonstrate that the lectin (TSP) mediates, at least in part, the secretion-dependent aggregation of platelets in response to a variety of agonists.

A particularly interesting aspect of our study is that the inhibitory antibodies were produced against a defined fragment of TSP, not the entire molecule. Gartner and Walz (21) have reported that TSP binds to the erythrocytes used to assay the endogenous platelet lectin via its amino-terminal heparin binding domain(s), the same heparin binding domain(s) present in the 23Kd heparin binding fragment. Since this fragment of TSP has a single amino terminus, the same region of TSP which mediates its binding to erythrocytes appears to play a direct role in mediating secretion-dependent aggregation of platelets. This part of the TSP molecule does not bind fibrinogen [Walz, unpublished and (22)], nonetheless fibrinogen is thought to be a receptor for the endogenous platelet lectin (23) and is known to bind the TSP (24). Thus, at least two domains of TSP may play critical roles in mediating the secretion-dependent aggregation of platelets.

ACKNOWLEDGEMENTS: This work was supported in part by grants HL 23010 and HL 27073 from the National Institutes of Health, and a Grant-in-Aid from the American Heart Association of Michigan.

REFERENCES

1. Gartner, T.K., Williams, D.C., Minion, F.C., and Phillips, D.R. (1978) Science 200:1281-1283.
2. Gartner, T.K., Phillips, D.R., and Williams, D.C. (1980) FEBS Lett. 113:196-200.
3. Gartner, T.K., Gerrard, J.M., White, J.G., and Williams, D.C. (1981) Blood 58:153-157.
4. Gartner, T.K., and Walz, D.A. (1983) Thromb. Res. 29:63-74.
5. Jaffe, E.A., Leung, L.L., Nachman, R.L., Levin, R.I., and Mosher, D.F. (1982) Nature 295:246-248.
6. Gartner, T.K., Doyle, M.J., and Mosher, D.F. (1983) Thromb. Haemostas. 50:124a.
7. Lawler, J.W., Slayter, H.S., and Coligan, J.E. (1978) J. Biol. Chem. 253:8609-8616.
8. George, J.N. (1978) J. Lab. Clin. Med. 92:430-446.
9. Hagan, I. (1975) Biochim. Biophys. Acta 392:242-254.
10. George, J.N., Lyons, R.M., and Morgan, R.K. (1980) J. Clin. Invest. 66:1-9.
11. Phillips, D.R., Jennings, L.K., and Prasanna, H.R. (1980) J. Biol. Chem. 255:11629-11632.
12. Gartner, T.K., and Dockter, M.E. (1983) Thromb. Res. 33:19-30.
13. Ciaglowski, R.E., Snow, J.W., and Walz, D.A. (1981) Ann. N.Y. Acad. Sci. 370:668-679.
14. Coligan, J.E. and Slayter, H.S. (1984) J. Biol. Chem. 259:3944-3948.
15. Brown, T.R., Ho, T.T.S., and Walz, D.A. (1980) Clin. Chim. Acta 101: 225-233.
16. Cuatrecasas, P. and Anfinson, C.B. (1971) Methods Enzymol. 22:345-378.
17. Charo, I.F., Feinman, R.D., and Detwiler, T.C. (1977) J. Clin. Invest. 60:866-873.
18. Huang, E.M. and Detwiler, T.C. (1981) Blood 57:685-891.
19. Nurden, A.T., Hasitz, M., and Rosa, E.P. (1983) Thromb. Haemostas. 50:132a.
20. Leung, L.L., Polley, M., and Nachman, R.L. (1983) Blood 62:suppl. 1, 261a.
21. Gartner, T.K. and Walz, D.A. (1984) J. Cell. Biol. Abstract, in-press.
22. Dixit, V.M., Grant, G.A., Frazier, W.A., and Santoro, S.A. (1984) Biochem. Biophys. Res. Commun. 119:1075-1081.
23. Gartner, T.K., Gerrard, J.M., White, J.G., and Williams, D.C. (1981) Nature 289:688-690.
24. Leung, L.L.K. and Nachman, R.L. (1982) J. Clin. Invest. 70:542-549.