Agkistin, a Snake Venom-derived Glycoprotein Ib Antagonist, Disrupts von Willebrand Factor-Endothelial Cell Interaction and Inhibits Angiogenesis*

Received for publication, April 7, 2000, and in revised form, April 17, 2000
Published, JBC Papers in Press, April 21, 2000, DOI 10.1074/jbc.C000234200

Chia-Hsin Yeh‡, Wen-Cheng Wang‡, Tsang-Tang Hsieh§, and Tur-Fu Huang‡‡
From the 3Department of Pharmacology, College of Medicine, National Taiwan University and 3Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Taipei 100, Taiwan

Glycoprotein (GP) Ib, an adhesion receptor expressed on both platelets and endothelial cells, mediates the binding of von Willebrand factor (vWF). Platelet GPIb plays an important role in platelet adhesion and activation, whereas the interaction of vWF and endothelial GPIb is not fully understood. We report here that agkistin, a snake venom protein, selectively blocks the interaction of vWF with human endothelial GPIb and inhibits angiogenesis in vivo. Agkistin specifically blocked human umbilical vein endothelial cell (HUVEC) adhesion to immobilized vWF in a concentration-dependent manner. Fluorescein isothiocyanate (FITC)-conjugated agkistin bound to HUVECs in a saturable manner. AP1, a monoclonal antibody (mAb) raised against GPIb, specifically inhibited the binding of FITC-conjugated agkistin to HUVECs in a dose-dependent manner, but other anti-integrin mAbs raised against αβ3, αβ5, and β1 did not affect this binding reaction. However, neither agkistin (2 μg/ml) nor AP1 (40 μg/ml) apparently reduced HUVEC viability. Both agkistin and AP1 exhibited a profound antiangiogenic effect in vivo when assayed by using the 10-day-old embryo chick chorioallantoic membrane model. These results suggest endothelial GPIb plays a role in spontaneous angiogenesis in vivo, and the antiangiogenic effect of agkistin may be because of disruption of the interaction of endogenous vWF with endothelial GPIb.

Angiogenesis, the development of new capillaries from pre-existing blood vessels, plays a critical role in a variety of physiological processes and pathological conditions, including embryonic development, wound healing, tumor growth, metastasis, and various inflammatory disorders (1). Interactions between endothelial cells and extracellular matrices (ECMs)1 including fibrinogen, vitronectin, collagen, laminin, and vWF, through cell surface adhesion receptors, are involved in the multiprocesses of neovascularization (2). For example, integrin αβ3 is not readily detectable in quiescent vessels but becomes highly expressed in angiogenic vessels (3). The dependence of angiogenesis on vascular cell adhesion events in vivo is evidenced by the observation that antibody and snake venom proteins antagonizing integrin αβ3 blocked angiogenesis in the chick chorioallantoic membrane (CAM) model (4, 5).

GPIb is an adhesive receptor expressed both on platelets and endothelial cells. Binding of plasma vWF to platelet GPIb plays a critical role in the earliest phase of primary hemostasis, which consists of the anchoring of platelets to injury vessel walls (6). In contrast to the platelets, the functions of endothelial GPIb for vWF are not well understood. Several studies have demonstrated the presence of GPIb complex expressed on the endothelial cell surface and up-regulation of endothelial GPIb by cytokines, i.e. tumor necrosis factor and interferon γ (7–10). It has been shown that endothelial GPIb participates in HUVEC binding to sickle cell erythrocytes (11) and also promotes platelet bridging to endothelial cells (12). Recently, Lian et al. (13) assumed that endothelial GPIb may contribute to mediate the process of endothelial cell migration during wound repair in vivo.

Snake venoms contain many unique components that affect cell-matrix interaction. Disintegrins represent a family of low molecular weight, cysteine-rich polypeptides that bind specifically to integrins αβ3, αβ5, and αβ1 expressed on platelets and other cells including vascular endothelial cells and some tumor cells, leading to inhibition of platelet aggregation, inhibition of cell adhesion, migration, and angiogenesis (14). On the other hand, some GPIb-binding venom proteins modulate vWF-GPIb interaction, leading to platelet activation, inhibition of platelet aggregation (15), or mediating HUVEC adhesion (16). Agkistin (also named agkicetin (17)), belonging to C-type lectin polypeptide derived from the viper venom of Agkistrodon acutus, specifically inhibited human platelet aggregation and agglutination triggered by ristocetin in the presence of vWF in vitro by acting as the platelet GPIb antagonist (18). Furthermore, agkistin is a potent anti-thrombotic agent because it pronounces blocked platelet plug formation in an experimental model in vivo.2 In this study, we showed that agkistin specifically inhibited HUVEC adhesion to immobilized vWF and it was shown to bind endothelial GPIb in a saturable manner. The role of endothelial GPIb in angiogenesis in vivo was thus evaluated.

EXPERIMENTAL PROCEDURES

Materials—Agkistin was purified from crude venom of Formosan Agkistrodon acutus by means of gel filtration and ion exchanger as described previously (15). The homogeneity of agkistin was judged by both sodium dodecyl sulfate-polyacrylamide electrophoresis and N-terminal amino acid sequencing. Rhodostomin was isolated from Agkistro-

1 The abbreviations used are: ECM, extracellular matrix; BCECF/AM, 2’,7’-bis(2-carboxyethyl)-5-and (6)-carboxyfluorescein acetoxymethyl ester; BSA, bovine serum albumin; CAM, chorioallantoic membrane; FITC, fluorescein isothiocyanate; GP, glycoprotein; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; vWF, von Willebrand factor.
2 C-H. Yeh, M-C. Chang, H-C. Peng, and T-F. Huang, manuscript in preparation.

* This work was supported by National Science Council of Taiwan Grant NSC 84-2331-B002-112BC and National Health Research Institute Grant NHRI-GT-EX99B920L. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Pharmacology, College of Medicine, National Taiwan University, No. 1, Sec. 1, Jen-Ai Rd, Taipei 100, Taiwan.
§ From the 3Department of Pharmacology, College of Medicine, National Taiwan University and 3Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Taipei 100, Taiwan.
¶ This paper is available on line at http://www.jbc.org

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
ECMs. HUVECs (5 × 10^6 cells/well) were added to 96-well plates, which were precoated with collagen type I (80 μg/ml), vitronectin (10 μg/ml), fibronectin (30 μg/ml), or vWF (10 μg/ml) in the absence or presence of various concentrations of agkistin. Results are expressed as percentage inhibition of adhesion compared with control cells in the absence of agkistin. All experiments were conducted in quadruplicate and repeated at least three times. Data are presented as mean ± S.E. (n = 3–6).

**Results and Discussion**

**Inhibition of HUVEC Adhesion to Immobilized vWF by Agkistin**—Multiple adhesive receptors expressed on endothelial cells mediate adhesion to extracellular matrices. First, we examined the effect of agkistin on HUVEC adhesion to a number of immobilized ECMs including collagen type I (80 μg/ml), fibronectin (30 μg/ml), vitronectin (10 μg/ml), and vWF (10 μg/ml). As shown in Fig. 1, agkistin specifically and dose dependently inhibited HUVEC adhesion to immobilized vWF (48.98 ± 4.12% inhibition at 0.066 μM) but apparently little affected those adhesive events toward other matrices used (less than 10% inhibition). Agkistin at a high concentration of 0.2 μM did not exhibit a further inhibition on HUVEC adhesion to immobilized vWF (about 52% inhibition, data not shown). These results indicate that agkistin does not interfere with the adhesion of integrins αvβ3, αvβ5, and αvβ1 and their respective ligands, vitronectin, collagen, and fibronectin. The interaction of vWF with HUVECs is mediated by endothelial integrin αvβ3 and by GPIb complex (16). Therefore, agkistin may process a similar effect. A similar result (40–50%) was reported with echistatin, another GPIb antagonist (23).

**Inhibition of FITC-conjugated Agkistin Binding to HUVECs**—To further explore the binding receptor of agkistin on HUVECs, we conjugated agkistin and BSA with FITC by a previously described method (24) and then examined the binding reaction of FITC-conjugated agkistin toward HUVEC by flow cytometry. Nonspecific binding was performed using FITC-BSA as a probe. After incubation of FITC-conjugated agkistin with HUVECs, the increment of relative fluorescence intensity of FITC-agkistin bound cells was concentration-dependent and reached a saturated binding at a concentration of 1 μM (Fig. 2). The different concentration range of agkistin used in this binding assay and in the cell adhesive assay (Fig. 1) is attributable to the different number of cells used in the two independent systems (i.e. 1 × 10^6 cells for agkistin binding and underlying CAM by the method described previously (22). A filter paper disc (1.3 cm, Minipore) saturated with test compounds or an equal aliquot of PBS (final volume in 20 μl) was applied to the top of CAM. The window was covered with sterile cellophane tape, and the embryos were incubated for a further 48 h at 37 °C with 60% humidity to develop spontaneous angiogenesis.

**FIG. 1. Effect of agkistin on HUVEC adhesive to immobilized ECMs.** HUVECs (5 × 10^6 cells/well) were added to 96-well plates, which were precoated with collagen type I (80 μg/ml), vitronectin (10 μg/ml), fibronectin (30 μg/ml), or vWF (10 μg/ml) in the absence or presence of various concentrations of agkistin. Results are expressed as percentage inhibition of adhesion compared with control cells in the absence of agkistin. All experiments were conducted in quadruplicate and repeated at least three times. Data are presented as mean ± S.E. (n = 3–6).

**FIG. 2. Binding isotherm of FITC-conjugated agkistin to HUVECs.** HUVECs (1 × 10^6 cells) were incubated with various concentrations of FITC-conjugated agkistin or FITC-conjugated BSA for 30 min and then analyzed by flow cytometry. Specific binding (●) is calculated by subtracting the nonspecific binding (○, as probed by FITC-BSA) from total binding (▲, as probed by FITC-agkistin). This is a representative one of three similar results.
cell proliferation. Thus, it is interesting to investigate if the blockade of endothelial GPIb reduces the viability of primary endothelial cells. HUVEC viability assay was performed by determining the cell metabolic activity with MTT. As shown in Fig. 4, neither agkistin (at a high concentration of 2 μg/ml, i.e. 0.066 μM), which exerted a maximal effect in blocking vWF-HUVEC adhesion (Fig. 1), nor AP1 (40 μg/ml) affected HUVEC viability. However, rhodostomin (2 μg/ml), a member of disintegrins, significantly reduced HUVEC viability. These data suggest that blockade of the vWF-endothelial GPIb interaction is not sufficient to induce cell death and endothelial GPIb receptor is not essential for cell survival.

Inhibition of Spontaneous Angiogenesis by Agkistin and AP1 in CAM Model—Several useful models have been used to study the role of cell adhesion molecule in angiogenesis. One of the most common in vivo models is the chick embryo CAM assay (29). To evaluate the role of endothelial GPIb complex in neovascularization in vivo, the effects of agkistin and anti-GPIb mAb AP1 on spontaneous angiogenesis occurring in the chick CAM model were examined. Upon dissection of the CAM of 12-day-old chick embryo, both agkistin (Fig. 5, B and C) and
A GPIb antagonist (agkistin) significantly inhibited angiogenesis in vivo. Furthermore, blockade of vWF-GPIb interaction either by anti-GPIb mAb (i.e. API) or by polyepitide (i.e. agkistin) markedly inhibited this process. It remains to be elucidated whether agkistin affects the multisteps in the endothelial GPIb in spontaneous angiogenesis, and GPIb antagonists are quite different from those of integrin. This is different from the previous observation with integrin αvβ3, which inhibited angiogenesis by acting as endothelial integrin αvβ3 antagonist and by inducing apoptosis (5). Taken together, snake venom constituents affect cell-matrix interaction via multiple mechanisms, resulting in modification of platelet and vascular cell behaviors, an important cellular process in thrombosis, hemostasis, and angiogenesis (30). Furthermore, at a higher dose (2 μg/embryo), both agkistin and API significantly disrupted preexisting blood vessels as evidenced by disappearance of large vessels (Fig. 5, C and E).

In conclusion, agkistin specifically inhibited HUVEC adhesion to immobilized vWF through selective binding of endothelial GPIb, resulting in a blockade of interaction between vWF and endothelial GPIb. Agkistin binds to endothelial GPIb in a specific and saturable manner. Furthermore, both agkistin and API significantly inhibited spontaneous angiogenesis in the chick CAM model in vivo but apparently did not affect HUVEC viability. In addition to their anti-thrombotic activity of agkistin and crotalin, another venom platelet GPIb antagonist (31), these GPIb antagonists may be useful tools for developing a new therapeutic strategy in angiogenesis-related diseases, such as inflammation and tumor metastasis.

Acknowledgments—We appreciate very much the generous supply of monoclonal antibodies from Dr. R. Montgomery (API) and Dr. B. Coller (TE3). We also thank S. C. Huang for preparing HUVECs.

REFERENCES
1. Folkman, J. (1986) N Engl. J. Med. 315, 1775–1783
2. Stromblad, S., and Cheresh, D. A. (1996) Chem. Biol. 3, 881–885
3. Brookes, P. C., Clark, R. A. F., and Cheresh, D. A. (1994) Science 264, 569–571
4. Brookes, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. (1994) Cell 79, 1157–1163
5. Yeh, C. H., Peng, H. C., and Huang, T. F. (1996) Blood 92, 3268–3276
6. Sadler, J. E. (1991) J. Biol. Chem. 266, 18172–18178
7. Wu, G., Essex, D. W., Meloni, F. J., Takafuta, T., Fujimura, K., Konkle, B. A., and Shapiro, S. S. (1997) Blood 90, 2660–2669
8. Beacham, D. A., Tran, L.-P., and Shapiro, S. S. (1997) Blood 88, 4071–4077
9. Konkle, B. A., Shapiro, S. S., Aesch, A. S., and Nachman, R. L. (1990) J. Biol. Chem. 265, 19833–19838
10. Rajagopalan, V., Essex, D. W., Shapiro, S. S., and Konkle, B. A. (1992) Blood 80, 153–161
11. Wick, T. M., Moake, J. L., Uddén, M. M., and McNtir, L. V. (1993) Am. J. Hemtol. 42, 284–292
12. Bomel, T., Schwartz, B. R., and Harian, J. M. (1998) J. Exp. Med. 187, 329–339
13. Lian, J., Guoping, C., Shapiro, S. S., Tran, L.-P., and Beacham, D. A. (1999) Exp Cell Res 252, 114–122
14. Gould, R. J., Polokoff, M. A., Friedman, P. A., Huang, T. F., Holt, J. C., Cook, J. T., and Niewiarowski, S. (1990) Proc. Soc. Exp. Biol. Med. 185, 168–171
15. Fujimura, Y., Kawasaki, T., and Tintian, K. (1996) Thromb. Haemostas 76, 633–639
16. Tan, L., Kowalska, M. A., Romo, G. M., Lopez, J. A, Darzykiewics, Z., and Niewiarowski, S. (1999) Blood 93, 2655–2661
17. Chen, Y. L., and Tsai, I. H. (1995) Biochem. Biophys. Res. Commun. 210, 472–477
18. Huang, T. F., and Yeh, C. H. (1994) Abstracts of Third Sino-Japanese Symposium on Coagulation, Fibrinolysis and Platelets, Kobe, Japan, Abstr. 51, p. 79
19. Huang, T. F., Ouyang, C., and Tec, C. M. (1990) Abstracts of Eleventh International Congress on Thrombosis, Ljubljana, Yugoslavia
20. Oikita, J. R., Pidard, D., Newman, P. J., Montgomery, R. R., and Kunicki, T. J. (1985) J. Cell Biol. 100, 317–321
21. Coller, B. S. (1985) J. Clin. Invest. 76, 101–108
22. Masoud, R., McGarvey, M. E., Zheng, T., Cai, J., Arora, N., Smith, D. L., Sloane, N., and Gill, P. S. (1999) Blood 92, 1038–1044
23. Peng, M., Lu, W., Beviglia, L., Niewiarowski, S., and Kirby, E. P. (1993) Blood 81, 2521–2528
24. Liu, C. Z., Wang, Y. W., Shen, M. C., and Huang, T. F. (1994) Thromb. Haemostas 72, 919–925
25. Andrews, R. K., and Berndt, M. C. (1998) Histol. Histopathol. 13, 837–844
26. Ruoslahti, E., and Reed, J. C. (1994) Cell 77, 477–478
27. Meredith, J. E., Fazeli, B., and Schwartz, M. A. (1993) Mol. Biol. Cell 4, 953–961
28. Feng, S., Christodoulides, N., and Kroll, M. H. (1999) Blood 93, 4256–4263
29. Stromblad, S., and Cheresh, D. A. (1996) Trends Cell Biol. 6, 462–468
30. Huang, T. F. (1998) Cell Mol. Life Sci. 52, 527–540
31. Chang, M. C., Lin, H. K., Peng, H. C., and Huang, T. F. (1998) Blood 91, 1582–1589
Agkistin, a Snake Venom-derived Glycoprotein Ib Antagonist, Disrupts von Willebrand Factor-Endothelial Cell Interaction and Inhibits Angiogenesis
Chia-Hsin Yeh, Wen-Cheng Wang, Tsang-Tang Hsieh and Tur-Fu Huang

J. Biol. Chem. 2000, 275:18615-18618.
doi: 10.1074/jbc.C000234200 originally published online April 21, 2000

Access the most updated version of this article at doi: 10.1074/jbc.C000234200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 14 of which can be accessed free at http://www.jbc.org/content/275/25/18615.full.html#ref-list-1