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DECREASED THROMBIN AFFINITY OF CELL-SURFACE THROMBOMODULIN FOLLOWING TREATMENT OF CULTURED ENDOTHELIAL CELLS WITH β-D-XYLOSIDE

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Thrombomodulin, an endothelial cell-surface anticoagulant, has been postulated to contain a glycosaminoglycan. Thrombomodulin function was therefore studied in endothelial cells treated with β-D-xyloside, an inhibitor of glycosaminoglycan attachment to proteoglycan core proteins. β-D-xyloside caused a reproducible 3 to 5-fold increase in the K_m of thrombomodulin for thrombin and a 20-30% decrease in the rate of protein C activation by the thrombin-thrombomodulin complex. These results support a role for glycosaminoglycans in thrombomodulin function and suggest that β-D-xylosides can be used to investigate both the anticoagulant mechanisms and the biosynthesis of cell-surface thrombomodulin.

Formation of a high-affinity complex between membrane-bound thrombomodulin (TM) and thrombin at the endothelial cell surface is a crucial step in the protein C anticoagulant pathway (1). Complex formation alters the macromolecular specificity of thrombin, resulting in acceleration of thrombin-dependent protein C activation and concomitant inhibition of thrombin's procoagulant activities (2,3). Activated protein C (APC) is an anticoagulant serine protease which proteolytically inactivates clotting factors Va and VIIIa (4). Endothelial cell TM thus functions to convert thrombin from a procoagulant to an anticoagulant enzyme.

Structure-function relationships in TM have been greatly advanced by proteolysis studies with purified rabbit TM (5-7) and by cloning of the human, bovine and mouse TM genes (8-11). The primary thrombin binding site and the minimum fragment of the extracellular domains of TM required for protein C activation have been identified (6,7,12,13). In addition to functional characterisation of the domain structure of TM, other studies have suggested that chondroitin sulphate glycosaminoglycans are also essential for TM anticoagulant function (14-17).

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Abbreviations used in the text are: 4-MUBDX, 4-methylumbelliferyl-β-D-xyloside; APC, activated protein C; DMSO, dimethylsulphoxide; rHPC, recombinant human protein C; TM, thrombomodulin.

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Correlation of the functional properties of detergent-solubilised purified TM with the behaviour of the protein in its natural membrane environment is problematic. This is exemplified by the alteration of the kinetic properties of purified rabbit TM when reconstituted into artificial phospholipid vesicles (18). Likewise, the role of glycosaminoglycans in the functional properties of cell-surface TM may differ from that of the purified protein. In the present studies 4-methylumbelliferyl-β-D-xyloside (4-MUBDX), a metabolic inhibitor of proteoglycan biosynthesis, was used to establish an experimental model in which the role of glycosaminoglycans in cell-surface TM function could be investigated. It was demonstrated that the kinetic properties of cell-surface TM were altered by exposure of endothelial cells to 4-MUBDX, indicating that glycosaminoglycans may indeed be involved in the regulation of the functional properties of this crucial membrane-bound anticoagulant cofactor.

MATERIALS and METHODS

Materials. Bovine thrombin (2400 NIH units/mg protein) was from Enzyme Research Laboratories, South Bend, IN. Recombinant human protein C (rHPC) was provided by Dr. S. Betty Yan, Lilly Laboratories, Indianapolis. This reagent has been fully described elsewhere (19). Hirudin and 4-MUBDX were from Sigma Chemical Co., St. Louis, MO. Chromogenic substrate S-2366 was from Kabi-Vitrum, Franklin, OH.

Cell Culture. Primary cultures of human umbilical vein endothelial cells were isolated and maintained as described (20), and used during passages 1-4. Primary cultures of bovine pulmonary aorta endothelial cells were purchased from the American Type Culture Collection, maintained as previously described (21) and used during passages 16-22. Confluent cell monolayers were treated with either 1 mM 4-MUBDX (from 1 M stock solution in DMSO) or 0.1 % v/v DMSO as a control by addition to culture medium for 24 hours.

Thrombomodulin Assays. Cell-surface TM activity was assayed using a 2-stage amidolytic assay essentially as described (22). Assay buffer was medium-199 (Gibco, Long Island, NY) supplemented with $CaCl_2$ to give a final concentration of 3 mM. Confluent endothelial cell monolayers (24-well clusters, ~10^5 cells/well) were washed twice with assay buffer. Monolayers were incubated at 37°C for 1 hour with 200 ul assay buffer containing 25 ug/ml rHPC and increasing concentrations of thrombin. Incubations containing rHPC and thrombin in the absence of cells were used as controls. Incubations were terminated by addition of 25 ul of sufficient hirudin to give a 10-fold excess to the thrombin concentration used. After further incubation for 5 minutes, 50 ul samples were mixed with 150 ul of assay buffer in 96-well plates. Chromogenic substrate S-2366 (0.4 mM final concentration) was then added and the O.D. at 405 nm determined at timed intervals using a UVmax Kinetic Microplate Reader (Molecular Devices, Palo Alto, CA). The amount of rHPC activated was determined by reference to standard curves of fully activated rHPC, prepared as described (23). Rates of APC formation were determined as ug APC/hr/10^6 cells and were corrected for APC generation in the absence of cells at each thrombin concentration used.

Kinetic Analyses. The dependence of the rate of APC formation on thrombin concentration was used to determine the apparent K_m for thrombin and the V_{max} at saturating thrombin concentrations. Kinetic parameters were calculated using the Enzfitter computer programme (Elsevier Biosoft,

Cambridge, U.K.). using averaged data from 3 separate experiments. Curve fitting was performed using simple weighting. K_m and V_{max} values given in the text are the calculated value +/- the standard error from the fitted data.

RESULTS

Endothelial cell-surface TM activity was measured as the rate of activated protein C formation and showed saturable kinetics with respect to the thrombin concentration used. The thrombin dependence of protein C activation by endothelial cells, both in control experiments and after treatment with 1 mM 4-MUBDX for 24 hours, is shown in Figure 1. Kinetic parameters (K_m and V_{max}) calculated from the thrombin saturation curves shown in Figure 1 are summarised in Table 1. In control experiments the apparent K_m of cell-surface TM for thrombin was found to be 0.12 +/- 0.01 and 0.74 +/- 0.09 nM on human and bovine endothelial cells, respectively. These values are in good agreement with those reported for human endothelial cell-surface TM (K_m = 0.5 nM, ref. 1). Previous studies have not compared the affinity of bovine and human endothelial cell-surface TM for thrombin. The present results suggest that human TM has a higher affinity for bovine thrombin than does bovine TM. At saturating thrombin concentrations, the V_{max} of protein C activation was calculated at 5.81 +/- 0.61 and 9.19 +/- 0.28 ug APC/hr/10^6 cells in human and bovine endothelial cells, respectively.

Significant changes in the thrombin-dependence of protein C activation were observed in both human and bovine endothelial cells after treatment

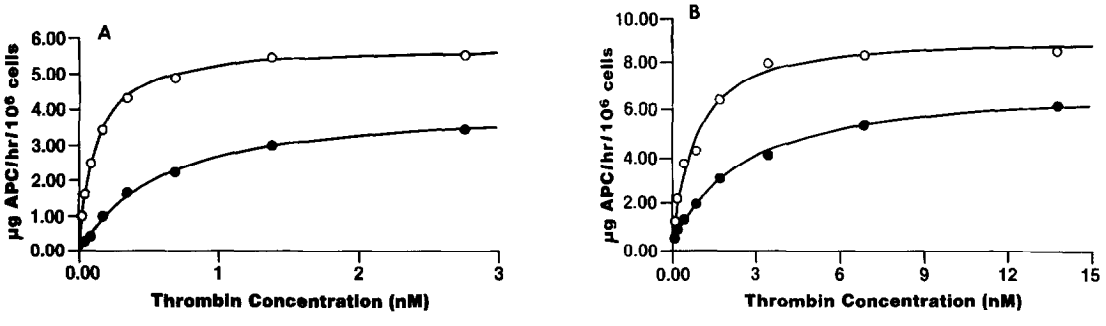

Figure 1. The effects of β-D-xyloside on thrombin-dependence of cell-surface thrombomodulin activity in cultured human and bovine endothelial cells. Human (Panel A) and bovine (Panel B) endothelial cell cultures were treated with 0.1% v/v DMSO as a control (open circles) or with 1 mM 4-methylumbelliferyl-β-D-xyloside (closed circles) for 24 hours. The activity of cell-surface thrombomodulin was assayed as described in "Materials and Methods" as the rate of protein C activation (ug APC/hr/10^6 cells) at increasing thrombin concentrations (nM). Data points represent the mean from 3 separate experiments. The line drawn through the data points was calculated by non-linear regression analysis as described in "Materials and Methods".

Table 1

Effects of 1 mM 4-MUBDX on Thrombin Kinetics of Cell-Surface Thrombomodulin

Kinetic Parameter[1]	Human Endothelium	Bovine Endothelium
K_m (DMSO Control)	0.12 +/- 0.01	0.74 +/- 0.09
K_m (1 mM 4-MUBDX)	0.60 +/- 0.05	2.21 +/- 0.21
V_{max} (DMSO Control)	5.81 +/- 0.61	9.19 +/- 0.28
V_{max} (1 mM 4-MUBDX)	4.22 +/- 0.12	7.09 +/- 0.23

[1]Apparent K_m for thrombin (nM) and V_{max} (ug APC/hr/10^6 cells) of protein C activation by the thrombin-TM complex at the surface of human and bovine endothelial cell cultures were calculated from the saturation curves shown in Figure 1. Non-linear regression analysis using the Enzfitter program was used as described in Materials and Methods. Calculated values are shown +/- standard error from the computed fit.

with 1 mM 4-MUBDX (see Figure 1 and Table 1). In human cells, the K_m for thrombin increased 5-fold to 0.6 +/- 0.05 nM, and in bovine cells the K_m increased 3-fold to 2.21 +/- 0.21 nM. V_{max} decreased to 73% and 77% of control in human and bovine cells, respectively. Dose-response effects of 4-MUBDX on the kinetic properties of cell-surface TM were not fully established. In bovine cells, 0.1 mM 4-MUBDX had no effect on the kinetic properties of cell-surface TM, whereas increasing the concentration of 4-MUBDX to 5 mM was found to increase the K_m for thrombin 10-fold and to further decrease V_{max} (data not shown). However, at high concentrations of 4-MUBDX, both human and bovine endothelial cells were observed to be retracted, indicating potentially cytotoxic effects of the inhibitor. The concentration of 1 mM 4-MUBDX used in the present study was not observed to cause any significant morphologic changes in endothelial cell monolayers which retained the tightly cobblestoned appearance of control cells.

DISCUSSION

The role of glycosaminoglycans in the anticoagulant functions of TM has received increasing attention (14-17). Studies with purified TM have shown that the ability to inhibit thrombin's procoagulant functions is sensitive to digestion with bacterial chondroitinases (15,16). Attachment of a chondroitin sulphate glycosaminoglycan chain was thus proposed as a post-translational modification essential to TM anticoagulant function. The presence of similar glycosaminoglycan chains in human TM has not been reported. However, in human TM, the short serine/threonine-rich domain which lies between the primary thrombin binding site and the transmembrane domain does contain a -Ser-Gly-Ser-Gly- structure similar to the consensus sequence required for glycosaminoglycan attachment to core proteins (24). Attachment of chondroitin sulphate glycosaminoglycan chains to proteoglycan core proteins typically occurs at serine residues and has the following

linkage region structure: Ser-Xyl-Gal-Gal-GlcUA-[GalNAc-GlcUA]$_n$. Attachment can be inhibited metabolically by β-D-xylosides, such as 4-MUBDX, which are competitive inhibitors of galactosyltransferase I, a Golgi membrane-bound enzyme, which attaches the first Gal of the linkage region to the Ser-Xyl acceptor (reviewed in ref. 25). Treatment of tissues and cells with 4-MUBDX thus results in the synthesis of glycosaminoglycan-deficient proteoglycans. The present studies demonstrated that 4-MUBDX can alter the properties of cell-surface TM in both human and bovine endothelial cells. These findings are consistent with the previous proposal that rabbit TM is a proteoglycan and suggest that rabbit, human and bovine TM may all contain glycosaminoglycans which contribute to the anticoagulant function of this important thrombin receptor.

Previous studies on the role of glycosaminoglycans in rabbit and bovine TM function focussed on inhibition of thrombin's procoagulant activities (15,16). The thrombin affinity of bovine TM and rate of protein C activation by the thrombin-TM complex were not found to be altered following digestion with chondroitinase (16). In the present studies the kinetic properties of cell-surface TM were altered following treatment of endothelial cells with 4-MUBDX. These findings suggest that the kinetic properties of the thrombin-TM complex at the endothelial cell surface are regulated by attachment of glycosaminoglycans. The discrepencies between the present findings and those reported for purified bovine TM may be explained by differences in the methodologies used. Chondroitinase digestion of proteoglycans leaves the linkage region intact, and quantitative removal of sulphated disaccharides from the glycosaminoglycan chain often requires several cycles of digestion with large amounts of fresh enzyme (27). With 4-MUBDX treatment, cell-surface TM would be expected to be expressed in a form lacking the entire glycosaminoglycan chain, including the linkage region.

The possibility that cells treated with 4-MUBDX for 24 hours may express a mixed population of TM isoforms at the cell-surface (i.e the native protein and the protein lacking glycosaminoglycan chains) should be considered. An estimate of 19 hours for the half-life of TM was obtained in a mouse hemangioma cell line using pulse-chase radiolabelling techniques (28). Assuming a similar half-life in endothelial cells, the 24-hour treatment with 4-MUBDX used in the present studies would be expected, at best, to replace only 50% of the native cell-surface TM with newly synthesised TM lacking glycosaminoglycans. The K_m values obtained in the presence of 4-MUBDX would thus represent an averaged value for a mixed population of cell-surface TM molecules and may in fact underestimate the effects of removal of glycosaminoglycan chains on the kinetic properties of the thrombin-TM complex. The decreased V_{max} values for protein C activation

seen in 4-MUBDX-treated endothelial cells may reflect a true alteration in the kinetic properties of cell-surface TM. However, V_{max} values, in contrast to K_m, would be dependent on the total amount of cell-surface TM available for thrombin-binding. It is thus also possible that the observed decrease in V_{max} might arise from a decrease in total cell-surface TM expression following treatment with 4-MUBDX.

In conclusion, the present studies provide support for the proposal that the functional properties of TM are regulated by attachment of a glycosaminoglycan. The present studies also demonstrate that β-D-xylosides should be useful tools for investigating the mechanisms of TM function in its natural endothelial cell membrane environment. β-D-xylosides may also be exploited for investigating the regulation and kinetics of TM biosynthesis.

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REFERENCES

(1). Esmon, C.T. (1989) J.Biol.Chem. **264**, 443-4746.
(2). Esmon, C.T. & Owen, W.G. (1981) Proc.Natl.Acad.Sci. **78**, 2249-2252.
(3). Esmon, N.L., Carroll, R.C. & Esmon, C.T. (1983) J.Biol.Chem. **258**, 12238-12242.
(4). Esmon, C.T. (1983) Blood **62**, 1155-1158.
(5). Kurosawa, S., Galvin, J.B., Esmon, N.L., & Esmon, C.T. (1987) J.Biol.Chem. **262**, 2206-2212.
(6). Kurosawa, S., Stearns, D. J., Jackson, K.W., & Esmon, C.T. (1988) J.Biol.Chem. **263**, 5993-5996.
(7). Stearns, D.J., Kurosawa, S., & Esmon, C.T. (1988) J.Biol.Chem. **264**, 3352-3356.
(8). Wen, D., Dittman, W.A., Ye, R.D., Deaven, L.L., Majerus, P.W., & Sadler, J.E. (1987) Biochemistry **26**, 4350-4357.
(9). Suzuki, K., Kusumoto, H., Deyashiki, Y., Nishioka, J., Maruyama, I., Zushi, M., Kawahara, S., Honda, G., Yamamoto, S., & Horiguchi, S. (1987) EMBO **6**, 1891-1897.
(10). Jackman, R.W., Beeler, D.L., Livingston, V., & Rosenberg, R.D. (1986) Proc.Natl.Acad.Sci. **83**, 8834-8838.
(11). Dittman, W.A., Kumada, T., Sadler, J.E. & Majerus, P.W. (1988) J.Biol.Chem. **263**, 15815-15822.
(12). Suzuki, K., Hayashi, T., Nishioka, J., Kosaka, Y., Zushi, M., Honda, G., & Yamamoto, S. (1989) J.Biol.Chem. **264**, 4872-4876.
(13). Zushi, M., Gomi, K., Yamamoto, S., Maruyama, I., Hayashi, T., & Suzuki, K., (1989) J.Biol.Chem. **264**, 10351-10353.
(14). Bourin, M.-C., Ohlin, A.-K., Lane, D.A., Stenflo, J. & Lindahl, U. (1988). J.Biol.Chem. **263**, 8044-8052.
(15). Bourin, M.-C., Boffa, M.-C., Bjork, I. & Lindahl, U. (1986) Proc.Natl.Acad.Sci. **83**, 5924-5926.
(16). Bourin, M.-C. (1989) Thromb. Res. **54**, 27-39.

(17). Parkinson, J.F., Grinnell, B.W., Moore, R.E., Hoskins, J.A., Vlahos, C.J. & Bang, N.U. (1990) J.Biol.Chem. In press.
(18). Galvin J.B., Korosawa, S., Moore, K., Esmon, C.T. & Esmon, N.L (1987) J.Biol.Chem. **262**, 2199-2205.
(19). Grinnell, B.W., Berg, D.T., Walls, J., & Yan, B.S. (1987) Biotechnology **5**, 1189-1192.
(20). Garcia, J.G.N., Painter, R.G., Fenton, J.W., English, D. & Callahan, K.S. (1990) J.Cell.Physiol. **142**, 186-193.
(21). Garcia, J.G.N., Dodson, R.F. & Callahan, K.S. (1989) Lab.Invest. **61**, 53-61.
(22). Moore, K.L., Andreoli, S.P., Esmon, N.L., Esmon, C.T. & Bang, N.U. (1987) J.Clin.Invest. **79**, 124-130.
(23). Vagano D'Angelo, S., Comp, P.D., Esmon, C.T. & D'Angelo, A. (1986) J.Clin.Invest. **77**, 416-425.
(24). Bourdon, M.A., Krusius, T., Campbell, S., Schartz, N.B. & Ruoslahti, E. (1987) Proc.Natl.Acad.Sci. **84**, 3194-3198.
(25). Schwartz, N.B. & Smalheiser, N.R. (1989) In "Neurobiology of Glycoconjugates" (Margolis, R.U. & Margolis, R.K. Eds.), pp 151-186, Plenum Press, New York, U.S.A.
(26). Schawrtz, N.B. (1979) J.Biol.Chem. **254**, 2272-2277.
(27). Sugahara, K., Yamashina, I., De Waard, P., Van Halbeek, H. & Vliegenthart, J.F.G. (1988) J.Biol.Chem. **263**, 10168-10174.
(28). Dittman, W.A., Kumada, T., Sadler, J.E. & Majerus, P.W. (1988) J.Biol.Chem. **263**, 15815-15822.