PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 253-256 (1976)

Fibrin Clot Retraction by Human Skin Fibroblasts: Effects of ADP and Thrombin[1] (39185)

STEFAN NIEWIAROWSKI AND SAMUEL GOLDSTEIN

Specialized Center for Thrombosis Research, Department of Medicine and Physiology, Temple University, School of Medicine, Philadelphia, Pa. 19140, and Departments of Medicine and Biochemistry, McMaster University Medical Center, Hamilton, Ontario, Canada

Platelet interaction with fibrin is of importance for the formation of hemostatic plugs and thrombi (1). Recent studies have demonstrated that cultured mouse fibroblasts, whether aneuploid and derived from the mouse (2), or diploid and of human origin (3), cause fibrin retraction. Moreover, clot retraction induced by fibroblasts and by platelets have many features in common. For example, both processes are inhibited by prostaglandin E_1, dibutyryl cyclic AMP, cytochalasin B, EDTA, and cellular disruption (3).

Clots formed upon addition of thrombin to fibrinogen and platelets retract readily. However, when *Bothrops atrox* thrombin-like enzyme (Reptilase, Defibrase) is substituted for thrombin no retraction occurs. Further, *Bothrops atrox* enzyme does not cause platelet aggregation and the platelet release reaction (3). Therefore, this clotting system is useful to study the mechanisms involved in platelet-dependent fibrin retraction. It has also been demonstrated that ADP and thrombin stimulate platelets to retract fibrin (4, 5), the effect of thrombin being mediated, at least in part, by ADP. Thus, thrombin releases ADP from platelet storage granules, and enzymes which destroy ADP inhibit clot retraction induced by low concentrations of thrombin (5). The purpose of the present experiments was to compare the effects of thrombin and *Bothrops atrox* thrombin-like enzyme on clot retraction induced by fibroblasts. We also explored whether ADP added exogenously or possibly released from fibroblasts following treatment with thrombin would have any effect on clot retraction.

[1] Supported by Grants Numbers HL15226 and 14217 from the National Institutes of Health and MA-3515 from the Medical Research Council of Canada during the tenure of a scholarship from the MRC (SG).

Methods. Cultures were established from 4-mm skin biopsies of the anterior forearm following informed consent of the patient or guardian and propagated as described earlier (3, 6). Cell strains used in these experiments were derived from a normal 22-year-old male and a 5-year-old boy with homocystinuria. The latter strain was examined because of the predisposition to vascular thrombosis that exists in homocystinuria (7). All experiments were performed on early-passage fibroblasts within two to five passages of harvesting primary skin explants. Fibroblast suspensions were prepared as described previously (3, 6). In brief, cells were harvested from monolayers following a 5-min exposure to 0.125% trypsin (Difco 1:250) at 37°, which was subsequently neutralized by soy bean trypsin inhibitor (Sigma) plus a 10 vol excess of growth medium containing the regular supplement in 15% fetal calf serum. Cells were then washed, resuspended in Tyrode's albumin solution containing 2×10^{-3} *M* calcium and 10^{-3} *M* magnesium, counted by means of a Cytograph (Biophysics, Inc., Mahopac, N.Y.), and the suspension adjusted to 2×10^6 cells/ml. In some experiments cells were harvested with a "rubber policeman" without exposure to trypsin.

Protein was determined by the method of Lowry *et al.* (8), and ADP and ATP concentrations were determined by the firefly method of Holmsen *et al.* (9). Clot retraction was tested as described previously (3, 5). Either bovine thrombin (Parke Davis, Detroit, Mich.) or *Bothrops marajoensis* thrombin-like enzyme (BM enzyme) was used as a clotting enzyme. The latter enzyme was prepared from the viper venom of *Bothrops marajoensis* (a subspecies of *Bothrops atrox*) and kindly supplied by Dr. K. Stocker (Pentapharm, Basel, Switzerland).

Human fibrinogen was supplied from Kabi (Stockholm, Sweden), ADP, creatine phosphate (CP), and creatine phosphate kinase (CPK) from Sigma, while apyrase was obtained by the method of Molnar and Lorand (10).

Results and discussion. Suspensions of fibroblasts detached from petri dishes nonenzymatically by means of a "rubber policeman" did not cause clot retraction. This was not related to physical damage since cells detached by this method had plating and growth capacities that were similar to replicate cultures detached by trypsinization (unpublished data). In all experiments fibroblasts harvested following exposure to trypsin were used.

Fibroblast-rich clots formed by means of thrombin showed more than 50% retraction at 37° during the 2-hr observation period, while clots formed by means of BM enzyme only retracted about 5% (Fig. 1). This retraction was not enhanced in either case when ADP (over the concentration range 10^{-7}–10^{-4} *M*) was added to the suspension of fibroblasts and fibrinogen prior to adding the clotting enzymes (Fig. 1). In fact, thrombin-induced retraction may have been inhibited by higher concentrations of ADP (10^{-4} *M*). Both normal and homocystinuric fibroblasts contributed similarly to clot retraction.

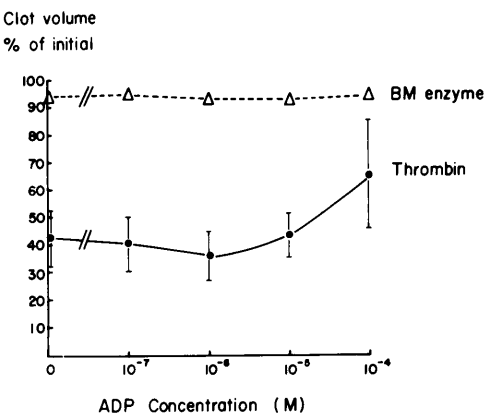

FIG. 1. Effect of ADP on fibrin retraction induced by cultured human fibroblasts. Each sample contained 0.7 ml of normal fibroblasts (2×10^6/ml), 0.1 ml of ADP (or saline), 0.1 ml of 0.5% human fibrinogen, and 0.1 ml of thrombin (20 units/ml, ●——●) or BM enzyme (10 units/ml, △——△). Suspensions of cells were preincubated for 1 min at 37° with ADP and fibrinogen before addition of the clotting enzyme. Samples were incubated at 37° for 2 hr. Values are the mean ± SD of four experiments.

The level of intracellular ATP averaged 5 nmole while the average level of ADP was 1.8 nmole per 10^6 cells (Table 1). Only a small fraction of the total ADP or ATP (1–2.5%) was present in the supernatants either of control cell suspensions or following treatment with thrombin and BM enzyme. This indicates that thrombin did not cause any significant release or leakage of adenine nucleotides from the fibroblasts. Additionally, we found that potato apyrase (5 units/ml), which degrades ADP to AMP, and the mixture of creatine phosphate (10 μmole) and creatine phosphokinase (3 units), which generates ATP from ADP, added to 1.0 ml of fibroblast suspension did not affect significantly clot retraction.

Fibroblasts (10^6) contained approximately 500 μg of protein. Thus, the value of ATP in fibroblasts was 12 nmole/mg of protein, the value of ADP was 3.6 nmole, and the value of both adenine nucleotides was 15.6 nmole/mg of protein. This closely resembles the value of nonmetabolic adenine nucleotides in human platelets, which was estimated recently by Holmsen (11) at 20 nmole/mg of protein.

In conclusion, thrombin stimulates fibroblast-dependent fibrin retraction but ADP does not appear to mediate this reaction. Moreover, exogenous ADP does not stimulate fibroblasts to retract *Bothrops atrox* fibrin in this experimental system. Although it has generally been accepted that cells other than platelets do not show this specific response to ADP (12, 13), few detailed investigations had been performed until now to support this statement. Our experiments were carried out in a system that allows comparison of contractile properties of platelets and fibroblasts, and the findings support the view that the response to ADP is a specific cellular property of platelets.

Recently Chen and Buchanan (14) found that thrombin stimulated the migration and mitoses of chick embryo fibroblasts. It is possible, therefore, that the stimulation by thrombin of fibroblast clot retraction and fibroblast motility both are mediated by an

TABLE I. EFFECT OF THROMBIN AND BM ENZYMES ON THE ATP AND ADP LEVEL OF HUMAN SKIN FIBROBLASTS (10-MIN INCUBATION AT 37°)[a]

	Normal strain				Homocystinuric strain			
	ATP		ADP		ATP		ADP	
Enzyme added	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
Thrombin 100 u	5.6	0.12	2.3	0.02	6.8	0.08	1.8	0.05
BM enzymes 10 u	6.1	0.11	1.7	0.02	6.6	0.09	2.1	0.05
0.9% NaCl (control)	5.2	0.12	1.8	0.01	5.2	0.18	1.2	0.01

[a] Samples of fibroblast suspensions (0.5 ml) were incubated with 0.1 ml of thrombin (100 u), 0.1 ml of BM enzyme (10 u) or 0.1 ml of 0.9% NaCl for 10 min at 37°. The incubation mixture was then spun down in an Eppendorf centrifuge for 2 min at 7600*g*. Sediments were resuspended to the original volumes with 0.9% NaCl and treated along with the supernatants with the EDTA-ethanol mixture followed by determination of the levels of ATP and ADP. Solutions of thrombin and BM enzyme did not contain any detectable adenine nucleotides. Results are expressed as nmole of ATP or ADP per 10^6 cells.

effect on contractile proteins in these cells.

The effect of thrombin on the fibroblasts should be differentiated from that of trypsin. In our experiments exposure of fibroblasts either to trypsin or to thrombin alone was not sufficient to stimulate contractile ability of the fibroblasts, but exposure to both enzymes was a necessary requirement. Recent observations by Tang and Chen (15) show that effects of thrombin and of trypsin on chick embryo fibroblasts are different. Thrombin did not digest a high molecular weight protein associated with fibroblast surface, but it caused a large increase in the incorporation of [^{3}H]thymidine into DNA. On the other hand, trypsin digestion resulted in the disappearance of this surface protein but incorporation of [^{3}H]thymidine into DNA was low. It is difficult to define the role of trypsin in our system. However, we observed that suspension of fibroblasts detached by means of rubber policemen contained more clumps than suspension of cells detached with trypsin. de Gaetano *et al.* (16) demonstrated that clot retraction is inhibited by previous *in vitro* platelet aggregation. It is possible that fibroblast aggregation might have a similar effect.

Summary. Thrombin stimulated human skin fibroblasts to retract fibrin clots. When *Bothrops marajoensis* thrombinlike enzyme was substituted for thrombin, no retraction occurred. Fibroblasts were found to contain 12 nmole of ATP and 3.6 nmole of ADP/mg of protein, a value closely resembling that of nonmetabolic adenine nucleotides in platelets. Thrombin caused neither release of adenine nucleotides from the suspension of fibroblasts harvested enzymatically nor did addition of ADP stimulate fibroblasts to retract fibrin clots.

The authors wish to thank Drs. H. Holmsen and G. J. Stewart for helpful discussions, and Ms. Elena Moerman and Ms. Maxine Millman for technical assistance.

1. Niewiarowski, S., Regoeczi, E., Stewart, G. J., Senyi, A. F., and Mustard, J. F., J. Clin. Invest. **51,** 685 (1972).
2. Niewiarowski, S., Recoeczi, E., and Mustard, J. F., Proc. Soc. Exp. Biol. Med. **140,** 199 (1972).
3. Niewiarowski, S., and Goldstein, S., J. Lab. Clin. Med. **82,** 605 (1973).
4. de Gaetano, G., Bottecchia, D., and Vermylen, J., Thromb. Res. **2,** 71 (1973).
5. Niewiarowski, S., Stewart, G. J., Nath, N., Sha, A. T., and Lieberman, G. E., Amer. J. Physiol. **229,** 737 (1975).
6. Goldstein, S., Littlefield, J. W., and Soeldner, J. S., Proc. Nat. Acad. Sci. **64,** 155 (1969).
7. Gerritsen, T., and Waisman, H. A. *in* "Metabolic Basis of Inherited Disease" (J. B. Stanberg *et al.*, eds.) p. 404. McGraw Hill, N.Y. (1972).
8. Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
9. Holmsen, H., Storm, E., and Day, H. F., Anal. Biochem. **46,** 489 (1972).
10. Molnar, J., and Lorand, L., Arch. Biochem. Biophys. **93,** 353 (1961).
11. Holmsen, H., *in* "Biochemistry and Pharmacology

of Blood Platelets" CIBA Foundation Symp., No. **36,** 175 (1975).
12. Spaet, T. H., and Lejnieks, I., "Biochemistry of Blood Platelets" (E. Kowalski and S. Niewiarowski, eds.), pp. 99–104. Academic Press and PWN, London, New York, Warsaw (1967).
13. Born, G. V. R., Ann. N.Y. Acad. Sci. **201,** 4 (1972).
14. Chen, L. B., and Buchanan, J. M., Proc. Nat. Acad. Sci. USA **72,** 131 (1975).
15. Teng, N. N. H., and Chen, L. B., Proc. Nat. Acad. Sci. USA **72,** 413 (1975).
16. de Gaetano, G., Donati, M. B., Vermylen, J., and Verstraete, M., Thrombos. Diathes. Haemorrh. **26,** 449 (1971).

Received April 3, 1975. P.S.E.B.M. 1976, Vol. 151.