Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 83-88

A PLATELET FACTOR THAT STIMULATES THE PROLIFERATION OF VASCULAR ENDOTHELIAL CELLS

Kohei Miyazono, Tetsuro Okabe, Akio Urabe, Manabu Yamanaka* and Fumimaro Takaku

The Third Department of Internal Medicine and
*the Department of Laboratory Medicine,
University of Tokyo, Hongo, Tokyo 113, Japan

Received October 31, 1984

SUMMARY: The effects of platelet factors on the growth of cultured porcine aortic endothelial cells were studied. Human platelet lysate stimulated the incorporation of [^{3}H] thymidine into DNA. Gel chromatography on Sephadex G-75 revealed at least two peaks of activity on endothelial cells, the major peak being at an apparent molecular weight of 20,000. This activity was heat-labile and trypsin-sensitive, and did not stimulate the growth of fibroblasts.

Platelets are widely known to play an important role in maintaining the integrity of vascular endothelium (1). In thrombocytopenic states, ultrastructural changes develop in endothelial cells and permeability to blood elements increases (2). Maca et al. disclosed that intact platelets enhance DNA synthesis and replication of cultured endothelial cells, and that this growth-promoting effect does not occur in leukocytes or erythrocytes but is limited to platelets (3). Although platelets have been shown to contain some growth factors including platelet-derived growth factor (PDGF) (4-6), peptide growth factors on endothelial cells from platelets have not been fully elucidated. Recently, we have noticed that outdated platelets had much less activity on endothelial cells, compared with high activity found in freshly isolated platelets (unpublished observations). This study is an attempt to see whether freshly isolated platelets contain growth factor(s) on vascular endothelial cells.

MATERIALS AND METHODS

Preparation of Platelet Lysate: Fresh platelet rich plasma from normal volunteers was obtained from blood collected in citrate-phosphate-dextrose

Abbreviations: PDGF, Platelet-derived growth factor; EGF, Epidermal growth factor; FBS, Fetal bovine serum; NRK, Normal rat kidney; VEPF, Vascular endothelial cell proliferation factor.

0006-291X/85 $1.50

solution by centrifuging at 160 x g for 20 min at 20°C. The upper four-fifth portion of platelet rich plasma was centrifuged at 1,700 x g for 20 min at 20°C, and the platelet pellet was suspended in 10 mM Tris HCl (pH 7.4)/150 mM NaCl/0.01% polyethylene glycol. The platelets were washed twice with the same buffer in the centrifuge and sonicated for 1 min. Erythrocyte and leukocyte contamination in the last platelet suspension was less than 0.1%. Sonicated platelets were centrifuged at 48,000 x g for 30 min at 4°C and the supernatant was used as platelet lysate. Further operations were performed at 4°C unless otherwise specified. PDGF and epidermal growth factor (EGF) were purchased from Collaborative Research.

Cell Cultures and Media: Endothelial cells were collected from a fresh porcine aorta using collagenase digestion as described by Booyse et al. (7). Cloning of endothelial cells was performed by single cell platings as described originally by Puck et al. (8). The endothelial cells were incubated with Ham's F-10 medium containing 10% fetal bovine serum (FBS) and subcultured into 25 cm^2 culture flasks with 0.25% trypsin solution (Gibco) when the cells reached confluency. There was no evidence of transformation or loss of endothelial monolayer under these conditions for more than 6 months. Normal rat kidney (NRK) fibroblasts were obtained by the method described by Duc-Nguyen et al. (9) and cultured in Dulbecco's modified Eagle's medium containing 10% FBS.

Assay for Growth-Promoting Activity: Growth-promoting activity was estimated as the incorporation of [^{3}H] thymidine into serum-deprived cells (10). Cells were seeded in 24-well (2 cm^2) tissue culture plates (Falcon) at a cell density of 10,000 per well. Cells were incubated for 24 hr in 500 µl of media containing 10% FBS to allow for attachment, and then for 24 hr in media containing 0.5% FBS. At this point, platelet lysate or other growth factors were added to the wells and after a further 16 hr incubation, all wells were pulsed for 8 hr with 0.4 µCi of [^{3}H] thymidine (6.7 Ci/mM, New England Nuclear, Boston) per well. The cells were then washed and extracted with 5% trichloroacetic acid. The resulting precipitates were washed twice with ethanol-ether (ratio of vol. 3:1) and solubilized with 1.0 N NaOH. Radioactivity was determined by a liquid scintillation counter.

Gel Chromatography: One milliliter of platelet lysate (10 mg of protein/ml) was applied to a Sephadex G-75 column (2.0 x 82.0 cm) equilibrated with 10 mM Tris HCl (pH 7.4)/150 mM NaCl/0.01% polyethylene glycol and eluted at a flow rate of 10.4 ml/hr at 4°C. The column was calibrated using bovine serum albumin (Mr, 67,000), ovalbumin (Mr, 43,000) and ribonuclease A (Mr, 12,700). Three-milliliter fractions were collected and assayed for growth-promoting activity.

Characterization of the Growth-Promoting Activity on Endothelial Cells: The major fractions (No.47-49) containing the growth-promoting activity were mixed prior to testing. Heat stability was tested by separate treatment of aliquots of the active fractions at 56°C for 30 min, and at 100°C for 10 min. Trypsin sensitivity was tested by incubation of an aliquot with 50 µg of trypsin (specific activity, 11,700 U/mg, Sigma) at 37°C for 2 hr, followed by inactivation using 100 µg of soybean trypsin inhibitor (Sigma). As a reference, 50 µg of trypsin was inactivated with 100 µg of soybean trypsin inhibitor for 30 min and then incubated at 37°C with an aliquot for 2 hr.

RESULTS AND DISCUSSION

Human platelet lysate was examined for its growth-promoting activity on cultured endothelial cells. As shown in Fig. 1, platelet lysate stimulated the incorporation of [^{3}H] thymidine into DNA in a dose-dependent manner.

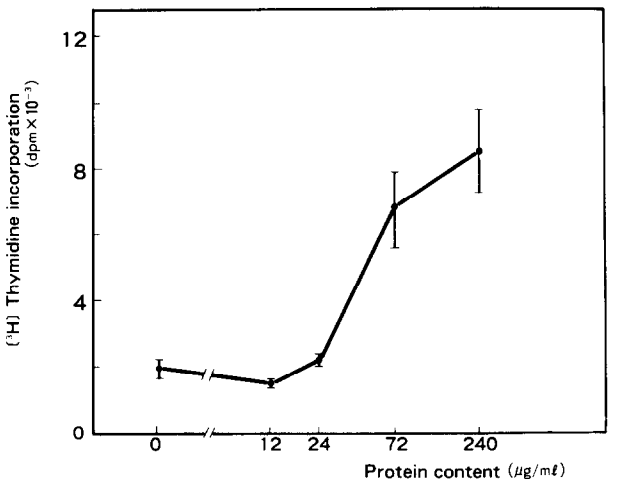

Fig. 1. Effect of platelet lysate on DNA synthesis of endothelial cells. The growth-promoting effect was active in the range of 72-240 μg of protein per ml.

When the platelet lysate was fractionated on a Sephadex G-75 column, at least two peaks of growth-promoting activity on endothelial cells were observed, the major peak being at an apparent molecular weight of 20,000 (Fig. 2). Growth-promoting activity on NRK cells in the fractionated platelet lysate was also examined. Activity was detected at an apparent molecular weight of 30,000, while the active fractions for endothelial cells did not significantly stimulate the growth of NRK fibroblasts (Fig. 2). This factor (Vascular endothelial cell proliferation factor: VEPF) strongly stimulated the incorporation of [^{3}H] thymidine into DNA in a dose-dependent fashion, while

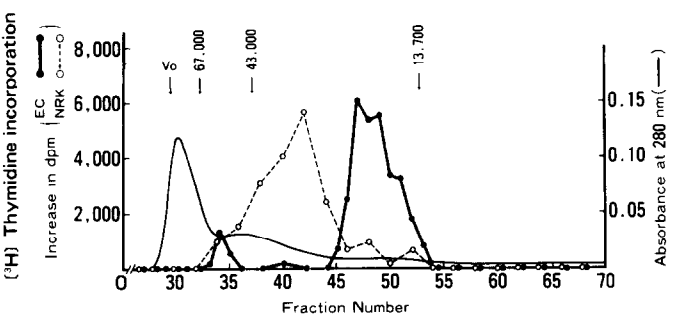

Fig. 2. Sephadex G-75 gel chromatography of the platelet lysate. Growth-promoting activity on endothelial cells was assayed (●). Two peaks of activity were seen, the major peak being at an apparent molecular weight of 20,000. Radioactivity in the control culture was 2,564 ± 370. Activity on the growth of NRK fibroblasts was measured as a reference (o). The major peak was seen at an apparent molecular weight of 30,000, corresponding to PDGF.

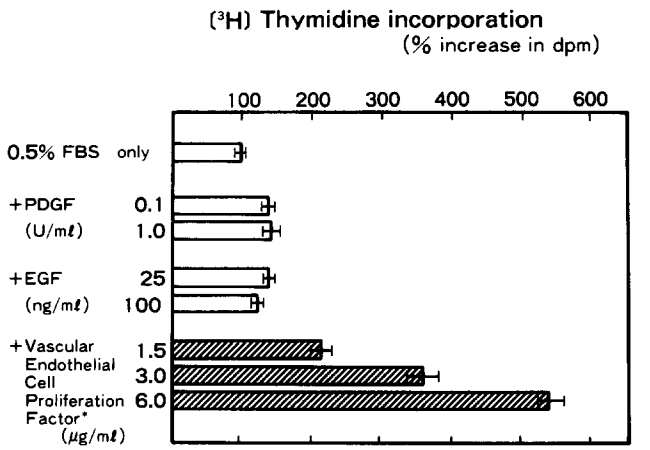

Fig. 3. Effects of various growth factors on the growth of endothelial cells. Vascular endothelial cell proliferation factor (the major part of the growth-promoting activity on endothelial cells obtained by gel chromatography) enhanced the incorporation of [^{3}H] thymidine into DNA in a dose-dependent manner, while other factors tested did not enhance incorporation significantly.

PDGF or EGF did not significantly enhance the incorporation (Fig. 3). VEPF activity was destroyed following incubation at 56°C for 30 min, incubation at 100°C for 10 min, or treatment with trypsin (Table I).

PDGF is a well-known growth factor active on various types of mammalian cells including fibroblasts and vascular smooth muscle cells. However,

Table I. Characterization of Vascular endothelial cell proliferation factor

Treatment	Biological * activity (%)
Not treated	100.0
Heat	
56°C, 30 min	16.9
100°C, 10 min	-12.1
Enzyme	
trypsin	12.7
inactivated trypsin	126.8

* Biological activity was calculated as follows:

$$\text{Biological activity (\%)} = \frac{\text{dpm in treated VEPF - dpm in controls}}{\text{dpm in not treated VEPF - dpm in controls}} \times 100\ \%$$

PDGF lacks the ability to stimulate the growth of endothelial cells (11). In contrast, VEPF potently stimulated the growth of endothelial cells, but did not significantly stimulate the growth of fibroblasts (Fig. 2). These observations suggest that VEPF is functionally different from PDGF. PDGF has been shown to be stable at 100°C, and has molecular weight of 28,000-31,000 with pI of 9.8-10.2 (4-6, 12). VEPF was heat-labile and the apparent molecular weight was 20,000. In addition, isoelectric focusing revealed that the pI of VEPF was 4.0-5.0 (data not shown). These observations strongly suggest that VEPF is biochemically as well as functionally different from PDGF. Thus, it is suggested that fresh platelets contain at least two distinct types of growth factors; PDGF, the growth factor active on vascular smooth muscle cells, and VEPF, the growth factor active on vascular endothelial cells.

Recently, King et al. (13, 14) have isolated a cationic endothelial-growth factor from outdated platelets with an apparent molecular weight of 65,000. In our experiments, we also observed similar growth-promoting activity on endothelial cells in fractions of such a higher molecular weight. However, VEPF activity was much stronger on endothelial cells. The activity of VEPF was present only when the platelet lysate was taken from fresh platelets, suggesting the extreme lability of VEPF at room temperature. Our evidence suggests that VEPF differs from the endothelial-growth factor of higher molecular weight. However, more concrete evidence to this extent requires further purification studies.

ACKNOWLEDGEMENT: We thank Miss Miho Omote for her excellent technical assistance.

REFERENCES

1. Johnson, S.A., Balboa, R.S., Dessel, B.H., Monto, R.W., Siegesmund, K.A., and Greenwalt, T.J. (1964) Exp. Mol. Pathol. 3, 115-127.
2. Kitchens, C.S., and Weiss, L. (1975) Blood 46, 567-578.
3. Maca, R.D., Fry, G.L., Hoak, J.C., and Loh, P.T. (1977) Thromb. Res. 11, 715-727.
4. Ross, R., Glomset, J., Kariya, B., and Harker, L. (1974) Proc. Natl. Acad. Sci. USA 71, 1207-1210.

5. Antoniades, H.N., Sher, C.D., and Stiles, C.D. (1979) Proc. Natl. Acad. Sci. USA 76, 1809-1813.
6. Heldin, C.-H., Westermark, B., and Wasteson, A. (1979) Proc. Natl. Acad. Sci. USA 76, 3722-3726.
7. Booyse, F.M., Sedlak, B.J., and Rafelson, M.E. (1975) Thromb. Diathes. haemorrh. (Stuttg.) 34, 825-839.
8. Puck, T.T., Marcus, P.I., and Cieciura, S.J. (1956) J. Exp. Med. 103, 273-289.
9. Duc-Nguyen, H., Rosenblum, E.N., and Zeigel, R.F. (1966) J. Bacteriol. 92, 1133-1140.
10. Pierson, R.W., and Temin, H.M.J. (1972) J. Cell. Physiol. 79, 319-330.
11. Heldin, C.-H., Westermark, B., and Wasteson, A. (1981) Proc. Natl. Acad. Sci. USA 78, 3664-3668.
12. Deuel, T.F., Huang, J.S., Proffit, R.T., Baenziger, J.U., Chang, D., and Kennedy, B.B. (1981) J. Biol. Chem. 256, 8896-8899.
13. King, G.L., and Buchwald, S. (1984) J. Clin. Invest. 73, 392-396.
14. King, G.L., White, M.F., and Buchwald, S. (1984) Clin. Res. 32, 401A.