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INHIBITION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR BY CONDITIONED MEDIUM FROM CULTURED HUMAN AND PORCINE VASCULAR ENDOTHELIAL CELLS

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Summary. Conditioned serum-free medium both from confluent porcine aortic endothelial cells and from human venous and arterial umbilical cord endothelial cells will inhibit plasminogen activation by tissue-type plasminogen activator. Inhibitory activity in the conditioned medium increases with time, and depends upon protein synthesis. Also, the conditioned medium directly inhibits the amidolytic activity of tissue-type plasminogen activator. Inhibitory activity can be removed from conditioned medium by absorption with immobilized tissue-type plasminogen activator.

Plasminogen activator activity, by histochemical methods, has been shown to be associated, in vivo, with endothelial cells lining blood vessels (1). Cultured vascular endothelial cells give an opportunity to study the synthesis and release of plasminogen activator(s) in vitro. Such studies so far have had conflicting results: some authors have shown the synthesis of plasminogen activators by endothelial cells in culture (2-10), but then others have been unable to do so (5,11-13). One reason for an inability to detect the presence of plasminogen activator activity could be the concomitant secretion into culture medium of substances preventing the detection of plasminogen activators, by inhibiting their enzymatic activity (14). Of the two types of plasminogen activator (tissue-type and urokinase-like), indeed inhibition of urokinase by endothelial cells has been described (3,4,11,12,15-17).

In the present paper we will demonstrate that cultured human and porcine vascular endothelial cells both synthesize and secrete an inhibitor of tissue--type plasminogen activator.

Abbreviations used. BSA: bovine serum albumin; CM: conditioned medium; DMEM: Dulbecco's modification of Eagle's medium; TA: tissue-type plasminogen activator.

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Materials and methods

Materials. Collagenase CLS type II (Worthington, Freehold, USA). DMEM, fetal calf serum, Medium 199 and antibiotics (Flow Laboratories, Irvine, UK). S-2251 and S-2444 (Kabi, Stockholm, Sweden). Human plasminogen was purified from Cohn fraction III by lysine-Sepharose chromatography as described by Deutsch and Mertz (18). Human plasmin (prepared by activation with immobilized urokinase) was a gift from Dr. D.W. Traas, Gaubius Institute. Soluble fibrin digest was prepared as described (19). Tissue-type plasminogen activator (TA) was purified from supernatant of Bowes melanoma cells (20). TA was immobilized by coupling to Eupergit C (Röhm Pharma, Weiterstadt, FRG) according to the manufacturers' instructions. Bovine serum albumin (type V), cycloheximide and Tween 80 (Sigma, St. Louis, USA).

Cells. Porcine aortic endothelial cells were isolated from descending aorta by collagenase digestion (1 mg/ml in DMEM, 15-20 min, 37°C; ref. 21). The isolated cells were resuspended in DMEM supplemented with 20% (v/v) fetal calf serum (heat-inactivated), 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml) and grown to confluency under 5% CO_2 in tissue culture wells that had previously been coated with porcine fibronectin (50 µg/ml). Confluent cells were subcultured by treatment with trypsin/EDTA at a split ratio of 3:1. Human arterial and venous endothelial cells were isolated by collagenase digestion from human umbilical cords, and grown to confluency in fibronectin-coated tissue culture wells in Medium 199, supplemented with 20% (v/v) pooled human serum (not heat-inactivated), 15 mM Hepes, glutamine, penicillin and streptomycin. Subcultures were obtained at a split ratio of 3:1 by trypsin/ EDTA.

Conditioned media (CM) were obtained by incubating cells, after two washes with serum-free medium, with 0.15 ml/cm^2 serum-free medium, containing 0.3 mg/ml BSA, for the indicated periods of time. CM were centrifuged at 1000 x g for 15 min, and stored at -20°C. Cell protein was determined by the method of Lowry et al. (22), using BSA as a standard.

Inhibition of TA activity by CM was determined by three methods.

Method A: In an indirect spectrophotometric assay, TA activity in the presence or absence of CM was measured by a modification of an assay described in detail elsewhere (19). In brief, to wells of a 96-well microtiter plate were added 100 µl buffer (0.1 M Tris.HCl, pH 7.5, containing 0.01% Tween 80), 20 µl soluble fibrin digest (1 mg/ml), 3 µl tissue activator (14 IU/ml), 5 µl conditioned medium, 100 µl S-2251 (0.66 mM) and finally 25 µl plasminogen (1.11 µM). The microtiter plates were incubated at 25°C, and the absorption at 405 nm read in a Titertek Multiscan spectrophotometer after 30, 45 and 60 min. TA activity was calculated according to Drapier et al. (23).

Method B: In a direct spectrophotometric assay (see ref. 24), CM (100 µl) was added to 125 µl buffer containing tissue activator (10 IU/ml) and incubated at 37°C. Residual amidolytic activity on S-2444 was determined spectrophotometrically at 405 nm and 37°C by adding 25 µl S-2444 (1 mM).

Method C: Inhibition of TA by CM was also determined with a clot lysis method, as described elsewhere (24).

Plasmin inhibition by CM was measured as described above for the indirect spectrophotometric inhibitor assay, substituting 10 µl of human plasmin (0.2 CU/ml) for plasminogen and TA, and by the clot lysis method (24).

Results

Cells. Confluent endothelial cell cultures consisted of closely opposed, polygonal cells, lending the cultures a typical cobble-stone appearance (21). Porcine aortic endothelial cells would reach confluency in 4-5 days, with a final cell density of (mean ± s.d., n = 15) 1.11 ± 0.13 x 10^5 cells/cm^2; cell protein 35 ± 4 µg/cm^2. These figures did not alter under normal culture

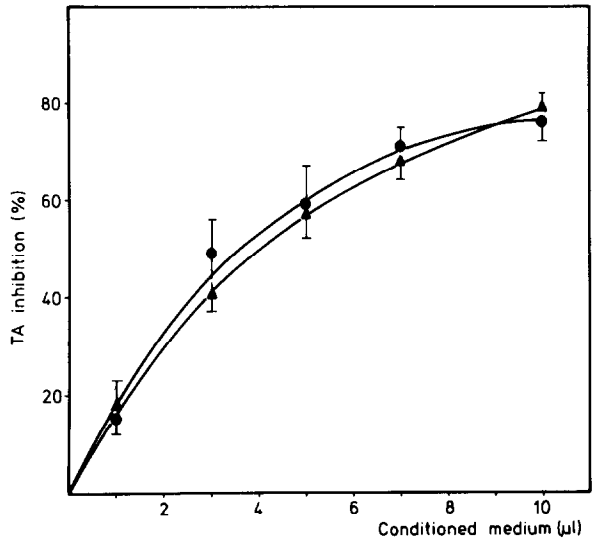

Fig. 1. Inhibition of TA by 24 hour conditioned medium from secondary human venous endothelial cells (●, n = 5) and primary porcine aortic endothelial cells (▲, n = 8). Data represent mean ± SEM. TA inhibition was measured by method A (see Materials and Methods).

conditions during the next days, and remained stable during incubation for 24 hours in serum-free medium. Furthermore, the cells were characterized as endothelial cells by the presence of angiotensin-converting enzyme. No F VIII R:Ag, however, in porcine aortic endothelial cells could be demonstrated by

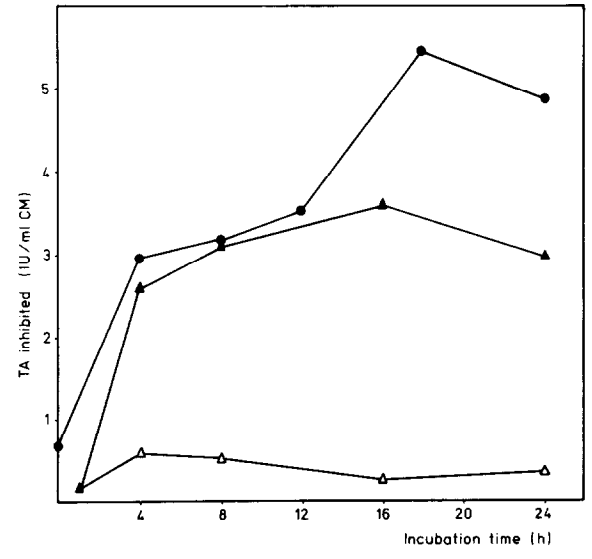

Fig. 2. Time course of increase in TA inhibitory activity in CM from human secondary arterial cells (●, n = 3) and porcine secondary arterial cells (▲, n = 10). In the presence of cycloheximide (3 µg/ml), no increase in inhibition by CM from porcine cells is seen (△, n = 4). TA inhibition was measured by method A (see Materials and Methods).

Table 1

Inhibition of TA by conditioned medium from cultured endothelial cells, and by non-conditioned medium used for cell growth.

		Number of determinations	TA inhibited (IU/ml medium)
A. Conditioned medium (24 hours)			
Cell source of CM	Passage		
Human artery	Secondary	5	4.82* ± 0.78
Human vein	Secondary	9	3.95 ± 0.54
Porcine aorta	Primary	14	3.47 ± 0.40
Porcine aorta	Secondary	16	2.34 ± 0.54
Porcine aorta	Tertiary	6	1.18 ± 0.32
B. Non-conditioned medium			
DMEM/0.03% BSA		17	0.17 ± 0.09
DMEM/20% fetal calf serum		12	1.01 ± 0.26
M199/20% human serum		5	2.44 ± 0.86

*Mean ± SEM. TA inhibition was measured by method A (see Materials and Methods). TA inhibition is expressed as IU of TA inhibited by 1 ml of medium.

indirect immunofluorescence (compare ref. 8). Human venous and arterial endothelial cells contained both angiotensin-converting enzyme and F VIII R:Ag.

Inhibition of TA activity. Endothelial cell CM obtained after 24 hours, dose-dependently, would inhibit TA, as shown in Fig. 1 for human and porcine endothelial cells. TA was inhibited by CM from primary, secondary and tertiary porcine cell cultures and by CM from venous and arterial human cells (Table 1). The amount of inhibitory activity in CM increased with incubation time, reaching a plateau around 17 hours (Fig. 2). This increase was abolished when cells were cultured in the presence of 3 µg/ml cycloheximide (Fig. 2).

In order to exclude the possibility of the inhibitory activity being due to components of the serum used during cell culture prior to the production of CM, the inhibition of TA by media containing 20% serum was measured. A minor inhibitory activity was found (Table 1).

As we wanted to see whether the observed inhibition of TA by CM might be due to inhibition of the plasmin generated during the indirect assay, plasmin

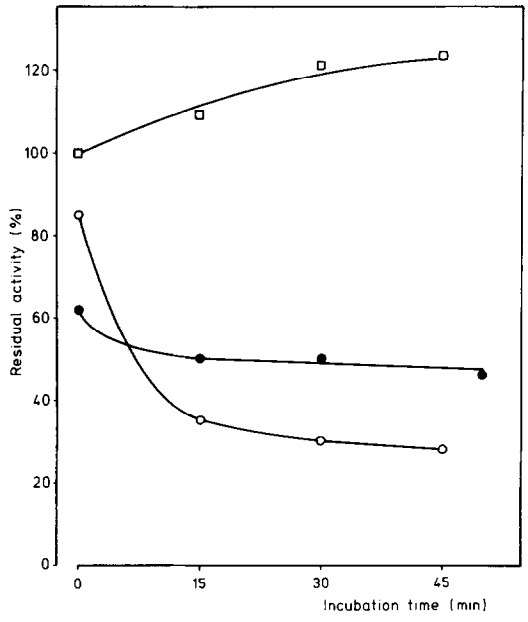

Fig. 3. Effects of human 24-hour conditioned medium on the activity of plasmin (□), and TA (○), as measured in the clot lysis method (method C). Also shown is the inhibition of TA, as measured in the direct spectrophotometric method (●; method B). Assay conditions as described in Materials and Methods.

inhibition by CM was determined. Using a plasmin concentration giving the same ΔOD/min as the one generated during the activator assay, no inhibition by porcine CM was observed (residual plasmin activity = 100 ± 2%; n = 17), while human CM showed a slight plasmin inhibition (residual activity = 94 ± 3%; n = 15) only.

In the clot lysis method, also, no inhibition of plasmin by CM was found, whereas TA activity appeared inhibited progressively by CM (Fig. 3). As shown in the same figure, CM would directly inhibit the amidolytic activity of TA on S-2444 as well.

Absorption of human CM with Eupergit-immobilized TA for 17 hours at 20°C completely removed the inhibitory activity of CM, as measured by the indirect spectrophotometric assay. Absorption with solely Eupergit had no effect.

Discussion

The association of urokinase-inhibition with cultured endothelial cells has been reported before: Dosne et al. (11,16) showed that (serum-containing)

CM from human umbilical vein endothelial cells inhibited urokinase, and Loskutoff et al. demonstrated the presence of an intracellular urokinase inhibitor in various types of endothelial cells (3,4,15). The present data show that serum-free CM from both human and porcine endothelial cells will inhibit tissue-type plasminogen activator. Urokinase-inhibition, occasionally, was found in our serum-free CM, although serum-containing CM would inhibit urokinase as well as TA (unpublished observations).

As cycloheximide was able to suppress the increase in inhibitory activity during culture, the secretion of inhibitory activity must depend on protein synthesis. This does not exclude the possibility of the increase with time being due to a - protein synthesis-dependent - regurgitation of stored serum components. However, it is most unlikely, as the serum-containing culture media themselves would contain little inhibitory activity only. Whether the synthesis of plasminogen activator inhibitor(s) is an artefact of cell culture, or the endothelial cells will synthesize inhibitor(s) in vivo as well, remains to be established.

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References

1. Todd, A. S. (1959) J. Pathol. Bacteriol. 78, 281-283.
2. Buonassisi, V., and Venter, J. C. (1976) Proc. Natl. Acad. Sci. USA 73, 1612-1616.
3. Loskutoff, D. J., and Edgington, T. S. (1977) Proc. Natl. Acad. Sci. USA 74, 3903-3907.
4. Levin, E. G., and Loskutoff, D. J. (1979) Thromb. Res. 15, 869-878.
5. Laug, W. E., Tokes, Z. A., Benedict, W. F., and Sorgente, N. (1980) J. Cell Biol. 84, 281-293.
6. Shepro, D., Schleef, R., and Hechtman, H. B. (1980) Life Sci. 26, 415-422.
7. Booyse, F. M., Scheinbuks, J., Radek, J., Osikowicz, G., Feder, S., and Quarfoot, A. J. (1981) Thromb. Res. 24, 495-504.
8. Booyse, F. M., Quarfoot, A. J., Radek, J., and Feder, S. (1981) Blood 58, 573-583.
9. Laug, W. E. (1981) Thrombos. Haemostas. 45, 219-224.

10. Bykowska, K., Levin, E. G., Rijken, D. C., Loskutoff, D. J., and Collen, D. (1982) Biochim. Biophys. Acta 703, 113-115.
11. Dosne, A. M., Dupuy, E., and Bodevin, E. (1978) Thromb. Res. 12, 377-387.
12. Moscatelli, D., Jaffe, E., and Rifkin, D. B. (1980) Cell 20, 343-351.
13. Goldsmith, G. M., Ziats, N. P., and Robertson, A. L. (1981) Exp. Mol. Pathol. 35, 257-264.
14. Roblin, R. O., Young, P. L., and Bell, T. E. (1978) Biochem. Biophys. Res. Commun. 82, 165-172.
15. Loskutoff, D. J., and Edgington, T. S. (1981) J. Biol. Chem. 256, 4142-4145.
16. Esnard, F., Dupuy, E., Dosne, A. M., and Bodevin, E. (1982) Thrombos. Haemostas. 47, 128-131.
17. Smariga, P. E., and Maynard, J. R. (1982) Blood 60, 140-147.
18. Deutsch, D. G., and Mertz, E. T. (1970) Science 170, 1095-1096.
19. Verheijen, J. H., Nieuwenhuizen, W., and Wijngaards, G. (1982) Thromb. Res. 27, 377-385.
20. Rijken, D. C., and Collen, D. (1981) J. Biol. Chem. 256, 7035-7041.
21. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) J. Clin. Invest. 52, 2745-2756.
22. Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951) J. Biol. Chem. 193, 265-275.
23. Drapier, J. C., Tenu, J. P., Lemaire, G., and Petit, J. F. (1979) Biochimie 61, 463-471.
24. Wijngaards, G. (1979) In: Progress in Chemical Fibrinolysis and Thrombolysis (J. F. Davidson, V. Cêpelàk, M. M. Samama, and P. C. Desnoyers, eds), Vol. 4, pp. 368-373, Churchill Livingstone, Edinburgh.