Local synthesis of plasminogen by the seminiferous tubules of the testis

O. Saksela and K.K. Vihko+

Department of Virology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki and +*Institute of Biomedicine, Department of Anatomy, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland*

Received 6 June 1986

Plasminogen activation occurs through conversion of plasminogen to plasmin by plasminogen activators. In adult mammals, liver has been the only known site for plasminogen synthesis. Seminiferous tubules secrete plasminogen activator, but are behind a barrier that excludes the entrance of many macromolecules to this tissue. Therefore, it became of interest to study the existence of plasminogen in this system. After metabolic labeling, a 90 kDa lysine-binding polypeptide was found. This protein could be immunoblotted by anti-plasminogen antibody, and was shown to possess urokinase-dependent proteolytic activity. The findings suggest that plasminogen is synthesized by seminiferous tubules. We propose that local plasminogen synthesis may occur in tissues which are separated by specific anatomical barriers.

Plasminogen synthesis *(Seminiferous tubule, Testis)*

1. INTRODUCTION

Plasminogen is a proenzyme, which is proteolytically activated by specific serine proteinases, the plasminogen activators, to plasmin, another serine proteinase capable of degrading e.g. polymerized fibrin in blood clots and known glycoproteins of the extracellular matrix [1,2]. The site of synthesis of plasminogen in circulation remained unconfirmed for a long time, though this zymogen represents the quantitatively most prominent proteolytic activity of plasma and of most extravascular fluids. It was not until 1980, when Raum et al. [3] demonstrated that liver is the principal source of human plasminogen. Simultaneously, Bohmfalk and Fuller [4] published their article describing synthesis of plasminogen by cultures of primary adult rat hepatocytes. Since then no new sites of synthesis have been detected in human. Valinsky and Reich [5] showed metabolically labeled plasminogen in the chick embryo and concluded that de novo synthesis occurs during the early phases of embryonic life. Isseroff and Rifkin [6] found plasminogen in the peripheral intracytoplasmic area of the basal keratinocytes of the skin, but failed to find any signs of plasminogen synthesis in cultures of epidermal cells.

Spermatogenesis occurs in seminiferous tubules separated from circulation. The effectiveness of the barrier is clearly demonstrated by the fact that polypeptide structures of the developing spermatogenic cells are highly immunogenic to the host. It is therefore a matter of interest that Sertoli cells inside the tubular wall are capable of secreting plasminogen activators [7–9]. The only well-characterized substrate apart from enzyme inhibitors [10] of these highly specific proteinases is plasminogen, and free diffusion of this zymogen or of other plasma macromolecules into the intratubular compartment has not been demonstrated [11]. This led us to look for possible local synthesis of plasminogen by the seminiferous tubules and here we demonstrate the accumulation of metabolically labeled plasminogen in the culture medium of rat seminiferous tubules.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2. MATERIALS AND METHODS

Male adult Wistar rats (2–12 months) were anesthesized with ether and killed by cardiac puncture. The testes were sterilely removed, and the seminiferous tubules were mechanically separated from the interstitial tissue in a petri dish under a stereomicroscope. The characteristic transillumination pattern of the tubules [12] was identified and 2 mm pieces of tubules were dissected representing stages II–VI, VII–VIII, IX–XII and XIII–I of the seminiferous epithelial cycle [13]. In each stage 1 mm of the tubule corresponds to about 1 mg tissue protein [7]. For the experiments, 50 pieces of each pool of stage were dissected. The tubule preparations do not contain significant amounts of interstitial Leydig cells as judged from their inability to respond to stimulation by the luteinizing hormone [14]. The tissue pieces were washed and incubated in 1 ml Eagle's minimal essential medium lacking methionine and supplied with antibiotics, at 32°C in humidified 5% CO_2 atmosphere. For metabolic labeling of the peptides, 40 μCi L-[^{35}S]-methionine/ml (800 Ci/mmol, Amersham, England) and the proteinase inhibitor aprotinin (100 IU/ml, Bayer, Leverkusen, FRG) were added. After incubations of 24, 48 and 72 h, the supernatants were collected and centrifuged, first at $500\times g$ for 15 min and then at $10000\times g$ for 30 min. Lysine-Sepharose (Pharmacia, Uppsala, Sweden) was added to the medium samples (100 μl of 50% (v/v) particles/ml) and incubated overnight at 4°C. The beads were washed several times with phosphate buffer (0.05 M, pH 7.5) and finally with 0.3 M NaCl dissolved in the same buffer. Next, the beads were taken in nonreducing Laemmli sample buffer [15] or, when the activity of the preparation was not tested, the bound material was eluted with ϵ-aminocaproic acid in distilled water (4×100 μl, 0.3 M) [16], and the proteins were precipitated by adding trichloroacetic acid to a final concentration of 5% and dissolved in the sample buffer. Plasminogen was identified from the samples by polyacrylamide gel electrophoresis followed by immunoblotting the protein on a nitrocellulose filter [17] with antihuman plasminogen (Dako, Denmark). Immunoprecipitation was carried out with the IgG fraction of the above antiserum (purified by the protein A method) followed by capture with protein A-Sepharose (Pharmacia).

3. RESULTS

The lysine-Sepharose method used for obtaining sufficient amounts of plasminogen for the immunological and radiographical analyses was sensitive enough to concentrate plasminogen from 500 μl rat plasma diluted 1:10000 (approx. 10 ng) for visualization in immunoblotting (fig.1a). The polypeptide doublet [18] bound from the culture medium of the seminiferous tubules comigrated with the one obtained from rat plasma (fig.2a). For the most part, it obviously represented plasminogen derived from the extratubular structures such as disrupted capillaries and contaminating plasma. It was the only polypeptide visualized

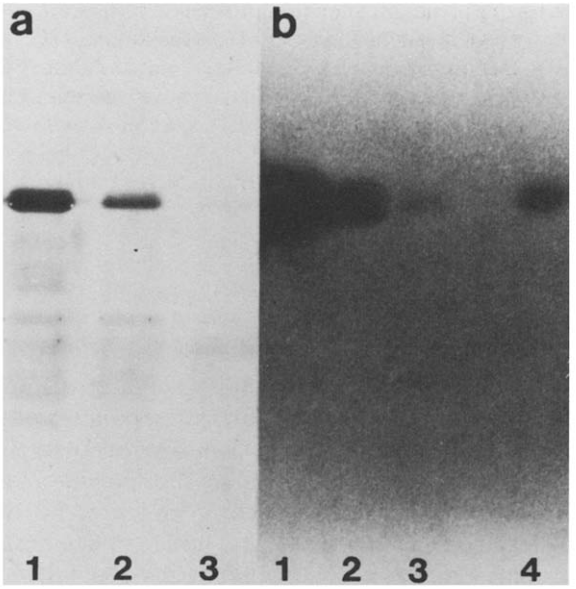

Fig.1. (a) Immunoblotting of plasminogen from rat plasma. Diluted samples of rat plasma (vol. 500 μl) were treated with lysine-Sepharose and the immobilized proteins were electrophoresed in a 8% polyacrylamide gel, transferred to nitrocellulose and visualized by immunoblotting. The samples contained about 1 μg (1), 100 ng (2), and 10 ng (3) plasminogen. (b) The above samples (1–3) showed proteolytic activity after treatment with plasminogen activator in the zymography assay. Sample 4 contains plasminogen bound from the conditioned medium of seminiferous tubules. The zymography assay [20] involves detection of proteolytic activity present in a polyacrylamide gel by an agarose gel overlay, which contains an opalescent substrate such as casein. Degradation of the substrate becomes visible as a clear band over the proteinase.

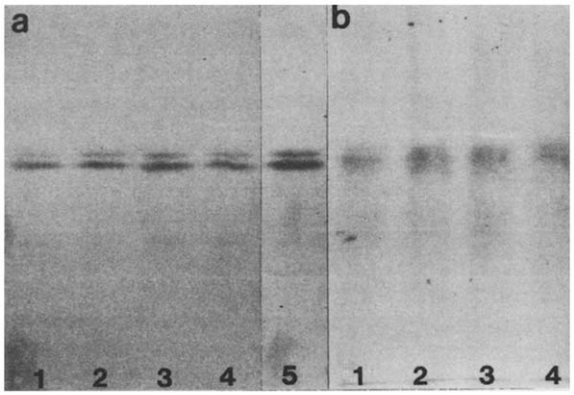

Fig.2. (a) Immunoblotting of plasminogen in the conditioned medium of seminiferous tubules. The samples represent stages II–IV (1), VII–VIII (2), IX–XII (3), and XIII–I (4) of the seminiferous epithelial cycle. Sample 5 represents plasminogen from rat plasma (about 100 ng). The seminiferous tubules (100 mg) were cultured in serum-free medium (1 ml) for 72 h and the plasminogen present in the growth medium was bound to lysine-Sepharose particles. The bound material was eluted with ϵ-aminocaproic acid followed by precipitation with trichloroacetic acid. The proteins were dissolved in sample buffer and electrophoresed in a 5–16% gradient polyacrylamide gel with a 3.5% stacking gel. (b) Autoradiography of the same nitrocellulose sheet as in a. The radioactivity (samples 1–4) comigrates with the plasminogen immunoreactivity.

after protein staining of the polyacrylamide gel (not shown). After autoradiography of the immunoblotting filter, a metabolically labeled protein was seen to comigrate with the immunoreactive protein (fig.2b).

The growth media from all four stage pools (II–VI, VII–VIII, IX–XII and XIII–I) contained roughly the same amount of the labeled protein, although electrophoretic analysis of the precipitated total proteins after 72 h labeling with [^{35}S]-methionine showed differences between the proteins secreted into the conditioned media (fig.3) thus confirming the separation of the various stages of the seminiferous tubule. The secretion of the labeled protein increased with time, and it could be removed from the conditioned medium prior to the lysine-Sepharose concentration by immunoprecipitation with purified anti-plasminogen-IgG (fig.4).

The lysine-Sepharose bound protein both from rat plasma and from the medium samples was

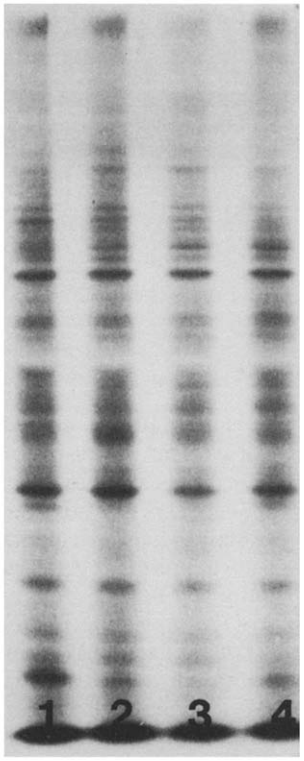

Fig.3. Autoradiography of the proteins secreted during 72 h by the same stage groups as in fig.2 (1–4). The samples were electrophoresed in an 8% polyacrylamide gel. Differences are seen between the proteins secreted by the various stage groups.

found to possess proteolytic activity when tested in the zymography assay (fig.1b), but only after treatment with plasminogen activator (54 kDa urokinase-type plasminogen activator, 60 000 IU/mg, Calbiochem, La Jolla, CA). Since aprotinin was present during the sample collection, the activity detected in the zymography assay was due to activation of plasminogen to plasmin in the gel. Plasminogen activator (1.0 IU/ml) was present in the casein-agarose overlay to carry out this activation.

Taken together, we find in the spent media of rat seminiferous tubular segments a lysine-binding ϵ-aminocaproic acid eluting protein reacting in immunoblotting with antiserum against plasminogen and possessing proteolytic activity after activation with plasminogen activator. This protein comigrates with a metabolically labeled polypeptide precipitable with antiplasminogen antiserum. These

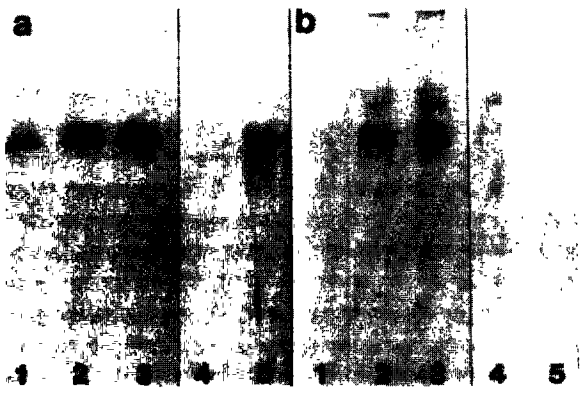

Fig.4. (a) Immunoblotting of plasminogen showing accumulation of the protein during incubation of the seminiferous tubules. The incubation periods were 24 h (1), 48 h (2), and 72 h (3). The antigenicity is removed by treatment of the conditioned medium with anti-human plasminogen and protein A-Sepharose (4). Sample 5 shows plasminogen derived from rat plasma. (b) Autoradiography of the same nitrocellulose sheet as in a. The metabolically labeled band (b,1–3) comigrating with rat plasma plasminogen (a,5) is removed by antiplasminogen (b,4) together with the plasminogen immunoreactivity (a,4). The lysine-Sepharose bound material was directly subjected to electrophoresis without elution with ϵ-aminocaproic acid. This resulted in the appearance of some additional metabolically labeled proteins with higher molecular mass into the autoradiography.

properties strongly suggest that the synthesized protein is plasminogen.

4. DISCUSSION

The seminiferous tubules consist of myoid cells surrounding the tubules, a thick basement membrane and cells inside the tubules. The spermatogonia, preleptotene spermatocytes and the large Sertoli cells rest on the inner side of the basement membrane. During the very early phases of the maturation of the spermatogenic cells, the preleptotene spermatocytes are detached from the basement membrane and become totally wrapped by the surrounding Sertoli cells [20]. These cells are tightly bound to each other by special attachment structures, which form the most effective part of the blood-testis barrier [21]. Urokinase-like plasminogen activator can be immunochemically localized in Sertoli cells, where it is seen in Golgi-like structures of the cytoplasm [22]. The secretion of this proteinase from the separated seminiferous structures is cyclic and is most prominent from the stages VII–VIII [7–9,22]. The secretion of plasminogen activator is at least partially regulated by the preleptotene primary spermatocytes [7]. In addition, Sertoli cells increase their plasminogen activator secretion after stimulation with the follicle stimulating hormone and cyclic AMP [8]. Potential targets for the action of plasminogen activation-mediated proteolysis are the matrix components during detachment of the primary spermatocytes from the basement membrane or dissolution of the intercellular junctions between the Sertoli cells, which occurs during the transport and maturation of the spermatocytes into the lumen of the tubules [7]. A role for the proteolytic activity in the modulation of cell-secreted proteins could also be anticipated [23,24]. The presence of plasminogen during all stages of maturation suggests that the regulation of proteolysis may occur via the action of plasminogen activator and/or an inhibitor of plasminogen activators.

The cells secreting plasminogen remain unknown. In immunofluorescence or immunoperoxidase studies (not shown) the vascular and extracellular areas outside the seminiferous tubules could be stained with antiplasminogen. The amount possibly located inside the tubules may, however, be too small to be visualized with the staining methods and antisera currently at use. It is possible that the observed synthesis might occur in the metabolically multipotent Sertoli cells, which are known to secrete e.g. transferrin [25] and ceruloplasmin [26].

To our knowledge, the seminiferous tubule is the first extrahepatic site of plasminogen synthesis in mammals. Such local production could occur at places not reached by the circulating pool of plasminogen, such as the nervous system. Cells located behind the blood-brain barrier secrete plasminogen activators [24,27], which suggests that either these enzymes have another substrate apart from plasminogen or the local synthesis of plasminogen takes place in that organ as well.

REFERENCES

[1] Danø, K., Andreasen, P.A., Grøndahl-Hansen, J., Kristensen, P., Nielsen, L.S. and Skriver, L. (1985) Adv. Cancer Res. 44, 139–266.

[2] Saksela, O. (1985) Biochim. Biophys. Acta 823, 35–65.
[3] Raum, D., Marcus, D., Alper, C.A., Levey, R., Taylor, P.D. and Starzl, T.E. (1980) Science 208, 1036–1037.
[4] Bohmfalk, J.F. and Fuller, G.M. (1980) Science 209, 409–410.
[5] Valinsky, J.E. and Reich, E. (1981) J. Biol. Chem. 256, 12470–12475.
[6] Isseroff, R.R. and Rifkin, D.B. (1983) J. Invest. Dermatol. 80, 297–299.
[7] Vihko, K.K., Suominen, J.J.O. and Parvinen, M. (1984) Biol. Reprod. 31, 383–389.
[8] Lacroix, M., Smith, F.E. and Fritz, I.B. (1977) Mol. Cell Endocrinol. 9, 227–236.
[9] Vihko, K.K., Toppari, J., Saksela, O., Suominen, J.J.O. and Parvinen, M. (1986) Acta Endocrinol., in press.
[10] Nielsen, L.S., Andreasen, P.A., Grøndahl-Hansen, J., Skriver, L. and Danø, K. (1986) FEBS Lett. 196, 269–273.
[11] Fritz, I.B. (1985) in: Recent Progress in Cellular Endocrinology of the Testis. INSERM 123, 15–54.
[12] Parvinen, M. and Vanha-Perttula, T. (1972) Anat. Rec. 174, 435–450.
[13] Parvinen, M. and Ruokonen, A. (1982) J. Androl. 3, 211–220.
[14] Parvinen, M., Nikula, H. and Huhtaniemi, I. (1984) Mol. Cell. Endocrinol. 37, 331–336.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Deutsch, D.G. and Mertz, E.T. (1970) Science 170, 1096–1097.
[17] Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[18] Powell, J.R. and Castellino, F.J. (1983) Biochemistry 22, 923–927.
[19] Granelli-Piperno, A. and Reich, E. (1978) J. Exp. Med. 146, 223–234.
[20] Runell, L. (1977) Am. J. Anat. 148, 313–328.
[21] Dym, M. and Fawcett, D.W. (1970) Biol. Reprod. 3, 308–326.
[22] Vihko, K.K., Kristensen, P., Toppari, J., Saksela, O. and Danø, K. (1985) J. Cell Biol. 101, 367a.
[23] Virji, M.A.G., Vassalli, J.-D., Estensen, R.D. and Reich, E. (1980) Proc. Natl. Acad. Sci. USA 77, 875–879.
[24] Granelli-Piperno, A. and Reich, E. (1983) J. Cell Biol. 97, 1029–1037.
[25] Skinner, M.K. and Griswold, M.D. (1980) J. Biol. Chem. 225, 9523–9525.
[26] Skinner, M.K. and Griswold, M.D. (1983) Biol. Reprod. 28, 1225–1229.
[27] Kristensen, P., Nielsen, L., Grøndahl-Hansen, J., Andreasen, P., Larsson, L.-I. and Danø, K. (1985) J. Cell Biol. 101, 305–311.