Biochimica et Biophysica Acta, 525 (1978) 180—185

BBA 68489

INHIBITOR OF TRYPSIN AND CHYMOTRYPSIN IN CULTURES OF HeLa CELLS

MARIA B. BERNIK

Section of Nephrology and Hypertension, Department of Medicine, Northwestern University Medical School and Northwestern Memorial Hospital, Chicago, Ill. (U.S.A.)

(Received January 12th, 1978)

Summary

An inhibitor of trypsin and chymotrypsin with apparent molecular weight of 68000 and a mobility similar to α_1-globulin on polyacrylamide gel electrophoresis, was isolated from serum-free supernatant preparations from HeLa cells. Immunoelectrophoresis assays indicated that the inhibitor differed serologically from known inhibitors of serine proteinases in plasma and urine but shared antigenic determinants with an unidentified protein in these body fluids and with an inhibitor recently isolated from cultures of lung.

Introduction

An inhibitor of trypsin (EC 3.4.21.4) chymotrypsin (EC 3.4.21.1) and plasmin (EC 3.4.21.7) with an estimated molecular weight of approximately 68000 and mobility similar to α_1-globulin, was isolated from supernatant preparations of primary cultures and subcultures of human adult and fetal lung [1]. Immunoassays with various antisera indicated that this inhibitor is immunologically distinct from other proteinase inhibitors in the circulation: α_1-anti-trypsin, α_1-antichymotrypsin, inter-α-trypsin inhibitor, antithrombin III, CI′ esterase inhibitor, α_2-macroglobulin, and the recently described α_2-antiplasmin [2,3,4]. The present study indicates that an inhibitor with properties similar to those of the inhibitor from lung tissue may be produced in large concentrations by HeLa cells.

Methods and Results

Accumulation of antitryptic activity in serum-free supernatant preparations of HeLa cells (S3 clone, Grand Island, Biol. Co., Long Island, N.Y.) was examined as described for cultures of lung tissue [1]. Cells growing as monolayers in plastic flasks were washed with Eagle's Basal Medium and grown in this me-

dium containing 0.5% lactoalbumin hydrolysate. Cultures were incubated at 37°C and the supernatant medium assayed at various intervals for antitryptic activity on a fibrin substrate. Trypsin (bovine, twice crystallized, 13000 Bz-Arg-OEt U/mg protein, Sigma Chemical Co., St. Louis, Mo.) was incubated with equal volumes of supernatant preparations (diluted serially as necessary with 0.75 M sodium barbital, pH 7.75) or with buffer alone for 20 min at 37°C and then assayed on fibrin plates prepared with purified plasminogen-free fibrinogen (Miles Laboratories, Elkart, Ind.). Inhibitory activity was expressed in Bz-Arg-OEt U/ml of trypsin inhibited on this substrate.

Cultures were studied until cells began to slough, 3—5 days after they received serum-free medium. Inhibitor bioactivity was elicited in 16 of the 20 cultures studied. In these cultures, accumulation was progressive reaching levels of up to 400 Bz-Arg-OEt U/ml on days 3—5 (Fig. 1). The supernatant solutions were collected, concentrated in an Amicon filter cell using Diaflo PM 30 membranes and chromatographed on Sephadex G-100 or Sepharcryl S-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) 0.9 × 55 cm columns, equilibrated in 0.2 M phosphate buffer, pH 6.5 [1] with essentially quantitative recovery of inhibitor bioactivity, eluting near the elution volume of human albumin monomer standard (M_r = 67500) (Miles Laboratories), as observed previously [1] for inhibitor from lung. Fractions containing 75% of the applied bioactivity were concentrated and examined by polyacrylamide gel electrophoresis. As shown in Fig. 2, the preparation from HeLa cells resolved into 2 major and several minor bands. Inhibitor bioactivity was located throughout one of the major bands, which exhibited the mobility of α_1-globulin similar to that of the inhibitor from lung. This band accounted for about 40% of the protein applied to the gel

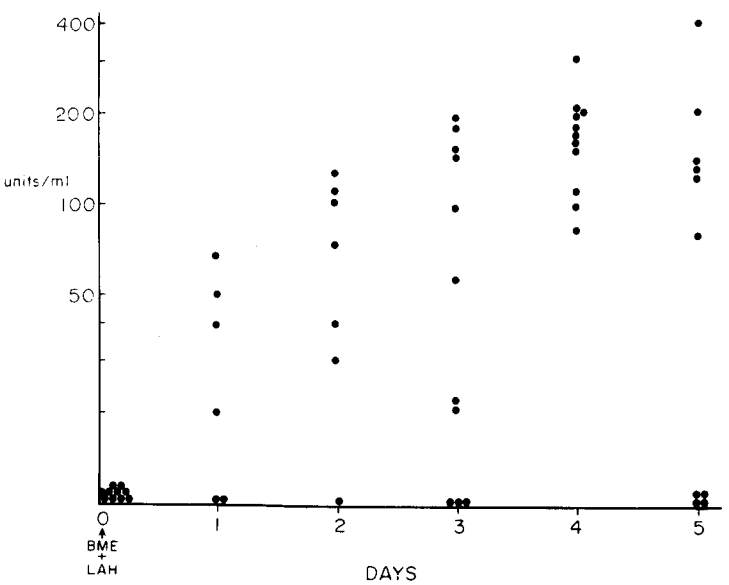

Fig. 1. Accumulation of antitryptic activity in serum-free supernatant preparations of HeLa cells. Arrow indicates feeding of the cultures with Eagle's Basal Medium (BME) and lactoalbumin hydrolysate (LAH). Days of observation are shown on the abscissa and inhibitory activity in the supernates on the ordinate in Bz-Arg-OEt U/ml. Fibrin plate assays.

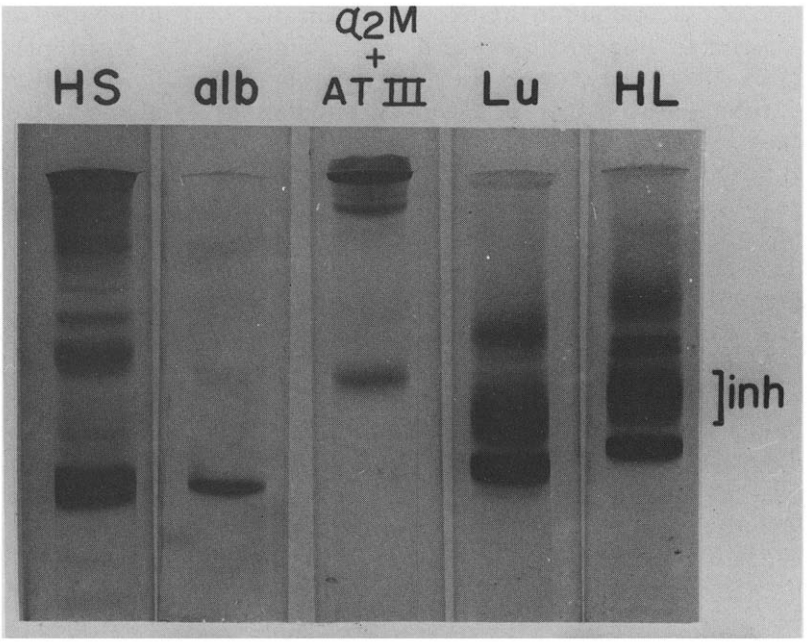

Fig. 2. Polyacrylamide gel electrophoresis (7.5% gel) of G-100 chromatographed preparations from HeLa (HL). Other preparations are: human serum (HS), albumin (alb), α_2-macroglobulin and antithrombin III (α_2M, AT III) and a chromatographed preparation of inhibitor from lung (Lu). Inhibitory activity from HeLa cells and lung tissue, determined by slicing gels longitudinally and incubating 1 mm sections with trypsin on fibrin substrate, is located in the band marked "Inh". Proteins were stained with Coomassie Blue.

as estimated by densitometry scanning (Beckman model R-100 Microzone Densitometer). The inhibitorcontaining band was sliced out from identical unstained gels (to remove approx. 60% protein from the band). The sections were sliced longitudinally and used in counter immunoelectrophoresis assays (Fig. 3).

Immunologic identity of the inhibitor from HeLa cells to proteinase inhibitors in human serum was examined using specific antisera to α_1-antitrypsin, α_1-antichymotrypsin, inter-α-trypsin inhibitor, CI′ esterase inhibitor, antithrombin III, α_2-macroglobulin (from Behring Diagnostics, Sommerville, N.J.) and α_2-antiplasmin (provided by Dr. D. Collen, Leuven, Belgium) [2]. Antiserum to inter-α-trypsin inhibitor was also used to examine immunologic identity to inhibitor in urine which has been shown to react with this antiserum [5,6]. In immunodiffusion, immunoelectrophoresis and counter immunoelectrophoresis, the antisera formed visible banding with their respective antigens in human serum standards (Behring Diagnostics) down to concentrations of 1—3 μg antigen per well. In assays performed with the inhibitor from HeLa cells, using a wide range of concentrations (2—20 μg inhibitor protein per well), there was no reaction with any of the antisera. There was also no reaction when the inhibitor band (sectioned from gels) was assayed with the antisera on counter immunoelectrophoresis. However, an immunoprecipitation reaction occurred with polyvalent antisera to urinary and serum proteins (Fig. 3), indicating that

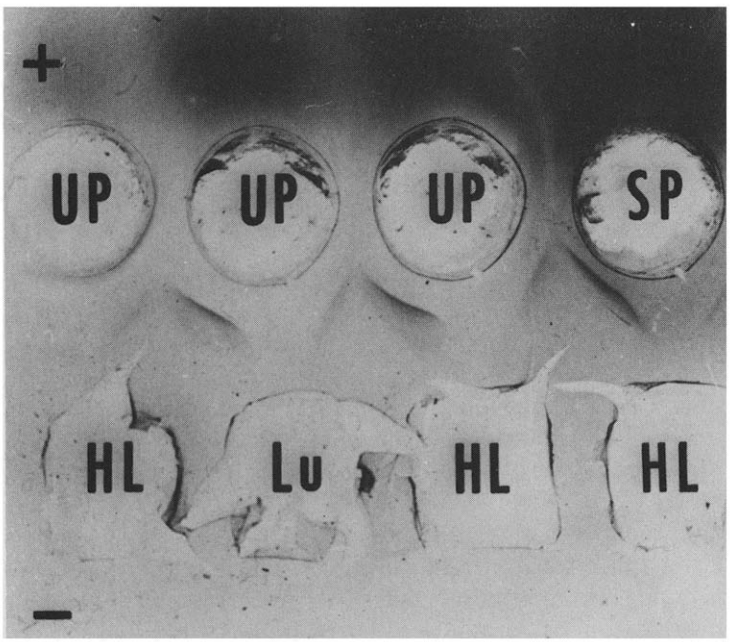

Fig. 3. Counter immunoelectrophoresis in agarose (0.9% agarose, pH 8.6 phosphate buffer, 30 V/cm for 20 min) of inhibitor from HeLa cells (HL) and lung tissue (Lu). Each band contains about 5 μg protein. Antibodies, placed in the anodic wells, are antiserum to urinary proteins (UP) or to whole human serum (SP). Proteins were stained with Amido Black.

these antisera contained antibody to this inhibitor material. Immunoprecipitin bands formed by the inhibitor from HeLa cells and the two antisera showed reaction of identity to one another and to the band of inhibitor from lung tissue as evidenced by fusion without spurring of the respective bands (Fig. 3).

In assays on fibrin and casein substrate (casein-agar plates, Worthington Biochemicals, Freehold, N.J.) chromatographed inhibitor preparations inhibited the activity of both trypsin and chymotrypsin (bovine three times crystallized, Worthington Biochemicals). Inhibition of human plasmin, (20 CTA * U/mg protein, kindly provided by L. Summaria, Michael Reese Medical Center, Chicago, Ill.) on the same substrates was weak or absent. Thus, at inhibitor concentrations which were adjusted to inhibit 20 μg trypsin (M_r = 23300), there was inhibition of about 15 μg chymotrypsin (M_r = 25000) but inhibition of only 12—14 μg plasmin (M_r = 73500) by some preparations and no inhibition by other preparations.

Discussion

The inhibitor of serine proteinases presently found in cultures of HeLa cells differed immunologically from known inhibitors in serum or urine, but showed several similarities to an inhibitor recently isolated from cultures of lung tissue

* CTA refers to the unit adopted by the Committee on Thrombolytic Agents, National Heart Insitute, National Institutes of Health, Bethesda, Md., U.S.A. [7]. The unit is based on the activity of a standard plasmin preparation (CTA standard) prepared by the Committee.

[1]. Similarities included: elution on Sephadex G-100 or Sephacryl S-200 columns in the region of albumin, a mobility similar to α_1-globulin on electrophoresis, non-reactivity with antisera to known proteinase inhibitors, formation of an immunoprecipitation band with polyvalent antisera to urinary and serum proteins and, perhaps most significantly, reaction of serologic identity between the band from HeLa cells and that from lung tissue. Whether the inhibitor from these tissues is similar or identical in other parameters remains to be determined.

Reactivity with the polyvalent, but not the specific, antisera indicates that the inhibitor from lung tissue and HeLa cells is related serologically to an antigen in urine and serum distinct from known inhibitors of proteinases in these biologic fluids. The identity of the antigen, however, is not apparent from the present observations. Thus, it may only be sepculated whether the antigen represents a known or a previously unrecognized protein and, further, whether this protein may be functionally, as well as serologically, similar or identical to the inhibitor from tissues.

Other aspects not apparent from the present study include the precise physiologic function(s) of the inhibitor and relationship, if any, to trypsin inhibitor(s) described recently in animal tumor tissues [8] and to small molecular weight (3000—13000) inhibitors of serine or other proteinases elicited in various mammalian tissues [9—12]. Larger-sized inhibitors (44000—50000) in human tissues such as skin [13,14] appear to differ from the present inhibitor in molecular weight as well as in immunologic identity with inhibitors in serum [13], or lack of inhibitory activity towards trypsin [14]. Specificity towards thiol dependant proteinases and a molecular weight of 90000 also distinguish a newly-described inhibitor in human serum [15] from the present inhibitor in HeLa cells and lung tissue. The inhibitor from these tissues appears to have a molecular weight of approx. 68000 as estimated by Sephadex and Sephacryl chromatography and, in the case of inhibitor from lung tissue, by SDS polyacrylamide gel electrophoresis [1]. The limited observations on the biological activity indicate that, in common with most previously recognized inhibitors of serine proteinases, the present inhibitor affects the activity of more than one enzyme. Whether the inhibitor participates in the regulation of known proteolytic processes in the blood (i.e. clotting, fibrinolysis, kinin generation) [9,15] or performs some other function(s) in tissues or at the cellular level remains to be determined.

Acknowledgements

This work was supported by U.S.P.H.S. Grants AM 14018 and RR 05370, and by a Grant from the Otho S.A. Sprague Memorial Institute.

References

1 Bernik, M.B. (1977) Thrombos. Res. 11, 739—746
2 Collen, D. (1976) Eur. J. Biochem. 69, 209—215
3 Moroi, M. and Aoki, M. (1976) J. Biol. Chem. 251, 5956—5965
4 Mullertz, S. and Clemenssen, I. (1976) Biochem. J. 159, 545—552
5 Hochstrasser, K., Bretzel, G., Feuth, H., Hilla, W. and Lempart, K. (1974) Hoppe Seylers Z. Physiol. Chem. 357, 153—162

6 Sumi, H., Takada, Y. and Takada, A. (1977) Thrombos. Res. 11, 747—754
7 Johnson, A.J., Kline, D.L. and Alkjaersig, M. (1969) Thrombos. Diathes. Hemorrh. 21, 259—262
8 Steven, F.S., Podrazky, V. and Itzhaki, S. (1977) Biochim. Biophys. Acta 483, 211—214
9 Werle, E. and Zickgraf-Rudel, G. (1972) Z. Klin. Chem. Klin. Biochem. 10, 130—150
10 Eisenstein, R., Kuettner, K.E., Neapolitan, G., Sobie, L.W. and Sorgente, M. (1975) Am. J. Pathol. 81, 337—346
11 Katunuma, M. and Kaminaki, E. (1977) in Proteinases in Mammalian Tissues and Cells (Barrett, A.J., ed.), p. 151—180, Elsevier/North Holland Biomedical Press, Amsterdam
12 Harris, Jr., E.D. and Cartwright, E.C. (1977) in Proteinases in Mammalian Tissues and Cells (Barrett, A.J., ed.), p. 249—283, Elsevier/North Holland Biomedical Press, Amsterdam
13 Fraki, J.E. and Hopsu-Havu, V.K. (1972) Arch. Derm. Forsch. 243, 155—163
14 Strickling, G.P., Bauer, E.A., Jeffrey, J.J. and Eisen, A.Z. (1977) Biochemistry 16, 1607—1615
15 Sasaki, M., Minakata, K., Yamamoto, H., Niwa, M., Kato, T. and Ito, N. (1977) Biochem. Biophys. Res. Commun. 76, 917—924.
16 Heimburger, N., Haupt, H. and Schwick, R.G. (1970) in Proceedings International Research Conference on Proteinase Inhibitors (Hans, H. and Tschesche, H., eds.), p. 1—21, Walter de Guyter, New York