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Secretion of antileucoprotease from a human lung tumor cell line

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Two human tumor cell lines were analyzed for the production of human antileucoprotease (ALP). One of them, a human squamous lung carcinoma cell line (HS-24) synthesized, as confirmed by Western blot analysis, high amounts of ALP in serum-free medium. The supernatant inhibited elastase, chymotrypsin and trypsin. Northern blot analysis with an 18-mer radiolabelled oligonucleotide, derived from an ALP specific cDNA clone, revealed a specific mRNA of about 700–800 nucleotides in HS-24 tumor cells. In contrast, a secondary human lung tumor cell line (SB-3), derived from the adrenal cortex, did not synthesize ALP when assayed under identical conditions. The supernatant inhibited only trypsin and chymotrypsin.

Secretion; Serine protease inhibitor; Antileucoprotease; (Lung tumor cell)

1. INTRODUCTION

Serine protease inhibitors are secreted not only from normal tissue but also from cancerous tissue and different tumor cell lines [1–3]. It has been previously shown that the HPSTI, primarily a pancreatic secretory product, is also secreted from different human tumor cell lines in serum-free medium. Interestingly, this inhibitor seems to act as a growth stimulating factor for human endothelial cells [1]. A further human serine protease inhibitor, antileucoprotease (ALP), has been isolated from various human mucous tissues and their secretions [4–9]. The inhibitor is acid-stable and has a strong affinity for neutrophil elastase, cathepsin G, trypsin and chymotrypsin, enzymes playing key roles in causing lung emphysema [10,11]. It has been shown that ALP is secreted from nonciliated (non-goblet) cells in the lung tissue [12] and recently its primary structure has been elucidated by the cloning of its cDNA [13]. The molecular mass has been determined as 11 726 Da.

In this report we show for the first time that a primary human lung tumor cell line (HS-24) secretes ALP in the serum-free medium, whereas a secondary lung metastasis cell line (SB-3), produces a serine protease inhibitor different from ALP.

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Abbreviations: TCM, tumor-conditioned medium; ALP, antileucoprotease; PBS, phosphate-buffered saline; HPSTI, human pancreatic secretory trypsin inhibitor; SSC, sodium chloride, sodium citrate; synthetic substrates are abbreviated according to the IUPAC-IUB rules for abbreviation of amino acids; substituents: MeO, methoxy; Suc, 3-carboxypropionyl; Bz, benzoyl; pNa, *p*-nitroanilide

2. EXPERIMENTAL

2.1. *Serum-free supernatants of lung tumor cell lines*

For production of serum-free tumor-conditioned medium (TCM), two human lung tumor cell lines (a primary cell line, designated HS-24 [14,15], and a secondary cell line,

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designated SB-3) were cultured in serum-free medium and their supernatants were harvested using a method similar to that of Fett et al. [16]. The cell lines were routinely grown in serum-conditioned RPMI-1640 medium. After reaching confluence, 2×10^7 tumor cells/175 cm^2 tissue flask, the cells were washed extensively with PBS and cultured in serum-free medium for 2–3 days. Collection and storage of the serum-free TCM were performed as described in [16]. For production of cell lysates 2×10^7 tumor cells were lysed in double distilled water and the supernatants were collected after centrifugation.

2.2. *Western blot analysis*

Western blotting was carried out as described in [17–19]. Rabbit anti-ALP antiserum was a gift from Professor H. Fritz (Inst. Klin. Chemie und Klin. Biochemie der Chirurgischen Klinik Innenstadt der Universität München). The second antibody, a goat anti-rabbit IgG-peroxidase conjugate, was purchased from Sigma.

2.3. *Enzyme inhibition assays*

Human elastase was a gift from Dr R. Geiger (Inst. Klin. Chemie und Klin. Biochemie der Chirurgischen Klinik Innenstadt der Universität München). Bovine trypsin and bovine chymotrypsin were purchased from Merck. The trypsin inhibition assay was performed as described in [20–22]. Inhibition of bovine chymotrypsin and human elastase was carried out according to DelMar et al. [23] and Geiger et al. [24]. Inhibition of elastase was measured with the substrate MeOSuc-Ala-Ala-Pro-Val-pNA. Measurements and inhibition of chymotrypsin were performed with Suc-Ala-Ala-Pro-Phe-pNA substrate and Bz-L-Arg-pNA, respectively.

2.4. *RNA isolation and Northern blotting*

Total cellular RNA from human HS-24 tumor cells was prepared according to the procedure of Chirgwin [25]. 10 μg poly(A)$^+$ RNA was denatured [26], electrophoresed and transferred onto a nitrocellulose filter as described [27]. As hybridization probe an 18-mer oligonucleotide was synthesized on an automated DNA synthesizer (Applied Biosystems). The nucleotide sequence was derived from the cloned ALP cDNA [13] and codes for amino acids 50–55 of the mature ALP. Hybridization was performed with a 10^6 cpm/ml hybridization solution at 51°C for 12 h. After hybridization the filter was washed at 42°C in 0.2 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate) for 20 min and exposed for 2 days.

3. RESULTS

ALP has been shown to be secreted by mucous secreting cells in the lung, especially the non-goblet cells [12]. In order to determine, whether human lung tumor cell lines also secrete ALP two different human tumor cell lines were tested, firstly, a primary squamous carcinoma cell line (HS-24) and secondly, a secondary lung tumor cell line (SB-3), which is derived from the adrenal cortex. These human cell lines were grown in serum-free medium and their supernatants were harvested as described [16]. Proteins present in the supernatant were then analyzed in a Western blot assay (fig.1), using antiserum against isolated ALP from human seminal plasma. Proteins of both supernatants were separated on 15% SDS gels and were then blotted onto nitrocelluloses by electrophoretic transfer. Fig.1 shows that two proteins in the HS-24 supernatant are detected by anti-ALP antibodies. In contrast to the HS-24 TCM, no cross-reactivity was found with proteins secreted from the SB-3 cell line. Furthermore, the immunoreactive proteins present in the HS-24 supernatant comigrated with two ALP forms isolated from human seminal plasma. The smaller molecular mass form probably results from a limited proteolysis at the early stage of serum collection. For comparison, proteins of lysed cells were also analysed in the Western blot assay. As shown in fig.1 four proteins cross-reacted with anti-ALP antiserum; one protein comigrated with the 11 700 Da form.

Interestingly, two proteins with molecular masses above 11 700 Da and one with a molecular mass smaller than native ALP were also immunoreactive with the antibodies. However, these proteins have not yet been characterized.

Because ALP has inhibitory activities against trypsin, chymotrypsin and elastase, supernatants of HS-24 and SB-3 tumor cells were tested for the inhibition of these three proteases. 80 pmol bovine trypsin were preincubated with increasing amounts of tumor cell supernatants and the release of

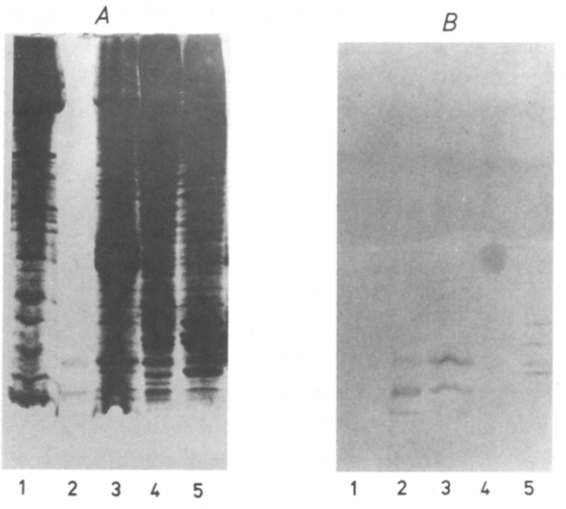

Fig.1. SDS-polyacrylamide gel electrophoresis of SB-3 supernatant (1), of SB-3 cell lysate (4), of HS-24 supernatant (3) and of HS-24 cell lysate (5). For comparison 1 μg ALP from seminal plasma was also electrophoresed on a 15% gel (2). (A) The proteins were stained according to the method of Oakley et al. [28] after electrophoresis. (B) Western blot analysis [17–19] of the electrophoresed proteins was performed with rabbit anti-ALP antiserum and goat anti-rabbit IgG-peroxidase conjugate as described in section 2.

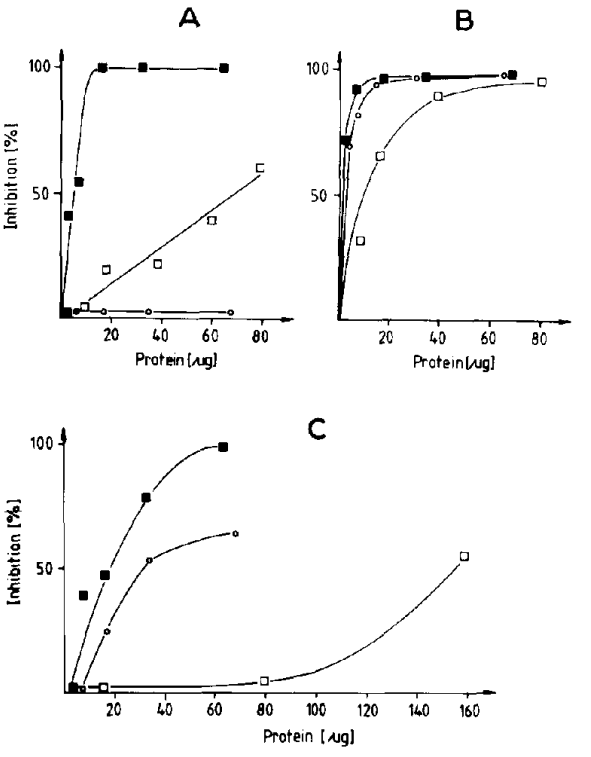

Fig.2. Enzyme inhibition assays of human elastase (A), bovine chymotrypsin (B) and bovine trypsin (C) with HS-24 supernatant (■—■), HS-24 cell lysate (□—□) and SB-3 supernatant (○—○) were performed as described in section 2.

4-nitroaniline from Bz-L-Arg-pNa was measured at 405 nm. Similarly, the inhibition of 11 pmol human elastase and 3 pmol bovine chymotrypsin was measured with their specific substrates as described in section 2. The protein concentration of both supernatants was determined according to Bradford [29]. For inhibition measurements both supernatants were concentrated 15-fold.

Fig.2 shows that 5–15 μg total protein of HS-24 TCM exhibited 50% inhibition of elastase, trypsin and chymotrypsin. Control experiments with ALP, isolated from seminal plasma, have shown that 200–300 ng purified ALP exhibits about 50% inhibition of chymotrypsin, trypsin and elastase. From these data and on the basis of our Western blot results we conclude, that 1% of the total protein in the HS-24 supernatant is antileucoprotease. In contrast to the inhibitory activities seen with HS-24 TCM, no inhibition of human elastase was detected with SB-3 supernatant; however, SB-3 TCM inhibited chymotrypsin and trypsin to a similar extent as obtained with HS-24 TCM (fig.2).

Furthermore, comparing the inhibitory activities between similar amounts of secreted protein and cytoplasmic protein from lysed HS-24 cells, a significant reduction of inhibitory activity was observed with the latter (fig.2).

In order to prove whether the ALP mRNA of cervix uterus is identical to that of human squamous lung carcinoma cell line (HS-24), we performed Northern blot analysis using a specific radiolabelled 18-mer oligonucleotide. 10 μg human lung tumor cell poly(A)$^+$ RNA were tested. After hybridization and exposure of the hybridized filter for 2 days, a 700–800 nucleotide long mRNA species could be detected. As shown in fig.3 the length of tumor-derived mRNA agreed with the reported length of ALP mRNA in cervix uterus [13].

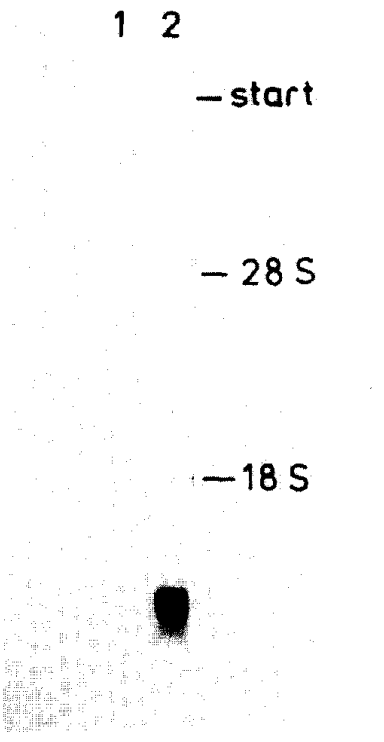

Fig.3. Northern blot analysis of human RNA from HS-24 tumor cells. RNA samples were denatured with glyoxal, separated on a 1.2% agarose gel and transferred to nitrocellulose. The RNA was hybridized to 5′-labelled synthetic 18-mer oligonucleotide. 10 μg poly(A)$^+$ RNA from HS-24 tumor cells (lane 2) were loaded. As a control 25 μg total celluar RNA from human spleen was transferred (lane 1).

4. DISCUSSION

Human squamous lung carcinoma cells (HS-24) in culture synthesize antileucoprotease that is structurally related or identical to ALP isolated from human seminal plasma. The evidence for this structural relationship is demonstrated by the facts, that (i) anti-ALP antiserum cross-reacts with ALP from HS-24 TCM, (ii) immunoreactive proteins comigrate with the ALP forms, isolated from seminal plasma, (iii) HS-24 TCM exhibits inhibition of the same proteases as ALP from seminal plasma. Finally, Northern blot analysis detects an mRNA transcript, which hybridizes with the ALP oligonucleotide and is identical in length to ALP mRNA from cervix uterus. Further purification and sequence analysis of tumor-derived ALP will confirm whether the tumor-derived protein is identical to normal ALP.

Cytoplasmatic proteins of HS-24 lysed cells cross-reacted with antibodies against ALP and one protein comigrated with the native ALP form: however, three other species had different molecular masses. It is possible that the higher molecular mass forms are ALP precursors which are located inside the cell and are processed during secretion. It might also be possible, that the immunoreactive proteins from the cell lysate, those not comigrating with the ALP forms, are, in fact, different proteins that share only structural similarities to ALP.

Whether the presence of ALP reflects lung cell character or tumor cell character or both is not clear. However, the fact that (i) ALP is a normally occurring protein in lung tissue; (ii) the HS-24 tumor cell line originating from lung produces ALP and (iii) tumor metastasis cell line (SB-3) not originating from lung produces no ALP, might imply that ALP secretion is a character inherent to lung cells.

With the enzyme inhibition assay inhibitory activities against chymotrypsin and trypsin could be identified in the supernatants of HS-24 and SB-3 cell lines. These results together with the blot analysis suggest, that SB-3 TCM also contains a serine protease inhibitor differing, however, from ALP. Further studies will concentrate on the identification of the SB-3 cell line specific inhibitor. Although it is not yet known whether these tumors in vivo exhibit the same high production of inhibitors, a detailed characterization of serine protease inhibitors from human lung tumor cell lines might be important in understanding the control of tumor cell proliferation.

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