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Biosynthesis and distribution of leucocyte elastase inhibitor. Production of recombinant inhibitor*

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The horse leucocyte elastase inhibitor (HLEI), present in neutrophils, monocytes and bone marrow cells, is apparently a cytoplasmic protein which is not released from cells even in response to stimulation with lipopolysaccharide, phorbol ester, tumour necrosis factor alpha, interleukin-1 or elastin degradation products. Although no expression of the inhibitor was detected in neutrophils, both monocytes and bone marrow cells were efficient in its synthesis. Using a new expression vector pREST5d, recombinant inhibitor was produced in a large quantity in a soluble form, with a yield of 88 mg per 10 litres of *E. coli* culture. A two-step purification procedure, consisting of ion-exchange chromatography and gel filtration, yielded 36 mg of the recombinant inhibitor of a purity higher than 95%, as judged by SDS/PAGE. The recombinant protein had physicochemical and kinetic properties indistinguishable from those of the natural one, including irreversible elastase inhibition with an association rate constant $k_{ass} > 10^7$ $M^{-1}s^{-1}$. Both proteins were eliminated from rat circulation at the same ratio, and within the first 20 min 70% of the protein was removed. Such a short half-life in the circulation suggests that local delivery of HLEI directly to lungs in the form of aerosol could be a more efficient therapeutic approach than its intravenous injection.

Unrestricted activity of elastases is believed to be responsible for the development of both chronic obstructive lung disease and emphysema in humans and horses [1]. The cytosol of blood leucocytes contains a very potent elastase inhibitor (HLEI) belonging to the ovalbumin family of serpins [2–4]. HLEI, the oxidation sensitive elastase inhibitor is a typical intracellular protein absent in plasma and in culture media of monocytes or bone marrow cells. Like most other members of the ovalbumin family, HLEI does not contain a typical cleavable signal sequence and is not glycosylated. The lack of a signal peptide and glycosylation explains its intracellular location and suggests that HLEI may have an intracellular function. At present, the physiological function of intracellular serpins is unknown (for more details see [5]). Cloning and expression of the horse leucocyte elastase inhibitor [4] makes possible the trials of its application in treatment of horse lung emphysema, as the new express-

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Abbreviations: HLEI, leucocyte elastase inhibitor; rHLEI, recombinant leucocyte elastase inhibitor; HNE, human neutrophil elastase; pNA, *p*-nitroanilide; MeOSuc, methoxysuccinyl; RSA, rat serum albumin; Suc, succinyl.

ion system described here allows production of large quantities of the recombinant protein. Therefore, this work has been designed to study distribution, synthesis and secretion of HLEI and to provide a method for large scale isolation and purification of the recombinant inhibitor.

MATERIALS AND METHODS

Cells. Mono- and polymorphonuclear cells were prepared from horse blood by Percoll (Sigma, St. Louis, MO., U.S.A.) density gradient centrifugation as described earlier for human blood [6]. The mononuclear and polymorphonuclear cell suspensions collected on a top of 60%, and on the border between 60% and 80% Percoll, respectively, were plated on 35 mm plastic culture dishes (5×10^6 cells/well) in RPMI-1640 media lacking methionine (Gibco, Grand Island, N.Y., U.S.A.) and incubated for 2 h under 5% CO_2 at 37°C to allow adherence of monocytes. Nonadherent cells were removed.

Crude bone marrow cells were isolated from the horse breast bone as described by Kordula *et al.* [4].

Biosynthesis. The cells were starved in media lacking methionine and then labelled for 6 h with 50 μCi/ml per well of L-[^{35}S]methionine (spec. act. 1175 Ci/mmol, Du Pont, Dreirch, Germany) with or without the tested factors. The medium as well as cell extract were immunoprecipitated and analysed by SDS/PAGE and fluorography. The presence of inhibitor in cells homogenates was also studied by SDS/PAGE and Western blotting technique.

Plasmid transformation. A 1.3 kb *NcoI/Hind* III fragment containing the entire open reading frame of HLEI [4] was cloned into the bacterial expression vector pRSET5d [7]. *Escherichia coli* BLZIDE3 cells (containing a marker plasmid plysS1CA which made it resistant to chloramphenicol) were put into an ice-water bath for 20 min, followed by suspension in 50 mM $CaCl_2$, pH 8.0, to render them competent for plasmid transformation. The competent bacteria were incubated with pRSET5d-HLEI plasmid. Transformants were selected on a medium containing ampicillin and chloramphenicol.

Protein expression. Each 1000 ml flask containing 50 mg of ampicillin and 100 mg of chloramphenicol was inoculated with bacteria transformed with pRSET5d-HLEI plasmid and placed into a 37°C shaking water bath. The bacterial suspension was incubated until A_{600} was 0.6. At that time 100 μl of 1 M isopropyl β-D-thiogalactopyranoside (IPTG) was added, followed by 4 h incubation in the shaking water bath. Bacterial cells were collected by centrifugation (3000 × *g*, 10 min), frozen in liquid nitrogen, then, after being resuspended in 50 ml of 20 mM Tris/HCl buffer, pH 8.0, they were subjected to sonification (5 × 10 s). The crude homogenate was clarified by centrifugation (10000 × *g*, 10 min), and the supernatant containing soluble recombinant HLEI protein retained.

Recombinant HLEI (rHLEI) isolation and purification. The following subsequent chromatography steps were used to purify rHLEI: ion-exchange chromatography on Whatman DE-52 cellulose, ion-exchange chromatography on TSK-DEAE 3SW (21.5 mm × 150 mm, LKB AB, Bromma, Sweden), ion-exchange chromatography on MonoQ HR 5/5 (Pharmacia LKB, Uppsala, Sweden) and gel filtration on TSK G-3000 SWG (21.5 mm × 300 mm, LKB AB, Bromma, Sweden). The HLEI inhibitory activity after each consecutive purification step was determined by incubation with a target proteinase HNE (human neutrophil elastase).

Assays of enzymatic activity. Amidolytic activity of the proteinases tested was determined in 0.2 M Tris/HCl, pH 8.0, using enzyme and substrate final concentrations of 4.8 nM and 0.2 mM, respectively. The substrates S-2238 (Kabi-Vitrum, Stockholm, Sweden), Suc-Ala-Ala-Pro-Phe-pNA and MeOSuc-Ala-Ala-Pro-Val-pNA (Sigma, St. Louis, MO., U.S.A.) were used to assay trypsin, chymotrypsin and elastase, respectively. The release of *p*-nitroaniline was monitored spectrophotometrically at 405 nm.

Measurement of the association rate constant (k_{ass}). The determination of k_{ass} was performed according to Bieth [8]. In brief, equimolar mixtures of enzyme and inhibitor (based on the active site titration of each protein) were incubated for increasing time periods at room temperature in a total volume of 0.75 ml of 0.1 M Tris/HCl, pH 8.0. Residual enzyme activity was then measured by addition of saturating amounts of a suitable chromogenic substrate and measuring the amount of released *p*-nitroaniline at 405 nm. The data obtained were subjected to computer calculations of k_{ass}.

Miscellaneous techniques. SDS/PAGE (12% gels, reducing conditions), as described by Laemmli [9], was performed in Mini Protean II cell (Bio Rad, U.S.A.).

Human neutrophil elastase (HNE) and horse leucocyte elastase inhibitor (HLEI) were prepared as previously described ([10, 11] respectively).

Rat serum albumin, native HLEI and rHLEI were labelled with [^{131}I]KI (Institute of Atomic Energy, Otwock-Świerk, Poland) using chloramine T [12].

RESULTS AND DISCUSSION

It has been demonstrated that both mature circulating horse blood leucocytes and bone marrow cells contain HLEI mRNA [4]. We confirmed these results on the protein level, showing by Western blot analysis the presence of HLEI in bone marrow cells and both polymorpho- and mononuclear cells (Fig. 1). However, the active *de novo* synthesis of HLEI was detected only in bone marrow cells and monocytes (Fig. 2), whereas neither bone marrow cells nor leucocytes released HLEI during 6 h of incubation, as determined by metabolic radiolabelling with [^{35}S]methionine and immunoprecipitation. Similarly, neither treatment of the cells with lipopolysaccharide, phorbol ester, tumour necrosis factor, interleukin-1 or elastin degradation products induced secretion of HLEI. The resistance of HLEI to being released from the cells under a number of conditions explains its absence from horse plasma and implies that the inhibitor has an important intracellular function. This is in agreement with the fact that HLEI, as most other members of the ovalbumin family [14], does not contain the

Fig. 1. Western blot analysis of elastase inhibitor (HLEI) from horse cells.
The proteins were resolved by SDS/PAGE, transferred to nitrocellulose membranes and reacted with rabbit monovalent antisera to HLEI, followed by alkaline phosphatase-labelled goat IgG against the Fc fragment of rabbit IgG. Line 1, HLEI (0.3 µg); line 2, bone marrow cells homogenate; line 3, polymorphonuclear and line 4, mononuclear cells homogenate (equivalent of 500 cells each).

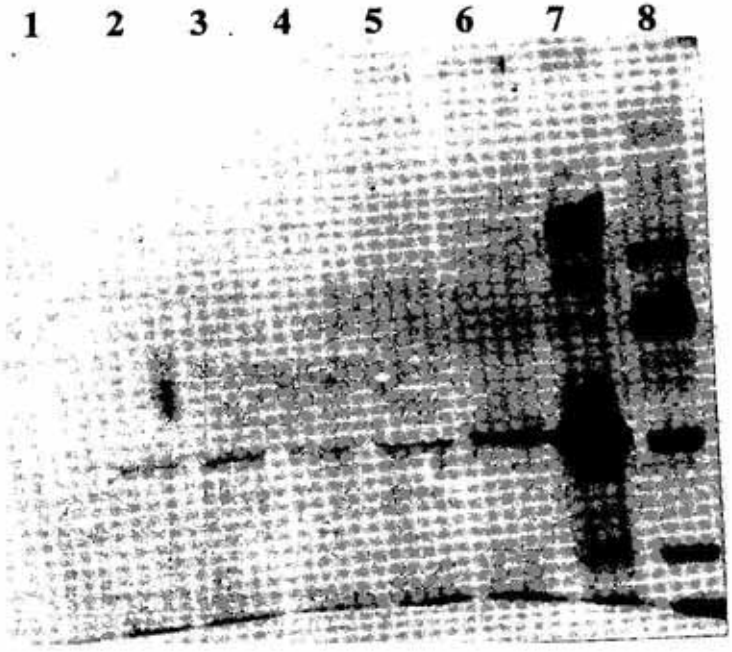

Fig. 2. Synthesis of horse leucocyte elastase inhibitor (HLEI) by mononuclear cells.
The protein were immonoprecipitated with rabbit monovalent antisera to HLEI, resolved by SDS/ PAGE and fluorography. Line 1–6, horse mononuclear cells homogenate (equivalent of 10^5 cells) after 1 h incubation with L-[^{35}S]methionine followed by 0 h (line 1), 1 h (line 2), 2 h (line 3), 3 h (line 4), 4 h (line 5) and 5 h (line 6) incubation in medium without L-[^{35}S]methionine; line 7, HLEI (0.1 µg) control; line 8, Pharmacia relative molecular mass markers (94000, 67000, 43000, 20100 and 14400 from the top, respectively). These markers and HLEI control were labelled by colyophilization with L-[^{35}S]methionine [13].

typical cleavable signal sequence and is not glycosylated [2]. Physiological target proteinases for most of the intracellular serpins are not known and it is expected that, in addition to enzyme inhibition, some of them might have additional physiological functions. Several members of the ovalbumin-serpin family have been cloned and efficiently expressed by the recombinant technology (for review see [5]). Especially promising are the recently identified novel intracellular serpins. They usually occur in small quantities, are confined to one specific location in the organism, are difficult to isolate from a natural source and, what is most important, their exact physiological function is still ambiguous. The available methods of large scale expression and purification of recombinant serpins from bacterial cultures render their possible use as therapeutically active agents. These techniques are of special importance if large amounts of specific serpins are needed for testing on large animal models. HLEI is an example of a promising, intracellular serpin, able to control neutrophil elastase activity *in vivo* [15] and, therefore, it can be used as a medication for horse pulmonary emphysema. The new protein expression system of *E. coli* transformed with the expression vector pREST5d-HLEI enables to obtain 88 mg of active rHLEI from 10 litres of the bacterial culture, that is equivalent to the amount of inhibitor which is present in about 10^{11} neutrophils (100 litres of horse blood) [15]. A simple purification procedure using two ion-exchange chromatography steps yielded 36 mg of rHLEI of more than 95% purity (Table 1).

The expressed protein acted as a rapid and irreversible elastase inhibitor with an association rate constant $k_{ass} > 10^7$ $M^{-1}s^{-1}$ and was found to be indistinguishable from the natural HLEI in its physicochemical properties (relative molecular mass, target proteinase, reaction with antibody and lack of glycosylation). However, for a potential medical application of rHLEI, the most important factor is the turnover rate of both proteins in rat circulation. The natural as well as recombinant HLEI were eliminated from the rat circulation with the same kinetics, which was distinctly different from that of rat serum albumin (Table 2).

When compared to HLEI, albumin was retained in rat circulation for a much longer period of time, being eliminated in only 36% during one hour. The natural and recombinant HLEI are eliminated from rat circulation by 70% within the first 20 min. Such a short half-life in the circulation suggests that the main function of HLEI is to control intracellular protein turnover, rather than proteolysis outside the cell. The pharmacokinetic behaviour of HLEI is important for possible therapeutic applications including treatment of experimentally induced emphysema in experimental animals through neutrophil elastase inhibition in lungs. The pharmacokinetic behaviour of recombinant HLEI indicates that, if injected intravenously, it would probably reach the target lung tissue only in small amounts and would be less effective than elastase inhibitors present in blood plasma, including alpha-1-proteinase inhibitor. It is very probable that, when applied locally, directly to the lung tissue, for instance in aero-

Table 1

Purification of recombinant horse leucocyte elastase inhibitor from 10 litres of the bacterial culture.
Protein content (mg) was estimated from A_{280} measurements and rHLEI concentration was determined by rocket immunoelectrophoresis. One unit of rHLEI is equivalent of 10 μg to natural purified inhibitor that was 100% active when titrated with neutrophil elastase.

Purification steps	Total protein		rHLEI		Purification factor
	mg	%	units	units/mg	
E. coli supernatant	6120	100.00	8800	1.43	1.0
DE-52 Cellulose	1203	19.65	4850	4.03	2.8
TSK-DEAE 3SW	54	0.88	3450	63.88	44.6
Mono Q HR 5/5	37	0.60	3400	91.89	64.2
TSK G-3000 SWG	36	0.59	3375	93.75	65.5

Table 2

Comparison of plasma clearance of natural (HLEI) and recombinant (rHLEI) horse leucocyte elastase inhibitor versus rat serum albumin (RSA)

Time (min)	Radioactivity remaining in circulation (%)		
	HLEI	rHLEI	RSA
10	60	62	92
20	32	30	90
30	28	27	86
40	22	22	80
50	20	20	72
60	18	18	64

sol, rHLEI might prove more effective in preventing pulmonary emphysema by virtue of its high antiproteinase activity and due to the fact that its main specificity is directed against elastase. This, however, remains to be tested. Alternatively, experiments of introducing HLEI cDNA with a viral vector into the cells of the respiratory tract might soon provide another therapeutic possibility for treatment of the diseases caused by proteinase-antiproteinase imbalance.

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