Characterization of recombinant human HBP/CAP37/azurocidin, a pleiotropic mediator of inflammation-enhancing LPS-induced cytokine release from monocytes

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Abstract Neutrophil-derived heparin-binding protein (HBP) is a strong chemoattractant for monocytes. We report here for the first time the expression of recombinant HBP. A baculovirus containing the human HBP cDNA mediated in insect cells the secretion of a 7-residue N-terminally extended HBP form (pro-HBP). Deletion of the pro-peptide-encoding cDNA sequence resulted in correctly processed HBP at the N-terminus. Electrospray mass spectrum analysis of recombinant HBP yielded a molecular weight of 27.237 ± 3 amu. Consistent with this mass is a HBP form of 225 amino acids (mature part +3 amino acid C-terminal extension). The biological activity of recombinant HBP was confirmed by its chemotactic action towards monocytes. Furthermore, we have shown that recombinant HBP stimulates in a dose-dependent manner the lipopolysaccharide (LPS)-induced cytokine release from human monocytes.

Key words: HBP/CAP37/azurocidin; Baculovirus; N-terminal processing; Mass spectrometry; Lipopolysaccharide; Cytokine

1. Introduction

Heparin-binding protein (HBP), also termed CAP37 [1] and azurocidin [2], is a human neutrophil-derived serine protease homologue exhibiting 45% sequence identity to neutrophil elastase, and 30–37% identity to several other granule serine proteases [3,4]. Despite these similarities, HBP is devoid of proteolytic activity because the active site residues serine and histidine have been replaced. Beside its lipopolysaccharide (LPS) binding capacity and antimicrobial action [2,5], HBP has been shown to be a specific and strong chemoattractant for monocytes in vitro and to induce longevity and differentiation of these cells towards a macrophage phenotype [1,3]. In addition, HBP has been shown to induce detachment and homotypic aggregation on confluent monolayers of endothelial cells and fibroblasts [6]. On the basis of these observations it has been hypothesized that HBP acts as a mediator of the second wave of inflammation [7].

The cDNA for HBP has been cloned and characterized [8,9], and in order to produce the protein by recombinant techniques we have tried different expression systems (yeast and mammalian cells) with very limited success. By usage of the baculovirus/insect cell system we report in the present paper the successful expression and characterization of biological active recombinant HBP. Furthermore, we have shown that recombinant HBP under serum-free conditions stimulates in a dose-dependent manner the LPS-induced release of IL-6 and TNF-α from human monocytes.

2. Materials and methods

2.1. Materials

The baculovirus transfer vector, pBlueBacIII, *Autographica californica nuclear polyhedrosis virus* (AcMNPV) DNA, *Spodoptera frugiperda* (SF9) cells, Baculovirus agarose, Grace media+supplements and Cationic liposome solution were from Invitrogen. Fetal calf serum (FCS) and the serum-free insect cell culture medium SF-900 II were from GIBCO BRL/Life Technologies. Trypsin was obtained from Sigma.

2.2. Construction of transfer vectors

Based on the human HBP amino acid sequence [3] and the CAP37/azurocidin cDNA sequence [8,9] a 770 bp *Bam*HI–*Hin*dIII fragment was constructed by PCR technology by usage of a human bone marrow cDNA library (Clontech). This fragment contains the entire coding region of HBP, including a 19-residue signal peptide, a 7 amino acid pro-peptide, a mature part of 222 amino acids, and a 3 amino acid C-terminal extension. The fragment was inserted into pBlueBacIII resulting in the transfer plasmid pSX556. For deletion of the pro-region an oligonucleotide linker of 99 bp, covering the signal peptide and the first 4 amino acids of mature HBP (from *Bam*HI to *Eag*I), was substituted for the original *Bam*HI–*Eag*I fragment in pSX556 giving rise to pSX559.

2.3. Transfection and isolation of recombinant baculoviruses

Transfection was performed according to the manufacturer's protocol (Invitrogen). Briefly, 1 μg of linear AcMNPV DNA and 3 μg of transfer plasmid were transfected into SF9 cells (2×10^6 cells in 60 mm dishes) seeded in complete Grace medium (Grace medium+supplements+10% (v/v) heat-inactivated FCS). The resulting culture supernatant was collected after 5 days. Fresh monolayers of SF9 cells (6×10^6 cells in 100 mm dishes) were infected with virus supernatant at various dilutions and then overlaid with 1.5% agarose containing complete Grace medium with 150 μg/ml X-gal. After 6 days presumed recombinant plaques were identified by their blue colour and used to infect 6-well plates containing SF9 cells. After 5 days the corresponding virus DNA was purified and subjected to a PCR reaction [10,11]. Recombinant viruses were subjected to another round of plaque purification to ensure that the recombinant virus stock was free of wild-type virus.

2.4. Expression of HBP in SF9 cells

The SF9 cell line was maintained in complete Grace medium at 27°C in suspension culture. For HBP production, cells in logarithmic phase were shifted to serum-free SF900-II medium resulting in a cell density of 1×10^6 cells/ml and the recombinant baculovirus was added to the cells at a multiplicity of infection (MOI) of 1. Medium was typically harvested 3–4 days postinfection. In time-course experiment cultures were terminated 6 days postinfection.

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2.5. Purification of HBP

Insect cell culture medium was passed through a glass microfibre filter (Whatman GF/A) to remove cells and cell debris and subsequently applied on a CM-Sepharose cation-exchange column (Pharmacia), previously equilibrated with 50 mM sodium phosphate, pH 7.3. Unbound and loosely bound materials were eluted with equilibration buffer until baseline was achieved measured by on-line UV detection at 280 nm. The column was then developed with a linear gradient from 0 to 1 M sodium chloride in equilibration buffer. HBP eluted with about 0.7 M sodium chloride and fractions were combined based on UV absorption. Pooled fractions were diluted with two volumes of distilled water and applied on a new CM-Sepharose column. Following equilibration HBP was step-eluted with 1 M sodium chloride in equilibration buffer and fractions combined based on absorption at 280 nm. Highly concentrated and pure HBP was obtained by this procedure. Final purification was carried out on a Sephadex G-25 gel-filtration column (Pharmacia) equipped with a UV-flow cell and equilibrated and eluted with 0.02% trifluoroacetic acid. HBP was collected based on absorption at 280 nm. The gel filtration served mainly as a buffer exchange step to produce a stable preparation of HBP that was kept at 4°C until use.

2.6. Analytical assays

HBP concentration in insect cell culture medium and in fractions collected during purification was measured either by a specific sandwich-ELISA or by reverse-phase HPLC. Quantification was obtained relative to a standard preparation in which the concentration of HBP had been determined by amino acid analysis. For analysis and quantification of HBP by reverse-phase HPLC aliquots were injected on a Vydac 214TP54 column (The Separations Group, Hesperia US) using 0.1% trifluoroacetic acid as eluent A and 0.07% trifluoroacetic acid in acetonitrile as eluent B. Equilibration was performed with 25% eluent B at room temperature at a flow rate of 1.5 ml/min. A linear gradient from 25 to 51% B was pumped through the column over 13 min and HBP eluted after 9.7 min.

Amino acid sequence analysis was determined by automated Edman degradation using an Applied Biosystems Model 477 gas-phase sequencer as described by the manufacturer.

Electrospray mass spectrum was recorded on API III LC/MS/MS system (Perkin-Elmer Sciex instrument, Thornhill Canada). The triple quadropole instrument has a mass-to-charge (m/z) range of 2400 and is fitted with an articulated, pneumatically assisted electrospray (also referred to as ion-spray) interface and an atmospheric pressure ionization source. Sample introduction was done by a syringe infusion pump (Sage instruments, Cambridge, MA) through a fused capillary (75 μm i.d.) with a liquid flow-rate set at 0.5–1 μl/min. The instrument m/z scale was calibrated with selected (covering m/z 50–2400) ammonium adduct ions of poly(propylene glycols) (PPG) under unit resolution. The accuracy of mass measurements is generally better than 0.02%.

Reduction and alkylation of recombinant HBP dissolved in 6 M guanidinium chloride, 0.1 M Tris-HCl (pH 8.5) was carried out using a 10-fold molar excess of dithiotreitol (reaction for 120 min at 37°C). The alkylated protein was HPLC purified and subsequently digested with trypsin. Enzyme/substrate ratio was 1 : 50 and the reaction time was 12 h at 37°C.

2.7. Chemotaxis assay

The chemotaxis assay was performed in a 48-well modified Boyden chemotaxis chamber (Neuro Probe) [12,13] using a Millipore MF membrane with 5 μm pore size for separating the upper and lower wells. Human mononuclear cells were harvested from buffy-coats of normal blood [14] and the migration of the monocytes was determined over six different fields on the same slide. Gays' buffered saline (GBSS, Gibco) containing 2% human serum albumin (HSA) served as the negative control, while 5% zymozan (Sigma) activated serum (ZAS) acted as a positive control.

Table 1
Chemotactic effect of recombinant HBP towards human monocytes

Additive to GBSS/HSA buffer	Distance migrated as a percent of negative control
–	100
Recombinant HBP (200 ng/ml)	171.4 ± 2.4
Native HBP (200 ng/ml)	169.0 ± 4.8
5% ZAS	164.3 ± 4.7

All results are the means as a percent of the negative control (GBSS/HSA) ± S.D. ($n = 3$).

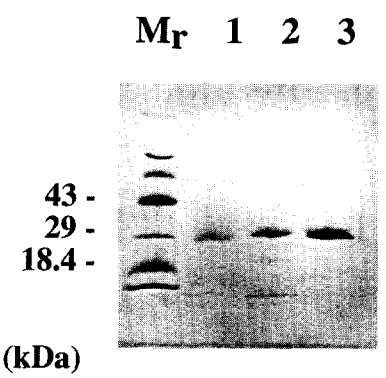

Fig. 1. Coomassie-stained SDS-PAGE (8–25%) comparing different HBP forms. Lane 1: native HBP purified from polymorphnuclear neutrophile leucocytes (PMN) [3]. Lanes 2 and 3: two recombinant HBP forms, pro-HBP and HBP, respectively.

2.8. Cytokine release from monocytes

Human monocytes were isolated from buffy-coats of normal blood [14,15]. In 24-well plates 2×10^5 cells per well were seeded in 1 ml of DMEM medium containing 2 mM Glutamax, 1% non-essential amino acids, and 1 mg/ml BSA. LPS (from *E. coli*, Sigma) and recombinant HBP were added as indicated in Fig. 4. After incubation for 24 h at 37°C (5% CO_2) cell supernatants were collected, clarified by centrifugation, and stored at −20°C until used for assay of their cytokine content. TNF-α, IL-1β, and IL-6 determinations were carried out by using the Biotrak ELISA systems (Amersham).

3. Results and discussion

3.1. Generation of baculovirus expressing HBP with correctly processed N-terminus

Secreted HBP from insect cells infected with recombinant baculovirus, containing the entire human HBP coding region (originating from pSX556), was purified and tested on SDS-PAGE (Fig. 1), demonstrating a molecular weight slightly larger than native HBP purified from human blood. When the N-terminal sequence was determined it appeared that nearly 100% of the produced material contained the pro-peptide of 7 amino acids in front of the mature part indicating that the insect cells are not able to process the pro-form (pro-HBP) in the same manner as the human myeloid neutrophil precursor cells in the bone marrow.

As an attempt to get HBP processed correctly at the N-terminus, the DNA sequence encoding the pro-peptide in the HBP cDNA was deleted (pSX559) and a new baculovirus was generated. After SF9 cells had been infected for production, it was noticed that the cells started to lyse at an earlier time-point (after 2 days) compared to cells producing pro-HBP, which first began to lyse 3 days postinfection. However, also here the medium was harvested after 3 days and the secreted protein was purified and tested on SDS-PAGE (Fig. 1). After N-terminal sequence determination it was verified that now 90% had been processed correctly starting with Ile^1 (the remaining 10% had been processed further downstream starting with Arg^5, see also Fig. 3B).

In order to compare the expression of pro-HBP and HBP a

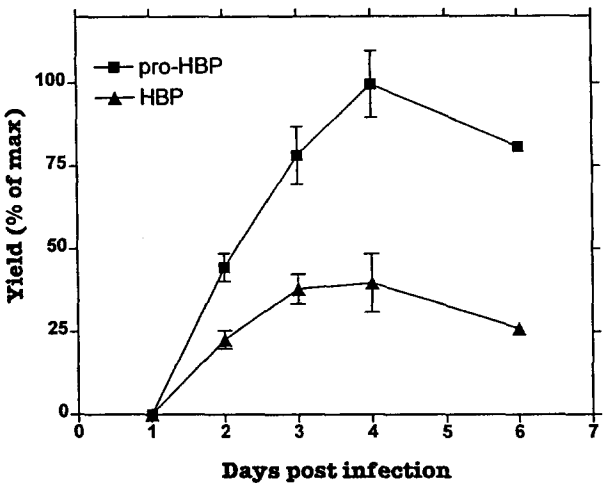

Fig. 2. Time-dependent expression of pro-HBP and HBP in SF9 cells. Each of the first 6 days (minus day 5) postinfection aliquots of the culture media were removed and tested in HBP-specific ELISA. The mean value ($n = 3$) of the maximal pro-HBP yield (on day 4) was set to 100%.

time-course experiment was carried out (Fig. 2), demonstrating that HBP is expressed 2–3 times less efficiently than the pro-form. The optimal yield of pro-HBP was obtained 4 days postinfection whereas the yield of HBP remained nearly unchanged from day 3 to 4.

3.2. Structural analysis of recombinant HBP

Electrospray mass spectrum (ESMS) analysis of recombinant HBP showed a molecular weight of 27.237 ± 3 amu (Fig. 3A,B). The calculated value for the 225 amino acid HBP form (the mature part plus 3 amino acid C-terminal extension) is 24.268,6 amu. As HBP contains three potential glycosylation sites (Asn^{100}, Asn^{114}, and Asn^{145}) this corresponds to a mass of 2.968 for the glycan part(s). This is in turn consistent with the theoretical value for two Man_3-[Fuc]$GlcNAc_2$ units and one $Man_3GlcNAc_2$ unit. These structures are characteristic for glycosylation patterns observed in recombinant proteins expressed in insect cells [16]. The glycosylation patterns at the three potential glycosylation sites in HBP have not been analysed individually.

Verification of the C-terminal as Ala^{225} was performed using MS analysis of a tryptic digest of alkylated HBP. The C-terminal tryptic peptide consists of amino acids 211–225 with a mass of 1521.7 amu. A peptide with the corresponding molecular weight was found in the digest mixture and proven by subsequent sequence analysis to have the expected Asp^{211} at the N-terminus (data not shown). These findings do not exclude the possibility of a heterogeneous C-terminal, but the ESMS spectrum of intact recombinant HBP (Fig. 3A,B) strongly suggests the absence of any significant C-terminal processing.

3.3. Chemotactic activity of recombinant HBP

In order to asses the biological activity of recombinant HBP we determined its chemotactic action towards human monocytes. As seen in Table 1, the chemotactic activity of recombinant HBP is similar to the activities of the positive controls (native HBP and 5% ZAS).

3.4. Recombinant HBP enhances the LPS-induced cytokine release from human monocytes

LPS-binding protein (LBP), an acute-phase protein present in small amounts in serum, is known to be necessary for LPS responses of CD14 receptor-bearing cells, such as neutrophils and monocytes [17–19]. LBP lowers the threshold stimulatory concentration of LPS and markedly enhances the LPS-induced cytokine release [20,21]. Taking into account that HBP, besides being a chemoattractant for monocytes, is able to bind LPS it was tempting to examine, under serum-free conditions, the effect of recombinant HBP on LPS-induced cytokine release from isolated human monocytes. As seen in Fig. 4 recombinant HBP, when added together with 10 ng/ml LPS, is able to mediate in a dose-dependent manner a drastic enhanced release of IL-6 and TNF-α whereas the release of IL-1β is nearly unaffected. How HBP mediates the LPS signal to the monocytes remains to be clarified. Perhaps an unknown receptor present on the monocytes binds the HBP-LPS complex or HBP simply mediates the internalization of LPS. Another explanation could be that HBP act as priming factor making the monocytes much more sensitive to small amounts of LPS.

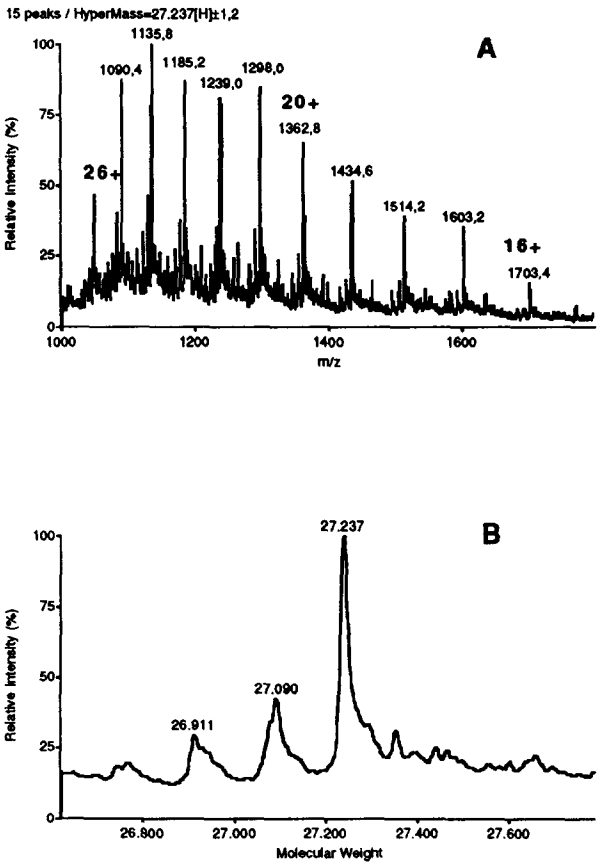

Fig. 3. ESMS spectrum of recombinant HBP (20 pmol consumed during analysis). The raw data (A) display a series of multiple protonated molecular ions (from charge-state 16+ to 26+) from which the molecular weight of the main component can be determined to 27.237 ± 3 amu. The reconstructed spectrum (B) (with a true mass scale) reveals the presence of minor components with masses 148 amu (fucose) and 326 amu (N-terminal I-V-G-G sequence) lower than the main component.

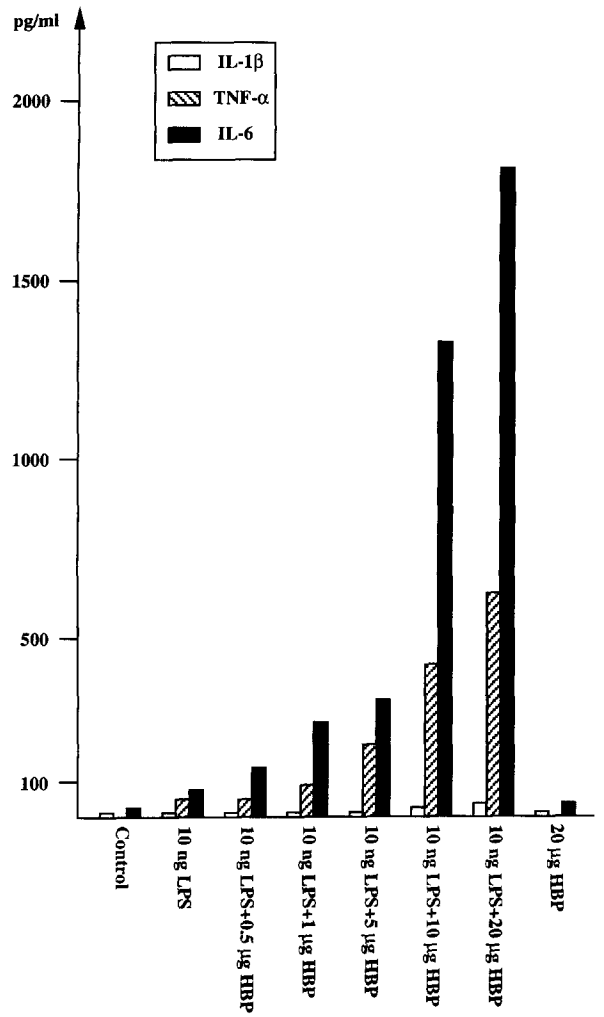

Fig. 4. TNF-α, IL-1β, and IL-6 release from human monocytes. The monocytes were cultured in 1 ml of serum-free medium for 24 h in the presence in the presence of LPS and/or recombinant HBP in the amounts indicated in the figure. Control was culture medium alone.

In an in vivo situation during an inflammatory event free LPS could act in a concerted fashion with HBP secreted from polymorphonuclear neutrophils and trigger the release of the very potent pro-inflammatory cytokine TNF-α from monocytes recruited to the injury area. The higher secretion of IL-6 would also be beneficial as it is an initial trigger for the induction of hepatic acute-phase protein production [22,23].

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