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Effects of heparin-binding protein (CAP37/azurocidin) in a porcine model of *Actinobacillus pleuropneumoniae*-induced pneumonia

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Abstract

Heparin-binding protein (HBP; CAP37/azurocidin) is secreted from neutrophil leukocytes early during inflammation and plays a central role in early capillary leakage and extravasation of neutrophils. Furthermore, HBP is chemotactic towards monocytes and lymphocytes and protects against stress-induced apoptosis, e.g. induced by oxygen radicals released during inflammation. Thus, administration of HBP as an adjunct to antibiotics increased survival of mice with peritonitis. In the present study, the effects of recombinant HBP as an adjunct to standard antibiotics were examined in a porcine model of *Actinobacillus pleuropneumoniae*-induced pneumonia. Beneficial and possible adverse effects of HBP were evaluated clinically and pathologically as well as by measurement of clinical chemical variables and markers of inflammation (interleukin-6 and C-reactive protein) and oxidative stress (ascorbic acid and α-tocopherol). Pigs receiving HBP (0.55 mg kg^{-1}, $n = 11$) as a 6-hourly subcutaneous infusion starting 1-h post-infection had a faster decrease in rectal temperature compared to control animals receiving a carrier-infusion ($n = 11$), with a significant lower temperature at 32 h post-infection ($p < 0.05$). This difference was, however, transient and the temperature curves had a similar course from 38 h and onwards. The transient effect of HBP might be explained by the dosage regimen that was used. No signs of adverse effects of the HBP-infusion were observed. More studies are needed to elucidate of the effects of HBP further and to optimise the dosage regimen for further improvement the efficacy and safety.

Keywords: Heparin-binding protein; *Actinobacillus pleuropneumoniae*; Pig; Pneumonia

1. Introduction

Despite the availability of a wide spectrum of potent antibiotics, pneumonia remains a frequent cause of morbidity and mortality. Consequently, development of new treatment strategies and therapeutic agents against bacterial infections are needed. Due to the pivotal role of neutrophils in the early defence against bacteria, agents that stimulate or mimic the function of neutrophils (e.g. protegrins) or increase their number (G-CSF), may conceivably enhance the elimination of bacteria and are currently evaluated for prevention and treatment of pneumonia. Neutrophils contain heparin-binding protein (HBP), also known as azurocidin or CAP37, which plays a major role in defence against bacterial infections [1–3]. HBP is released after adhesion of neutrophils to endothelial cells and mediates an increase in the capillary permeability, facilitating extravasation of macromolecules and neutrophils [4,5]. In the tissue, neutrophils release more HBP, which by chemotaxis contributes to accumulation of monocytes and neutrophils [6,7]. Furthermore, HBP enhances the inflammatory response of monocytes [3,8]. Thus, HBP supposedly have antibacterial properties due to immunostimulation, initiation and enhancement of the inflammatory response and protection against apoptosis [9,10]. Thus, recombinant HBP (rHBP) used as adjunct to antibiotics increased survival during severe peritonitis in mice [1], indicating that HBP is beneficial for defeat of bacterial infections.

The purpose of the present study was to evaluate the effect of human rHBP on selected variables defining morbidity res-

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olution during pneumonia and to observe for side effects of rHBP administration using a porcine model of *Actinobacillus pleuropneumoniae* (Ap) pneumonia [11]. This model appeared suitable, as HBP previously was found in pigs, in contrast to, e.g. mice, and has a sequence homology of 68% with human HBP [12]. Moreover, pigs have many anatomic and physiologic similarities with humans, making the Ap-model a clinically relevant model for pneumonia.

2. Material and methods

2.1. Animals

Twenty-four male pigs (31.5 ± 6 kg) were obtained from a herd free from Ap and *Mycoplasma hyopneumoniae*, verified by clinical and serological examination. The pigs were housed in an isolated unit and were fed twice daily with antibiotic-free commercial feed. Water was provided ad libitum. The study was approved by the Danish Animal Experiments Inspectorate.

2.2. Drugs and chemicals

Human recombinant HBP (rHBP) was expressed in baculovirus transformed SF9 insect cells and purified, as described by Rasmussen et al. [13], or expressed in HEK293 cells [14] and purified by cation exchange chromatography. The two types of human rHBP, each used for six animals, differed slightly in molecular weight due to difference in glycosylation, but displayed no significant difference in their ability to enhance LPS-mediated interleukin-6 (IL-6) secretion from swine monocytes in vitro, and were consequently considered to be equivalent. A similar test in human blood was used to test for the specific activity of the HBP. Before use both types of rHBP was further purified by size-exclusion chromatography to achieve a homogenous monomeric protein and underwent sterile filtration to exclude bacterial contamination. All HBP preparations were tested for pyrogenes (including LPS and LTA) in a whole blood assay for IL-6 production. Only preparations not inducing IL-6 production in this test, and therefore being considered pyrogene free, were used in the animal experiments. Tiamulin (200 mg ml^{-1}, Tiamutin Vet., Novartis) was obtained commercially.

2.3. Experimental infection

The pigs were infected with Ap (strain 4226, biotype 1, serotype 2) in a closed infection chamber by inhalation of aerosols containing bacteria [11]. The strain was stored at $-80\,^{\circ}$C and was cultured for 19 h at $30\,^{\circ}$C on modified pleuropneumonia-like organism (PPLO)-agar before use. A saline suspension of $1.7 \pm 0.6 \times 10^6$ (mean ± S.D.) colony forming units (CFU) of Ap per ml at a volume of 27.1 ± 0.7 ml were aerosolized into the closed infection chamber to groups of three or four pigs. The exposure time was 12.7 ± 0.4 min.

2.4. Study design

One hour after inoculation, the animals received either a subcutaneous (s.c.) infusion of HBP in a carrier-solution containing 0.1% bovine serum albumin in PBS, or the carrier-solution alone (control animals). The infusions were given between the scapulae by the use of an elastomeric infusion pump (C1073KJ, Baxter Healthcare Corp., IL, USA), running over 6 h with flow rate of 5 ml h^{-1}. The 12 HBP-treated animals received 0.55 ± 0.03 mg kg^{-1} of HBP in a total volume of 31.7 ± 0.3 ml. Twenty hours post-infection (p.i.), all animals received tiamulin in dose of 9.3 ± 0.1 mg kg^{-1} intramuscularly to prevent mortality. One control animal died 31 h after inoculation and one HBP-treated animal was euthanased after 33 h due to severe clinical symptoms. Data from these two animals were excluded from the analysis. All other animals were killed after 68 h followed by necropsy, whereby it was verified that the animals had pneumonia.

2.5. Clinical observations and blood sampling

The pigs were examined clinically and venous blood samples were obtained through a jugular vein catheter prior to and at 7, 20, 26, 32, 44, 50 and 68 h p.i. Furthermore, the animals were clinically examined 38 h p.i. Plasma was obtained by centrifugation of the blood samples ($3000 \times g$, 10 min, $4\,^{\circ}$C), and similarly while serum was obtained after 1 h of clotting. Plasma for ascorbic acid analysis was obtained as previously described [11]. All samples were stored at $-80\,^{\circ}$C until analysis.

2.6. Efficacy and safety variables

The therapeutic effect of HBP was monitored by clinical and pathological observations, while an increased haematocrit and pro-thrombin time as well as a decrease in platelet count a priori were defined as signs of adverse effects. Furthermore, plasma interleukin-6 (IL-6) and C-reactive protein (CRP) were used to monitor the inflammatory response, and standard clinical chemistry (alanine aminotransferase, albumin, aspartate aminotransferase, bilirubin, carbamide, creatine kinase, creatinine, fibrinogen, lactate dehydrogenase, potassium, protein, sodium and zinc) and haematology variables were measured as indicators of organ damage. Plasma ascorbic acid and α-tocopherol were used as biomarkers of oxidative stress.

2.7. Analytical procedures

Plasma CRP was measured by ELISA applying rabbit anti-human-CRP antibodies (Dako, Glostrup, Denmark) [11]. Plasma IL-6 was quantified by a commercial ELISA-kit using polyclonal goat-anti-porcine antibodies and a porcine