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Research paper

Lipopolysaccharide-binding protein: Local expression in bovine extrahepatic tissues

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ABSTRACT

Lipopolysaccharide-binding protein (LBP) is an acute phase protein involved in host response to Gram-negative and Gram-positive pathogens. It is synthesized by hepatocytes and released as 60–65 kDa glycoprotein in plasma. Little is known about the distribution of LBP in non-pathological bovine tissues. The aim of the present study was to investigate the extra hepatic expression of LBP in different bovine tissues by qualitative and quantitative real time (RT) PCR. The presence of the protein was also confirmed by immunohistochemistry using an anti-human LBP antibody preliminarily validated by cross-reactivity in bovine tissues. While a wide panel of organs and tissues was investigated, the attention was focused on the digestive tract and mammary gland.

Moderate amount of mRNA was detected in most of the tissues involved in this study. Extra hepatic LBP mRNA expression was particularly high in parotid and submandibular salivary glands. Remarkably, LBP mRNA was found in rumen, reticulum and omasum. High expression was also found in the mammary gland. Intensity of protein staining paralleled mRNA expression in most tissues, with the exception of lung, ovary and thyroid gland. The presence of LBP throughout epithelial mucosal tissues is indicative of an important role of LBP in mucosal immunity at sites of bacterial exposure. These results suggest that ruminant forestomachs may mount a local acute phase reaction.

1. Introduction

The acute phase protein LBP plays important roles in modulating the innate immune response against bacteria (Zweigner et al., 2006). LBP is a 50 kDa polypeptide which, after post-translational processing, is released as a 60–65 kDa glycoprotein in the bloodstream (Schumann et al., 1990). Together with bactericidal permeability-increasing protein (BPI), cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PTP), LBP belongs to the small family of lipid transfer/LPS binding proteins (LT/LBP), whose role is to participate in host defence (Bingle and Craven, 2004). LBP's classical function includes the binding to the amphipatic lipid A moiety of LPS, that facilitates the process of LPS presentation to $CD14^+$ cells. This, in turn, leads to the activation of Toll like receptor 4 (TLR4) pathway, thus triggering a pro-inflammatory response (Zweigner et al., 2006). The presentation of LPS to $CD14^+$ cells through LBP is believed to increase the LPS-mediated innate immunity pathways from 100- to 1000-fold (Martin et al., 1992). Although historically dedicated to Gram-negative infection, LBP can also recognize

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and discriminate other pathogen-associated molecular patterns (PAMPs), such as pneumococcal and staphylococcal lipoteicoic acid (LTA), a class of amphiphilic molecules anchored to the cytoplasmic membrane of Gram-positive bacteria, eventually activating the cellular response via TLR2 pathway (Schroder et al., 2003). Thus, the overall function of LBP is crucial to amplify the immune response against both classes of bacteria. But the immunomodulatory activity of LBP is probably more complex and not fully understood, because high concentrations of LBP inhibits both LBP- and LTA-induced cell activation (Zweigner et al., 2001; Schroder et al., 2003; Fierer et al., 2002). This inhibitory effect of high concentrations of LBP may possibly reduce an excessive defensive response which ultimately leads to systemic inflammatory syndromes such as septic shock and acute respiratory distress syndrome (ARDS) (Zweigner et al., 2001; Lamping et al., 1998; Wurfel et al., 1994).

From a clinical perspective, LBP is regarded as an acute phase protein, i.e. it belongs to a non-structurally related group of proteins whose plasma concentration is increased, or decreased, during systemic reaction to inflammation (Gabay and Kushner, 1999). In humans, LBP plasma concentration is increased during some neonatal and pediatric diseases (Behrendt et al., 2004; Oude Nijhuis et al., 2003) as well as in adults, during endocarditis (Vollmer et al., 2009).

The importance of LBP as an acute phase marker for diseases is an increasingly interesting topic in bovine medicine. Bovine LBP (bLBP) has been purified and its structure determined (Khemlani et al., 1994; Horadagoda et al., 1995). Its aminoacid sequence revealed an 86% similarity compared to human LBP (hLBP).

Plasma levels of LBP increase from 2 μg/ml (non-pathological concentration) up to seven folds in calves – but up to 25 μg/ml in adult animals after experimental infections with *Mannheimia haemolytica (Pasteurella)* (Schroedl et al., 2001), consistent with results by Horadagoda et al. (1995). Plasma and milk levels of LBP also increase during experimental mastitis (Bannerman et al., 2003).

LBP is mainly synthesized by hepatocytes, but extra hepatic expression has been reported. Other sources of LBP include respiratory type II epithelial cells (Dentener et al., 2000), epithelial cells of the skin, lung, kidney, heart, intestine (Su et al., 1994) and gingival tissues from human, mouse and rat (Ren et al., 2004, 2005). A recent report focused on mouse intestine localized LBP in secretory granules of Paneth cells (Hansen et al., 2009).

Although apparently expressed at basal level in some tissues, LBP local expression is strongly up regulated by pro-inflammatory challengers such as turpentine, LPS or cytokines (Su et al., 1994; Dentener et al., 2000). Despite the acknowledged importance of LBP as a component of the first line of defense, particularly in intestinal mucosa, the extra hepatic origin of bLBP has not been investigated. Information regarding local bLBP expression might provide insights on the interplay between the triggering and dampening of inflammation in organs such as the gastrointestinal tract and mammary gland, which is of paramount importance for initiation and final outcome of the inflammatory reaction.

The objective of this study was to evaluate the extra hepatic expression of bLBP. While a wide panel of organs and tissues was investigated, attention was focused primarily on digestive tract and mammary gland.

The expression of LBP gene was determined by qualitative and quantitative RT-PCR studies and the protein was detected by means of immunohistochemistry following validation of a monoclonal antibody (MAB) raised against human LBP.

2. Materials and methods

2.1. Tissue collection and preservation

Bovine tissues were collected from a local slaughterhouse from five clinically healthy animals. Clinical status of the animals was assessed by antemortem inspection and the absence of gross findings recorded during common slaughterhouse autopsy procedures. Sections from liver were used as positive controls for LBP expression studies in other tissues. Portions of each tissue were preserved in RNA*later*® (Sigma–Aldrich, Milano, Italy) and stored at −80 °C before RNA extraction. Sections for immunohistochemistry were taken from tissues preserved in liquid nitrogen immediately after collection and stored at −80 °C.

2.2. Validation of MAB cross-reactivity against bovine tissues by Western Blotting

A mouse anti-human LBP monoclonal antibody (biG42, Biometec Ltd, Greifswfal, Germany) was used through out the experiments. Because the cross-reactivity of this antibody utilized had never been validated before in the bovine, a preliminary study to assess the possible utilization of biG42 for bLBP detection was carried out. These experiments were performed by testing bovine serum (1 μl) with SDS-PAGE on a 12% polyacrylamide gel, and Western Blotting onto nitrocellulose membrane. The membranes were incubated with different concentrations of biG42 primary antibody for 45 min at room temperature, before fixing the optimal antibody concentration at 1:200 (5 μg/ml). Serum LBP positive bands were visualized after immunostaining with enhanced chemiluminescence (Immobilon™ Western Chemiluminescence HRP Substrate – Millipore, Vimodrone (Mi), Italy). Recombinant human LBP (10 ng/lane) (Biometec Ltd, Greifswfal, Germany) and healthy human serum (0.5 μl) were used as a positive control.

In order to further assess the specificity of the primary antibody, the Western Blotting experiments were repeated after 3 h incubation of biG42 antibody with recombinant human LBP using a 1:1 molar ratio, in order to block every anti-LBP specific reactive sites and to detect any possible non-specific reactions. The solution was then utilized as primary antibody in immunostaining procedure.

2.3. mRNA expression studies

All mRNA expression studies were carried out with multiple tissues from two animals. Each bovine tissue sample was preserved in RNA*later*® until RNA extrac-