Haptoglobin gene expression in bovine and human leukocytes in blood and bovine somatic cells in milk

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Abstract

Local expression of haptoglobin (Hp) in tissue samples collected from different sites of the bovine mammary gland has recently been established (Hiss et al., 2003). The aim of this study was to investigate whether bovine leukocytes are capable of synthesising Hp and might thus contribute to the Hp mRNA transcripts found in the mammary gland through migration into this organ. For this purpose, RT-PCR was carried out with RNA extracted from mononuclear and polymorphonuclear cells in blood and from somatic cells in milk of dairy cows. In comparison, human leukocytes were examined. Both subpopulations of bovine blood cells and the bovine somatic cells in milk exhibited Hp mRNA transcripts in contrast to human leukocytes in which no Hp mRNA transcripts were detectable by our method. In conclusion, blood derived leukocytes express Hp and, hence, might at least be partly responsible for the Hp mRNA transcripts found in the bovine mammary gland. It remains to be clarified whether other cells within the mammary gland are also capable of Hp synthesis.

Introduction

Acute phase proteins (APP) are known to play an important role within the response to infection, inflammation, injury or tumour growth (Heinrich et al., 1990). They are generated mainly by the liver leading to a significant rise in blood concentrations during an acute phase reaction (APR). Hp has been described as a major APP in cattle (Eckersall and Conner, 1988; Eckersall et al., 2001; Grönlund et al., 2003).

Several recent studies on mastitis in dairy cows have provided data on Hp concentrations not only in blood but also in milk to further elucidate its role in the animal's response to this disease (Eckersall et al., 2001; Grönlund et al., 2003; Hiss et al., 2003; Pedersen et al., 2003). In particular, a significant increase of Hp concentration in milk was observed only hours after mastitis had been induced experimentally (Grönlund et al., 2003; Hiss et al., 2003; Pedersen et al., 2003). Moreover, compared to blood Hp concentrations recorded simultaneously, the increase in milk occurred earlier (Hiss et al., 2003). These findings indicate that Hp might be synthesised locally in the mammary gland. Indeed, Hiss et al. (2003) showed Hp mRNA expression in tissue homogenates obtained from different sites of the bovine mammary gland. Immune cells from blood are potential candidates for Hp mRNA expression. We therefore aimed to investigate the presence of Hp mRNA in bovine leukocytes from blood and in somatic cells from milk. Since Wagner et al. (1996) reported that Hp is not expressed in human leukocytes, these cells were also examined in comparison.

Materials and Methods

Blood collection and processing

For leukocyte preparation, venous whole blood was collected into tubes preloaded with heparin (5000 IU/l blood; Heparin-Natrium-5000-ratiopharm, Ratiopharm GmbH, Ulm, Germany) from four dairy cows (German Holstein) and five human donors. Leukocytes were isolated by a 20 min centrifugation (4°C) at 1100 *g* (bovine blood) or 700 *g* (human blood), respectively, and subsequent use of the resulting buffy coat. Contaminating erythrocytes were eliminated by two consecutive 20 sec hypotonic lyses in water. After adding an equal volume of 2 x PBS, the solution was centrifuged at 100 *g* for 8 min at 4°C. The pellets obtained were washed in PBS and stored at -80°C.

The isolation of mononuclear cells (MNC) and polymorphonuclear cells (PNC) was performed by density gradient centrifugation. From another four cows heparinised whole blood was diluted 1:2 with PBS, layered in a 2:1 ratio on Histopaque®-1077 (Sigma,

Deisenhofen, Germany) and centrifuged (1100 *g*, room temperature) for 30 min. The interface containing the MNC was removed and washed once, followed by a hypotonic lysis as above and another washing step. The pellet was stored at -80°C. The PNC were presented together with the erythrocytes as a pellet in the original tube. After erythrocyte lysis the remaining cells were pelleted, washed once and stored at -80°C.
For all blood cell samples, cell count and cell viability were estimated by Trypan Blue staining, and cells were differentiated in a smear stained by Pappenheim method (Romeis, 1968). The viability of the isolated blood cells were assessed at above 85 %, and the purity of the MNC and PNC fractions was above 85 %.

Milk collection and processing
Milk samples were obtained from six dairy cows with somatic cell counts >120,000 cells/ml one week prior to sampling. After disposal of the foremilk, milk was collected aseptically, mixed with an equal volume of PBS and centrifuged (1000 *g*, 15 min, 4°C). The cell pellet was washed twice and stored at -80°C.

RNA extraction
Total RNA was prepared from the blood and milk derived cells as described by Chomczynski and Sacchi (1987). After treatment with DNase (DNase I RNase free, Roche, Mannheim, Germany), total RNA was quantified by determining the optical density (OD) at 260 nm. RNA integrity was verified by the OD_{260}/OD_{280} absorption ratio > 1.6 and by denaturing gel electrophoresis.

Reverse transcription-polymerase chain reaction (RT-PCR)
One microgram of total RNA was reversely transcribed into cDNA with 50 pmol random hexamer primers (Invitrogen, Karlsruhe, Germany), 4 µl 5x reaction buffer, 500 µM dNTPs each, 20 U ribonuclease inhibitor and 200 U reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany) at 65°C for 5 min, 27°C for 10 min, 42°C for 60 min and 99°C for 1 min in a 20 µl volume. Three microlitres of this cDNA were used for the PCR together with 20 pmol of the respective primers (Invitrogen, see Table 1), 100 µM dNTPs each, 5 µl 10x reaction buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$ and 1 U Taq DNA polymerase (all MBI Fermentas) in a final volume of 50 µl. PCR conditions of the respective primers are summarised in Table 2. Amplified products (10 µl) were visualised on a 2 % agarose gel by ethidium bromide staining (FluorImager SI, Amersham Pharmacia Biotech, Uppsala, Sweden) and analyzed by image analysis software (Peak Finder, Image QuaNT™, Version 4.1, Molecular Dynamics).

Table 1: Sequence of PCR primers used (forward: F; reverse: R), PCR product length and reference source

Gene	NIH GenBank accession no.	Product length (bp)	Primer sequence 5'-3'
b-Hp	AJ 271156	174	F = GTCTCCCAGCATAACCTCATCTC
			R = AACCACCTTCTCCACCTCTACAA
h-Hp	NM 005143	338	F = CCTGAATGTGAAGCAGTATGT *
			R = TTCTGTTTGAGTTTGATGAGC *
b-β-actin	AY141970	226	F = CGTGGGCCGCCCTAGGCACCA †
			R = GGGGGCCTCGGTCAGCAGCAC †
b-18S rRNA	AF176811	170	F = GAGAAACGGCTACCACATCC
			R = CACCAGACTTGCCCTCCA

* Chen et al., 1998; † Fitzpatrick et al., 2002

Table 2: Temperature and time conditions for the PCR

		Fragment							
		b-Hp		h-Hp		b-β-actin		b-18S RNA	
Denaturation		95°C	1 min	95°C	1 min	95°C	1 min	95°C	1 min
Cycle	Denaturation	94°C	40 sec	94°C	40 sec	94°C	30 sec	94°C	40 sec
	Primer annealing	55°C	20 sec	55°C	30 sec	55°C	30 sec	55°C	30 sec
	Extension	72°C	20 sec	72°C	40 sec	72°C	1 min	72°C	20 sec
Number of cycles		37		34		30		22	
Final extension		72°C	5 min	72°C	5 min	72°C	5 min	72°C	5 min
Cooling		4°C	∞	4°C	∞	4°C	∞	4°C	∞

Initially, all b-cDNA together with one h-cDNA for comparison were amplified using b-Hp-primers. The negative PCR result of this human sample led us to use specific primers from the human Hp sequence (Table 1). Both b- and h-cDNA samples were amplified separately with b-β-actin and b-18S rRNA primers for internal control.

Results

Based on the initial evidence of Hp mRNA signals in the total leukocyte preparations from all four cows (results not shown), RNA from MNC and PMC was investigated separately. Bovine Hp transcripts were clearly detectable in both leukocyte subpopulations obtained from three cows (cow 79, 80, and 83; Figure 1). For cow 49, no band was visible but b-Hp transcripts could be identified for this animal by the image analysis software. For the internal controls b-β-actin and b-18S rRNA, transcripts were obtained from all samples, except for b-β-actin in cow 49, indicating poor RNA quality of this particular sample (results not shown).

No Hp signal was detectable in human leukocyte RNA when using primers for b-Hp (Figure 1). When using h-Hp primers, transcripts were obtained from h-liver used as positive control, while in all h-leukocyte samples no valid Hp signal was yielded by our method (Figure 2). However, transcripts were detected for both b-18S rRNA and b-β-actin fragments (results not shown).

Hp PCR products could be demonstrated in RNA from somatic cells in milk (results not shown).

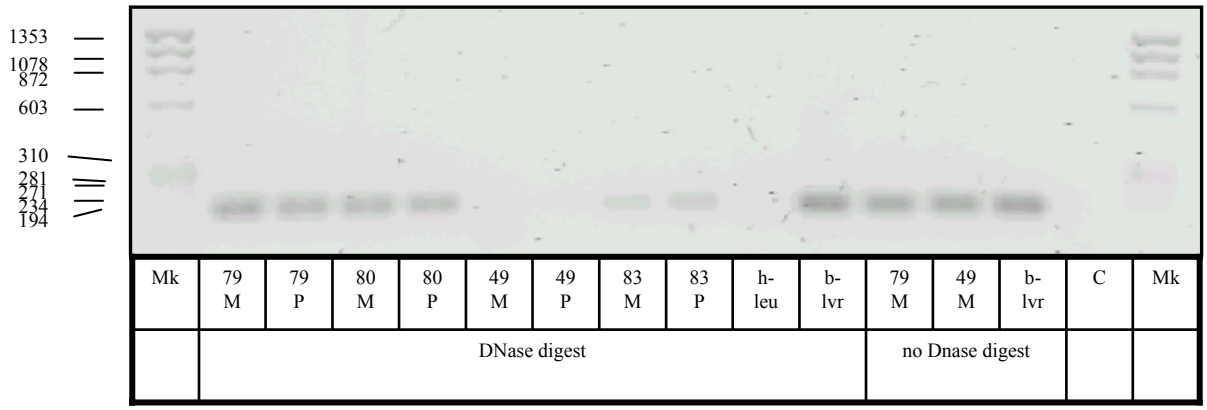

Fig. 1: Qualitative detection of b-Hp RT-PCR products in RNA extracted from b-MNC and b-PNC of four dairy cows and in h-leukocytes of one test person. Numbers are referring to individual test animals. Mk = marker, M = MNC, P = PNC, h-leu = h-leukocytes (test person 1), b-lvr = b-liver = positive RT-PCR control, C = water = negative RT-PCR control. Some samples are shown with and without DNase digest as control for potentially contaminating transcripts originating from genomic DNA.

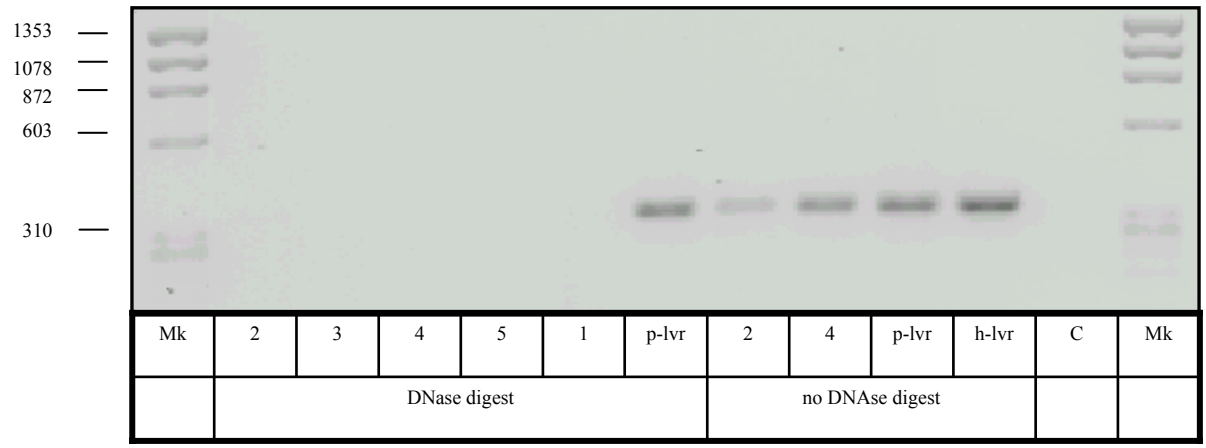

Fig. 2: Qualitative detection of h-Hp RT-PCR products in RNA extracted from h-leukocytes. Numbers are referring to individual test persons. Mk = Marker, p-lvr = porcine liver = positive RT-PCR control, h-lvr = h-liver = positive RT-PCR control, C = water = negative RT-PCR control. Some samples are shown with and without DNase digest as control for potentially contaminating transcripts originating from genomic DNA.

Discussion

We have demonstrated that haptoglobin mRNA is expressed in leukocytes, in particular in both subpopulations of mononuclear cells and polymorphonuclear cells, and in somatic milk cells from cows, while in human leukocytes no Hp mRNA could be detected. These findings are substantiated by the quality of the RNA preparations used; in particular, the significance of our findings is validated by the results of the control genes b-β-actin and 18S rRNA.

This is the first report on Hp gene expression in bovine leukocytes and in bovine somatic milk cells. In advancing our recent observation that b-Hp mRNA is present in tissue homogenates from different parts of the mammary gland, namely the teat, cisternal region and glandular parenchyma (Hiss et al., 2003), we could herein assign leukocytes as one possible cellular source of mammary Hp mRNA expression. Since somatic milk cells generally comprise about 90 % blood-derived cells (Boutinaud and Jammes, 2002), the detected Hp expression in these cells confirms the results obtained from the blood cells.

There is evidence that the mammary gland expresses other acute phase proteins such as lactoferrin (Molenaar et al., 1996), mammary-associated serum amyloid A 3 (McDonald et al., 2001) and mammary serum amyloid A 3 homologue (Molenaar et al., 2002). Moreover, leukocytes investigated in our study might contribute to Hp found at the protein level in milk (Eckersall et al., 2001; Grönlund et al., 2003; Hiss et al., 2003; Pedersen et al., 2003). However, it is unknown whether bovine leukocytes in fact translate Hp mRNA or secrete the protein. In humans, granulocytes can store Hp and release the protein after their exposure to *Candida albicans* (Wagner et al., 1996). In addition, no Hp expression in h-leukocytes could be demonstrated by the Northern blot method (Wagner et al., 1996), in agreement with our results by the more sensitive RT-PCR method.

The species difference between man and cattle in the expression of the Hp gene in blood leukocytes demonstrated herein is novel, however, the difference between the blood concentrations and the extent of increase during APR is well known. In cattle haptoglobin is considered to be the most prominent indicator of inflammation alongside serum amyloid A (Eckersall at al., 1988) whereas in man C-reactive protein is the most sensitive acute phase protein (Heinrich et al., 1990). The effects of Hp have been described as modulating the immune response based on trials with human and bovine sera showing inhibition of lymphocyte proliferation after exposure to Hp (Chase and Miller, 1972; Murata and Miyamoto, 1993). Similarly, specific binding of Hp to human neutrophils even inhibited their respiratory burst activity after stimulation by diverse agonists (Oh et al., 1990). The significance for cattle of synthesising Hp de novo in leukocytes rather than up-taking and storing remains to be clarified.

In summary, we were able to demonstrate that bovine peripheral blood leukocytes and somatic cells in milk synthesise Hp in contrast to human leukocytes. Further research is required to determine whether also cells of the mammary gland itself produce Hp.

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