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Transcriptional Activity of Il-8 in Healthy Bovine Mammary Gland at Mid and Late-lactation

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Abstract: mRNA profiles of IL-8 in the bovine mammary gland were investigated using newly developed TaqMan® real-time PCR systems. The transcription of bovine IL-8 between middle and late stages of the lactation period revealed no significant differences. The constant transcription of IL-8 at both stages could be related to the low need for neutrophils recruitment to the healthy udders. The possible interaction of GM-CSF and IL-12 with the IL-8 in the bovine mammary gland was discussed.

Key words: IL-8, midlactation, latelactation, bovine, cytokine

Introduction

Interleukin-8 (IL-8) is a well known neutrophil-chemotactic cytokine that is produced by stimulated monocytes, T lymphocytes, macrophages, neutrophils, endothelial cells and number of tumor cells lines (Matsushima and Oppenheim, 1989).

The chemotactic property of bovine IL-8 in attracting neutrophils was established using bovine recombinant IL-8 (Caswell *et al.*, 1999). Evidence on the expression IL-8 mRNA in bovine mammary gland was established using bovine mammary epithelial cells pulsed with lipopolysaccharide (LPS) (Boudjellab *et al.*, 1998). The chemotactic activity of IL-8 in bovine mammary secretions was also shown to be blocked with anti-IL-8 antibodies. Nevertheless, the inhibition of IL-8 activity was only obvious in the mastatic, but not nonmastatic, secretions (Barber and Yang, 1998). Recently, with the aid of the newly developed TaqMan® real-time PCR systems, IL-8 was detected in milk cells and peripheral blood mononuclear cells (PMBC) of healthy cows (Leutenegger *et al.*, 2000).

IL-8 considered one of the most important chemokines in regulating the neutrophils migration by enhancing the expression of adhesion molecules (Baggiolini *et al.*, 1989). Augmentation of IL-8 in the infected glands was considered as an important factor in enhancing the neutrophils chemotaxis, which play a major role in amplification of inflammatory responses (Shuster *et al.*, 1996).

The main purpose of this study was to compare the normal transcription of IL-8 between mid lactation, the resistant stage and late lactation, a stage were mammary glands undergoing dramatical changes.

Materials and Methods

Animals and Milk cells preparation: One litre composite milk samples selected from the same samples that were referred by Alluwaimi and Cullor (2002) and collected from 15 (Holstein cows), in their second and third lactations, obtained from the last milking before they dried off and from 10 cattle of the same age and breed at their 3rd –4th month of their lactation period (BandG Dairy Farm, Dixon, CA, U.S.A). The milk samples were screened for bacterial contamination at the Dairy Food Safety Laboratory, Haring Hall, UC Davis, CA and the total somatic cells were counted (Siliker Laboratories, Modesta, CA). Each milk sample was centrifuged at 700 g for 20 min and the pellet washed twice with 50 ml phosphate buffered saline. Using commercial kit (RNeasy mini kit, Qiagen, Valencia, CA) 5×10^6 cells were lysed with 350 μl of the lysis buffer according to the manufacturer's recommendations and kept at $-80°C$ until extraction of RNA and synthesis of complementary DNA (cDNA).

RNA extraction: The total RNA (tRNA) was extracted from the frozen lysed milk cells using a commercial RNeasy® total RNA extraction kit (RNeasy mini kit, Qiagen, Valencia, CA). To remove contaminating genomic DNA (gDNA), the extracted tRNA was treated with 10 U/μl of RNase free DNase I (DNase, Amersham Pharmacia Biotech Inc, Piscataway, NJ) at $37°C$ for 10 min heat inactivating at $95°C$ for 5 min and chilling on ice.

Reverse transcription (RT) of total RNA: For the synthesis of cDNA, 20 μl RT mix containing 50 U MuLV reverse transcriptase, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 μM random hexadeoxyribonucleotide (pd $(N)_6$) primers, 0.5 U/μl

Rnase inhibitor (GeneAmp® RNA PCR kit, PE-Biosystems, Foster City, CA) and 1 mM dNTPs (Amersham Pharmacia Biotech, Piscataway, NJ), was heated to 42°C for 60 min and then inactivated at 95°C for 5 min. The final volume of the mix was adjusted to 100 µl with RNase free water. The cDNA was analyzed immediately or stored at -30°C until used.

Real-time TaqMan® PCR for quantifying cytokine cDNA:
The details of the real-time Taq-Man® PCR system and the design of bovine IL-8 primers and probes were described by Leutenegger *et al.* (2000). The sequences of IL-8 primers and probe are as follows, forward primer, IL-8.177f 5'-CACTGTGAAAAATTCAGAAATCATTGTTA 3', reverse primer, IL-8.282r 5'-CTTCACCAAATACCTGCACAACCTTC-3' and the probe, [IL-8.214p 5'-AATGGAAACGAGGTCTGCTTAAACCCCAAG-3'] (GenBank accession no. S74436). Bovine IL-8 cDNA was assayed for the cytokine profile and for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as endogenous control in separate wells [forward primer GAPDH.463f. 5'-GGCGTGAACCACGAGAAGTATAA-3'], [reverse primer, GAPDH.582r 5'-CCCTCCACGATGCCAAAGT-3'] and [probe GAPDH.489p 5'-ATACCCTCAAGATTGTCAGCAATGCCTCCT-3'] (GenBank accession no. AF022183). The PCR reaction was carried out in 25 µl PCR mixtures containing final concentration of 400 nM primer, 80 nM probe and a commercially available PCR mastermix (TaqMan® Universal PCR Mastermix, PE-Biosystems) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625U of AmpliTaq Gold DNA polymerase per reaction, 0.25U AmpErase UNG per reaction and 10 µl of the diluted cDNA sample. The samples were placed in 96 well plates and amplified in an automated fluorometer (ABI Prism 7700 Sequence Detection System, PE-Biosystems, Foster City, CA). The amplification conditions were 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 60 s at 60°C. The level of IL-8 was calculated according to the comparative C_T (threshold cycle) method (Leutenegger *et al.,* 1999).

Statistical analysis: Statistical analysis was performed using BMDP statistical software (BMDP Statistical software, Inc., Statistical Solution Limited, Crosses Green, Ireland). One and two-factor analysis of variance, the t-test for equality of 2 independent samples and the Mann-Whitney nonparametric test for equality of 2 independent samples were used.

Results
IL-8 transcriptional activity was detected in 100% of the samples collected at mid- and late-lactation (Fig. 1). The two-sample t-test analysis of transcription of IL-8 between mid- and late-lactation revealed no significant differences. The result was confirmed further with the Mann-Whitney non-parametric test for equality of 2 independent samples. Since this study was conducted on the same milk samples that were referred to previously (Alluwaimi and Cullor, 2002), Pearson correlation analysis between IL-8 and interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 subunit 40 (IL-12), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and granulocytes monocyte-colony stimulating factor (GM-CSF) revealed that IL-8 has significant positive correlation with GM-CSF in mid- (P < 0.033) and late-lactation (P < 0.001) and with IL-12 only in late-lactation (P < 0.031).

Discussion
Numerous studies indicated that IL-8 is a neutrophil specific chemotactic factor (Baggiolini *et al.,* 1989; Ribeiro *et al.,* 1991; Caswell *et al.,* 1999). Bovine IL-8 chemotactic activity was detected in the mammary secretions of glands infected with *E. coli* and *Staphylococcus aureus* (Barber and Yang, 1998; Shuster *et al.,* 1996) and in normal milk cells (Leutenegger *et al.,* 2000).
In this study the transcriptional activity of IL-8 was detected in healthy bovine milk cells at mid- and late-lactation. However, no significant difference was recorded in the transcription of IL-8 between these two stages.
The predominant somatic cells in the healthy lactating mammary gland are lymphocytes and macrophages (Sordillo *et al.,* 1997). Nevertheless, the somatic milk cells constituent could vary during certain stages of the lactation period, particularly at early and late stages of involution (Sordillo *et al.,* 1997). However, neutrophils content of normal milk hardly differs at different stages of the lactation period, even in the dry period (Nickerson, 1989). It was shown that neutrophil percentage slightly decreased from 19% at onset of the dry period to 15% at the steady state of involution (Nickerson, 1989). Therefore, it appears that the major source of IL-8 in the healthy mammary gland are macrophages and to a lesser extent lymphocytes. Hence, the non-significant difference in the transcription of IL-8 between the two stages most likely reflected the low need of neutrophils recruitment to the normal mammary gland.
IL-8 expressed positive correlation with IL-12 in late-lactation. It was reported that IL-8 responses was influenced by IL-12 (Trinchieri, 1995). Therefore, the positive correlation of IL-12 with IL-8 might indicate certain regulatory role for IL-12 in the induction of IL-8 in the healthy bovine mammary gland. IL-8 also expressed a correlation with GM-CSF at both stages. GM-CSF and IL-8 are produced by macrophages and lymphocytes and they share common target cells (McDonald *et al.,* 1993). It was shown that IL-8 induced lipid mediators, leukotrien-B4, platelate activating factor and others in GM-CSF
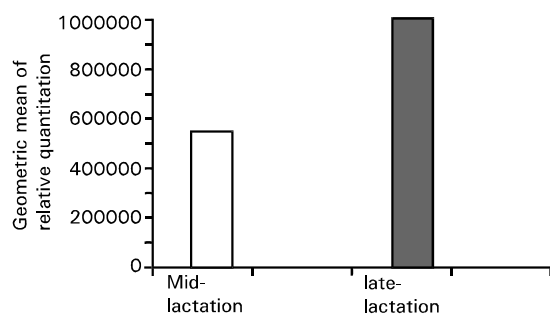
Fig. 1: Geometric mean of relative quantitation of IL-8 in mid- and late-lactation. No significant difference was seen in the transcription of IL-8 between these two stages

pretreated human neutrophils (McDonald *et al.,* 1993). Furthermore, the pattern of the transcriptional activity of GM-CSF in mid and late-lactation was similar to that of IL-8, which was observed in this study (Alluwaimi and Cullor, 2002). The positive correlation of IL-8 with GM-CSF at both stages could be attributed to a possible synergistic function of IL-8 and GM-CSF in the healthy bovine mammary gland. However, the exact synergistic mechanism and its possible immunoregulatory role need to be investigated. In general, correlation of IL-8 with GM-CSF at both stages and with IL-12 in late-lactation could be envisaged in parallel to overwhelming information of synergistic action of these pro-inflammatory cytokines in blood and other tissues (Paludan, 2000).

In conclusion, it was possible to confirm the transcription of IL-8 in the healthy lactating bovine mammary gland and to compare its transcriptional activity between mid- and late-lactation. However, no significant difference in the transcription of IL-8 between these two stages was observed.

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