High epidermal growth factor concentration associated with somatic cell count in milk of cows with subclinical mastitis¹⁾

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Summary

The aim this study was to determine the milk epidermal growth factor (EGF) concentration in cows with subclinical mastitis and its relationship with the somatic cell count (SCC). The animal material of this study was composed of 40 lactating cows aged 3-6 years. Subclinical mastitis was diagnosed using the California Mastitis Test and SCC in milk. The study group consisted of 20 cows with SCC > 200.000 cells/ml, and the control group comprised 20 cows with SCC < 200.000 cells/ml. EGF concentration in milk was determined using a bovine-specific enzyme-linked immunosorbent assay (ELISA) kit. The mean EGF concentration was 6.08 ± 2.91 ng/ml in the study group and 2.85 ± 1.87 ng/ml in the control group ($P < 0.001$). The results also indicated a significant correlation between SCC and EGF concentration in the study group ($r = 0.965$, $P < 0.01$). The findings of this study suggest that a milk EGF assay together with SCC could be useful for diagnosing mastitis as well as for monitoring udder health.

Keywords: cow, epidermal growth factor, milk, subclinical mastitis

Subclinical mastitis is considered to be one of important diseases of dairy cows because it results in production losses and low profitability (3, 20, 39, 53). Inflammation of the mammary gland is a major cause of decreased milk yield and reproductive performance, combined with increased veterinary costs (5, 26, 34, 40, 45, 50). The diagnosis of subclinical mastitis is difficult because changes in both the udder and milk are invisible. Therefore, the evaluation of somatic cell counts and laboratory analysis of the composition of udder milk are required to diagnose subclinical mastitis (20, 22, 23, 27, 28, 31, 37, 38, 42, 44).

Epidermal growth factor (EGF) is a 6 kDa protein with 53 amino acids, and its receptors have been found mainly in endothelial, mesodermal, fibroblast and smooth muscle cells. EGF is known to play a role in the development of mammary glands (1, 7, 17, 36, 46, 57). It has been reported that the normal development and homeostasis of the mammary gland are critically

dependent on regulated EGF receptor signaling (55). Paracrine activation of stromal EGF receptors is required for ductal morphogenesis of the mammary gland (51). In addition, EGF supplementation was found to modify lymphocyte composition in mesenteric lymph nodes and to contribute to immune maturation in suckling rats (54). A relationship has been demonstrated between EGF and folliculogenesis, embryogenesis, pre-implantation and peri-implantation, as well as a potential growth-promoting effect of that relationship on the implanted embryo and endometrium in various mammalian species, such as cow, sheep, mare, pig, rabbit and cat (8, 14, 18, 21, 25, 32, 52). It has been determined that EGF expression in the bovine mammary gland with mastitis increases due to inflammation, and this increase is thought to be a part of a cellular process and tissue repair (47). An increase in mRNA expression of insulin-like growth factor and vascular endothelial growth factor in mammary gland tissue after experimental infection with *Staphylococcus aureus* showed that these growth factors play important roles in the

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inflammatory process of bovine mastitis (9). However, there is no literature concerning the concentrations of EGF in subclinical mastitis.

The purpose of this study was to evaluate milk EGF concentration in cows with subclinical mastitis and the relationship between EGF concentration and SCC.

Material and methods

Animals and study design. A total of 40 lactating cows aged 3 to 6 years were used in the study. The study group consisted of 20 cows with subclinical mastitis, and the control group was made up of 20 clinically healthy and CMT-negative cows with no apparent abnormalities in the udder or milk (CMT). Subclinical mastitis was diagnosed by a California Mastitis Test on the basis of the somatic cell count (SCC) in milk. The study group included 20 cows with $SCC > 200,000$ cells/ml, and the control group included 20 cows with $SCC < 200,000$ cells/ml. Milk samples were collected from each quarter of every cow into glass tubes of 10 ml for SCC and into plastic vials of 10 ml for ELISA analysis.

Counting of somatic cells. Somatic cells in raw milk were counted by the direct microscopic method (30). Briefly, a 10 ml milk sample taken into a glass tube was centrifuged at 3000 rpm for 10 minutes. After the fatty layer had been removed, the tube was inverted and allowed to stand for 20 minutes. Sediment at the bottom of the tube was carefully removed and spread over a microscope slide with a drop of saline. Slides, dried at the laboratory ambient temperature, were stained with 0.2% toluidine blue. A drop of immersion oil was dripped carefully onto the slide, and somatic cells were counted within about 20 random microscope fields, and the mean cell number in 1 ml of milk was calculated as presented in Table 1.

Separation of milk serum. The method of Alais (2) was used to obtain milk serum. One millilitre of 0.3% chymosin was added to 10 ml of raw milk, which was then maintained for 20 minutes in a water bath at 37°C for clotting. After 80 minutes, separated milk serum was filtered into tubes and centrifuged at 3000 rpm for 5 minutes. After centrifugation, the fatty layer was removed, and clear milk serum was obtained.

Analysis of EGF concentrations. The concentrations of EGF in milk serum were determined using a bovine-specific enzyme-linked immunosorbent assay (ELISA) kit (MBS706122, MyBioSource, Inc. San Diego, CA, USA) according to a procedure recommended by the manufacturer. Analysis was performed concurrently in duplicate. The absorbance of each plate was determined with a microplate reader (Infinite F50, Tecan Austria GmbH, Austria), and EGF concentrations were calculated.

Statistical analysis. The results were analyzed with a statistical package program (SPSS Statistics V21.0, IBM

Corporation, Armonk, NY). Group differences for EGF were determined using the Mann-Whitney U test, and the results were presented as mean $\pm$ standard deviation. Pearson's correlation was calculated to determine the relationship between the somatic cell count and EGF concentration.

Results and discussion

Milk serum EGF concentrations for the study and control groups are shown in Figure 1. The EGF concentration amounted to 6.08 ± 2.91 ng/ml (from 2.09 ng/ml to 10.75 ng/ml) in the study group and 2.85 ± 1.87 ng/ml (from 0.33 ng/ml to 8.43 ng/ml) in the control group. The mean milk serum EGF concentration in the study group was higher than that in the control group ($P < 0.001$). In addition, a significant correlation was found between milk EGF concentration and SCC in the study group ($r = 0.965$, $P < 0.01$) (Fig. 2).

Economic consequences of subclinical mastitis due to changes in milk quality and quantity are a major

Fig. 1. EGF concentration in milk serum of the groups
Explanation: * $P < 0.001$, Mann-Whitney U test

Fig. 2. Correlation between milk EGF concentration and SCC in the study group
Explanations: $r = 0.965$, $P < 0.01$, Pearson's correlation

Tab. 1. Evaluation of somatic cell count

Mean cell number	Evaluation	Cell number per ml
1-5	+	< 200,000
6-20	++	> 200,000
> 20	+++	< 1,000,000

problem in the dairy industry (3, 4, 20, 24, 26, 39, 50, 53). Early diagnosis of subclinical mastitis and close monitoring of affected cows is of great importance to control the disease in the herd (33). The inflammatory process of mastitis should be well understood in order to develop more effective treatment strategies. EGF has receptors in healthy mammary glands and plays a role in the development of mammary tissue by stimulating DNA synthesis (47). Therefore, evaluation of the EGF level could be an alternative diagnostic method.

Growth factors are believed to be important in the inflammatory process in the mammary tissue (9, 47). They participate in the normal development of the mammary gland by controlling growth and differentiation (7, 11, 48). However, there is no study concerning changes in milk EGF during subclinical mastitis. EGF precursor arises in the alveolar cells of lactating mammary glands and is transferred to the cell membrane (6). In addition, it has been reported that mutation in EGF receptors leads to impairment in lactation and in the development of the mammary gland (16). The number of EGF receptors increases during pregnancy in the cow (49). The concentration of EGF in goat milk was found to be influenced by the pregnancy and lactation status (10). The milk EGF was shown to stimulate the differentiation of intestinal epithelial cells in suckling animals (15) and contribute to the repair of mucosa (12, 29, 43, 56). The contents of breast EGF may be influenced by lactation periods and maternal diet (35). It has been postulated that maternal colostrum and milk are the main sources of EGF for developing intestinal mucosa (13, 41). Besides, EGF expression in the mammary tissue of mastitis cows was reported to be increased, and it is believed to have a potential role in tissue repair and the cellular process during mastitis (47). Similarly, our study revealed that milk EGF concentration in cows suffering from subclinical mastitis was higher than that in healthy ones. A significant positive correlation was determined between the milk SCC and EGF of cows with subclinical mastitis, and this significance suggests a role of EGF in the inflammatory process in the mammary gland. To the best of our knowledge, this is the first study showing association between increased EGF concentration and SCC in milk during subclinical bovine mastitis. In our study, milk EGF concentration and SCC were positively correlated, and this finding suggests that a milk EGF assay could be a useful tool for the diagnosis of mastitis, as well as for monitoring udder health.

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