BRIEF COMMUNICATION

Decreased gene expression of integrins in epithelial cells during mammary engorgement

K. SINGH, J. DOBSON, K. STELWAGEN, C.G. PROSSER, V.C. FARR, A. MOLENAAR, S.R. DAVIS[1]

AgResearch, Ruakura Agricultural Research Centre, P.B. 3123, Hamilton

Keywords: involution; bovine; mammary; apoptosis; integrins.

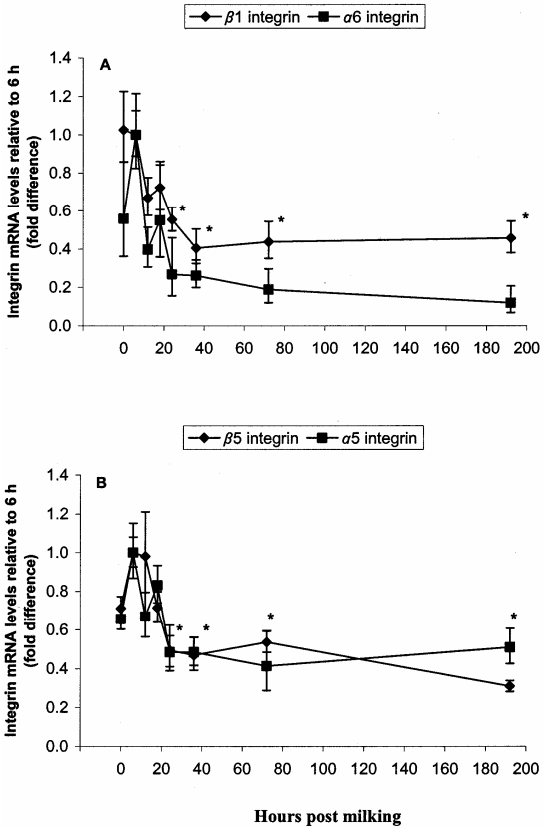
In ruminants, the molecular mechanisms regulating mammary involution and apoptosis (programmed cell death) are not well characterised, but extensive research in rodents has established that many cell types, including mammary epithelial cells, require anchorage to the extracellular matrix for survival. This anchorage is mediated via the integrins, a family of transmembrane glycoproteins that bridge intra- and extracellular compartments enabling direct communication between adaptor molecules in the cytoplasm and specific receptor motifs on the ECM proteins (Clark & Brugge, 1995; Giancotti & Ruoslahti, 1999; Hynes, 1992). Integrins form heterodimers between α and β subunits to produce more than 20 different receptors, and many subtypes have been identified in mammary glands (Clark & Brugge, 1995). We have shown previously that one such integrin, β1, is reduced rapidly as a result of induced weaning in rodents, and these changes are followed by apoptosis (McMahon *et al*., 2004). The aim of this study was to investigate the molecular mechanisms that may regulate mammary involution in the bovine by examining cell-matrix interactions that may be involved in survival of mammary epithelial cells, ultimately to improve lactational persistency and tolerance of extended milking intervals. Involution of the bovine mammary gland was induced by abrupt termination of milking in 48 non-pregnant Friesian dairy cows at mid-lactation (average days in milk, 92 ± 3.0). The cows were in their first lactation with an average daily milk yield of 14.3 ± 0.3 kg/cow. Somatic cell count at the start of the experiment was 1.96 ± 0.29 x 10^5 cells/ml. Alveolar mammary tissue was obtained following slaughter at 0, 6, 12, 18, 24, 36, 72 and 192 h (n=6 per group) after the last milking. In a second trial, two non-pregnant multiparous Jersey and Jersey x Friesian crossbred dairy cows at late-lactation were milked unilaterally. Somatic cell count at the start of the experiment was 0.65 ± 0.31 x 10^5 cells/ml. The left and right udder halves of each cow were randomly assigned to two milking frequencies; either twice daily milking (at 0800 and 1600 h; control) or once daily milking (at 0800 h only) for 4 days.

Animals were slaughtered by captive bolt and exsanguination and samples (approximately 30 g) of secretory alveolar mammary tissue were obtained from the middle of the upper one-third of the gland of the rear quarter and snap-frozen in liquid nitrogen. Total RNA was extracted from an aliquot of ground tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and converted to cDNA using a SuperScript II First-Strand Synthesis system as described by manufacturer (Invitrogen). PCR primer sequences for detection of integrins (β1, α6, β5 and α5) were from Coussens & Nobis (2002) and were generated for bovine genes and ortholog-selected bovine EST sequences. Quantitative real-time PCR analysis was carried out using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Detection of the product was by SYBR Green I using the Universal PCR Master Mix (Applied Biosystems, Sequence Detection System, Chemistry Guide, 2003). For each assay two control reactions were included; a reverse transcriptase-negative control, and omission of the template (no template control). Any amplification occurring in these control reactions would indicate the presence of non-RNA template. The thermal cycling program was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec, 56 °C (β1 and α5 integrin) or 58 °C (β5 and α6 integrin) for 30 sec, 72 °C for 30 sec, 78 °C for 10 sec. β-actin was used as an internal control. Dissociation curve analysis confirmed that only 1 product was amplified for each of the genes. The amplified products were verified by sequencing and on 2% agarose gels. In experiment 1, the 6 h post-milking time point is the most representative of actively lactating mammary glands; therefore results were expressed as the fold change relative to the 6 h mean and in experiment 2 the results were relative to the twice daily milked mean. The threshold cycles (C_T) were generated by real-time PCR and used to quantify the relative abundance of each gene using the relative standard curve method (Applied Biosystems, Sequence Detection System, Chemistry Guide, 2003). Differences between means were analysed using analysis of variance in Minitab (Minitab Release 13 for Windows, 2000). The least significant differences identify the means significantly different from each other (* $P<0.05$, ** $P<0.01$). Data were expressed as mean ± the standard error of the mean (SEM).

The mRNA levels of β1 and α6 integrins were decreased ($P<0.05$) by 24 h after last milking relative to 6 h (1.8- and 3.7-fold decrease, respectively, see Figure 1A). There was no further decrease after 24 h.

The mRNA levels of β5 and α5 integrins were both decreased 2-fold (P<0.05), by 24 h after last milking relative to 6 h (see Figure 1B).

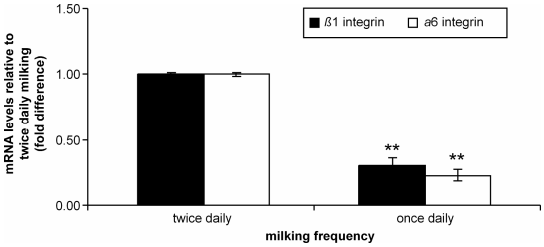
FIGURE 1: Changes in mRNA levels of integrins (β1, α6, β5, and α6) with time in mammary alveolar tissue of lactating cows at mid lactation following the last milking (n=6 per time point). Data are expressed as mean ± SEM and P values (* P<0.05) are relative to 6 h time point for respective genes.



The β1 and α6 integrin mRNA expression was also lower (P<0.01) in alveolar tissue from udders milked once daily relative to those milked twice daily (3.3- and 4.5-fold decrease, respectively, see Figure 2).

This study shows a decrease in expression of multiple integrin genes induced by either abrupt cessation of milk removal (ie termination of milking), or milking frequency. These changes are a critical early event in the cell survival pathway and are consistent with a role for integrins in involution of the mammary gland of lactating cows. These data agree with our earlier findings that after 18-20 h of milk accumulation in goats and cows, physiological changes occur in the gland (Stelwagen *et al.*, 1994; 1995; 1997). In rodents, the abrupt cessation of milk removal induced by sealing teats is associated with decreased β1 integrin levels and cytochrome C levels from mitochondria as early as 6 hours (McMahon *et al.*, 2004). Studies have also shown an increase in apoptotic cells in rodent mammary tissue within 24 h of litter removal (Quarrie *et al.*, 1995). This study shows a very early decrease in the expression of different integrin genes (β1, α6, β5 and α5) 24 h after last milking in mammary alveolar tissue, suggesting factors influencing apoptosis and mammary cell survival during involution also occur rapidly in lactating cows. However, ruminants may be more tolerant to the effects of prolonged milk accumulation with the capacity to reinstate milk production in dairy cow quarters unmilked for 12 days (Hamann & Reichmuth, 1990). Thus in ruminants, the commitment to apoptosis may be delayed, thus retaining some capacity to re-initiate lactation.

FIGURE 2: Changes in mRNA levels of β1- and α6-integrins in mammary alveolar tissue from glands milked unilaterally with one udder half once daily and the other twice daily for 4 days, during late lactation. Data are expressed as mean ± SEM and P values (** P<0.01) are relative to twice daily milked means for respective genes.



Our study further shows variation in integrin expression as a result of variation in the frequency of milking. There was a decrease in expression of β1 and α6 integrins in glands of cows milked once daily relative to the glands in the same animal milked twice daily. This suggests the cell survival pathway is sensitive to frequency of milking and the loss in adhesion of mammary alveolar cells to the extracellular matrix may be related to the reduction in milk yield due to an increase in cell loss by apoptosis. Increased rate of secretory tissue loss from the udder during once-daily milking has been demonstrated previously (Carruthers *et al.*, 1993).

Extensive studies *in vitro* (Streuli & Gilmore, 1999) have described signal transduction pathways associated with cell adhesion. Mammary cells require adhesion to extracellular matrix proteins of the basement membrane to suppress apoptosis. Recent studies in our laboratory (McMahon *et al.*, 2004) suggest the role of β1 integrin in this pathway. Previous studies also suggest α6 integrin is an anti-apoptotic signal *in vivo*, promoting the survival of metastatic human breast carcinoma cells (Wewer *et al.*, 1997). Results from our study also implicate two other integrins, β5 and α6, as having a role in cell survival *in vivo* in mammary of lactating cows. Overall, this study suggests that in dairy animals milk accumulation stimulates apoptosis in bovine mammary tissue by regulating a decrease in the cell survival pathway by

causing a loss in communication between the integrins and the extracellular matrix.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the contributions of Harold Henderson for providing statistical advice; and Kim Oden and Craig Smith in RNA preparation for experiments 1 and 2, respectively.

REFERENCES

Carruthers, V.R.; Davis, S.R.; Bryant, A.M.; Henderson, H.V.; Morris, C.A.; Copeman, P.J.A. 1993: Response of Jersey and Frisian cows to once-daily milking and prediction of response based on udder characteristics and milk composition. *Journal of Dairy Research 60*: 1-11.

Clark, E.A.; Brugge, J.S. 1995: Integrins and signal transduction pathways: the road taken. *Science 268*: 233-239.

Coussens, P.M.; Nobis, W. 2002: Bioinformatics and high throughput approach to create genomic resources for the study of bovine immunobiology. *Veterinary Immunology and Immunopathology 86*: 229-244.

Giancotti ,F.G.; Ruoslahti, E. 1999: Integrin signaling. *Science 285*: 1028-1032.

Hamann, J.; Reichmuth, J. 1990: Compensatory milk production within the bovine udder: effects of short-term non-milking of single quarters. *Journal of Dairy Research 57*: 17-22.

Hynes, R.O. 1992: Integrins – versatility, modulation, and signalling in cell-adhesion. Cell 69: 11-25.

McMahon, C.D.; Farr, V.C.; Singh, K.; Wheeler, T.T.; Davis, S.R. 2004 Decreased expression of β1-integrin and focal adhesion kinase in epithelial cells may initiate involution of mammary glands. *Journal of Cellular Physiology* (in press).

Minitab Release 13 for Windows (2000), Minitab Inc.

Pullan, S.; Wilson, J.; Metcalfe, A.; Edwards, G.M.; Goberdhan, N.; Tilly, J.; Hickman, J.A.; Dive, C.; Streuli, C.H. 1996: Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium. J. Cell Sci. 109: 631-642.

Quarrie, L.H.; Addey, C.V.P.; Wilde, C.J. 1995: Apoptosis in lactating and involuting mouse mammary tissue detected by nick-end DNA labelling. *Cell and Tissue Research 281*: 413-419.

Stelwagen, K.; Davis, S.R.; Farr, V.C.; Prosser, C.G.; Sherlock, R.A. 1994: Epithelial cell tight junction integrity and mammary blood flow during an extended milking interval in goats. *Journal of Dairy Science 77*: 426-432.

Stelwagen, K.; Farr, V.C.; Davis, S.R.; Prosser, C.G. 1995: EGTA-induced disruption of epithelial cell tight junctions in the lactating caprine mammary gland. *American Journal of Physiology 269*: R848-R855.

Stelwagen, K.; Farr, V.C.; McFadden, H.A.; Prosser, C.G.; Davis, S.R. 1997: Time course of milk accumulation-induced opening of mammary tight junctions and blood clearance of milk components. *Journal of American Physiology 273*: R379-R386.

Streuli, C.H.; Gilmore, A.P. 1999: Adhesion-mediated signalling in the regulation of mammary epithelial cell survival. *Journal of Mammary Gland Biology and Neoplasia 4*: 183-191.

Wewer, U.M.; Shaw, L.M.; Albrechtsen, R.; Mercurio, A.M. 1997: The integrin alpha 6 beta 1 promotes the survival of metastatic human breast carcinoma cells in mice. *Am. J. Path. 151*: 1191-1198.