

Mammary mechanisms for lactoferrin: interactions with IGFBP-3

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Lactoferrin (Lf) is an iron-binding protein found in high concentrations in mammary secretions but synthesized by many tissues. Bovine mammary tissue secretes $\mu\text{g/ml}$ mass of Lf in milk, but during involution and prepartum periods, 20–80 mg/ml concentrations may be observed. While a number of functions have been ascribed to lactoferrin, only the antimicrobial and lymphocyte interactions have compelling experimental evidence of support. We report a new finding that lactoferrin binds to insulin-like growth factor binding protein-3 (IGFBP-3) and not to other mammary secreted IGFBPs (IGFBP-2, -4, & -5). Furthermore, bovine Lf (bLf) is found associated with membranes of mammary cells. We demonstrate that bovine Lf competes with IGF for binding to IGFBP-3 with ED_{50} competition of 3 $\mu\text{g/ml}$ and displacement of 1 mg/ml to monomeric bLf. The tetrameric form that is favored by high concentrations of Lf and calcium, does not appear to bind IGFBP-3. Both IGFBP-3 and Lf have nuclear localization sequences that are reported to be key components of nuclear localization of proteins. We demonstrate that extracellular IGFBP-3 binds to membrane Lf and that Lf is the key to the entry of IGFBP-3 to mammary cellular nucleus. Additionally, we have shown that the internalization of Lf requires the presence of retinoids that also induces both IGFBP-3 and Lf synthesis in primary cultures of bovine mammary epithelial cells. We hypothesize a new role for Lf in the regulation and integration into the IGF System.

Keywords. Mammary gland, lactoferrin, IGFBP-3.

Mécanismes d'action de la lactoferrine au niveau mammaire : interactions avec l'IGFBP-3. La lactoferrine (Lf) est une protéine de liaison du fer trouvée en concentrations élevées dans les sécrétions mammaires mais synthétisées également par de nombreux autres tissus. Les tissus mammaires bovins secrètent dans le lait des quantités de Lf à des concentrations de l'ordre du $\mu\text{g/ml}$ alors que pendant les périodes d'involution et *pre-partum*, ces concentrations fluctuent entre 20 et 80 mg/ml. Bien qu'un certain nombre de fonctions biologiques de la Lf ont été décrites, seules les activités antimicrobiennes et lymphocytaires ont été clairement démontrées expérimentalement. Nous décrivons une nouvelle propriété de la Lf laquelle se lierait uniquement à la protéine de liaison 3 de l'insulin-like growth factor-I (IGFBP-3) et non aux autres protéines de liaison de l'IGF-I sécrétées par la glande mammaire (IGFBP-2, -4 & -5). Par ailleurs, la lactoferrine bovine se fixe aux membranes des cellules de la glande mammaire. Nous avons démontré que la Lf bovine (bLf) entre en compétition avec l'IGF pour la liaison avec l'IGFBP-3 avec un ED_{50} de compétition de 3 $\mu\text{g/ml}$ et un déplacement de 1 mg/ml de la bLf monomérique. La forme tétramérique dont l'obtention est favorisée par des concentrations élevées en Lf et en calcium, ne paraît pas se lier à l'IGFBP-3. Tant l'IGFBP-3 que la Lf présentent dans leurs séquences des signaux de translocation dans le noyau qui militent en faveur d'une localisation nucléaire de ces protéines. Nous avons démontré que l'IGFBP-3 extracellulaire se fixe à la Lf de membranes et que la Lf joue un rôle central dans l'internalisation de l'IGFBP-3 dans le noyau des cellules mammaires. De plus, nous avons observé que l'internalisation de la Lf nécessite la présence de rétinoïdes lesquels induisent aussi bien la synthèse d'IGFBP-3 que de Lf par les cellules épithéliales mammaires placées en culture primaire. En conclusion, nous formulons l'hypothèse d'un nouveau rôle de la Lf dans la régulation et l'intégration du système IGF.

Mots-clés. Glande mammaire, lactoferrine, IGFBP-3.

1. INTRODUCTION

Lactoferrin (Lf) is an iron-binding glycoprotein found in high concentrations in mammary secretions and to a lesser extent in exocrine secretions of mammals and is released from neutrophilic granules during inflammation. However, Lf mRNA expression appears in almost all normal human tissues (Siebert, Huang, 1997). Although first isolated and characterized in the 1950s, evidence for a significant biological function for this protein remains inadequate except for the bacteriostatic and immune functions described below.

The many effects of Lf have been recently related to host defense mechanisms. Lactoferrin is associated with the activation of natural killer cells (Shau *et al.*, 1992), induction of colony-stimulating activity (Sawatzki, Rich, 1989), activation of leukocytes and monocytes (Gahr *et al.*, 1991), potentiation of macrophage cytotoxicity (Gahr *et al.*, 1991), maturation of splenic B cells (Zimecki *et al.*, 1995), among other functions (Baveye *et al.*, 1999).

While the bacteriostatic capacity of Lf stemming from its sequestering of iron is established, additional roles for lactoferrin have been described that appear to be independent of iron. Lactoferrin has been shown, in some cell systems, to stimulate growth of the cells (Nichols *et al.*, 1990). However, growth stimulation reports are positive (Azuma *et al.*, 1989), some demonstrating inhibition of growth (Rejman *et al.*, 1992), and still others with no effect (Amouric *et al.*, 1984). In support of a cellular function, Lf receptors have been reported in intestinal brush border membranes (Gislason *et al.*, 1993; Kawakami, Lonnerdal, 1991) with reported Kd of around 1–3 μM . Monocytes also have been shown to bind Lf with higher affinities (10^{-9}M) (Birgens *et al.*, 1984) and other leukocytes with lower affinity (Campbell, 1982).

2. REGULATION OF LACTOFERRIN AND IGFBP SYNTHESIS

The regulation of lactoferrin is tissue specific. While Lf synthesis is stimulated by 17 β -estradiol in uterine tissue at the transcription level, the mammary gland is unaffected by this hormone (Lonnerdal, Iyer, 1995). On the other hand, mouse mammary tissue Lf has been shown to be responsive to prolactin (Green, Pastewka, 1978). Interestingly, the expression of Lf is decreased or absent in most breast cancer cell lines compared to normal cells and tissue (Campbell *et al.*, 1992). Recent studies by Close *et al.* (1997) indicate that Lf expression is partially mediated by changes in cell shape and cytoskeleton.

Figure 1 demonstrates that the appearance of Lf in media from primary culture of bovine mammary cells (MEBo cells) takes more than four days when cultured

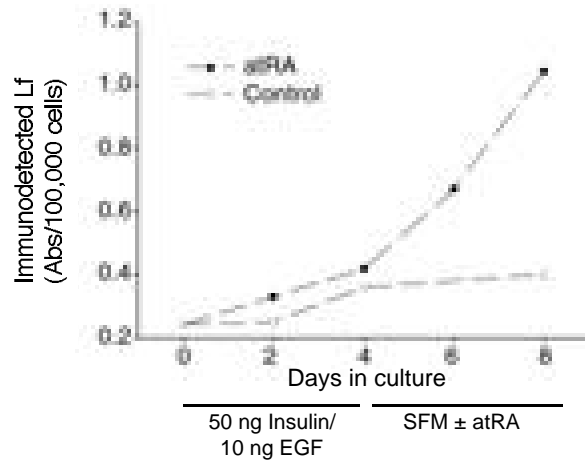


Figure 1. All trans retinoic acid (atRA) stimulates the appearance of Lf in primary cultures of MEBo cells. Cells were cultured in DME/F12 on plastic for 4 days with insulin and epidermal growth factor (EGF) to stimulate cellular growth. Culture for days 4 to 8 was conducted in serum free media (SFM) \pm 1 μM atRA. Lf was detected in Western blots with a bLf antibody donated by Dr. Floyd Schanbacher, Ohio State University — *Tous les acides rétinoïques trans (atRA) stimulent l'apparition de Lf dans les cultures primaires de cellules MEBo. Les cellules étaient cultivées sur plastique en présence de DME/F12 pendant 4 jours ainsi que d'insuline et du facteur de croissance épidermique (EGF) afin de stimuler la croissance cellulaire. La culture cellulaire allant de 4 à 8 jours était réalisée sur un milieu sans sérum (SFM) avec \pm 1 μM d'atRA. La Lf était détectée par Western blots avec un anticorps dirigé contre la Lf bovine et fourni par le Dr Floyd Schanbacher, Ohio State University.*

on plastic and is stimulated by the application of all trans retinoic acid (atRA). The time element of Lf expression is likely related to cytoskeleton interactions (Close *et al.*, 1997) as primary cells lay down an extracellular matrix with time in culture. The atRA response supports the report of a retinoic acid response element (RARE) in the 5' flanking region of the lactoferrin gene (Lee *et al.*, 1998).

Additionally, **figure 2** shows that the application of atRA to growth stimulated MEBo cells also results in a growth inhibition response reported for many cell cultures (Fontana *et al.*, 1991) and the bovine mammary cell line MAC-T cells (Woodward *et al.*, 1996). Furthermore, atRA also is known to stimulate the synthesis and secretion of IGFBP-3 (Adamo *et al.*, 1992) and this is also demonstrable with MEBo cells (Baumrucker *et al.*, 1999). The effects of atRA (cell growth inhibition; changes in levels of Lf and IGFBPs) on mammary cells (Adamo *et al.*, 1992; Gucev *et al.*, 1996; Liu *et al.*, 1997) appear to be associated. Our information presented herein provides direct evidence of a linkage between retinoic acid, Lf, IGFBP-3 and associates these changes with cell mechanisms for reduced growth or increased apoptosis.

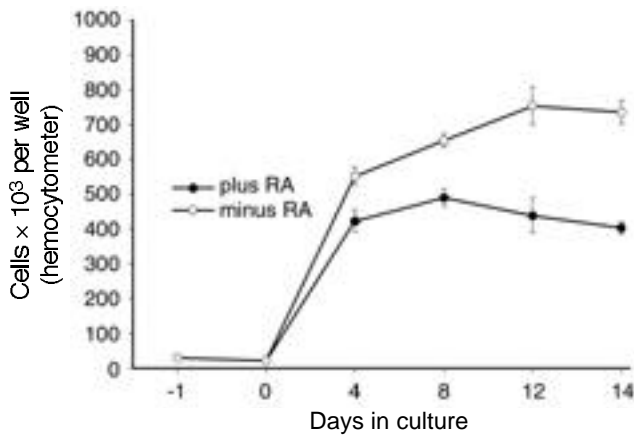


Figure 2. All trans retinoic acids (atRA) inhibit mammary cell proliferation. MEBo cells were grown on plastic and stimulated to grow with 50 ng/ml insulin and 10 ng/ml of EGF. Treatment with atRA (1 μ M) started on day 0 of culture — *Tous les acides rétinoïques trans (atRA) inhibent la prolifération des cellules mammaires. Les cellules MEBo croissent sur plastique et sont stimulées dans leur croissance par 50 ng/ml d'insuline et 10 ng/ml d'EGF. Le traitement au moyen d'atRA (1 μ M) débutait au jour 0 de la culture.*

3. CORRELATION BETWEEN IGFBP-3 AND LACTOFERRIN

Some interesting physiological connections have been reported between IGFBP-3 and Lf. For example, fluctuation in the levels of Lf in the bovine lactation cycle corresponds well to that of IGFBP-3 (Campbell *et al.*, 1991). During involution and prepartum periods, IGFBP-3 (Vega *et al.*, 1991) and Lf (Talhok *et al.*, 1990) are high in expression and high concentrations appear in mammary secretions. During involution, Lf concentration in the secretion of dairy cows is enormously variable and can reach extremely high concentrations (~10% solution of Lf) in some cows (**Figure 3**). Furthermore, in certain pathological conditions such as inflammation, an increased secretion of IGFBP-3 (Schuster *et al.*, 1995) accompanies the elevated secretion of Lf that is independent of the status of the epithelial tight junctions. **Figure 4** shows this timing relationship. While the uninfected lactating mammary gland has low levels of BSA, Lf and IGFBP-3 in milk, an *Escherichia coli* induced inflammation response shows the increase in serum BSA appearing in milk within 20 hours of treatment, indicative of the relaxation of epithelial tight junctions. This response is reversed within 60 hours and the milk concentration of BSA returns to the concentrations that existed prior to infection. Interestingly, bIGFBP-3 increases in milk with a pattern similar to BSA. IGFBP-3 is found in blood and extracellular fluids at concentrations greater than that of

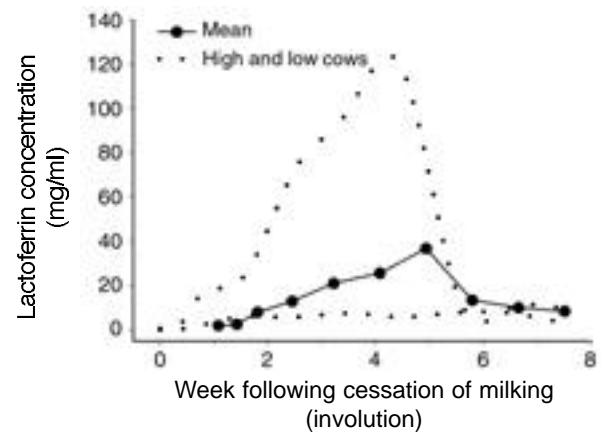


Figure 3. Variation of the concentrations of lactoferrin in the secretions of dairy cows during involution. Data are plotted from information obtained from Welty *et al.* (1976) — *Variation des concentrations de lactoferrine dans les sécrétions des vaches laitières pendant l'involution. Les résultats sont reproduits à partir d'informations obtenues de Welty et al. (1976).*

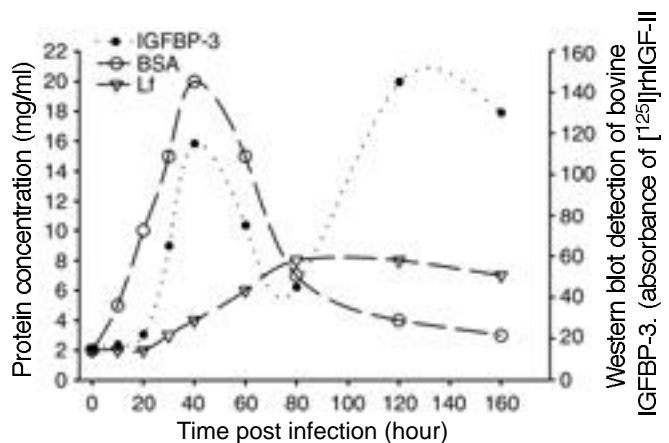
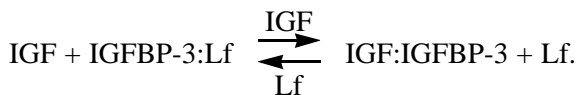


Figure 4. Appearance of BSA, Lf and IGFBP-3 in the secretions of dairy cows after an *E. coli* induced inflammation. Data are replotted from Harman *et al.* (1976) and Schuster *et al.* (1995) — *Apparition de sérum albumine bovine (BSA), lactoferrine (Lf) et d'IGFBP-3 dans les sécrétions de vaches laitières après induction d'une inflammation par E. coli. Les résultats sont reproduits à partir d'informations obtenues de Harman et al. (1976) et Schuster et al. (1995).*

milk (Gibson *et al.*, 1999). However after the tight junctions reform (> 60 h), IGFBP-3 continues to appear in milk at elevated concentrations. Lactoferrin, on the other hand, is low in blood and extracellular fluids and therefore does not show increased concentrations during relaxation of the tight junctions. However, after the tight junction recovery period, similar to IGFBP-3, Lf concentrations become elevated in milk for extended periods.

4. AN IGFBP-3 BINDING PROTEIN IN MAMMARY CELL MEMBRANES

At the 1998 Endocrine Meetings (Gibson *et al.*, 1998), we reported that [¹²⁵I] recombinant human (rh)IGFBP-3 bound to a glycosylated ~60 to 70 kDa membrane protein from primary cultures of bovine mammary cells. A report by Cohen (1998) recently suggested transferrin as a IGFBP-3 “partner protein” prompting us to consider the > 60 kDa glycosylated protein in mammary membranes to be lactoferrin. We have recently positively identified this membrane protein as lactoferrin by immunoprecipitation (Baumrucker *et al.*, 1999). Additionally, we have shown that [¹²⁵I] rhIGFBP-3 binds to Lf regardless of its source (Sigma vs. Fluka) or animal origin (human vs. bovine). It appears that iron-containing holo-Lf binds more [¹²⁵I] rhIGFBP-3 compared to apop-Lf and transferrin lacks the ability to bind [¹²⁵I]-rhIGFBP-3. The relationship of Lf to transferrin is not well understood (Baker *et al.*, 1998). While the two proteins share some functional roles, there are very distinct differences with specific roles ascribed to one or the other protein. Our finding that binding to IGFBP-3 is restricted to Lf but not to transferrin supports the protein differences and suggests a specific role of Lf



To demonstrate more aspects of [¹²⁵I] rhIGFBP-3 binding to Lf, we used a modified dot Western blot. Lactoferrin (20 µg) was spotted onto the membrane and [¹²⁵I]rhIGFBP-3 or [¹²⁵I]rhIGF-II was allowed to bind in the absence or presence of unlabeled rhIGFBP-3 or rhIGF-II. While [¹²⁵I]rhIGF-II did not bind to Lf, [¹²⁵I]rhIGFBP-3 did bind bLf. Binding specificity was demonstrated by the addition of unlabeled rhIGFBP-3 that blocked tracer binding. The application of rhIGF-II was shown to decrease [¹²⁵I]rhIGFBP-3 binding, presumably by binding to IGFBP-3 and preventing formation of the Lf:IGFBP-3 complex. The finding that IGF-II interacts with the IGFBP-3:Lf binding was an important observation.

To confirm that this is a competitive interaction, we again utilized a Western dot blot. Data indicated that Lf competes or displaces [¹²⁵I]IGFs for binding to nitrocellulose-bound IGFBP-3. We have shown that Lf competes with IGF-II:IGFBP-3 binding with an ED50 of approximately 3 µg/ml and displaces bound IGF-II from the IGF:IGFBP-3 binding with an ED50 of approximately 1 mg/ml. This is well within the range of Lf concentrations that exist within mammary tissue in various physiological states (Neville *et al.*, 1998). We propose that Lf interacts with IGFBP-3 and IGF-II

in the manner shown in the equation above and we also assume that such an interaction would also be shown for IGF-I.

Thus, the synthesis and secretion of mammary cell Lf will likely have a role in the IGF system and ultimately, cell growth and survival. We hypothesize that the enhanced production of Lf during the immediate *pre-partum* period and during involution (Nuijens *et al.*, 1996) will free IGFs (both IGF-I and IGF-II) from their association with IGFBP-3. This mechanism could provide an increased stimulation from type 1 IGF receptor and lead to increased cell growth and decreased apoptosis.

5. APPLICATION OF LF TO PRIMARY CULTURES OF MEB0 CELLS STIMULATES INCREASED CELL NUMBERS

Reports of Lf stimulatory effect upon epithelial cell growth are not clear with both positive and negative reports. **Figure 5** shows data that the application of holo bovine Lf (bLf) to a bovine mammary cell (MEBo) culture increases cell numbers compared to controls, while transferrin has no growth promoting effects. The application of insulin/EGF demonstrates a high level of growth promotion. We hypothesize that this Lf effect is one related to the competitive

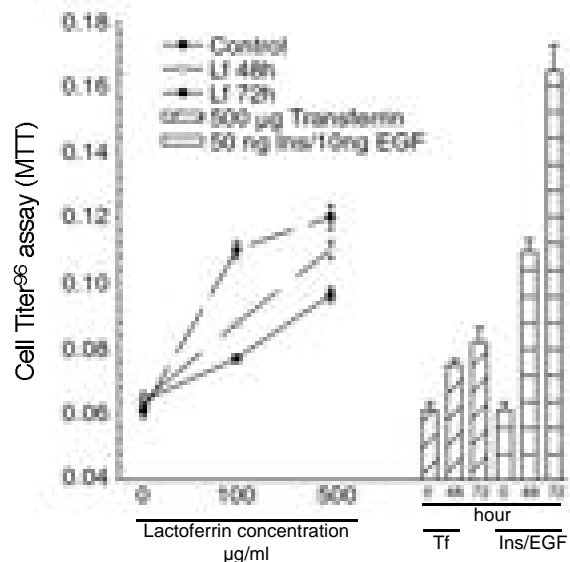


Figure 5. Lactoferrin stimulates the growth of MEBo cells. Cells were grown on plastic for 72 hours and were treated with Lf (0, 100 and 500 µg/ml), 500 µg/ml of transferrin, or 50 ng/ml of insulin and 10 ng/ml of EGF — *La lactoferrine stimule la croissance des cellules MEBo. Les cellules MEBo croissaient sur plastique pendant 72 heures et étaient mises en présence de Lf (0, 100 et 500 µg/ml), 500 µg/ml de transferrine ou 50 ng/ml d’insuline et 10 ng/ml d’EGF.*

interaction of the Lf/IGFBP-3/IGF diagramed above. MEBo cells synthesize and secrete both IGFBP-3 (Gibson *et al.*, 1999) and IGF-II (Baumrucker *et al.*, 1993). The application of Lf to the cultures would be expected to cause a competitive release of IGF-II from the IGFBP-3:IGF-II complex and allow for IGF-II stimulation of increased cell number *via* the mammary cell type I IGF receptor. However, this mechanism of action would be dependent upon the mass of IGF and IGFBP-3 that any cell secretes or has present in its environment. Because this is likely inconsistent, the variation of results concerning Lf growth promoting effects discussed earlier may be explained.

6. NUCLEAR APPEARANCE OF IGFBP-3:LF COMPLEXES

In recent studies with MCF-7 cells, we have shown that these breast cancer cells translocated extracellular IGFBP-3 into the nucleus that is dependent upon the presence of Lf and treatment with atRA (Baumrucker *et al.*, 1999). We utilized Texas Red (TR)-labeled rhIGFBP-3 and fluorescein (FITC)-labeled -Lf in studies with MEBo cells. Cells were cultured for 8 days in SFM \pm atRA and at the initiation of uptake studies, the culture media was changed to fresh SFM with the addition of the labeled proteins for 2 hours. Cells were then fixed, stained with DAPI (nuclear stain) and photomicrographs taken with an inverted fluorescent microscope.

Cells cultured without atRA(control) showed light traces of FITC-bLf in the cytoplasm of cells when FITC-bLf was applied. The control cells also show a small amount of TR-IGFBP-3 appearance in the nucleus when IGFBP-3 was applied. However when both FITC-Lf and TR-IGFBP-3 were added together under control conditions, a much stronger signal was observed in the cytoplasm of the cells. Some nuclear occurrence of both Lf and IGFBP-3 could also be observed

Differences in cellular uptake of Lf and IGFBP-3 when the cells are cultured in the presence of atRA were also observed. Cells take up the FITC-labeled Lf and the location is clearly more nuclear when treated with atRA. The uptake of TR-IGFBP-3 in atRA treated cells appears to be similar to that of the control cells with only some cellular nuclei showing the presence of the protein. However, when both FITC-Lf and TR-IGFBP-3 were added to atRA treated cells, a much stronger and simultaneous signal was observed in the nucleus of the treated cells. This was in contrast to the controls that showed strong cytoplasmic signal, but only minor nuclear signal.

The photomicrographs described above indicate that the Lf and IGFBP-3, which we have shown to form a complex, are cooperative in the capacity to be

taken up into bovine mammary epithelial cells. Additionally, atRA treatment causes changes in the distribution of these proteins that may or may not be linked to cellular mechanisms of action. Obviously, these descriptions demonstrate coincidental events and do not demonstrate specific mechanisms of action. However, we believe that these events may, at least partially, explain a mechanistic action of atRA upon nuclear IGFBP-3 and Lf in bovine mammary cells.

7. RETINOIC ACID/VITAMIN A IMPACTS IN DAIRY COWS

While we have shown a connection between atRA, bLf and IGFBP-3, little is known of potential *in vivo* effects of such interactions. All trans retinoic acid is part of the retinoic acid family of steroid endocrine factors. Both carotenes and vitamin A are dietary components that are metabolized to active retinoid components. Vitamin A and its derivatives have many functions including immune alterations, vision impacts, reproductive effects and epithelial cell maintenance (Weis, 1998). While supplementation of vitamin A or β -carotene has been prescribed during the dry period in order to increase colostrum vitamin A for the sake of transfer to the newborn, other impacts upon mammary function have been explored. While the mammary immune boost capacity of vitamin A during the dry period is controversial, the study of Oldham *et al.* (1991) documented an increase in milk production that was not related to differences in mammary gland health. These authors concluded that while milk production was not a major objective of their study, the surprising results warrant additional research. A later vitamin A/ β -carotene study with lactating cows (Michal *et al.*, 1994) did not support an overall increase in milk production; however, the high vitamin A treatment did result in greater increases when considering the initial week of production. The question of retinoid impact upon dairy cow lactational production is currently unknown, but with the evidence that we present herein, a possible mechanism could be acting via Lf and the IGF system.

The proposed model shown in **figure 6** is hypothesized from our findings with primary cultures of bovine mammary epithelial cells. Retinoids bind to nuclear retinoic acid receptors and one of their known actions is to stimulate the synthesis and secretion of IGFBP-3. Our data also shows that the appearance of Lf in conditioned media is also increased by atRA treatment. Extracellular IGFBP-3 is therefore available for binding to IGF originating from autocrine, paracrine or endocrine sources, and therefore indirectly has the potential to alter the binding of IGF to the type 1 receptor. When Lf is present in the extracellular domain it binds IGFBP-3,

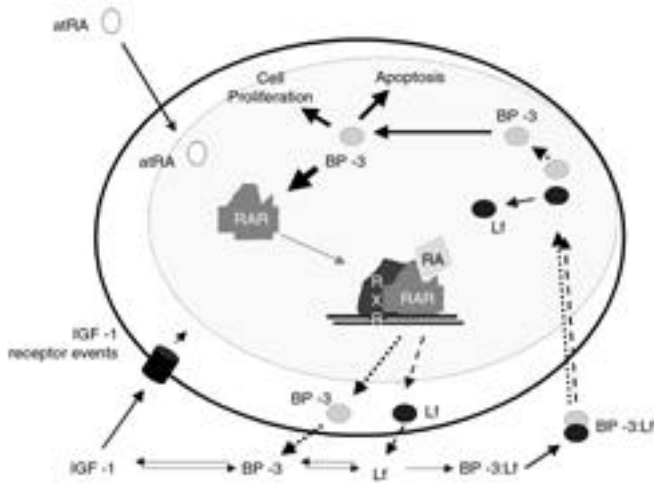


Figure 6. Hypothesized model of the mechanism linking retinoic acid, lactoferrin and IGFBP-3 in the growth regulation of bovine mammary epithelial cells — *Proposition du modèle action unissant l'acide rétinoïque, la lactoferrine et l'IGFBP-3 dans la régulation de la croissance des cellules épithéliales mammaires bovines.*

aRA = all trans retinoic acids — *tous les acides rétinoïques trans*
 RAR = retinoic acid receptor (α or β) — *récepteur de l'acide rétinoïque (α ou β)*

RXR = retinoic acid receptor (activated exclusively by 9-cis-retinoic acid) — *récepteur de l'acide rétinoïque activé uniquement par 9-cis-acide rétinoïque.*

RA = retinoic acid associated with the RAR:RXR heterodimer complex — *acide rétinoïque associé au complexe hétérodimère RAR:RXR*

BP-3 = IGFBP-3

Lf = lactoferrin — *lactoferrine*

causing the dissociation of IGF and results in an IGFBP-3:Lf complex.

We envision two modes of mechanistic action for this complex. The IGFBP-3:Lf complex enters the cell (**Figure 6**) and nucleus, and together or dissociated, the protein(s) have impact upon the cell cycle (inhibition of cell growth) or alteration of apoptotic pathways. Recently we have shown that the retinoic acid receptors (α & β) are co-immunoprecipitated when anti-bovine IGFBP-3 is applied to nuclear extracts of aTRA treated MCF-7 cells (data not shown). This finding of an nuclear IGFBP-3:RAR and IGFBP-3:RXR association is supported by a report of a direct functional interaction between IGFBP-3 and retinoid receptors (Liu *et al.*, 1999). In this report, IGFBP-3 is reported to bind to the RXR receptors in F9 embryonic carcinoma cell line as well as RARE transfected COS7 cells and leads to modulation of the transcriptional activity of RXR favoring homodimerization over heterodimerization, the latter being the typical signaling mechanism in mammary epithelial cells (Shang *et al.*, 1998; Shang *et al.*, 1999a; Shang *et al.*, 1999b).

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