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Insulin-like growth factor binding proteins initiate cell death and extracellular matrix remodeling in the mammary gland

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Abstract

We have demonstrated that insulin-like growth factor binding protein-5 (IGFBP-5) production by mammary epithelial cells increases dramatically during forced involution of the mammary gland in rats, mice and pigs. We proposed that growth hormone (GH) increases the survival factor IGF-I, whilst prolactin (PRL) enhances the effects of GH by decreasing the concentration of IGFBP-5, which would otherwise inhibit the actions of IGFs. To demonstrate a causal relationship between IGFBP-5 and cell death, we created transgenic mice expressing IGFBP-5, specifically, in the mammary gland. DNA content in the mammary glands of transgenic mice was decreased as early as day 10 of pregnancy. Mammary cell number and milk synthesis were both decreased by approximately 50% during the first 10 days of lactation. The concentrations of the pro-apoptotic molecule caspase-3 was increased in transgenic animals whilst the concentrations of two pro-survival molecules Bcl-2 and Bcl-x were both decreased. In order to examine whether IGFBP-5 acts by inhibiting the survival effect of IGF-I, we examined IGF receptor- and Akt-phoshorylation and showed that both were inhibited. These studies also indicated that the effects of IGFBP-5 could be mediated in part by IGF-independent effects involving potential interactions with components of the extracellular matrix involved in tissue

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remodeling, such as components of the plasminogen system, and the matrix metallo-proteinases (MMPs). Mammary development was normalised in transgenic mice by R3-IGF-I, an analogue of IGF-I which binds weakly to IGFBPs, although milk production was only partially restored. In contrast, treatment with prolactin was able to inhibit early involutionary processes in normal mice but was unable to prevent this in mice over-expressing IGFBP-5, although it was able to inhibit activation of MMPs. Thus, IGFBP-5 can simultaneously inhibit IGF action and activate the plasminogen system thereby coordinating cell death and tissue remodeling processes. The ability to separate these properties, using mutant IGFBPs, is currently under investigation.

Keywords: Insulin-like growth factor binding protein; IGFBP-5; Mammary gland; Apoptosis; Transgenic; Extracellular matrix; Plasmin

1. Introduction

Cell death is a major determinant of the decline in milk yield in ruminants and a better understanding of this process clearly offers the opportunity to achieve sustained levels of milk production over a prolonged lactation. This concept of extended lactation has been examined in numerous recent studies and is considered to be an economically viable alternative to the annual lactation cycle, which is widely used. Bovine mammary gland involution is a key remodeling transformation, which occurs both during and at the end (drying off period) of lactation and is characterised by a loss of mammary epithelial cells (more moderate than the intensive loss of cells which occurs during forced involution which has been extensively studied in the rodent mammary gland). This process is accompanied by extensive proteolytic degradation of the extracelluar matrix [1–4]. During this process, cell removal occurs in the absence of inflammation through the process of programmed cell death or apoptosis [2–5]. There are several hallmarks of the apoptotic process including caspase activation and one of the final stages, DNA fragmentation [1].

In dairy cows, genetic selection has dramatically increased two interrelated factors, peak yield and lactation persistency. Peak yield is determined in part by the number of secretory cells and in part by the secretory activity per cell. Studies in goats [6] have revealed that parenchymal cell numbers increase during pregnancy and into early lactation, after which cell loss is largely responsible for the decline in milk yield, whilst activity per cell is maintained [7].

2. Prolactin (PRL) and GH are major influences on cell death in the mammary gland

The role of PRL on epithelial cell survival has been clearly demonstrated in rodents, whilst GH seems to have a lesser effect [8]. Reduction in serum prolactin concentrations resulted in a decrease of milk yield and a loss of around 20–25% of the secretory cell population of the mammary gland within 48 h [9]. Conversely, prolactin treatment following litter removal delayed mouse mammary apoptosis [10], whilst PRL suppression by bromocriptine during lactation also rapidly induced DNA laddering and cell loss, even in the absence

of milk stasis [11,12]. Although GH plays a less significant role in rodents, induction of mammary apoptosis by anti-serum to GH in lactating rats [13] demonstrate that it does play a supporting role [12]. Thus, it has been proposed that (GH) and prolactin play an interactive role in maintaining mammary gland function and cell survival in rodents [11,14–17] although the precise mechanism of this interaction was unclear until recent observations provided such a link. In contrast to rodents, GH appears to play the major galactopoietic role in ruminants. Serum GH concentrations are elevated in the dairy cow during the first 2–3 month post-partum when peak milk yield occurs and decline during late lactation [18]. Numerous studies have shown that administration of GH induces an increase in milk yield of 10–40% of normal, confirming that GH clearly plays an important role [14,15]. In cow and goat, GH seems to maintain the number of mammary cells [19] stimulating proliferation [20] rather than limiting cell death [21,22]. While PRL seems to act directly on mammary tissue, the effects of GH are considered to be indirect, mediated through stimulation of IGF-I production [9].

3. GH/IGF-I axis as a cell survival system

IGF-I is predominantly synthesised by liver, but it is now recognised that in many other tissues it is produced and acts locally, including the mammary stroma. GH treatment increases serum concentrations of IGF-I and activates the expression of both hepatic and mammary stromal IGF-I mRNA [23,24]. Thus, GH could represent a survival factor for mammary epithelial cells [7,18]. Considerable data exists to support a role for IGF-I in the regulation of mammary cell survival both in vitro and in vivo. It has a stimulatory effect on DNA synthesis and mammary cell proliferation (fibroblast and epithelial cell), casein gene expression and glucose transport [23,25–27]. In addition, IGF-I can act as a survival factor, inhibiting apoptosis induced in cultured cells. IGF-I has been shown to promote mammary epithelial cell survival in vitro in primary cultures [28] and in vivo IGF-I or IGF-II have been shown to inhibit mammary cell death in transgenic mouse models [29–31].

4. IGF-binding proteins (IGFBPs) influence IGF actions

The actions of IGF-I are modulated by a family of six proteins, the IGF-binding proteins or IGFBPs [32]. Their actions are complex, depending upon the tissue, species and concentrations of the IGFBP, and in the case of IGFBP-5, its ability to interact with proteins in the extracellular matrix (ECM), such as heparan sulphate proteoglycans, collagens, fibronectin and laminin. IGFBPs can inhibit or augment the actions of IGF-I and, in addition, have IGF-independent effects, particularly in relation to apoptosis. There is evidence that a number of IGF binding proteins, as well as IGFs themselves, are synthesised by the mammary epithelial cells. During the process of lactational involution there is a 4-fold increase in IGFBP-2 mRNA and dramatic 6- and 50-fold increases in the expression of IGFBP-4 and IGFBP-5 message, respectively, along with a significant increase in IGFBP-5 protein, all within 48 h after weaning in rats [13,33,34] and mice [35]. When the mammary gland ceases to produce milk it undergoes massive apoptosis resulting in a return of the gland to a rudimentary state.

We showed that the mammary epithelium secretes increased concentrations of IGFBP-5 at this time, which we proposed served to inhibit IGF-mediated cell survival. Furthermore, we provided the first mechanistic explanation for the interactive effects of GH and PRL, which act in concert to support mammary gland development through a process involving stimulation of IGF-I synthesis by GH and inhibition of IGFBP-5 production by PRL [36]. In addition, two recent studies have shown that part of the effect of prolactin may be to increase IGF-II production in the mammary gland [37,38].

IGFBP-5 expression is also increased in the involuting thyroid [39], in ovarian follicles undergoing atresia [40] and in regressing prostate after androgen withdrawal [41]. We thus proposed that the secretion of IGFBP-5 is involved in the initial stages of apoptosis in many tissues by inhibiting IGF-I-mediated cell survival [13]. We subsequently showed that this increase in IGFBP-5 during mammary involution also occurs in mice during forced involution and in pigs following weaning at day 21 of lactation (our unpublished observations). In addition, we have demonstrated an increase in an unidentified IGFBP in the milk of sheep 48 h after weaning of lambs. Sejrsen et al. [42] also described increases in both IGFBP-4 and IGFBP-5 during the drying off period in cattle. A role for IGFBP-5 in bovine mammary apoptosis was also evident from in vitro studies of mammosphere cultures [42]. Around day 3 of culture, there is increased DNA laddering and DNA fragmentation in cells within the core of the mammosphere, which die in order to allow lumen formation and vectorial secretion of milk proteins. This event was preceded by an increase in IGFBP-5 secretion into the medium [43]. In a second study involving explants of bovine mammary tissue, we cultured explants in the presence of cortisol, 17β-estradiol, progesterone, insulin, GH, PRL and IGF-I. We omitted PRL, GH and IGF-I from the cultures and determined any subsequent changes in both the levels of apoptosis by evaluation of DNA laddering and IGFBP-5 message levels by RT-PCR. The results showed a high level of DNA laddering and an increase in IGFBP-5 mRNA content in mammary explants cultured under these conditions [44]. This offers further evidence of a close and direct correlation between levels of apoptosis and IGFBP-5 mRNA expression and confirms the importance of this binding protein in the initiation of programmed cell death in bovine mammary tissue.

5. IGFs and cancer: a causal relationship

IGF-I is a survival factor for many cell types and could, thus, be a determining factor in tumour growth, when cell death programmes are overridden. The hypothesis that IGF-I is a tumour promoter has been proposed as a result of several epidemiological studies, which have demonstrated that individuals with high plasma IGF-I concentrations have an increased risk of developing cancers of the mammary gland, intestine and prostate gland [45–48]. This association between IGFs and cancer risk is even stronger when corrected for concentrations of circulating IGFBPs. Thus, cancer risk is greatest for individuals with high IGF-I and low IGFBP-3 concentrations. Support for a role of IGFBPs as tumour suppressors also comes from demonstrations that agents which are effective in tumour therapy, anti-oestrogens, retinoids and Vitamin D analogues, stimulate IGFBP production by cells in vitro [49–51]. Furthermore, phosphatase and tensin homologue (PTEN), the second most commonly mutated tumour suppressor, when expressed as a transgene in the mammary

gland, leads to increased apoptosis and a 25-fold increase in IGFBP-5 [52]. Thus, PTEN as a phosphatidyl-inositol trisphosphate (PIP-3) phosphatase inhibits intracellular signalling via the IGF/PI3-kinase/Akt pathway and inhibits extracellular IGF signalling indirectly by increasing IGFBP-5 secretion. This combination of epidemiological, clinical and basic scientific data has led to the suggestion that IGFBPs could be used to inhibit tumour cell proliferation.

6. IGFBP-5 is a causal factor in cell death

The issue of a causal relationship between IGFBP-5 synthesis and cell death in the mammary gland has not been possible in ruminants because of the lack of sufficient quantities of the protein or the routine application of transgenesis, and thus, not surprisingly, the demonstration of causality between IGFBP-5 and cell death has been exclusively achieved in rodent models. Several studies have examined the effects of exogenous IGFBP-5, including in vitro and in vivo approaches, and we have also developed a transgenic model examining the effects of over-expression of IGFBP-5 using a mammary-specific promoter. Streuli and co-workers [53] have demonstrated that addition of exogenous IGFBP-5 or IGFBP-3 to mammary epithelial cells suppresses IGF-I-mediated survival, resulting in three-fold greater apoptosis in cells compared to the rates seen with IGF-I alone, involving decreased phosphorylation of Akt (a downstream signalling molecule for IGF-I) and the forkhead transcription factor, FKHRL1. Recent studies using human breast cancer cells have also revealed that IGFBP-5 can inhibit their growth both in vivo and in vitro [54]. Administration of IGFBP-5 during late pregnancy resulted in impaired development of mammary alveoli and reduced invasion of the mammary fat pad, indicative of increased rates of apoptosis [55]. Perhaps the most convincing evidence for this role of IGFBP-5 comes from our studies in transgenic mice expressing IGFBP-5 in the mammary gland, using a mammary-specific promoter, β-lactoglobulin (BLG) [56]. We found that the DNA content in the mammary glands of transgenic mice was decreased by day 10 of pregnancy and mammary cell number and milk synthesis were both decreased by approximately 50% during the first 10 days of lactation. Around parturition the concentrations of the pro-apoptotic molecule caspase-3 and of plasmin (an indicator of the breakdown of the ECM) were both increased, whereas the concentrations of two pro-survival molecules, Bcl-2 and Bcl-xL were significantly reduced. The bcl-2 proteins are a family of proteins involved in the response to apoptosis. Some of these proteins (such as bcl-2 and bcl-XL) are anti-apoptotic, while others (such as Bad or Bax) are pro-apoptotic. The pro-apoptotic bcl-2 proteins are often found in the cytosol where they act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria, where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome C and other pro-apoptotic molecules from the intermembrane space. This in turn leads to the formation of the apoptosome and the activation of caspases.

Furthermore, the effects of IGFBP-5 on DNA content could be completely prevented by treatment with R3-IGF-I, an analogue of IGF-I, which binds with very low affinity to IGFBP-5. Despite this, the decrease in milk yield was only partially prevented. All of these

findings are strongly consistent with a pro-apoptotic effect of IGFBP-5, which is mediated principally by inhibition of IGF actions.

7. ECM remodeling—a role for IGFBP-5

During mammary involution degradation of the ECM occurs via activation of plasminogen and the matrix-metallo proteinases. IGFBPs also interact with a variety of molecules in the ECM and these interactions may influence the actions of the binding proteins. Interactions of cells with the ECM forms a crucial regulatory system, which when disrupted (such as during activation of matrix-metallo proteinases, MMPs), can lead to increased migratory activity and potential development of metastases. Mutant IGFBPs, which are either unable to bind to the ECM or to IGFs, have recently been developed in our laboratory to investigate these interactions in relation to biological function [57–60]. We have demonstrated in IGFBP-5 transgenic mice that plasminogen is cleaved to plasmin during mammary gland development at a time when plasmin generation is normally suppressed [56]. Plasminogen activators, tissue-type plasminogen activator and urokinase plasminogen activator (tPA and uPA, respectively), generate plasmin whereas plasminogen activator inhibitor-1 (PAI-1), which inhibits the proteolytic activation of plasminogen to plasmin by both tPA and uPA. Previously, others have demonstrated that IGFBP-5 is able to bind directly to PAI-1 with high affinity [61], and we have recently demonstrated that IGFBP-5 can inhibit the actions of PAI-1 and thus could influence both cell survival and tissue remodeling processes (unpublished results).

In conclusion, mammary cell survival is reflected by a balance between the survival effects of IGF-I, in part driven by GH and the death-inducing effects of IGFBPs. Several species exhibit increases in IGFBP production in the mammary gland during the natural involutionary process that occurs at the end of lactation and we have demonstrated, using exogenous IGFBPs and by expressing IGFBP-5 as a transgene in the mammary gland that IGFBP-5 can indeed induce apoptotic cell death in mammary epithelial cells. Furthermore, we identified a novel mechanism by which IGFBP-5 activates plasmin generation and thus potentially initiates the proteolytic cascade, which results in remodeling of the extracellular matrix. This provides an intriguing mechanism whereby the dying epithelial cell produces a suicide protein, which serves to inhibit its two principal survival signals, growth factor and integrin-mediated events. Such a mechanism may provide important protection against the inappropriate disruption of cell anchorage (by activation of proteases) in the presence of sustained growth factor survival signals, an event which would favour metastatic processes.

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