Prolactin inhibits cell loss and decreases matrix metalloproteinase expression in the involuting mouse mammary gland but fails to prevent cell loss in the mammary glands of mice expressing IGFBP-5 as a mammary transgene

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Abstract

Insulin-like growth factor-binding protein 5 (IGFBP-5) mediates involution of the mammary gland. The decrease in DNA content and mammary gland weight which accompanies involution was inhibited by prolactin (PRL) in wild-type but not transgenic mice expressing IGFBP-5. Phospho-STAT5 protein levels were significantly lower in IGFBP-5 transgenic mice during lactation suggesting that IGFBP-5 antagonises PRL signalling in the mammary epithelium. In contrast, phospho-STAT3 levels increased during involution to a similar extent in both wild-type and transgenic mice and were unaffected by PRL. PRL inhibited gene expression of matrix metalloproteinases (MMPs) 3 and 12 but not tissue plasminogen activator or plasmin in wild-type and transgenic animals. The effects of PRL on MMPs appear to be indirect since PRL failed to inhibit MMP-3, -7 or -12 expression in HC-11 cells or in a co-transfection including an activated PRL receptor, STAT5 and a MMP-3-luciferase reporter gene. PRL is a potent inhibitor, both of cell death, an effect which is suppressed by IGFBP-5, and of MMP expression, which is independent of the actions of IGFBP-5.

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Introduction

We have previously shown that growth hormone (GH) and prolactin (PRL) interact in regulating milk synthesis in the mammary gland and that their effects can be explained, at least in part, by enhancing mammary epithelial cell survival (Travers *et al.* 1996). We showed a large increase in the concentration of IGFBP-5 protein in the milk of rats after 48 h of mammary involution induced by removal of the suckling young, and proposed that this acts to inhibit insulin-like growth factor I (IGF-I) interaction with its receptor on the epithelial cells, resulting in cell death. More recently we have shown that IGFBP-5 mRNA levels are also significantly increased during involution in the mouse mammary gland (Boutinaud *et al.* 2004). In rats, the increase in IGFBP-5 protein was inhibited by 90% if they received concurrent PRL treatment (Tonner *et al.* 1997). We therefore proposed that GH and PRL interact to suppress apoptosis in the rat mammary gland by a mechanism in which GH increases IGF-I synthesis while PRL suppresses the production of IGFBP-5 in the epithelial cells, which would otherwise inhibit IGF-mediated cell survival. Subsequently we generated transgenic mice expressing IGFBP-5 from a

mammary-specific promoter and showed that this led to increased rates of apoptosis and impaired mammary gland development (Tonner *et al.* 2002). Given that PRL is a major survival factor for the mammary epithelium, we wished to examine whether it could prevent the effects of IGFBP-5 on mammary epithelial cell survival. Whereas our previous studies indicated evidence for inhibition of IGF signalling by IGFBP-5 we wished to examine whether IGFBP-5 could also influence PRL signalling pathways and thereby antagonize PRL's survival capability. Mammary involution involves two major phases. During the initial phase of involution there are dramatic increases in the rates of apoptosis whereas during the second phase activation of extracellular proteases occurs, including the plasmin and matrix metalloproteinases (MMPs), which are involved in degrading the extracellular matrix during tissue remodelling. In addition, IGFBP-5 binds to plasminogen activator inhibitor-1 (PAI-1), suggesting that IGFBP-5 may also be involved (in an IGF-independent fashion) in the regulation of tissue remodelling via the plasminogen system (Nam *et al.* 1997). PRL is capable of inhibiting both of these phases of involution in rats (Tonner *et al.* 2000). Thus we initially examined whether involution of the mouse mammary gland could be inhibited by PRL

in mice, as it is in rats, and whether IGFBP-5 could, in turn, influence the responses to PRL. We therefore measured parameters associated with the early phase (IGF and PRL signalling) and the later phase (plasmin and MMPs).

The aims of this study were therefore to determine whether PRL could inhibit cell death and prevent increased expression of plasmin and MMPs in mouse mammary gland and to examine whether the effects of PRL are influenced by expression of IGFBP-5 as a transgene. Our results demonstrate that IGFBP-5 can inhibit the cell-survival effects of PRL and inhibit phosphorylation of signal transducer of transcription 5 (STAT5), a downstream signalling molecule of PRL. In contrast PRL also inhibits MMP expression in a fashion which appears to be both indirect and independent of IGFBP-5.

Materials and methods

Animals

All animal studies were conducted under appropriate licence from the UK Home Office after approval by local ethical review committees. Wild-type or IGFBP-5 transgenic lactating CBA × C57Bl/6 mice had their litters adjusted to eight in number at parturition. Between days 10 and 14 of lactation some females had their litters removed and were either left untreated for 2 (LR2) or 4 (LR4) days or were injected subcutaneously twice daily with ovine PRL (Sigma; product code L5620) in sterile PBS containing 0.01 M NaHCO₃ administered at a dose of 200 µg in 100 µl for 2 (LR2+) or 4 (LR4+) days. Animals were then killed by cervical dislocation and mammary glands removed, weighed and stored at -80 °C before analyses were undertaken. Tissue was also fixed in 4% paraformaldehyde for preparation of histological sections. The frozen tissue samples were reduced to powder by pulverizing the frozen tissue in liquid nitrogen with a mortar and pestle before subsequent analyses as indicated below. DNA content was determined by the method of Labarca & Paigen (1980). Three or four animals (as indicated in the figure legends) were used to derive samples for a single point of measurement.

DNA plasmids

The plasmid pBK-MMP3-luc was generated by PCR amplification of a 1.3 kb MMP-3 promoter region using the oligonucleotides MMP3-1 (5'-CAA GCA CAA CCC TTA TTC TCA CAC-3') and MMP3-2 (5'-TAG GTC CAC CTT CTT AAG CCC AAG-3') and subsequent insertion of this fragment into the plasmid pBK-bcas2-luc (Kolb 2002) from which the β -casein promoter region was removed. The expression plasmids

encoding an activated PRL receptor (Gourdou *et al.* 1996) and STAT5 were gifts from Jean Djiane (Unité d'Endocrinologie Moléculaire, INRA, Jouy-en-Josas, France) and Bernd Groner (Georg-Spayer-Hans, Institute for Biomedical Research, Frankfurt/Main, Germany), respectively.

RNA extraction and cDNA synthesis

Total RNA was extracted from the mammary gland samples as described previously (Boutinaud *et al.* 2004). For the HC-11 cells, 300 µl RNeasy lysis buffer was added to 1×10^6 cells after removal of growth medium. The cells were harvested and 60 µl chloroform was added to the homogenate and after a 10-min incubation at room temperature the mixture was centrifuged at 12 000 *g* for 15 min at 4 °C. The aqueous supernatant containing total RNA was separated and precipitated by addition of isopropyl alcohol and the RNA was pelleted by centrifugation (12 000 *g* for 10 min at 4 °C), rinsed with 70% alcohol and finally dissolved in sterile distilled water (Life Technologies, Paisley, UK). The amount of total RNA extracted from either mammary gland tissue or HC-11 cells was measured by absorbance at 260 nm.

Quantitative PCR

Quantitative PCR was carried out using a Roche LightCycler system and a FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Lewes, E. Sussex, UK) according to the manufacturer's instructions. PCR amplifications were done with a final primer concentration of 0.5 µM and final MgCl₂ concentration of 3 mM. Primer design was done using the Primer Select program of the DNA Star software suite. The sequences, annealing temperatures and amplicon sizes of the oligonucleotides used in this study are provided in Table 1. Primers for IGFBP-5, IGF-I, IGF-II, the type 1 IGF receptor (IGFR) and the housekeeping gene cyclophilin were described previously (Boutinaud *et al.* 2004). The PCR products obtained by quantitative PCR were evaluated by melting point analysis and agarose gel electrophoresis.

Thermal cycling was carried out using the following conditions: initial denaturation was done by incubation at 95 °C for 10 min; subsequently 40 or 50 cycles of amplification were done with 15 s at 95 °C, 5 s at the specific annealing temperature for each gene (Table 1) and 30 s at 72 °C. A single fluorescence acquisition point was specified for each gene at the end of each cycle. At the end of the PCR amplification the melting curve of the products was analysed by incubation at 95 °C for 0 s, 57 °C for 15 s and a linear temperature transition at 0.05 °C/s from 57 to 95 °C with continuous fluorescence acquisition.

Table 1 Oligonucleotides used for quantitative PCR

Name	Length	Sequence	Annealing temp. (°C)	Amplicon length (bp)
b-actin1	23mer	5'-GTC GAC AAC GGS TCC GSC ATG TG-3'	60	908
b-actin2	23mer	5'-CTG TCR GCR ATG CCW GGG TAC AT-3'		
cyclophilin-f	23mer	5'-CAT CCT AAA GCA TAC AGG TCC TG-3'		
cyclophilin-r	20mer	5'-TCC ATG GCT TCC ACA ATG TT-3'	58	165
bcas21	23mer	5'-GAT GCC CCT CCT TAA CTC TGA AA-3'	56	226
bcas22	22mer	5'-TTG TGG AAG GAA GGG TGC TAC T-3'		
MMP3-3	24mer	5'-ACC CAG TCT ACA AGT CCT CCA CAG-3'	56	463
MMP3-4	21mer	5'-GCA GCA TCG ATC TTC TTC ACG-3'		
MMP12-1	20mer	5'-CCC AGC ACA TTT CGC CTC TC-3'	54	494
MMP12-2	24mer	5'-AGC TCC TGC CTC ACA TCA TAC CTC-3'		
MMP7-1	24mer	5'-TAC CCT CAT GAC TCT AAA ACA AAG-3'	54	486
MMP7-1	20mer	5'-TGC GTC CTC ACC ATC AGT CC-3'		

Calibration curves for the MMP-3, MMP-7, MMP-12, β -casein, IGFBP-5, tissue plasminogen activator (tPA), IGF-I, IGF-II, IGFR type 1, cyclophilin A and actin genes were generated from serial dilutions of a specific plasmid cDNA for each gene. The resulting calibration curves demonstrated good efficiencies according to the calculation $E = 10^{(-1/\text{slope})}$ (from 1.71 to 1.90). Samples were quantified in duplicate between different experiments, and demonstrated little variation between runs. Crossing points for cDNA samples were then correlated with this standard curve yielding a DNA concentration. A non-template negative control was incorporated in all PCR runs. Expression of all genes was correlated either with cyclophilin A expression (on a molecule-per-molecule basis for RNA isolated from mouse tissues) or with β -actin expression (on a fg-per-pg basis for RNA isolated from HC-11 cells).

Western blotting

Mammary gland homogenates were prepared by homogenization for 60 s on ice in a buffer consisting of 0.15 M NaCl and 50 mM Tris/HCl, pH 7.4, containing 0.5 mM EDTA and EGTA, 1% IgePal CA-630, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM orthovanadate, 1 mM NaF and 1 μ g/ml protease inhibitor cocktail (Sigma P8340) containing AEBSF (4-(2-aminoethyl) benzenesulphonyl fluoride), aprotinin, leupeptin, bestatin, pepstatin A and E-64. Protein concentrations were determined using the Bradford assay and 5 μ g protein were loaded per track for electrophoresis. After transfer onto nitrocellulose, the blots were blocked with 50 mM Tris/HCl and 0.15 M NaCl, pH 7.4, containing 5% dried skimmed milk and 0.05% Tween 80. Total and phosphorylated forms of STAT3, STAT5 and protein kinase B/Akt were determined using antibodies from Cell Signalling Technology (STAT3 and phospho-STAT3) and Upstate Biotechnology (Akt-1, STAT5 and

phospho-STAT5) diluted 1:1000. Secondary antibodies were peroxidase-labelled anti-mouse or anti-rabbit antibodies, as appropriate (Amersham Biosciences), diluted 1:2000. Detection involved the enhanced chemiluminescence (ECL) detection system. The Akt and STAT3 proteins were validated by running positive control extracts an A431 Cell Lysate (Upstate Biotechnology catalogue no. 12-301 for Akt) and an extract of interferon- γ -induced HeLa cells (Cell Signalling Technology catalogue no. 9133) side by side with the mammary gland-derived samples. The expected molecular masses of 60 and 86 kDa for Akt and STAT3 were observed. Molecular-mass markers were also used to confirm the appropriate molecular size for STAT5 (94–95 kDa).

Plasmin assay

Mammary homogenates, prepared in 110 mM NaCl, 50 mM E-amino-*n*-caproic acid (EACA) and 50 mM Tris/HCl, pH 8.0, were centrifuged at 2000 *g* for 15 min and defatted. Plasmin activity was then measured by incubating 10 μ l samples in 50 mM Tris/HCl, pH 7.4, 110 mM NaCl and 2.5 mM EACA containing the artificial plasmin substrate 0.6 mM Val-L-Leu-L-Lys-*p*-nitroanilide (Sigma). Generation of nitroaniline was monitored by measuring absorbance at 405 nm at 15-min intervals for 2 h. The rate of change of absorbance was used to calculate the activity of plasmin. RIA of IGFBP-5 was performed as described previously (Tonner *et al.* 2002).

Cell culture

HC-11 and BHK-21 cells (ECACC ref. 85011433) were cultivated as described previously (Kolb 2002). Transfections of BHK cells were done by using calcium phosphate methodology as described previously (Kolb *et al.* 1999).

Figure 1 Mammary gland involution in response to PRL treatment in wild-type and IGFBP-5 transgenic mice. Mammary gland weight, DNA content and β -casein mRNA expression levels (molecules of β -casein mRNA per 10^{-3} molecules of cyclophilin A) were determined in lactating (L) mice and mice from which litters had been removed for 2 days (LR2) or 4 days (LR4). PRL treatment is indicated by +. Values are means $\pm$ S.E.M. from three animals per group. Data were subjected to ANOVA followed by Bonferroni's test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$ compared with lactating control; a, significantly different ($P < 0.05$) for comparisons of PRL treatment vs corresponding control; +, significantly different from wild-type.

Statistical analysis

The data were analysed in a one-way analysis of variance (ANOVA) using MINITAB followed by supplementary *t* tests using Bonferroni correction.

Results

Mammary gland histology, DNA content and casein expression

To elucidate the role of IGFBP-5 in mammary gland development and function, we have generated transgenic

mice expressing IGFBP-5 from the mammary gland-specific β -lactoglobulin promoter (Tonner *et al.* 2002). Mammary gland weight, DNA content and β -casein mRNA expression were significantly lower in IGFBP-5 transgenic mice, consistent with an inhibitory effect of IGFBP-5 on mammary development and milk secretion (Fig. 1). IGFBP-5 transgene expression also led to an increase of apoptosis markers including caspase 3, DNA laddering and a reduction in the expression of cell-survival markers from the Bcl-2 family (Tonner *et al.* 2002). This was further supported by a decreased pup weight gain in transgenic dams compared with wild-type controls (Tonner *et al.* 2002). In the present study,

involution for 2 days led to dramatic decreases in β -casein expression in both wild-type and transgenic animals, which continued up to day 4 until levels of β -casein expression were extremely low. PRL treatment resulted in a 4-fold increase in β -casein expression in wild-type animals on day 4 of involution but had no significant effect in the transgenic animals. Involution also led to decreases in both mammary gland weight and DNA content in both groups. Concurrent PRL treatment in wild-type animals led to an increase in mammary gland weight on day 2 of involution (mainly due to increased milk accumulation) and inhibited the weight loss on day 4 of involution. DNA content was also maintained by PRL on day 2 of involution and although it declined by day 4 of involution, it was still significantly increased compared with untreated animals. Once again, in transgenic animals there were no significant effects of PRL on these parameters. These changes were confirmed histologically since, after 4 days of involution, the mammary parenchyma had been largely replaced by adipocytes in both transgenic and wild-type animals and, whereas PRL was able to inhibit this process in wild-type animals, resulting in the presence of numerous milk-filled alveoli, it was completely without effect in the transgenic animals (Fig. 2).

Analysis of gene expression using quantitative PCR

IGFBP-5 expression was increased 100-fold in transgenic animals compared with wild-type animals during lactation (Fig. 3). Two days after involution was induced by litter removal (LR2) IGFBP-5 expression increased in wild-type animals but decreased in transgenic animals, although levels still remained higher in transgenic animals, presumably due to contributions from both the endogenous IGFBP-5 gene and the transgene. By day 4 of involution (LR4) IGFBP-5 levels had decreased in both groups. There were no significant effects of PRL treatment on IGFBP-5 mRNA expression in either wild-type or transgenic animals. These changes in gene expression were paralleled by changes in IGFBP-5 protein content in milk, as determined by RIA. In wild-type animals IGFBP-5 concentrations in milk were low in lactation (9 ± 7 ng/ml; mean $\pm$ S.E.M.) and increased by day 2 of involution (irrespective of PRL treatment) to 341 ± 57 ng/ml before declining to 224 ± 69 ng/ml on day 4 of involution. In IGFBP-5 transgenic animals IGFBP-5 concentrations in lactation were already elevated (419 ± 81 ng/ml), and increased further during day 2 of involution (again independently of PRL treatment) to 578 ± 74 ng/ml before declining on day 4 of involution to 145 ± 89 ng/ml. We have shown previously that involution in wild-type animals is accompanied by a dramatic decrease in IGF-I mRNA expression by 2 days with a recovery by day 4 and a

reciprocal change in IGFR, leading to a dramatic increase in its expression by day 4 of involution (Boutinaud *et al.* 2004). In this study we show that very similar changes occurred in IGFBP-5 transgenic mice (Fig. 3) and that, furthermore, these changes were independent of PRL treatment (results not shown). In contrast, IGF-II expression was not significantly changed by any of the treatments in either wild-type or transgenic animals (Fig. 3). Note that expression of IGF-II was approximately 100-fold lower than expression of IGF-I.

Analysis of signalling molecules

To look for interaction of PRL with IGF-signalling pathways, we measured phosphorylation of Akt, a target of phosphoinositide 3-kinase activity in the IGF-I signalling pathway. Levels of both Akt and phospho-Akt were high and similar in wild-type and transgenic animals during lactation, decreased dramatically by day 2 of involution and were unaffected by PRL treatment (Fig. 4). In contrast, when we examined changes in the PRL-signalling pathway we identified an effect of IGFBP-5. Concentrations of both total and phosphorylated STAT5 were high during lactation in wild-type animals whereas phospho-STAT5 but not total STAT5 was significantly reduced in lactating IGFBP-5 transgenic animals (Fig. 5). The fact that total STAT5 is almost undetectable during involution whereas phospho-Stat5 can be readily detected is due to the different sensitivities of the two antisera used. During involution there was a significant decline in both total and phosphorylated STAT5 such that levels were not significantly affected by the transgene or by PRL treatment. In contrast, levels of STAT3 were unchanged during the transition from lactation to involution but levels of phospho-STAT3 increased dramatically, independent of transgene expression or PRL treatment (Fig. 6).

Proteinase expression

Involution of the mammary gland is accompanied by activation of numerous proteases and we had shown previously that plasmin activity is increased in IGFBP-5 transgenic mice. Plasmin activity was increased significantly on day 2 of involution but was unaffected by PRL treatment in both wild-type and transgenic animals (Fig. 7). Expression of tPA, one of the two major activators of plasminogen, increased approximately 4-fold by day 2 of involution, remained elevated on day 4 in both wild-type and transgenic animals and was also largely unaffected by PRL treatment (Fig. 7).

The expression patterns of MMP-3, -7 and -12 were examined by quantitative PCR. MMP-3 and MMP-12 are the most highly expressed MMP genes during mammary gland involution (Stein *et al.* 2004, Sorrell

Figure 2 Histological changes in the mammary gland of wild-type and transgenic mice during involution. Tissues were isolated from mice at peak lactation (a, b), 4 days after litter removal (c, d) and 4 days after litter removal plus PRL treatment (e, f) in wild-type (a, c, e) and IGFBP-5 transgenic (b, d, f) mice, fixed in 4% para-formaldehyde and embedded in paraffin. Sections of 5–7 μm were cut and stained with haematoxylin and eosin. Images were acquired as TIFF files using a digital camera (Leica) on an inverted microscope (Leica). Scale bar, 100 μm . Note the transition from a predominantly epithelial morphology to a tissue largely composed of adipocytes by day 4 of involution and the ability of PRL to inhibit this in wild-type animals, as indicated by the presence of alveolar epithelial tissue containing accumulated milk, but not in transgenic animals.

Figure 3 IGFBP-5, IGF-I, IGFR and IGF-II mRNA expression levels during involution as measured by quantitative PCR. Expression values of IGFBP-5, IGF-I, IGFR and IGF-II are correlated with cyclophilin A expression (molecules per molecule of cyclophilin). Values are means $\pm$ S.E.M. from three animals. Data were subjected to ANOVA followed by Bonferroni's test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$ compared with lactating control; +, significantly different from wild-type.

et al. 2005) and are expressed in the stromal cells of mammary tissue (Lund *et al.* 1996). MMP-7 is reported to be expressed in the epithelial rather than the stromal compartment of the mammary gland (Wilson *et al.* 1995). We therefore wanted to assess whether expression of the IGFBP-5 transgene or PRL treatment would have different effects on the stromal and epithelial proteases. Steady-state mRNA levels for all three MMPs increased during mammary involution. MMP-3, although low during lactation, increased 30-fold by day 2 of involution and a further 5-fold by day 4 of involution in wild-type animals (i.e. a 150-fold increase with respect to lactation). Transgenic animals also expressed low levels of MMP-3 in lactation but showed a much more modest rise (2-fold) by day 2 of lactation although by day 4 of involution they also exhibited a further 27-fold increase in expression (a 55-fold increase with respect to lactation).

PRL was effective in markedly suppressing the increase in MMP-3 expression in both wild-type and transgenic animals (Fig. 8). Although expressed at lower absolute levels than MMP-3, changes in MMP-12 were qualitatively similar to MMP-3 and were also markedly suppressed by PRL treatment (Fig. 8). MMP-7 expression was extremely low but it also showed a 6–9-fold increase by day 2 of involution and remained elevated on day 4 of involution. However, MMP-7

expression was unaffected by PRL treatment in either wild-type or transgenic animals (Fig. 8).

To further examine the inhibition of MMP-3 and -12 expression by PRL, we utilized HC-11 cells, a murine mammary cell line (Ball *et al.* 1988). These cells can be induced to differentiate in the presence of a lactogenic hormonal mix of insulin, PRL and glucocorticoids. The differentiation can be monitored by measurement of β -casein mRNA expression, which increases between 1000- and 10 000-fold after addition of all three hormones (Kolb 2002). In our studies the effectiveness of PRL was demonstrated by a 30-fold increase in the expression of β -casein in cells given PRL, insulin and glucocorticoids compared with treatment with insulin and glucocorticoid alone (Fig. 9). These cells also expressed MMPs 3, 7 and 12 and, as for *in vivo* observations, the expression of MMP-3 was higher than MMP-12 which was in turn considerably higher than MMP-7. The induction of differentiation also involves the removal of epidermal growth factor (EGF) from the medium. Unexpectedly, we found that withdrawal of EGF alone (in the absence of PRL and glucocorticoids) led to a pronounced increase in the expression of the three MMPs, identifying EGF as a major repressor of MMP expression in HC-11 cells (Fig. 9). To our surprise PRL did not significantly inhibit expression of any of the MMPs whereas, in accordance with published data

Figure 4 Akt expression and phosphorylation during involution in wild-type and IGFBP-5 transgenic mice as determined by western blot analysis. Each lane, containing 5 μ g total protein, is derived from an individual animal. Bar charts represent quantitative analysis of Western blots using densitometry and are expressed in arbitrary units. Values are means $\pm$ S.E.M. from three or four animals per group. Data were subjected to ANOVA followed by Bonferroni's test for multiple comparisons. * $P < 0.05$ compared with lactating control. L, lactating; LR2, litter removed for 2 days; LR2+, litter removed for 2 days plus PRL.

(Lund *et al.* 1996), glucocorticoid potently inhibited expression of all three MMPs (Fig. 9).

To corroborate these results we tested the ability of the PRL signalling pathway to influence the activity of an MMP gene promoter by using a co-transfection assay in BHK cells. BHK cells were transfected with an expression vector encoding an activated form of the PRL receptor (Gourdou *et al.* 1996), an expression vector encoding murine STAT5 and a luciferase reporter construct under the transcriptional control of the murine MMP-3 promoter. BHK cells transfected with this mix of plasmids showed no significant activation of the MMP-3 promoter (as measured by cytoplasmic levels of luciferase activity). In contrast, when the PRL receptor and STAT5 expression vectors were co-transfected

with a reporter construct carrying the luciferase gene under control of the murine β -casein promoter, the reporter gene was activated 10–50-fold dependent on the amount of co-transfected plasmid (Fig. 10). This is in accordance with previous studies (Kolb 2002).

Discussion

We have previously shown a large increase in the production of IGFBP-5 by the mammary gland during involution and subsequently proposed that IGFBP-5 exerts an apoptotic effect by abrogating the survival effects of IGF-I (Tonner *et al.* 1997). This hypothesis requires that IGFBP-5 has an inhibitory rather than an

Figure 5 STAT5 expression and phosphorylation during involution in wild-type and IGFBP-5 transgenic mice as determined by Western blot analysis. Each lane, containing 5 µg total protein, is derived from an individual animal. Bar charts represent quantitative analysis of Western blots using densitometry and are expressed in arbitrary units. Values are means $\pm$ S.E.M. of four animals per group. Data were subjected to ANOVA followed by Bonferroni's test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$ compared with lactating control; +, significantly different from wild-type. W, wild-type; T, transgenic; L, lactating; LR2, litter removed for 2 days; LR2+, litter removed for 2 days plus PRL.

enhancing effect on IGF-I action (Jones *et al.* 1993) and we have shown this to be the case in three independent studies involving transgenic animal models expressing IGFBP-5 in the mammary gland (Tonner *et al.* 2002) and exogenous treatment with IGFBP-5 both *in vivo* (Allan *et al.* 2002) and *in vitro* (Marshman *et al.* 2003). One of the objectives of this study was to examine whether the expression of IGFBP-5 as a transgene would further accelerate the involution process, over and above that induced by the endogenous gene, the expression of

which increases post-lactation. In addition, we wished to examine whether IGFBP-5 would impair the ability of PRL to inhibit involution. Whereas we obtained no evidence of accelerated cell loss or tissue remodelling during involution of the mammary gland in IGFBP-5 transgenic animals, the ability of PRL to prevent cell loss from the mammary gland (as determined by DNA content, mammary gland weight and β -casein expression) was completely blocked in the transgenic animals, whereas PRL was clearly effective, in this respect, in

Figure 6 STAT3 expression and phosphorylation during involution in wild-type and IGFBP-5 transgenic mice as determined by Western blot analysis. Each lane, containing 5 µg total protein, is derived from an individual animal. Bar charts represent quantitative analysis of Western blots using densitometry and are expressed in arbitrary units. Values are means±S.E.M. from three animals per group. Data were subjected to ANOVA followed by Bonferroni's test for multiple comparisons. * $P < 0.05$ compared with lactating control. L, lactating; LR2, litter removed for 2 days; LR2+, litter removed for 2 days plus PRL; MWM, molecular-mass marker (kDa).

wild-type animals. Although DNA content does not necessarily equate with rates of apoptosis, we have previously shown, in our IGFBP-5 transgenic mice, that it correlates very well with a number of parameters associated with apoptosis, including changes in the concentrations of caspase-3, Bcl-2 family members, DNA laddering and BrdU incorporation (Tonner *et al.* 2002). The manner in which IGFBP-5 induced this inhibition of PRL action was examined in several ways; including activation of the STAT5 and STAT3 pathways, the IGF-signalling pathway and the activation of proteases.

Although there were changes in the expression of IGF-I and the IGFR during involution, these were similar in wild-type and transgenic animals and were also unaffected, at least in the long-term (i.e. 2–4 days post litter removal) by PRL treatment. Furthermore, levels of both total and phospho-Akt were not significantly different in transgenic animals and were also unaffected by PRL. These results suggest that IGFBP-5 was not exerting its effects via secretion into the extracellular environment to inhibit IGF actions. This suggestion is further supported by the fact that we have shown that, although IGFBP-5 can inhibit IGF-signalling in pregnancy and early lactation when expressed as a transgene, this effect is lost by day 10 of lactation via decreased phosphorylation of the IGF-I receptor and Akt (Tonner *et al.* 2002). This is because tight junctions close and the majority of IGFBP-5 is secreted apically, rather than basolaterally, where it would need to be, to inhibit IGF-signalling. Further

support for the absence of basolateral secretion of IGFBP-5 was provided by the demonstration that circulating concentrations of IGFBP-5 were not increased in transgenic animals (Tonner *et al.* 2002). We were thus intrigued to observe in this study that, although IGF signalling was unimpaired during lactation in IGFBP-5 transgenic mice, PRL signalling, as judged by phosphorylation of STAT5, was markedly impaired. This suggests that intracellular IGFBP-5 may interact directly with the PRL-signalling pathway. Such a proposal is supported by the observation that IGFBP-5 has a nuclear localization signal, is transported into the nucleus, can induce apoptosis without a requirement for secretion and has been demonstrated to be capable of acting as a transcription factor (Schedlich *et al.* 1998, Schedlich *et al.* 2000, Xu *et al.* 2004). This is the first report of intracellular crosstalk between STAT5 and IGFBPs and adds another dimension to the complex extracellular and stromal–epithelial interactions these two families of survival factors exhibit. Activation of STAT3 via phosphorylation is known to accompany apoptotic cell death during involution and mammary gland-specific STAT3-knockout mice exhibit impaired mammary gland involution (Chapman *et al.* 1999). We were able to confirm this increase in phosphorylation of STAT3 during involution and, furthermore, that its activation was not influenced by either the transgene or PRL, arguing against a role for STAT3 as a mediator of the actions of IGFBP-5.

We had previously demonstrated that part of the action of PRL in the rat was to suppress the activation of

Figure 7 tPA mRNA expression levels and plasmin activity during involution as measured by quantitative PCR and enzymatic activity analysis, respectively. Expression values of tPA are correlated with cyclophilin A expression (molecules per molecule cyclophilin). Values are means $\pm$ S.E.M. from three animals (tPA) and five or six animals (plasmin). Data were subjected to ANOVA followed by Bonferroni's test for multiple comparisons. ** $P < 0.01$ compared with lactating control. L, lactating; LR2, litter removed for 2 days; LR2+, litter removed for 2 days plus PRL; LR4, litter removed for 4 days; LR4+, litter removed for 4 days plus PRL.

plasminogen and the subsequent proteolytic cascade, which includes activation of various MMPs (e.g. MMP-3 and -12). However, in the present study, in the mouse, PRL did not significantly influence plasmin generation, although it did act as a potent inhibitor of both MMP-3 and MMP-12 expression. MMP-3, or stromelysin-1, is well known to be associated with remodelling of the mammary gland during involution and transgenic animals expressing MMP-3 in the mammary gland demonstrate impaired mammary gland development (Thomasset *et al.* 1998), whereas inactivation of MMP-3 limits ductal branching and alveolar growth during pregnancy (Wiseman *et al.* 2003). The ability of IGFBP-5 to block the cell-survival action of PRL, however, did not appear to involve regulation of MMP expression, since PRL was able to suppress expression of both MMP-3 and MMP-12 equally effectively in wild-type or IGFBP-5 transgenic animals.

The inhibitory effects of PRL on MMP-3 and -12 expression were somewhat unexpected since the promoter sequences do not exhibit consensus binding

sites for STAT5, a major target of PRL signalling. We therefore examined this effect in cell culture using HC-11 cells. These cells are derived from the mid-pregnant mouse mammary gland and can be induced to differentiate and express β -casein in response to PRL (Ball *et al.* 1988). We were unable to demonstrate any significant effect of PRL on MMP-3 expression, even though there was a significant induction of casein expression by PRL in these same cells. These results were confirmed in a fibroblast cell line, BHK-21, which was co-transfected with expression constructs encoding an activated form of the PRL receptor and STAT5 along with luciferase reporter constructs under the control of the MMP-3 or the β -casein promoter. Whereas this strategy led to a significant activation of the β -casein promoter, there was again no effect on MMP-3 promoter activity. While we cannot rule out the possibility that other components of a PRL-signalling cascade (different from those required for β -casein expression) are absent in these cells, it is equally plausible that the effects of PRL are indirect, possibly

Figure 8 MMP mRNA expression during involution in wild-type and IGFBP-5 transgenic mice as measured by quantitative PCR. Expression values of MMP-3, MMP-12 and MMP-7 are correlated with cyclophilin A expression (molecules per molecule cyclophilin). Values are means $\pm$ S.E.M. from three animals per group. Data were subjected to ANOVA followed by Bonferroni's test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$ compared with lactating control; a, significantly different ($P < 0.05$) for comparisons of PRL treatment versus corresponding control. L, lactating; LR2, litter removed for 2 days; LR2+, litter removed for 2 days plus PRL; LR4, litter removed for 4 days; LR4+, litter removed for 4 days plus PRL.

involving mesenchymal expression of MMPs. Although direct effects of PRL on mammary epithelial cells are well documented, recently it has been shown that part of the survival pathway activated by PRL may involve stimulation of IGF-II, a survival factor for epithelial cells, from the mammary stroma (Briskin *et al.* 2002, Hovey *et al.* 2003), clearly indicating that PRL can influence the activity of cells other than the epithelial compartment. This situation would in fact be analogous to the effects of GH which are also believed to be indirect, inducing its survival actions by stimulating IGF-I production in the mammary stroma (Kleinberg *et al.* 1990). Given that any effects of PRL on stromal activities (such as MMP expression) were unaffected by IGFBP-5, whereas the survival effects of PRL on the

epithelium were inhibited by IGFBP-5, it seems likely that the effects of IGFBP-5 on PRL actions, at least in this model, are limited to the epithelium.

In summary, we have shown that IGFBP-5 expression increases dramatically in the involuting mammary glands of mice, as it does in rats, consistent with it having a pro-apoptotic role. Involution of the mouse mammary gland, induced by pup removal, led to cell loss within 2 days and this could be inhibited for up to 4 days by PRL. In contrast to the rat (Tonner *et al.* 1997), however, PRL failed to inhibit IGFBP-5 synthesis and only moderately suppressed plasminogen activation. Expression of IGFBP-5 as a transgene did not lead to accelerated loss of cells or accelerated activation of proteases involved in remodelling of the extracellular

Figure 9 β-Casein and MMP mRNA expression levels in HC-11 cells as measured by quantitative PCR. Expression values of, MMP-3 (pg/pg actin), MMP-12, MMP-7 and β-casein (all fg/pg actin) were correlated with β-actin expression. Cells were grown to confluence for 3 days and then treated with combinations of the following hormones and growth factors: insulin (I; final concentration 5 µg/ml), epidermal growth factor (E; 10 ng/ml), PRL (P; 5 µg/ml), hydrocortisone (G; 1 µg/ml).

matrix during involution, but it did prevent the survival effects of PRL on cell number and differentiated function. This adds further support to our belief that IGFBP-5 plays a significant physiological role as an inducer of apoptosis in the mammary gland by blocking the actions of PRL. We have also demonstrated that PRL inhibits the expression of two MMPs involved in

degradation of the extracellular matrix. While this may in part explain the survival role of PRL, this cannot be the mechanism by which IGFBP-5 inhibits PRL actions, since PRL was equally effective at inhibiting the expression of MMP-3 and -12 in wild-type or transgenic mice. We also provide evidence that the effect of PRL on MMP expression is likely to be indirect, possibly mediated via a stromal–epithelial interaction. Finally, we provide evidence that IGFBP-5 may act intracellularly to regulate PRL signalling by decreasing the phosphorylation of STAT5.

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Figure 10 Luciferase expression directed by the MMP-3 and the β-casein promoter in BHK cells transfected with 1 µg pBK-MMP3-luc or 1 µg pBK-bcas2-luc in the presence of 1 or 2 µg PRL receptor (PRLR) and STAT5 expression constructs. Control transfections were carried out in the presence of 2 µg pBluescript. Extracts for luciferase measurement were prepared 48 h post-transfection and measured in a luminometer (Berthold). Values are given as arbitrary units and are means ± S.D. from triplicate experiments. The data were subjected to a paired *t* test. ***P* < 0.02 compared with control transfection.

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