Hormonal control of plasmin and tissue-type plasminogen activator activity in rat milk during involution of the mammary gland

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Abstract

We have proposed that growth hormone (GH) and prolactin (PRL) interact to suppress apoptosis in the mammary gland. GH increases insulin-like growth factor-I (IGF-I) synthesis whereas PRL suppresses the production of insulin-like growth factor-binding protein-5 (IGFBP-5) in the epithelial cells, which would otherwise inhibit IGF-mediated cell survival. IGFBP-5 was present in milk from involuting glands at high concentrations (approximately 60 μg/ml) and had a high affinity ($8{\cdot}03 \times 10^{-10}$ M) for IGF-I, suggesting an inhibitory effect of IGFBP-5 in the mammary gland. IGFBP-5 was present in the micellar fraction of milk and binds specifically to α_{s2}-casein. Since α_{s2}-casein also binds plasminogen and tissue-type plasminogen activator (t-PA), resulting in the conversion of plasminogen to plasmin, and since IGFBP-5 binds to plasminogen activator inhibitor–1 (PAI-1), we investigated whether apoptosis and extracellular matrix (ECM) degradation might be coordinately controlled by GH and PRL possibly acting through IGFBP-5.

Litters were removed from lactating rats to initiate involution. Plasminogen activation and t-PA activity were both increased dramatically after 48 h and GH and PRL suppressed this response. By contrast, 17β-oestradiol, progesterone or corticosterone did not influence either process. An antiserum to IGF-I, which blocked systemic IGF-I effects, failed to inhibit the activation of plasminogen or the increase in t-PA, suggesting that paracrine effects of IGF-I may be more important. Teat-sealing, which led to the accumulation of milk without hormonal changes, also led to increases in plasminogen activation and t-PA activity, suggesting that locally produced factors (of which IGFBP-5 is one) are important in controlling ECM remodelling. We propose that GH and PRL inhibit apoptosis and ECM remodelling by a process that involves the control of IGF-I and PAI-1 availability by IGFBP-5, thus allowing these processes to be tightly coordinated.

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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, as enhancing mammary epithelial cell survival (Travers *et al.* 1996). We demonstrated that there is a large increase in the concentration of insulin-like growth factor-binding protein-5 (IGFBP-5) in milk after 48 h of mammary involution induced by removal of the suckling young, and we propose that this acts to inhibit the interaction between insulin-like growth factor-I (IGF-I) and its receptor on the epithelial cells, resulting in cell death. This increase in IGFBP-5 was inhibited by 90% if the rats received concurrent PRL treatment (Tonner *et al.* 1997). We therefore proposed a model whereby GH raises IGF-I concentrations whereas PRL suppresses the production of IGFBP-5 from the epithelium to provide conditions that are optimal for cell survival.

Involution of the mammary gland also involves extensive remodelling and degradation of the extracellular matrix (ECM), a process initiated by the activation of plasminogen to produce plasmin. Human and bovine caseins can bind tissue-type plasminogen activator (t-PA) and enhance plasminogen activation, raising the possibility that casein micelles may provide a matrix for plasmin generation in milk (Heegaard *et al.* 1997*a*,*b*). The IGFBP-5 in milk from the involuting mammary gland is also associated with the casein micelles (Tonner *et al.* 1997). In addition, IGFBP-5 binds to plasminogen activator inhibitor-1 (PAI-1), suggesting that IGFBP-5 may also be involved in the regulation of tissue remodelling via the plasminogen system (Nam *et al.* 1997). We therefore examined the nature of the interaction of IGFBP-5 with the casein micelle in more detail.

The principal aim of this study was to characterise the control of the plasminogen system in rats undergoing mammary gland involution. The cessation of milk production leads to rapid changes in serum concentrations of

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17β-oestradiol, PRL, progesterone and IGF-I. We therefore investigated the effects of these hormones and, in addition, examined the effects of GH and corticosterone because these have previously been implicated in mammary gland involution (Flint & Gardner 1994, Feng *et al*. 1995, Travers *et al*. 1996).

Materials and Methods

Animals

Female Wistar rats weighing approximately 250–300 g were mated; at parturition, the litters of these rats were each adjusted to 10 pups in number. All animals were allowed free access to food (Labsure-irradiated CRM diet; Labsure, Poole, UK) and water. Approval for these studies was granted under the Home Office Licence and Ethical Review Committee of the Hannah Research Institute.

Hormones and milk proteins

Recombinant bovine GH was a gift from Monsanto (St Louis, MO, USA), and ovine PRL-19 (oPRL-19) was a gift from National Institute for Diabetes, Digestion and Kidney Diseases (NIDDK). Recombinant human IGF-I was from Bachem UK (Saffron Walden, Essex, UK). Human IGFBP-5 peptide (201–218) and insect cell-baculovirus-expressed human IGFBP-5 were gifts from Dr D. Andress (Veterans' Affairs Medical Center, Seattle, WA, USA). Purified bovine milk proteins were obtained from Dr Jeff Leaver (Hannah Research Institute, Ayr, UK).

Liquid-phase assay of IGFBP-5 binding to ^{125}I-IGF-I

Recombinant wild-type rat IGFBP-5 was expressed in the insect cell-baculovirus system using the Bac-to-Bac system (Gibco-BRL, Paisley, UK) to produce recombinant virus as previously described (Bramani *et al*. 1999). Milk containing IGFBP-5 was from animals whose litters had been removed for 48 h. IGFBP-5 activity was assessed using a liquid-phase assay involving incubating diluted milk samples with ^{125}I-IGF-I (iodinated using Iodogen to a specific activity of appoximately 100 μCi/μg; 30 000 c.p.m. per tube) as described previously (Conover *et al*. 1989). IGFBP-5 concentrations in milk were determined by using increasing amounts of recombinant IGFBP-5 as a standard and the concentrations of IGFBP-5 in milk samples were determined by interpolation from the standard curve generated. The affinity of interaction of IGFBP-5 with IGF-I was derived by Scatchard analysis of assays in which increasing amounts of unlabelled IGF-I were used as a competitor (Scatchard 1949).

Ultracentrifugation of milk

Milk was centrifuged at 100 000 ***g*** for 15 min to provide a casein micelle pellet and a supernatant (whey fraction) or micelles were disrupted by incubation in 100 mM EDTA before centrifugation as above.

Binding of ^{125}I-IGFBP-5 to hydroxyapatite

^{125}I-IGFBP-5 (iodinated using Iodogen to a specific activity of approximately 150 μCi/μg; 30 000 c.p.m. per tube) was incubated overnight with 100 μl 0·2% (w/v) hydroxyapatite (Calbiochem-Novabiochem, Nottingham, UK) in the presence of increasing concentrations of various competitors, as described in the Results section. Samples were centrifuged at 1700 ***g*** for 10 min, the supernatant was discarded and then the pellet, containing ^{125}I-IGFBP-5 bound to hydroxyapatite, was counted in a gamma counter.

Western ligand blotting of bovine milk proteins with ^{125}I-IGFBP-5

Purified milk proteins and BSA (5 μg/well) were subjected to SDS-PAGE and Western ligand blotting with ^{125}I-IGFBP-5 as previously described (Hossenlopp *et al*. 1986). Briefly, Western blots were blocked in 3% (w/v) NP-40 in 50 mM Tris-HCl, pH 7·4, 150 mM NaCl (TBS) for 30 min, 1% (w/v) BSA in TBS for 2 h, then 0·1% Tween 20 in TBS for 10 min. The blots were then incubated overnight at 4 °C with ^{125}I-IGFBP-5 (2×10^6 c.p.m./ml) in TBS with 1% BSA, 0·1% Tween 20. After incubation the blot was washed twice in TBS/0·1% Tween and three times in TBS, then air-dried and exposed to a Phosphorimager screen and the image detected by Phosphorimager 445SI equipment (Molecular Dynamics Ltd, Kensing, UK).

Study 1: effects of 17β-oestradiol and teat-sealing on plasminogen activation in lactating rats

Lactating rats were treated on day 14 of lactation by daily s.c. injection of 17β-oestradiol (100 μg in safflower oil/injection). A second group had teats on one side sealed with tissue glue (Vetbond; Vet Drug Co., Falkirk, UK), and the pup number was reduced to six (one per unsealed gland). After treatment for 2 days, litters were removed from these groups for 4 h to allow milk to accumulate before a milk sample was obtained. Animals were anaesthetised by i.p. injection of 0·3 ml sodium pentobarbital (60 mg/ml; Sagatal; RMB Animal Health, Dagenham, UK), after which the dam received 1 U oxytocin subcutaneously (Intervet UK, Cambridge, UK) to induce milk ejection. Milk was obtained from the upper abdominal mammary glands by gentle pressure and stored at − 20 °C until used to determine t-PA and plasmin activities as described below.

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Study 2: effects of litter removal, PRL, GH, anti-IGF-I serum, progesterone and corticosterone on plasminogen activation

Animals had their litters removed on day 14 of lactation (time zero) and received one of the following treatments for 2 days (all by s.c. injection): recombinant GH (1 mg in 20% polyvinylpyrrolidone (PVP)/ injection) twice daily, PRL (1 mg in 20% PVP/ injection) twice daily, combined GH plus PRL (1 mg of each in 20% PVP/injection) twice daily, anti-IGF-I serum (equivalent to 3 ml/injection) once daily, corticosterone (1 mg in safflower oil/injection once daily, or progesterone (5 mg in safflower oil/injection once daily). The dose of anti-IGF-I was based on a previous study (Spencer *et al.* 1991) in which it was found to be effective. In fact it was used at three times the dose previously demonstrated to neutralise the IGF-I bio-activity in plasma. The IGF-I antiserum was administered to inhibit the potential effects of the increase in serum IGF-I that occurs after litter removal, but was unlikely to block effects of locally produced IGF-I. A control group of animals had their litters removed and were untreated. After treatment for 2 days, a milk sample was obtained as described above and stored at −20 °C until used to determine t-PA and plasmin activities as described below.

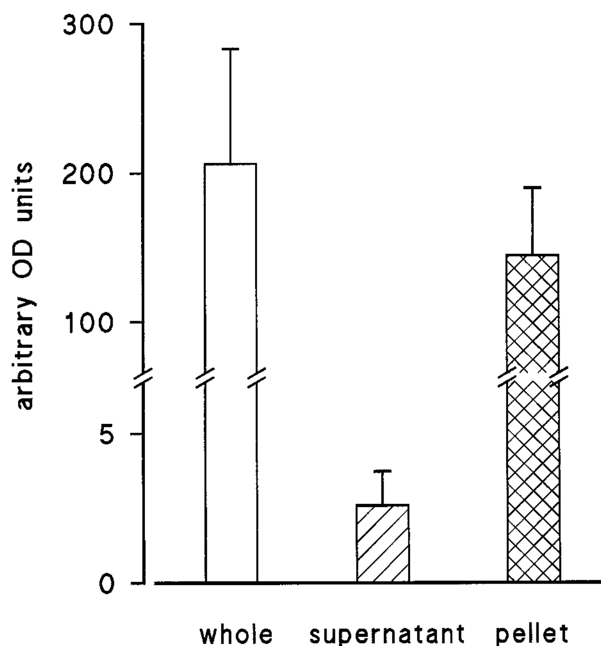

Figure 1 Densitometric analysis of Western ligand blot of fractionated milk derived from animals whose litters had been removed for 48 h. Values are means ± S.E.M. for five animals.

Assay of plasmin and t-PA activities

Milk samples were centrifuged at 2000 ***g*** for 15 min and defatted. Plasmin activity was measured by incubating milk samples in 50 mM Tris-HCl, pH 7·4, 110 mM NaCl, 2·5 mM epsilon-amino-n-caproic acid (EACA), 0·6 mM Val-L-Leucyl-L-Lys-*p*-nitroanilide (Sigma, Poole, Dorset, UK). The absorbance at 405 nm was measured at 15 min intervals for 3 h, and the generation of nitroaniline was determined by the rate of change in absorbance. Total plasmin plus plasminogen activity was determined after the conversion of plasminogen to plasmin by incubation in the presence of urokinase (600 plough units (894 IU)/ml).

The t-PA activity was measured by incubating milk samples in 60 mM Tris-HCl, pH 8·5, 0·09% Tween 20, with 0·375 mg/ml *N*-methylsulphonyl-D-Phe-Gly-Arg-4-nitroanilide acetate substrate (Chromozym t-PA; Boehringer Mannheim UK, East Sussex, UK). The absorbance at 405 nm was measured at 5 min intervals for 40 min and the generation of nitroaniline was determined by the rate of change in absorbance.

Statistical analysis

The data were analysed by the Kruskal–Wallis test (a non-parametric test, analogous to the one-way analysis of variance method) followed by post-hoc tests for multiple comparisons (Conover 1999). A correction for the number of significance tests was made using the Bonferroni method as described by Bland & Altman (1995).

The data were also analysed by one-way analysis of variance (ANOVA) using MINITAB followed by supplementary *t*-tests using the residual mean square (Cochran & Cox 1957), which gave results similar to those in the Kruskal–Wallis test. However, even after square-root transformation of the plasmin activity and $\log_e$-transformation of t-PA activity there was some heterogeneity of variances or deviation from normality.

The interactive effect of combined GH plus PRL treatment was analysed in a two-way ANOVA. The t-PA activity data were $\log_e$-transformed to reduce the heterogeneity of variances.

Results

IGF-binding properties of IGFBP-5

IGFBP-5 concentrations were estimated in milk derived from five animals whose litters had been removed for 48 h. Milk IGFBP-5 concentrations were 59 ± 9·0 µg/ml when compared in a binding assay with known amounts of recombinant IGFBP-5. Milk IGFBP-5 had an affinity for IGF-I (K_d=8·03 × 10^{-10} M) which was similar to that of baculovirus-produced IGFBP-5 (K_d=6·92 × 10^{-10} M) (results not shown).

Ultracentrifugation of milk

Ultracentrifugation of milk produced a pellet containing the casein micelles and a supernatant containing the whey

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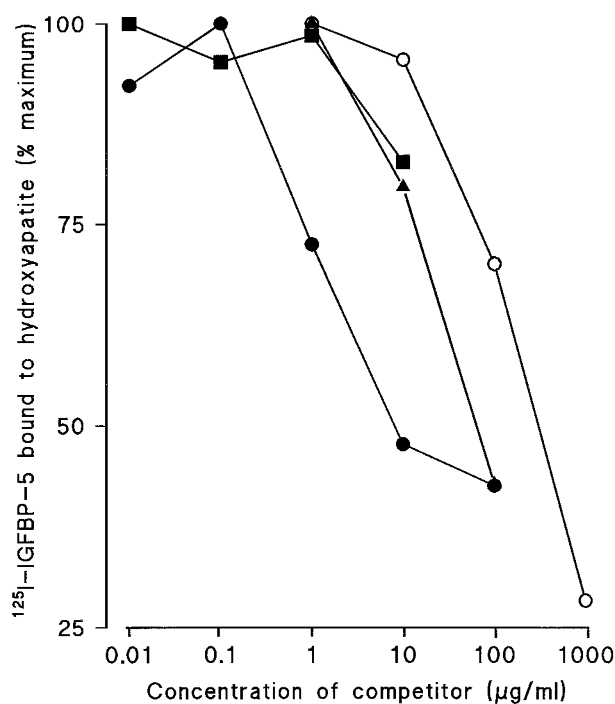

Figure 2 Inhibition of ^{125}I-IGFBP-5 binding to hydroxyapatite by heparin (●), IGFBP-5 (■), sodium caseinate (▲) and a peptide consisting of the sequence 201–218 of IGFBP-5 (○).

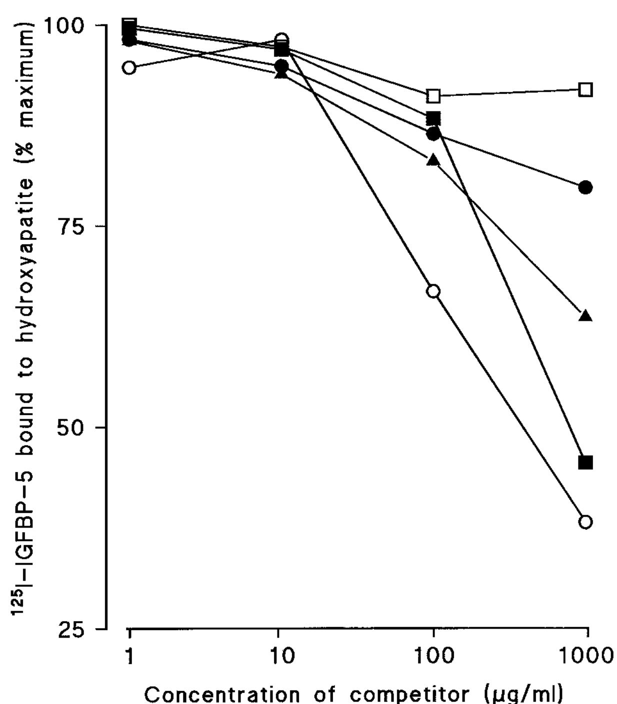

Figure 3 Inhibition of ^{125}I-IGFBP-5 binding to hydroxyapatite by bovine α_{s2}-casein (○), α_{s1}-casein (■), β-casein (▲), κ-casein (●) and sheep IgG (□).

proteins. When these fractions were analysed for IGFBP content approximately 98% of the IGFBP was present in the casein micelle fraction (Fig. 1). Disruption of the micelles by the use of EDTA displaced all of the IGFBP-5 into the supernatant (results not shown).

Binding of IGFBP-5 to hydroxyapatite

^{125}I-IGFBP-5 binding to hydroxyapatite was inhibited by heparin, a peptide consisting of the sequence 201–218 of IGFBP-5, and sodium caseinate (Fig. 2). Unlabelled IGFBP-5 also partially inhibited binding of ^{125}I-IGFBP-5, although we had insufficient IGFBP-5 to test it at higher concentrations. However, IGFBP-5 appeared to be at least 10-fold more potent than the peptide IGFBP-5 (201–218). When individual caseins were assessed, the potency of displacement of IGFBP-5 was α_{s2}-casein> α_{s1}-casein>β-casein>κ-casein (Fig. 3). This correlates with the number of phosphorylated residues in these proteins (α_{s2}-casein, 10–13 phosphates; α_{s1}-casein, 8–9 phosphate residues; β-casein, 5 phosphate residues; κ-casein, 1 phosphate residue).

Binding of ^{125}I-IGFBP-5 to bovine milk proteins

Radiolabelled IGFBP-5 bound to α_{s2}-casein in both its monomeric and dimeric forms, weakly to α_{s1}-casein, but not to a range of other milk proteins, or to BSA or Immunoglobulin G (Fig. 4).

Study 1: effects of 17β-oestradiol and teat-sealing on plasminogen activation in lactating rats

Plasmin activity (Fig. 5*a*) and total plasmin plus plasminogen activity (Fig. 5*b*) were unaffected by 17β-oestradiol administration (given to mimic the rise in plasma oestradiol that occurs when the oestrous cycle resumes after litter removal). Sealing of half the mammary glands (to allow milk accumulation to occur in the presence of an unchanged hormonal environment) dramatically increased plasmin activity (Fig. 5*a*), whereas total plasmin plus plasminogen activity was unchanged (Fig. 5*b*). Consistent with this change in plasminogen activation, t-PA activity was dramatically increased by teat-sealing but unaffected by 17β-oestradiol (Fig. 5*c*).

Study 2: effects of litter removal, PRL, GH, anti-IGF-I serum, progesterone and corticosterone on plasminogen activation

Involution of the mammary gland induced by litter removal resulted in a marked increase in plasmin activity (P<0·001), and concurrent treatment with PRL, GH or PRL plus GH (P<0·001) inhibited this increase (Fig. 6*a*).

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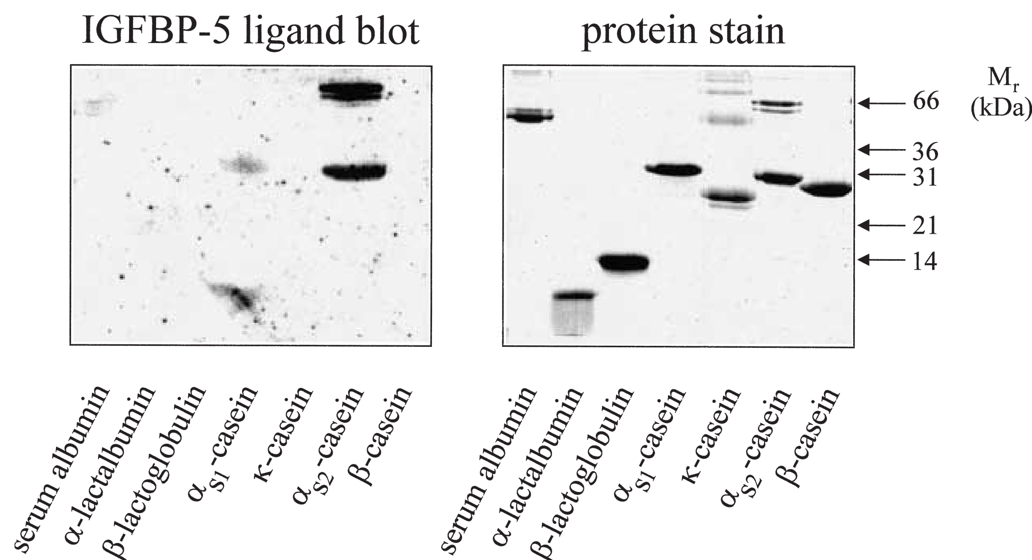

Figure 4 SDS-PAGE and western ligand blot analysis of bovine milk proteins. Milk proteins and BSA (5 μg) were electrophoresed on a 12% SDS-polyacrylamide gel and were either Western ligand blotted with ^{125}I-IGFBP-5 (left) or stained with Coomassie brilliant blue (right).

Treatment with progesterone ($P<0{\cdot}01$) or corticosterone ($P<0{\cdot}05$) produced smaller decreases in plasminogen activation, but an antibody to IGF-I (to neutralise the rise in IGF-I that occurs after litter removal) failed to prevent the increase in plasmin activity. Total plasmin plus plasminogen activity was slightly increased by progesterone treatment ($P<0{\cdot}05$) but was unchanged by the other treatments when compared with lactating glands (Fig. 6*b*). The t-PA activity also increased in response to litter removal ($P<0{\cdot}001$) and this increase was also reduced by concurrent treatment with PRL ($P<0{\cdot}05$), GH ($P<0{\cdot}05$) and PRL plus GH ($P<0{\cdot}001$) (Fig. 6*c*). Treatment with progesterone and corticosterone led to decreases in t-PA activity, though they did not reach statistical significance. An antibody to IGF-I failed to inhibit the increase in t-PA activity (Fig. 6*c*).

A separate two-way analysis of the interaction between PRL and GH treatment showed that GH or PRL treatment inhibited the increases in plasmin and t-PA activity but that there was no interactive effect of these hormones ($P<0{\cdot}498$ for plasmin activity and $P<0{\cdot}206$ for t-PA activity).

Discussion

We have previously shown a large increase in the production of IGFBP-5 by the mammary gland during involution and we have 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 enhancing, effect on IGF-I action. Here we report that IGFBP-5 in the involuting gland is of high affinity (similar to that of IGFBP-5 expressed in baculovirus) and of a higher affinity than that reported for the IGF-I receptor (Steele-Perkins *et al.* 1988). In addition, IGFBP-5 is present in high concentrations (approximately 60 μg/ml milk) in the involuting gland. These observations support an inhibitory, rather than an enhancing, role for IGFBP-5 in the mammary gland.

IGFBP-5 has been shown to interact with several ECM components and these interactions lower the affinity for IGF-I; in several studies this was associated with augmentation, rather than inhibition, of IGF-I action (Jones *et al.* 1993). We therefore investigated the binding of IGFBP-5 to casein micelles in greater detail and examined binding to various milk proteins. It is possible that IGFBP-5 binds to the calcium phosphate nanocluster present in the casein micelle since it is structurally similar to hydroxyapatite. However, we showed that IGFBP-5 binds to the micellar milk protein α_{s2}-casein, particularly in its dimeric form. Studies involving inhibition of IGFBP-5 binding to hydroxyapatite demonstrated that the potency of caseins in disrupting IGFBP-5 binding correlated with the extent of their phosphorylation. Whether the binding of IGFBP-5 to hydroxyapatite and to α_{s2}-casein involves similar molecular interactions remains to be determined and it will be interesting to explore the precise nature of molecular interaction of IGFBP-5 with α_{s2}-casein to examine whether, for example, this involves the heparin-binding domain of IGFBP-5 (Parker *et al.* 1998). However, the binding of IGFBP-5 to α_{s2}-casein did not reduce the high-affinity binding to IGF-I, which contrasts with the effects of the

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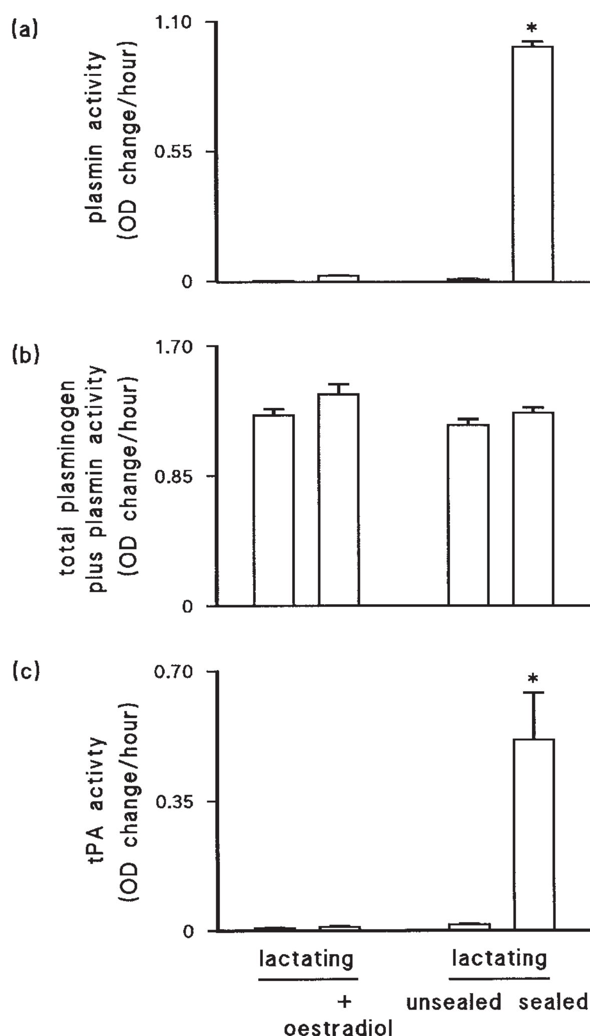

Figure 5 Effects of 17β-oestradiol and teat-sealing on milk plasmin (*a*), total plasmin plus plasminogen activities (*b*), and t-PA activity (*c*). Values represent the means ± S.E.M. of 4–6 animals. *P<0·001 compared with lactating animals.

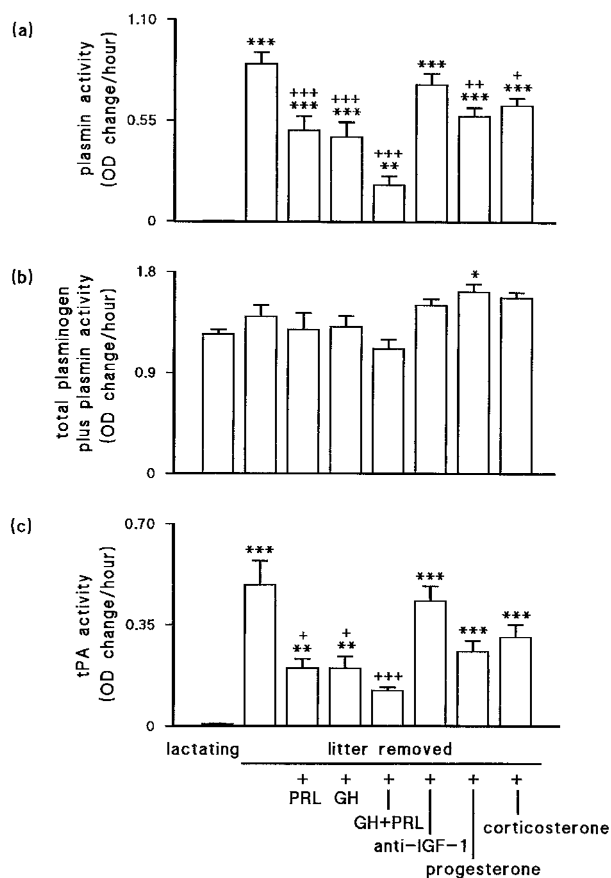

Figure 6 Effects of litter removal and concurrent treatment with PRL, GH, PRL plus GH, an antiserum to IGF-I, progesterone or corticosterone on milk plasmin (*a*) total plasmin plus plasminogen activities (*b*), and t-PA activity (*c*). Values represent the means ± S.E.M. of 4–6 animals. ***P<0·001; **P<0·01; *P<0·05 (compared with lactating animals); ^{+++}P<0·001; ^{++}P<0·01; ^{+}P<0·05 (compared with 'litter-removed' values).

interaction between IGFBP-5, via its heparin-binding domain, and various components of the ECM.

Dimeric α_{s2}-casein has also been reported to bind both plasminogen and t-PA, resulting in enhanced conversion of plasminogen to plasmin (Heegaard *et al.* 1997*a*). PAI-1, which inhibits PA activity, is a component of the ECM of several cell types and it has been shown that IGFBP-5 can bind to PAI-1; this interaction may serve to regulate PAI-1 activity by sequestering it from its interaction with t-PA (Nam *et al.* 1997). The plasminogen system has important physiological functions in the mammary gland and is under hormonal control. Plasmin generation is catalysed by two activators – urokinase-type plasminogen activator (u-PA) and t-PA – and they are considered to participate in separate processes. The u-PA binds to its cell-surface receptor, u-PAR, and is involved in cell migration and tissue remodelling, whereas t-PA, in association with fibrin, is active in fibrinolysis and thrombolysis. By analogy, it has been proposed that t-PA may serve to inhibit inappropriate clotting of milk in the mammary gland (Heegaard *et al.* 1997*b*). The ability of α_{s2}-casein to bind to plasminogen and t-PA and enhance conversion of plasminogen to plasmin suggests that, in addition to its role in fibrinolysis, t-PA may have a role in ECM degradation during mammary gland involution by activating plasminogen and eliciting the proteolytic cascade which results in degradation of the basement membrane.

The PA content of rodent mammary gland is correlated with involution (Ossowski *et al.* 1979). In rodents, an increase in PA production and a decrease in PAI-1 activity are correlated with the destruction of the basement membrane and degeneration of the secretory cells during gland involution (Ossowski *et al.* 1979, Busso *et al.* 1989,

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Talhouk *et al.* 1992). The results of the present study are in agreement with this, since litter removal led to a large increase in plasmin and t-PA activity. We have extended these studies to examine the hormonal control of plasminogen activation. The possibility that IGFBP-5 regulates plasminogen activation (through its ability to bind PAI-1) coupled with the ability of PRL to inhibit IGFBP-5 synthesis suggested that PRL might also influence t-PA activity and plasminogen activation. This was shown to be the case and the ability of PRL to suppress the increase in plasmin activity induced by litter removal confirms and extends previous studies in which PRL suppressed involution and plasminogen activation in mammary glands of mice (Ossowski *et al.* 1979). We demonstrate here, for the first time, the ability of GH to suppress the increase in plasmin induced by litter removal. This role of GH is consistent with its role as a survival factor. The failure of an antiserum to IGF-I to affect the plasminogen system implies that GH does not exert its effect via an endocrine effect of IGF-I. However GH stimulates IGF-I mRNA in the mammary stroma and therefore local production of IGF-I may be important in this respect (Kleinberg *et al.* 1990). The fact that there was no evidence for an interaction between PRL and GH in suppressing plasminogen activation suggests that they act through independent mechanisms, lending further support to the notion that PRL may act through regulation of IGFBP-5 whereas GH acts via stimulation of IGF-I which has been shown to raise PAI-1 mRNA expression (Fattal *et al.* 1992).

The failure of *in vivo* treatment with corticosterone to suppress t-PA activity and the modest degree of suppression of plasmin activity in milk contrast with studies in which hydrocortisone suppressed the plasminogen activator content of mouse mammary glands (Ossowski *et al.* 1979). This may reflect species differences or the use of different glucocorticoids. However, there are conflicting reports of the effects of glucocorticoids on plasminogen activators: for example, in a human mammary cell line, dexamethasone suppressed u-PA and raised the t-PA activity, whereas the production of plasminogen activator inhibitors was not affected (Busso *et al.* 1986).

Oestrogen treatment of lactating cows has previously been shown to increase the activation of plasminogen in mammary secretions (Athie *et al.* 1997) and oestradiol inhibits mRNA expression of uPA, PAI-1 and t-PA in breast-cancer cells (Levenson *et al.* 1998). However, in this study plasminogen or plasmin activity in lactating animals was unaffected by oestradiol treatment, suggesting that it is not a major physiological regulator of this system in the lactating rat.

Teat-sealing also increased plasmin activity dramatically by increasing plasminogen conversion within the gland. This observation was consistent with the increase in t-PA activity seen in teat-sealed, but not unsealed, glands and suggests that unidentified factors regulate this process locally within the gland. Intriguingly, we have previously shown that IGFBP-5 synthesis is raised in sealed glands, which extends the coincidental expression of IGFBP-5, t-PA activity and plasmin activity.

During the extensive tissue remodelling that occurs in the involuting gland, many proteases are activated and these could potentially proteolyse IGFBP-5. Although proteolytic cleavage may result in fragments of IGFBP-5 with no affinity for IGF-I, it may also result in fragments with a lowered affinity for IGF-I which, in some cases, can potentiate the effects of IGF-I. However, IGFBP-5 found in rat milk after litter removal was predominantly intact, suggesting that it may be protected from proteolysis, although Western immunoblot analysis demonstrated the presence of small amounts of proteolysed forms of IGFBP-5 which were undetectable by Western ligand blotting (Tonner *et al.* 1997). Binding of IGFBP-5 to ECM not only lowers its affinity for IGF-I but may also protect IGFBP-5 from proteolysis. Thus the binding of α_{s2}-casein to IGFBP-5 may also have a role in the protection of IGFBP-5 from proteolysis and the retention of its high affinity for IGF-I. Alternatively, the interaction of IGFBP-5 with PAI-1 may serve this function, as proposed by others (Nam *et al.* 1997).

IGFBPs can act as transport molecules for IGFs, so their secretion into milk, and in particular into colostrum, suggests a role for IGFBPs in the delivery/protection of IGFs for biological processes in the neonate. This has been the subject of much interest because of the concerns about increased levels of IGF-I in the milk of cows treated with GH (Epstein 1996). IGFBPs could conceivably increase the half-life of IGF-I in the gut by protecting it from degradation. Furthermore, casein can protect IGF-I from proteolysis in the gut (Xian *et al.*1995) and it is possible that complexes, for example between IGF-I, IGFBP-5, PAI-1 and casein, might increase this protection still further. However, during lactation the levels of IGFBP-5 mRNA and protein synthesis are very low and are dramatically enhanced by milk accumulation; thus IGFBP-5 induction occurs in response to involution, suggesting that it may be involved in this process.

In conclusion, we have shown that the IGFBP-5 present in involuting glands is present in extremely high concentrations and has a high affinity for IGF-I, lending further support to our belief that it is inhibitory to IGF-I action. GH and PRL are potent inhibitors of plasminogen activation and t-PA activity in the mammary gland and they are even more potent when given in combination. Thus GH and PRL regulate both cell death and tissue remodelling. Since α_{s2}-casein binds to plasminogen and t-PA, resulting in enhanced conversion of plasminogen to plasmin, and since IGFBP-5 binds to α_{s2}-casein and to PAI-1, it is tempting to speculate that GH and PRL regulate the plasminogen system by their effects on the IGF-system. In our model we propose that IGFBP-5 binds to PAI-1, sequestering it and inhibiting its interaction with

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t-PA. Treatment with PRL inhibits IGFBP-5 synthesis, which would result in the release of PAI-1, which in turn would suppress t-PA activation. GH acts by its ability to stimulate IGF-I synthesis, since IGF-I has been shown to increase PAI-1 mRNA expression (Fattal *et al.* 1992). This hypothesis provides a mechanism whereby activation of apoptosis and ECM degradation can be tightly coordinated by a single molecule – IGFBP-5. Thus IGFBP-5 would serve a dual role involving both IGF-dependent and IGF-independent actions. Our results also suggest a novel role for a milk protein, and presumably the casein micelle, as a matrix for the assembly of a multi-protein complex of IGFBP-5, t-PA, plasminogen and PAI-1.

Subsequent studies will examine this hypothesis by examining transgenic mice expressing IGFBP-5 in the mammary gland using the β-lactoglobulin promoter, and by investigating the ability of recombinant IGFBP-5 to regulate t-PA activity and plasminogen activation *in vitro*. It is intriguing that IGFBP-5 has also been implicated as a causal factor in ovarian follicular atresia (Liu *et al.* 1993) and that PRL has been shown to inhibit t-PA mRNA levels and increase PAI-1 activity in rat granulosa cells (Liu *et al.* 1998). Thus this mechanism may not be restricted to the mammary gland but could represent a common feature in the coordination of cell death and the degradation of ECM in a variety of tissues.

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