

Insulin-like growth factor binding protein-5 (IGFBP-5) induces premature cell death in the mammary glands of transgenic mice

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SUMMARY

We have previously demonstrated that IGFBP-5 production by mammary epithelial cells increases dramatically during involution of the mammary gland. To demonstrate a causal relationship between IGFBP-5 and cell death we created transgenic mice expressing IGFBP-5 in the mammary gland using a mammary-specific promoter, β -lactoglobulin. DNA content in the mammary glands of transgenic mice was decreased as early as day 10 of pregnancy. Histological analysis indicated reduced numbers of alveolar end buds, with decreased ductal branching. Transgenic dams produced IGFBP-5 in their milk at concentrations similar to those achieved at the end of normal lactation. Mammary cell number and milk synthesis were both decreased by approximately 50% during the first 10 days of lactation. BrdU labelling was decreased, whereas DNA ladders were increased in transgenic animals on day 1 of lactation. On day 2 postpartum, the epithelial invasion of the mammary fat pad was clearly impaired in transgenic animals. The concentrations of the pro-apoptotic molecule caspase-3 and of plasmin were both increased in transgenic animals whilst the concentrations of 2 prosurvival molecules Bcl-2 and Bcl-x_L were both decreased. In order to examine whether IGFBP-5 acts by inhibiting the survival effect of IGF-I we

examined IGF receptor phosphorylation and Akt phosphorylation and showed that both were inhibited. We attempted to “rescue” the transgenic phenotype by using growth hormone to increase endogenous IGF-I concentrations or by implanting minipumps delivering an IGF-1 analogue, R₃-IGF-1, which binds weakly to IGFBP-5. Growth hormone treatment failed to affect mammary development suggesting that increased concentrations of endogenous IGF-1 are insufficient to overcome the high concentrations of IGFBP-5 produced by these transgenic animals. In contrast mammary development (gland weight and DNA content) was normalised by R₃-IGF-I although milk production was only partially restored. This is the first demonstration that over-expression of IGFBP-5 can lead to; impaired mammary development, increased expression of the pro-apoptotic molecule caspase-3, increased plasmin generation and decreased expression of pro-survival molecules of the Bcl-2 family. It clearly demonstrates that IGF-I is an important developmental/survival factor for the mammary gland and, furthermore, this cell death programme may be utilised in a wide variety of tissues.

Key words: IGFBP-5, Mammary gland, Involution, Apoptosis, Transgenic, Caspase, Plasmin, Mouse

INTRODUCTION

Prolactin and growth hormone (GH) interact to promote cell survival in the mammary gland in rodents and we proposed that the mechanism involves the insulin-like growth factor (IGF) system (Flint and Gardner, 1994; Travers et al., 1996; Tonner et al., 1997). GH has been shown to increase insulin-like growth factor-1 (IGF-1) production from mammary stromal cells (Kleinberg et al., 1990) and IGF-1 has in turn been shown to promote mammary epithelial cell survival in transgenic mouse models (Hadsell et al., 1996; Neuenschwander et al., 1996). Prolactin may act by suppressing the production of insulin-like growth factor binding protein-5 (IGFBP-5) from the mammary epithelium. This is based upon the large increase in IGFBP-5 synthesis that occurs during involution of the

mammary gland, and the fact that only prolactin was able to markedly suppress this, coincident with an anti-apoptotic effect on the mammary epithelium (Tonner et al., 1997). A number of other studies have added support to this concept. For example, IGFBP-5 expression is reduced and involution delayed in STAT3 KO mice (Chapman et al., 1999) whilst in IRF-1 knockout mice involution and IGFBP-5 expression are both accelerated (Chapman et al., 2000). IGFBP-5 expression is also increased during involution of the prostate and thyroid glands and in ovarian follicles undergoing atresia (Guenette and Tenniswood, 1995; Phillips et al., 1997; Liu et al., 1993). Additional studies of ours and of others have demonstrated extensive expression of IGFBP-5 in the developing embryo, at sites that are consistent with it having a role in apoptosis (van Kleffens et al., 1998). Most striking has been the demonstration

of IGFBP-5 in the interdigital webbing, which is programmed to undergo apoptotic cell death during digit formation in the mouse embryo (Allan et al., 2001) and the unusual expression pattern of IGFBP-5 in the *Hypodactyly* mouse where digit formation is abnormal (Allan et al., 2000). All of this evidence indicates no more than an association of IGFBP-5 with the process of apoptosis, although we have shown, in preliminary studies, that beads coated in IGFBP-5 can induce apoptosis in the developing chick limb bud (Allan et al., 1998).

This largely circumstantial evidence in support of an inhibitory role for IGFBP-5 is countered by a number of studies that have indicated an enhancing effect of IGFBP-5 on IGF-I action in vitro (Jones et al., 1993) and studies that have questioned the role of IGFBP-5 in prostate involution (Miyake et al., 2000). Augmentation of IGF action typically involves proteolysis or reduction in the affinity of IGFBP-5 for IGF-1, often involving interaction with components of the extracellular matrix (Clemmons et al., 1998; Nam, 2000). We addressed aspects of this phenomenon in a recent study, in which we showed that, in the mammary gland, IGFBP-5 interacts with a milk protein, α_{s2} -casein (Tonner et al., 2000). Unlike its interaction with a number of other proteins or components of the extracellular matrix, the interaction of IGFBP-5 with α_{s2} -casein did not reduce its affinity for IGF-I and thus its affinity remained in excess of that of the type I-IGF receptor. In addition, the study showed that IGFBP-5 concentrations in milk were in excess of 50 mg/l, which is several orders of magnitude greater than that of IGF-1. Typically, augmentation of IGF actions takes place when IGFBP and IGF are approximately equimolar but, when IGFBP concentrations are in excess, IGF actions are inhibited (Ewton et al., 1998). Indeed, in all four transgenic mouse models described thus far (IGFBPs 1-4) growth inhibitory effects have been described (Murphy, 2000).

In this study we sought to provide evidence for a causal relationship between IGFBP-5 and apoptosis by producing transgenic mice expressing IGFBP-5 from a mammary-specific promoter in order to examine various aspects of mammary development and function. These included histological analysis, measurement of caspase-3 (a marker of mammary apoptosis) (Marti et al., 2000; Marti et al., 2001), plasmin activity (a marker of extracellular matrix degradation in the mammary gland) (Ossowski et al., 1979; Busso et al., 1989; Tonner et al., 2000) pro-survival members of the Bcl-2 family and IGF signalling cascade. In this study we provide the first evidence that IGFBP-5 inhibits cellular proliferation and induces cell death, when expressed in the mammary gland of transgenic mice.

MATERIALS AND METHODS

Animals

All animal studies were conducted under appropriate Licence from the UK Home Office and after approval by local Ethical Review Committees.

Production of IGFBP-5 transgenic mice

Production of transgenic mice was by injection of DNA into the pronuclei of fertilised eggs from superovulated F₁ (CBA × C57B1/6) female mice mated with F₁ stud male mice as described previously

(Simons et al., 1987). The transgene rescue strategy (Clark et al., 1992) was used, which involved the co-injection of the BLG transgene pSS1tgXS and pBJ69 containing the IGFBP-5 cDNA driven off BLG sequences. The IGFBP-5 cDNA (Shimasaki et al., 1991) was inserted as a blunted *EcoRI* fragment into the *EcoRV* site of pBJ41 (Sola et al., 1998). Transgenic mouse colonies were maintained on a mixed CBA × C57B1/6 background. Milk was collected at day 11 of lactation and processed as described (Simons et al., 1987). Various tissues were taken from day 10 lactating mice to examine whether transgene expression was limited to the mammary gland. Mammary tissue samples were also obtained at days 10 and 15 of pregnancy and day 2 of lactation to compare the level of transgene expression at these times, using northern blotting.

Identification of transgenic mice

6- to 8-week old mice were anaesthetised with halothane and the tip of the tail removed for identification of the BLG transgene by PCR. DNA was extracted from tail biopsies using a Wizard® Genomic Purification Kit (Promega, Southampton, UK) and resuspended in 10 mM Tris-HCl buffer containing 1 mM EDTA pH 8.0. Primer sequences for the transgene were 5'-GCT TCT GGG GTC TAC CAG GAA CCG-3' and 5'-GTC GTG CTT CTG AGC TCT GCA GGG-3'. Primers for acetyl-CoA carboxylase, which was used as a positive control were 5'-TCC GAG CTC GAT AAG AAG TGG GGA GGG AGA GCC-3' and 5'-TCC GAG CTC ACA CCA TAA GGT CAG AAA ATC TCC-3'. Oligonucleotides were synthesised by MWG Biotech UK Ltd (Milton Keynes, Bucks, UK). 24 µl of the following mix was added to 1.0 µl of DNA solution: 0.08 µl (0.4 U) of Taq DNA polymerase (Promega, Southampton UK), 2.5 µl of 10× buffer (supplied with enzyme), 0.25 µl of each oligonucleotide primer (25 pmoles of each), 1.0 µl of 25 mM MgCl₂, 4.0 µl of deoxynucleotide triphosphate (dNTP) mix (1.25 mM each dATP, dCTP, dGTP and dTTP), 2 µl DMSO (8% final), and distilled water to bring the final volume to 24 µl. PCR amplification was performed as follows: 95°C for 1 minute, 30 cycles of 30 seconds at 92°C and 5 minutes at 65°C, then 5 minutes at 65°C. Amplified products were analyzed by electrophoresis through a 2% agarose gel.

Northern blotting

RNA was isolated from tissue using the RNeasy total RNA isolation system (Promega, Southampton, UK). 5 µg aliquots of RNA were separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde and transferred to nylon membrane as described previously (Barber et al., 1992). Membranes were hybridised sequentially with cDNA probes to ovine β -lactoglobulin and rat IGFBP-5 or an antisense riboprobe to murine β -casein. The ovine β -lactoglobulin probe was a 300 bp *EcoRV*-*Bam*HI fragment of targeting vector pBJ41 (Sola et al., 1998) corresponding to exons 5, 6 and 7 (3' UTR). The IGFBP-5 cDNA corresponded to a *Sac*II-*Xho*I fragment within exon 1 of the rat cDNA (Shimasaki et al., 1991). The β -casein probe was an antisense riboprobe corresponding to nucleotides 55-382 of the mRNA (Yoshimura et al., 1986). The hybridisation was performed as described previously (Barber et al., 1992). The filters were exposed to a Kodak phosphor screen and the resulting images were scanned using a Molecular Dynamics (Sunnyvale, CA, USA) phosphorimager 445 SI.

Assessment of mammary development

Wild-type and transgenic dams were mated with male wild-type Tuck's No 1 mice and checked for vaginal plugs to determine the day of mating (day 1 of pregnancy). Dams were killed by cervical dislocation on days 10 or 15 of pregnancy or on day 2 of lactation (parturition=day 1 of lactation). The 4th mammary gland was removed to prepare a whole mount for histological examination as described below. After photography portions of the whole mount were embedded in paraffin for sectioning and staining with Haematoxylin

and Eosin. Glands were also frozen in liquid nitrogen for further analyses.

Assessment of lactation performance

All dams were mated with wild-type mice. At parturition, litters were adjusted to 8 in number. Pups were weighed daily to monitor growth and, if losses occurred, replacement pups were added to maintain litter size. In a second study, transgenic and wild-type dams were paired and their litters were exchanged on a daily basis to ensure that all of the effects on pup growth could be ascribed to the dam and not to her transgenic offspring. On day 10 of lactation the dams were killed by cervical dislocation and the second to fifth abdominal glands removed and weighed. Glands were either fixed for histological analysis as described below or stored in liquid nitrogen for determination of DNA content (Labarca and Paigen, 1980), acetyl-CoA carboxylase activity (the rate-limiting enzyme for de novo fatty acid synthesis) (Barber et al., 1992), plasmin and plasminogen activities (Tonner et al., 2000) and caspase-3 activity (R&D Systems Europe Ltd, Abingdon, UK). The caspase-3 assay was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide resulting in the release of the p-nitroaniline moiety. This was quantified spectrophotometrically at 405 nm. Tissues were homogenised in the lysis buffer provided (250 mM Hepes, pH 7.4, 1% Chaps, 50 mM DTT, 20 mM EDTA).

Treatment of transgenic mice with R3-IGF-I

Transgenic mice were mated and on day 8 of pregnancy were anaesthetised with halothane and a 100 µl osmotic minipump (Alza Corporation, Palo Alto, CA) was implanted subcutaneously in the interscapular region. The pump was designed to deliver 50 µg/day of R3-IGF-I (GroPep, Adelaide, Australia) in 0.1 M acetic acid for a period of 14 days, i.e. for the remaining period of pregnancy. At parturition litters were adjusted to 8 in number and weighed daily until dams were killed on day 10 of lactation.

Treatment of transgenic mice with GH

Transgenic mice were mated and from day 10 of pregnancy they received 300 µg of recombinant bovine GH (Monsanto) in 100 µl of 0.75% NaHCO₃ daily until parturition. At parturition litters were adjusted to 8 in number and weighed daily until the dams were killed on day 10 of lactation. Mammary glands were removed and used, as indicated in the results, as well as for whole-mount analysis and histology.

Preparation of whole mounts of mammary gland

Individual mammary glands were placed on microscope slides, carefully spread with forceps and allowed to air dry for 10 minutes. They were then fixed in 10% formalin for 2-4 hours and washed in 70% ethanol for 15 minutes followed by a gradual change into distilled water. They were then stained overnight in a solution of 0.2% carmine, 0.5% aluminium potassium sulphate. After overnight staining, the gland was washed for 15 minutes in 70%, 95% and 100% ethanol before immersion in HistoClear for 5 minutes and finally mounting in DPX (VWR International, Poole, UK).

Histological analysis of mammary tissue

Mammary samples were fixed overnight in 4% paraformaldehyde, embedded in paraffin and 5-7 µm sections were cut and stained in Haematoxylin and Eosin.

Determination of IGFBP-5 concentrations in milk and serum

In order to verify co-integration of BLG and IGFBP-5, transgenic females were mated and, on day 10 of lactation, a milk sample was obtained as described previously. Milk samples were then examined for the presence of IGFBP-5 using the ¹²⁵I-IGF-I ligand blotting technique as described previously (Hossenlopp et al., 1986) and a

radioimmunoassay for IGFBP-5 developed in our laboratory. Briefly, ¹²⁵I-rIGFBP-5, IGFBP-5 standards and unknown samples, were incubated with a sheep antiserum to rIGFBP-5. After overnight incubation antibody-bound radioactive label was precipitated with an anti-sheep serum, centrifuged at 2000 g for 30 minutes, decanted, and the pellets, containing the antibody-bound IGFBP-5, counted in a gamma counter. The assay sensitivity was <15 ng/ml.

Proliferation studies

In the initial study mice received a single injection of bromodeoxyuridine (BrdU: 150 µl of 1 mg/ml) 2 hours before death on day 15 of pregnancy. In a second study mice were injected from day 16-20 of pregnancy, twice daily before killing on the day of parturition.

Mammary tissue was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 µm. For the detection of BrdU incorporation, sections were pretreated with 50 µg/ml protease (S4508, Sigma, Dorset, UK) and any endogenous peroxidase activity was inhibited by treating with 2% (v/v) H₂O₂ in methanol for 10 minutes at room temperature. To denature the DNA the sections were incubated with 2 M HCl for 1 hour at 37°C. Sections were incubated with the primary antibody (M0744, DAKO, Cambridge, UK) diluted to 1:20 in 0.5% (w/v) bovine serum albumin in PBS for 1 hour and with the secondary antibody, antimouse biotinylated Ig, for 1 hour. Streptavidin biotin complex (K0377, DAKO, Cambridge, UK) was then applied to sections according to manufacturer's instructions and the peroxidase activity was detected by incubating sections with a 0.5 mg/ml solution of 3',3'-diaminobenzidine dihydrate (DAB). Cell nuclei were counterstained with Mayer's Haematoxylin. Cells that stained positive for BrdU was quantified by using an image analysis system (Leica Q500MC, Nussloch, Germany) where at least 1000 nuclei per slide were counted from at least 6 separate fields. Results are represented as the percentage of cells that stained positive for BrdU.

Immunoblotting

Preparation of tissue homogenates, determination of protein concentrations and western immunoblotting was carried out exactly as described previously (Jeay et al., 2000). Uniformity of protein loading and transfer was confirmed by Ponceau Red staining of the membrane prior to blocking and incubation with antibodies. The antibodies used were, anti-IGF receptor (α-IR-3) (Oncogene Research Products, Boston, USA), diluted 1:1000, anti-Akt and anti-phospho-Akt (Cell Signalling Technology), diluted 1:1000, anti Bcl-2 and anti Bcl-x_{S/L} (Santa Cruz Biotechnology Inc, USA) diluted 1:500. Secondary antibodies were peroxidase labelled anti-mouse or anti-rabbit, as appropriate (Amersham Pharmacia Biotech), diluted 1:5000 or 1:4000 respectively. Detection involved the enhanced chemiluminescence detection system of NEN Life Science Products Boston, US), according to the manufacturer's instructions.

For determination of IGF-receptor phosphorylation, 250 µg of mammary gland supernatants were incubated overnight with anti-IR-3 antibody (diluted 1:1000). The primary antibody was then precipitated using protein A Sepharose (Amersham Pharmacia Biotech), the precipitate was washed extensively and then solubilised in Laemmli buffer before performing western blotting as described above using an anti-phosphotyrosine antibody (Upstate Biologicals, Lake Placid, US) diluted 1:4000 followed by an anti-mouse antiserum and detection by chemiluminescence as above. Quantification of bands was performed using Scion Image analysis.

Oligonucleosomal laddering

DNA was isolated from mouse mammary glands using the Wizard Genomic DNA Purification Kit according to the manufacturers instructions (Promega, Southampton, UK) and the DNA was quantified by fluorimetric assay (Labarca and Paigen, 1980). DNA was labelled by incubating 2 µg DNA in 100 mM Tris-HCl, pH 7.2,

containing 10 mM MgCl₂ with 100 nCi [³²P]dCTP and 5 U of DNA polymerase (Klenow fragment) in a 50 µl volume at room temperature for 10 minutes. The reaction was stopped by adding EDTA (final concentration 10 mM), and the DNA was alcohol precipitated in the presence of ammonium acetate and 15 µg glycogen overnight at -80°C. DNA was recovered by centrifugation at (16,000 g), washed in 70% alcohol, air dried and resuspended in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA. DNA (1 µg) was subjected to electrophoresis on 1.8% agarose gels in TBE with 5 µg/ml ethidium bromide. The gel was then fixed in 10% methanol/10% acetic acid, dried and exposed to a phosphorimage cassette and the image analysed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Growth curves were analysed using repeated measures ANOVA after adjustment for the lack of independence between errors using the Greenhouse-Geisser method. A more detailed analysis was performed by fitting logistic curves to derive slope, upper and lower asymptotes to permit us to undertake independent sample *t*-tests for wild-type versus transgenic mice. Where multiple comparisons were required, Fisher's LSD test was used. For one data set REML was used because of imbalances in the data. In this case both the Wald test and the deviance were used to test for significance.

The remaining data was analysed by ANOVA with post-hoc testing by Fisher's LSD.

RESULTS

IGFBP-5 levels are normally undetectable in the lactating mammary gland but are dramatically upregulated 2 days after litter removal (Tonner et al., 1997). We generated transgenic mice that expressed IGFBP-5 during pregnancy and lactation. Of the 12 lines of transgenic mice generated, 11 expressed the BLG-driven IGFBP-5 transgene with IGFBP-5 clearly detectable in milk samples (Fig. 1). The highest level of expression in the transgenic lines reached values equivalent to, or greater than, those of the wild-type involuting gland. No correlation between copy-number and expression level was evident (data not shown). One line, BIP/114, was selected for further analysis because it had the highest levels of protein expression although these mice achieved levels only 2-fold higher than the maximum physiological concentrations occurring in wild-type mice. Southern blot analysis indicated that this line carried approximately 10 copies of the transgene at one integration site (data not shown). Expression of the transgene was evident in the mammary gland as early as day 10 of pregnancy, increasing to maximum steady-state levels of mRNA by day 10 of lactation (Fig. 2). Given that the transgene rescue expression strategy (Clark et al., 1992) was used, and

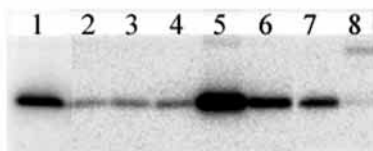


Fig. 1. ¹²⁵I-IGF-I western ligand blot analysis of IGFBP-5 expression in milk from transgenic mice. Lane 1: 48 hours involution (positive control); lanes 2-7: transgenic lines, 211, 285, 289, 114 (used in this study), 137 and 292 respectively. Lane 8: from lactating wild-type mice (negative control).

since one of the probes used for northern blot analysis consisted of a sequence present in the 3' UTR of both BLG and IGFBP-5 transgenes, two transcripts were detected. The larger transcript coded for IGFBP-5 while the smaller, 800 nucleotide, transcript corresponded to BLG. This was confirmed using a cDNA probe derived from the coding sequence of IGFBP-5 (Fig. 2). Transgene expression was not apparent in any other tissues examined.

IGFBP-5 concentrations in milk of transgenic animals were found to be 138±21 µg/ml (mean and s.e.m. of 6 animals) compared with values 1000-fold lower (170±58 ng/ml) in wild-type animals. IGFBP-5 concentrations in serum were 152±25 and 129±11 ng/ml on day 2 of lactation in transgenic and wild-type animals respectively. Serum IGFBP-5 concentrations were significantly decreased (*P*<0.01) during pregnancy although there was again no difference between transgenic (85±9) and wild-type (76±18) animals. These results suggest that IGFBP-5 does not act in an endocrine fashion after secretion from mammary cells.

Pregnancy rates and litter size were unaffected in transgenic females (results not shown). However, immediately postpartum it was evident that these females showed impairment of lactogenesis (Fig. 3A). During the first 24-48 hours the pups failed to thrive and even after 48 hours, although pup weight gain increased, it did not reach the same rate as that of wild-type pups. Subsequently the glands of transgenic mice produced milk at around half the rate of wild-type animals suggesting some amelioration of the phenotype (Fig. 3A). Statistical analysis indicated that the rate of weight gain of pups (gradient of curve) at day 5 was significantly greater (*P*<0.001) for wild-type than transgenic dams. The delay in pup growth was also identified statistically by the fact that curve fitting revealed that the point of inflexion was significantly greater (*P*<0.01) for transgenic dams than for wild-type dams. Pup weight gain was significantly greater (*P*=0.006) for wild-type animals within 24 hours of birth. In order to rule out the possibility that transgenic pups developed abnormally, despite receiving normal amounts of milk from the transgenic dams, a second study was initiated in which litters were exchanged on a daily basis between wild-type and transgenic females. This study clearly showed that all litters failed to thrive during the periods spent with the transgenic dam but grew normally while with the wild-type dam (Fig. 3B). Again, statistical analysis revealed that the difference in growth rate of the pups when nursed by the wild-type or transgenic dams was highly significantly different (*P*=0.011). This conclusively demonstrated that the transgenic dams were failing to produce either sufficient quantity or quality of milk.

Analysis of the milk derived from transgenic dams demonstrated no changes in the major milk proteins (results not shown). The poor pup growth rates could have been caused by reduced rates of milk production from a normal number of mammary epithelial cells, by a normal production rate from a reduced number of cells, or a combination of both. In Fig. 4 we clearly demonstrate a marked reduction in both the weight and DNA content of the mammary glands of transgenic dams, suggesting that the major effect is upon mammary cell number. Further support for this was provided by measurement of total acetyl-CoA carboxylase activity, which, although lower in transgenic animals, when expressed per mammary gland, [4.7±1.6 (mean ± s.e.m.) versus 9.4±1.2 µmol/minute/gland in

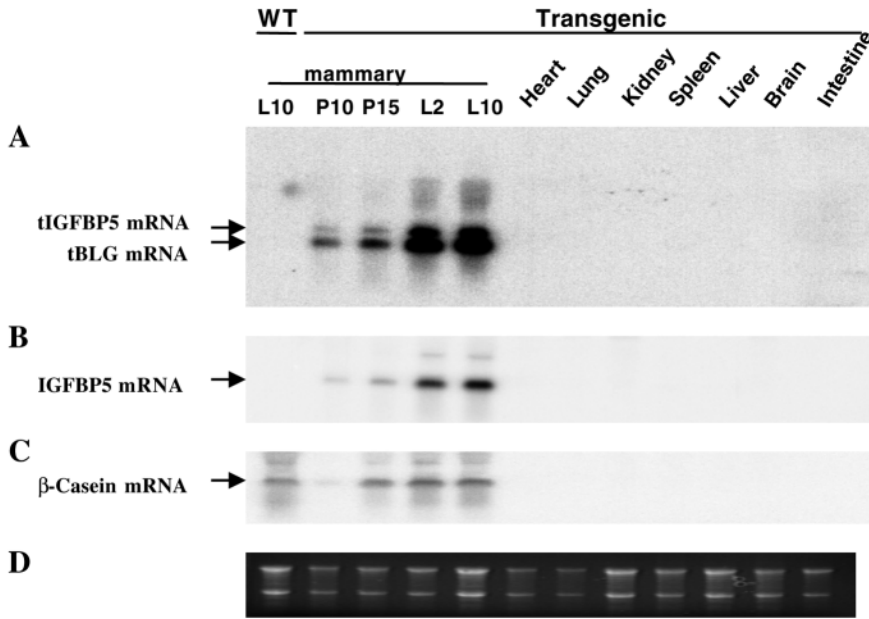


Fig. 2. IGFBP-5, BLG and β -casein mRNA expression in various tissue of transgenic mice. Blots were probed with (A) cDNA from the 3' UTR of BLG present in both the BLG and IGFBP-5 construct; thus two transcripts are present, the upper band representing IGFBP-5 and the lower band representing BLG; (B) with IGFBP-5 cDNA and (C) β -casein riboprobe. (D) Ethidium bromide stained RNA as loading control.

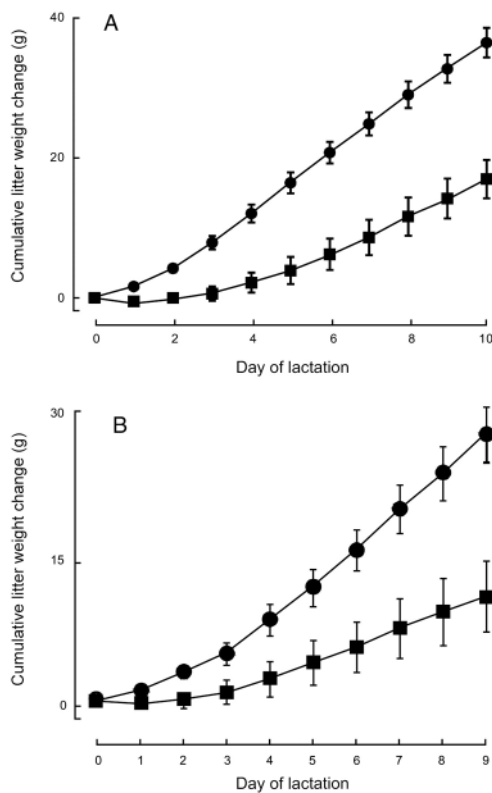


Fig. 3. Cumulative litter weight change (A) in pups suckled by wild-type dams (circles) or transgenic dams (squares), and (B) in pups alternately suckled by wild-type dams (circles) and transgenic dams (squares), where litters were exchanged on a daily basis between wild-type and transgenic dams. Note that the weight gains in B do not represent the actual weight gain of individual litters but represent the cumulative weight gain achieved by litters (whether transgenic or wild type) when being nursed by the wild-type or transgenic dam. Values are mean \pm s.e.m. of 5-6 mice.

transgenic and wild-type mice respectively], was normal in transgenic dams, when expressed on a per cell basis (1.4 ± 0.2 versus 1.5 ± 0.5 $\mu\text{mol}/\text{minute}/\text{mg}$ DNA), indicating that de novo fatty acid synthesis was unaffected in the surviving cells on day 10 of lactation.

Since mammary function was impaired during early lactation we also examined mammary development during pregnancy. As early as day 10 of pregnancy, mammary DNA content was less than with wild-type animals (Fig. 5). Statistical analysis revealed this difference between transgenic and wild-type animals to be significant ($P < 0.01$). Whole-mount analysis revealed that ductal branching and alveolar development were already impaired by day 10 of pregnancy (Fig. 6). Whereas alveolar structures were apparent on day 10 of pregnancy in wild-type animals and were relatively abundant on day 15, they were greatly reduced in transgenic animals and, instead, large ductular structures were evident

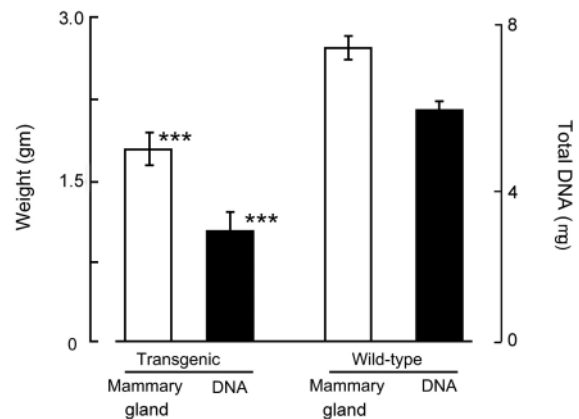


Fig. 4. Mammary gland weight and total DNA content on day 10 of lactation in IGFBP-5 transgenic and wild-type mice. Values are means \pm s.e.m. of 10-11 mice. *** $P < 0.001$ compared with wild-type mice.

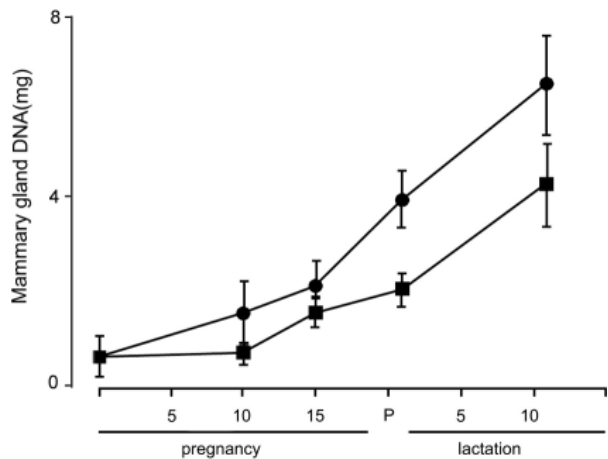


Fig. 5. DNA content in mammary glands of wild-type mice (circles) or IGFBP-5 transgenic mice (squares) at various stages of pregnancy and lactation. Values are means \pm s.e.m. of 3-7 mice.

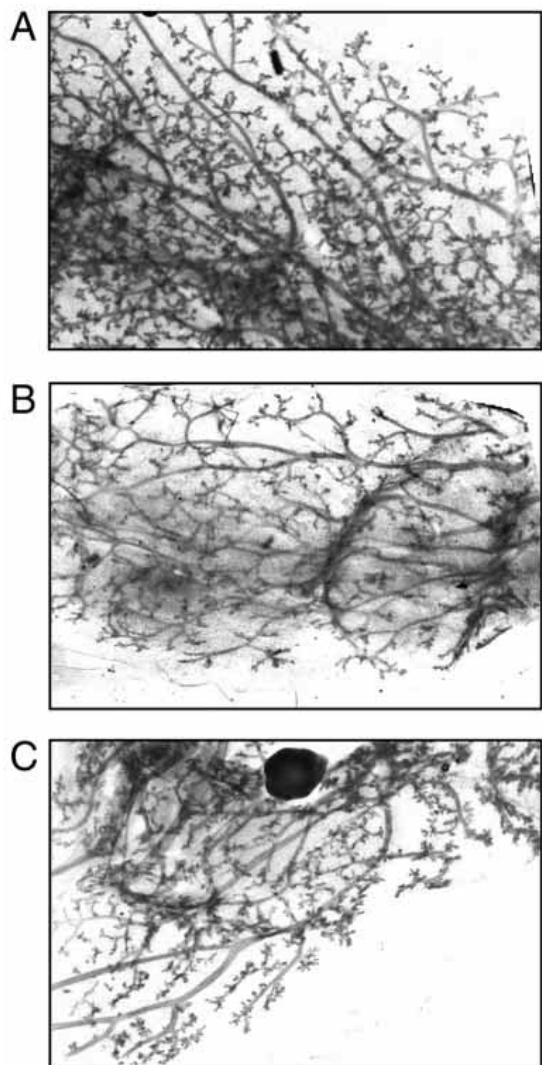


Fig. 6. Whole mounts of mouse mammary glands showing ductal branching on day 10 of pregnancy in a wild-type mouse (A) and two transgenic mice (B,C).

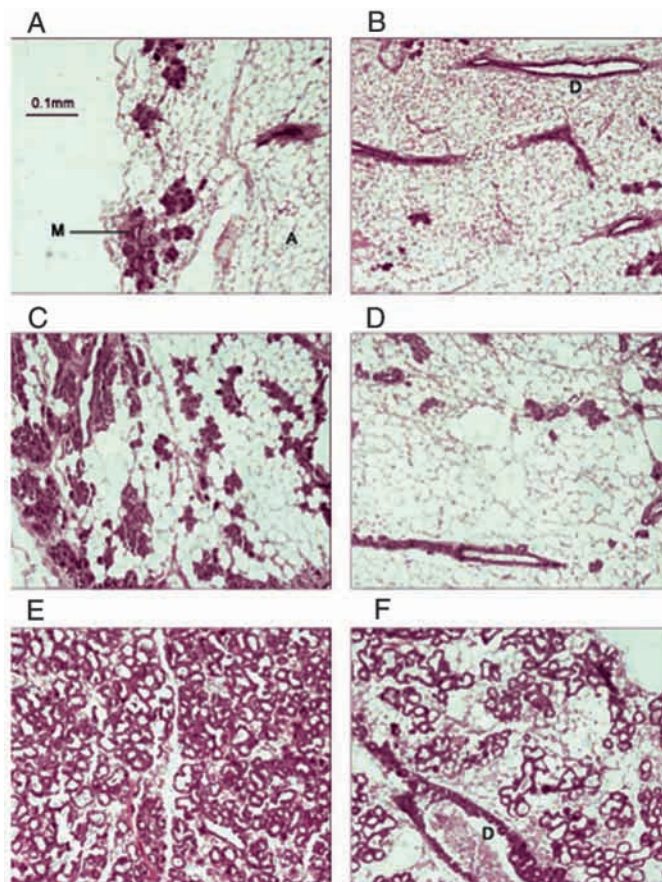


Fig. 7. Sections of wild-type (A,C,E) or IGFBP-5 transgenic (B,D,F) mammary gland on day 10 (A,B) or 15 (C,D) of pregnancy or day 2 of lactation (E,F). M, mammary epithelium, D, duct, A, adipose tissue.

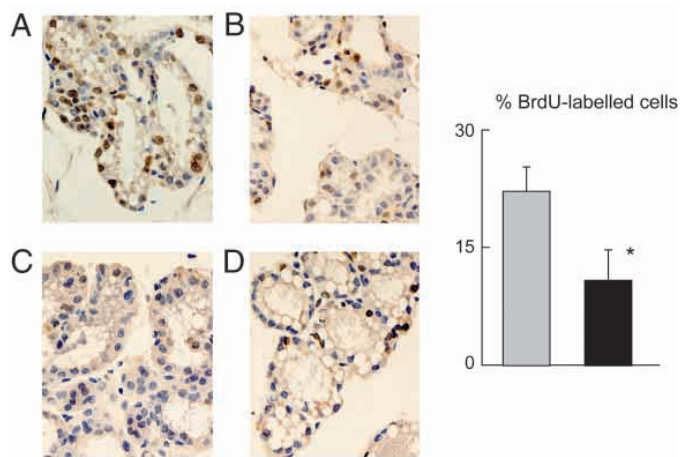


Fig. 8. BrdU-labelled cells in 2 wild-type mice (A,B) and two transgenic mice (C,D). Representative sections are shown for animals with the highest and lowest incorporations for each group. Right hand panel shows quantitative analysis of the data. Wild-type mice, grey bar; transgenic mice, black bar. At least 1500 nuclei were counted from at least three random fields. * $P < 0.05$ compared to wild type.

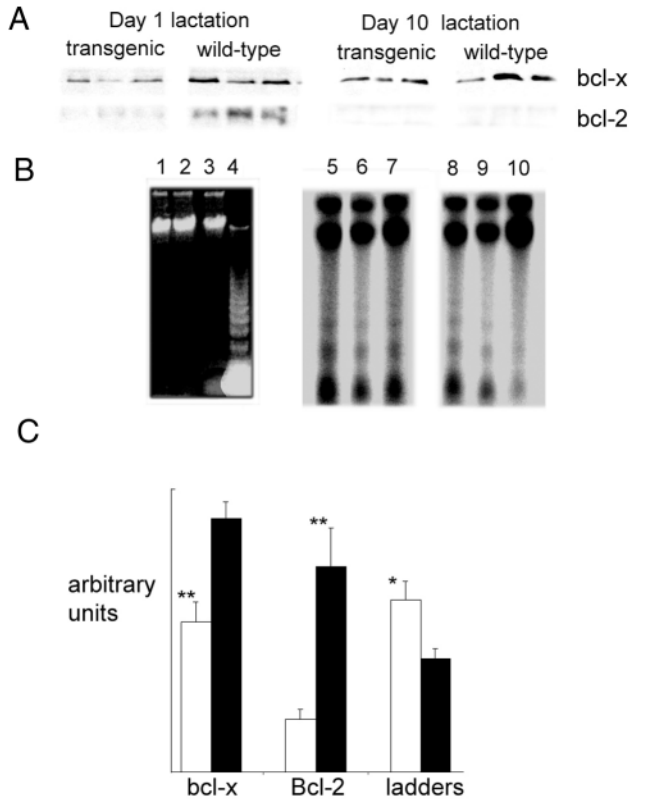


Fig. 9. (A) Western immunoblots for Bcl-2 and Bcl-x_L in day-1 and day-10 lactating mammary glands from wild-type and transgenic mice. Three representative animals are shown and the bar chart in C illustrates quantitative analysis of the data. (B) Endonucleosomal ladders in mammary gland DNA preparations. Lanes 1-4 show ethidium bromide detection, and lanes 5-10 show $[^{32}\text{P}]$ dCTP-end labelling. Lane 1, wild type, day 2 of lactation; lane 2, transgenic, day 2 of lactation; lane 3, wild type, day 10 of lactation; lane 4, day 2 of involution (positive control). Lanes 5-7 are samples from 3 representative day-1 lactating transgenic mice and lanes 8-10 are samples from 3 representative day-1 lactating wild-type mice. (C) Quantitative analyses for values on day 1 of lactation. White bars, transgenics, black bars, wild type. Values are mean \pm s.e.m. for 5-6 mice per group. * P <0.05, ** P <0.01 compared with wild-type mice.

(Fig. 7A-D). On day 2 of lactation, histological analysis indicated that, although alveolar development did occur in transgenic animals, invasion of the mammary fat pad was impaired (Fig. 7). Large areas of adipose tissue remained in the gland of transgenic mice, in contrast to wild-type animals, where almost the entire fat pad was filled by mammary epithelium (compare Fig. 7E and F).

In order to explore the mechanism of impairment of mammary gland development we initially examined rates of BrdU incorporation on day 15 of pregnancy, when proliferation rates are high. However, we were unable to demonstrate any significant difference in proliferation at this time (results not shown). We then conducted a study around parturition when transgene expression increases considerably and demonstrated a 50% decrease in proliferation rates in the transgenic animals (Fig. 8).

Preliminary observations using the in situ TUNEL technique

failed to demonstrate any significant increase in apoptotic rates in late pregnancy (results not shown). However, recognizing that expression of the transgene was greatly augmented at parturition we looked in greater detail at this time point, using quantitative DNA laddering techniques. In preliminary experiments we used a non-radioactive technique to examine endonucleosomal ladders. This clearly demonstrated DNA ladders during forced involution of the mammary gland on day 10 of lactation (when apoptotic indices are at their highest) but failed to show anything in transgenic animals on day 2 of lactation (Fig. 9B, lanes 1-4). We thus used a more sensitive radiolabelling technique to detect ladders and were able to demonstrate a significant 37% increase in DNA laddering at day 1 of lactation (Fig. 9B, lanes 5-10). These results were supported further by the demonstration of decreases in the concentrations of two pro-survival molecules, Bcl-2 and Bcl-x_L on day 1 of lactation (Fig. 9A,C). By day 10 of lactation Bcl-2 was no longer detectable in wild-type or transgenic animals, whilst the effect of IGFBP-5 on Bcl-x expression was lost.

In addition, on day 2 of lactation, when milk production was most strongly impaired, caspase-3 and plasmin activities were significantly increased (Fig. 10). The increase in plasmin was partly a result of increased plasminogen content of the tissue (results not shown) but principally due to increased conversion of plasminogen to plasmin (23 ± 3 versus $11 \pm 2\%$, mean \pm s.e.m. in transgenics and wild-types respectively; P <0.002). Increases in both caspase-3 and plasmin activation would be anticipated during impaired function of the mammary gland.

These effects upon caspase-3 and plasmin were also lost by day 10 of lactation supporting the concept that the phenotype is lost at this stage.

We next addressed the question of the mechanism of action of IGFBP-5 by examining aspects of the IGF signalling cascade. The concentration of IGF receptor (IGFR) was unaffected in transgenic animals whereas IGFR phosphorylation was significantly impaired on day 1 but not

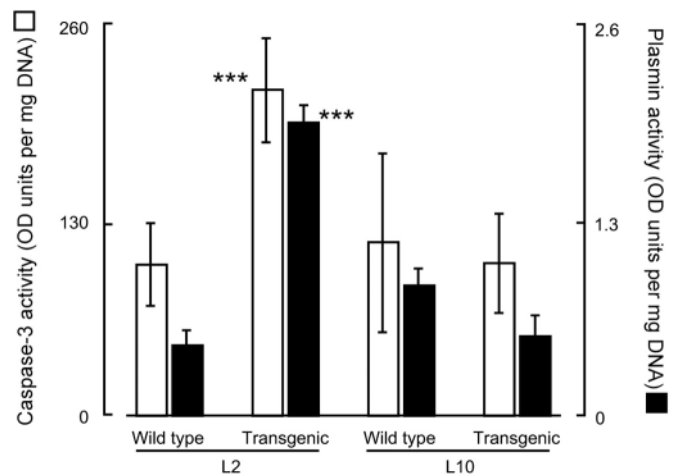


Fig. 10. Caspase-3 and plasmin activities in mammary homogenates of wild-type or transgenic females on day 2 (L2) or 10 (L10) of lactation. Values are means \pm s.e.m. of 3-7 mice. *** P <0.001 compared with wild type on day 2 of lactation for caspase-3 or plasmin activity.

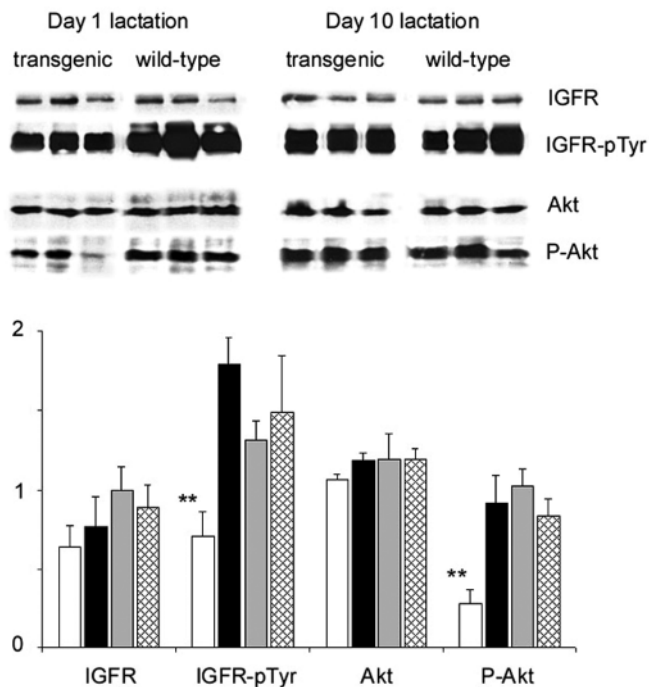


Fig. 11. Western immunoblots for IGF receptor, IGF receptor phosphorylation, Akt and phospho-Akt in mammary tissue from lactating wild-type and transgenic mice. Samples from three representative mice of each type are shown. Quantitative analysis is shown in the bar chart. White bars, transgenic L1; black bars, wild-type L1; grey bars, transgenic L10; cross-hatched bars, wild-type L10. Values are means \pm s.e.m. for 5 mice. ** $P < 0.01$ compared with wild-type mice.

day 10 of lactation (Fig. 11). Similarly, Akt levels were unaffected but phospho-Akt levels were significantly impaired on day 2 of lactation but not on day 10 (Fig. 11).

We finally conducted two studies to increase IGF concentrations, either using GH to increase endogenous IGF-I, or exogenous R3-IGF-I, in order to attempt to ‘rescue’

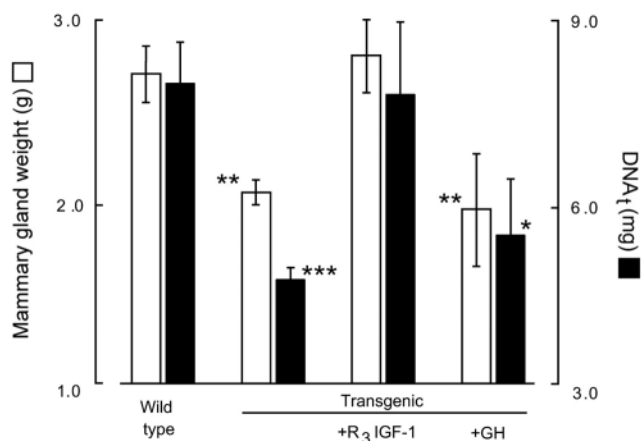


Fig. 12. Mammary gland weight and DNA content in wild-type mice and in transgenic mice receiving R3-IGF-I or GH. Values are means \pm s.e.m. of 4-13 observations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with respective wild-type values.

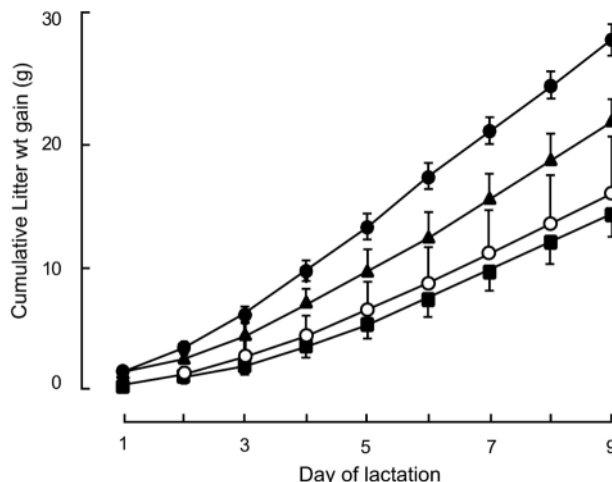


Fig. 13. Cumulative litter weight gain for wild-type dams (solid circles), transgenic dams (squares) or transgenic dams receiving R3-IGF-1 (triangles) or GH (open circles). Values are means \pm s.e.m. of 4-13 observations.

mammary development. Mammary gland weight was significantly greater ($P < 0.002$) in wild-type than transgenic animals, as was total mammary DNA content ($P < 0.001$). GH treatment of transgenic animals failed to affect mammary weight or DNA content compared with untreated transgenic animals (Fig. 12). In contrast, R3-IGF-I treatment increased mammary gland weight and DNA content, such that they were similar to those of wild-type animals. These changes in mammary gland parameters were reflected in pup weight gains (Fig. 13) where pups nursed by wild-type animals grew significantly faster than untreated transgenic animals ($P < 0.001$), or pups from GH-treated transgenic animals ($P < 0.05$). Pups from transgenic animals treated with R3-IGF-I grew at an intermediate rate, which was significantly greater than that of untreated transgenic animals ($P < 0.03$) but significantly less than pups nursed by wild-type animals ($P < 0.05$).

DISCUSSION

We have produced transgenic mouse lines expressing IGFBP-5 in the mammary gland, using a mammary-specific promoter, BLG, in order to test our hypothesis that IGFBP-5 is apoptotic in the mammary gland. The amount of IGFBP-5, produced in milk, varied between these lines, with one line (BIP/114) achieving concentrations in the range of 100-150 mg/l, similar to those achieved physiologically during normal mammary involution. Thus the phenotype of the IGFBP-5 transgenic mice was achieved with physiological, rather than pharmacological levels of the binding protein. When transgenic females were mated with wild-type males they showed impaired mammary function. This was particularly noticeable during the first 24-48 hours postpartum when pup weight gain was severely compromised. Subsequently pup growth was improved although it never achieved the levels of wild-type mice. Transgenic animals produced approximately 50% of normal milk yield, and this was shown to be principally

due to a reduction in the number of mammary epithelial cells, since DNA content of the glands was approximately 50% of wild-type values. In addition, acetyl-CoA carboxylase activity (the rate-limiting enzyme for de novo fatty acid synthesis) was equivalent in transgenic and wild-type animals when activity was expressed per cell, indicating normal synthetic capacity of the mammary gland.

Examination of the glands during pregnancy indicated that development was impaired, with reductions in both ductal branching, and alveolar end bud formation resulting in decreased invasion of the mammary fat pad and ultimately a decrease in the number of alveoli. At this time the alveolar epithelium is not fully polarised and tight-junctions are 'leaky' suggesting that IGFBP-5 would have free access to the basolateral surfaces where IGF-I acts. This situation also pertains immediately postpartum (consistent with the greatly impaired milk production at this time). Within 24-48 hours however, tight junctions restrict apical-basal transport via this paracellular route (Nguyen and Neville, 1998; Nguyen et al., 2001) and, since IGFBP-5 is secreted into milk, its biological effects are probably greatly attenuated. This would explain the recovery in pup weight gain that occurs around days 2-3 postpartum. The fact that these animals show only partial impairment of mammary development could reflect the fact that the transgene is expressed at relatively low levels during pregnancy and thus may not totally neutralise IGF bioavailability, or alternatively that compensatory mechanisms may exist to overcome the absence of IGF action in the mammary gland. Our data do not allow us to distinguish between these two possibilities.

When cellular function or alveolar integrity are compromised in the mammary gland, this is accompanied by an increase in caspase-3 activity (Marti et al., 2000; Marti et al., 2001) and an increased conversion of plasminogen to plasmin (Ossowski et al., 1979; Busso et al., 1989; Tonner et al., 2000). Both caspase-3 and plasmin activities were significantly increased on day 2 of lactation in transgenic mice providing strong evidence that expression of IGFBP-5 was able to promote inappropriate apoptosis and extracellular remodelling at this time similar to that which occurs during normal mammary involution (Marti et al., 2000; Marti et al., 2001; Ossowski et al., 1979; Busso et al., 1987; Tonner et al., 2000). The demonstration of abnormal levels of caspase-3 and plasmin in early lactation, but normal levels of caspase-3 and plasmin on day 10 of lactation, adds further support to the proposal that the eventual closure of tight junctions prevents access of IGFBP-5 to the serosal side of the mammary gland, with a consequent loss of its apoptotic capacity between day 2 and day 10 of lactation. Both Bcl-2 and Bcl-x_L are pro-survival molecules (Adams and Cory, 2001) and, recently, conditional deletion of the Bcl-x_L gene in the mammary gland has been shown to accelerate the rate of apoptosis (Walton et al., 2001). Both Bcl-2 and Bcl-x_L protein levels were decreased on day 1 of lactation consistent with increased cell death and we were able to confirm this by demonstrating an increase in the level of endonucleosomal ladder formation in the glands of IGFBP-5 transgenic mice at this time. Once again, the effects of the transgene were not apparent on day 10 of lactation, although in the case of Bcl-2 this was due to the fact that we were unable to detect Bcl-2 in either wild-type or transgenic animals at this time. These results are consistent with those of Metcalfe et al.

(Metcalfe et al., 1999) who demonstrated a loss of Bcl-2 expression in the mammary gland between pregnancy and day 9 of lactation. In addition these authors proposed that mammary epithelial cells are primed for apoptosis at parturition by de novo expression of Bak and Bad but that this is prevented by anti-apoptotic members of the Bcl-2 family. The decreased expression of two pro-survival Bcl family members in our transgenic animals adds weight to this hypothesis. The protection that IGF-I confers against apoptosis is known to occur through the activation of PI3-kinase and Akt, followed by phosphorylation-dependent inactivation of the proapoptotic protein Bad (Datta et al., 1997). Our findings of altered levels of other Bcl-2 family members in IGFBP-5 transgenic animals adds further support to the importance of this pathway in cell survival.

We also provided evidence for reduced rates of cell proliferation around parturition but not on day 15 of pregnancy. It is our belief that the effectiveness of the transgene increases as its expression increases, in particular as parturition approaches. Its effects are probably maximal immediately postpartum when gene expression is high but tight junctions have not yet formed. Subsequently, when tight junctions close, the majority of the IGFBP-5 is secreted into milk rather than into the extracellular environment, with a subsequent loss of all of the phenotypic changes described. This may explain the partial rather than full impairment of mammary function.

Certain actions of IGFBP-5 have been proposed to occur independently of its ability to inhibit IGF action (Andress, 1998) and we therefore decided to examine whether IGF signalling was impaired by IGFBP-5 and to determine if R3-IGF-1, an analogue of IGF-1, could 'rescue' the phenotype of the IGFBP-5 transgenic mouse. Clear evidence of impairment of IGF signalling was demonstrated by reduced phosphorylation of both the IGF receptor and reduced phosphorylation of Akt in IGFBP-5 transgenic mice. During involution of the mammary gland both the quantity and the phosphorylation status of the IGF receptor and Akt decrease (Hadsell et al., 2001; Schwertfeger et al., 2001). Furthermore Schwertfeger et al. (Schwertfeger et al., 2001) demonstrated that constitutive expression of Akt could delay mammary involution. R3-IGF-I is an analogue that binds very weakly to IGFBPs (King et al., 1992; Francis et al., 1992) and we showed that this was able to completely overcome the effects of IGFBP-5 on mammary growth in terms of weight and DNA content but, whilst improving milk synthesis, it could not fully restore it. These findings suggest that IGF-I serves to promote proliferation and survival of mammary epithelial cells but that, at the same time, it impairs differentiation. Increased IGFBP-5 secretion has been associated with cellular differentiation in myoblasts (Rotwein et al., 1995) and Schwann cells (Cheng et al., 1999) although the nature of its role is under debate with studies suggesting stimulatory (Ewton et al., 1998) or inhibitory (James et al., 1996) effects of IGFBP-5 on IGF-I action. It is conceivable that the response of the cell is dependent upon the relative balance between IGFBP-5 and IGF-1. Thus, whilst an excess of IGFBP-5 results in cell death by neutralizing IGF-1 (as in natural involution or the IGFBP-5 transgenics) and an excess of IGF-1 results in mitogenesis and a lack of differentiation, a balance between the two may result in cell survival and differentiation. This hypothesis is currently under investigation in our laboratory and is supported

by the studies of Ewton et al. (Ewton et al., 1998) who showed concentration-dependent effects of IGFBP-5 in myoblasts with high concentrations inhibiting and lower concentrations augmenting differentiation.

In contrast, our attempts to mimic the effect of R3-IGF-I with GH (to stimulate endogenous IGF-I) failed to influence the phenotype of the transgenic animals. It is likely that GH could not increase IGF-I concentrations sufficiently to overcome the inhibitory effects of IGFBP-5. This is because IGFBP-5 is produced at concentrations that are several orders of magnitude higher than IGF-I, and GH treatment typically increases serum IGF-I concentrations by only two- to threefold in this model (Flint and Gardner, 1994). The results are also consistent with our failure to inhibit the involutory process, after litter removal, using GH treatment (Tonner et al., 1997). This contrasts with the effectiveness of prolactin in delaying involution, and could be explained by the observation that prolactin dramatically suppresses IGFBP-5 synthesis (Tonner et al., 1997) which would enhance the effectiveness of endogenous IGF-I.

Although R3-IGF-I was very effective in stimulating mammary development, its failure to normalise milk yield might also reflect the fact that IGFBP-5 acts via an IGF-independent mechanism, acting through cell surface receptors of its own (Andress et al., 1998). We have also provided recent evidence that IGFBP-5 may act via an IGF-independent pathway involving interactions with plasminogen activator-inhibitor-1 (PAI-1) with resultant effects on tissue remodelling processes (Tonner et al., 2000). However, we cannot rule out the possibility that, since the BLG promoter is active before day 10 of pregnancy, irreversible phenotypic changes may have been produced before IGF-treatment commenced.

This is the first report of a transgenic mouse expressing IGFBP-5. Overexpression of IGFBP-1 results in intrauterine and postnatal growth retardation (for a review, see Murphy, 2000). In order to address the issue of whether IGFBP-3 has augmentary or inhibitory actions, IGFBP-3 transgenic mice were produced using the phosphoglycerate kinase or cytomegalovirus (CMV) promoter (Modric et al., 2001). Significant reductions in birthweight and postnatal growth were evident. Similarly, IGFBP-2, which has also been proposed as an enhancer of IGF effects, produced growth inhibition when expressed as a transgene on the CMV promoter, in growth hormone transgenic mice, which are double the normal size (Hoefflich et al., 2001).

Whatever the precise mechanism of action of IGFBP-5, our study conclusively demonstrates, for the first time, that IGFBP-5 induces impaired mammary development and function when expressed at a concentration equivalent to that which occurs during physiological involution of the mammary gland and that this effect involves increased activity of caspase-3, and plasmin, as well as decreased concentrations of Bcl-2 and Bcl-xL. These findings are consistent with increased apoptotic cell death and extracellular matrix degradation. It also represents the first demonstration that neutralization of the effects of endogenous IGF-I results in impaired mammary gland development. This adds weight to the importance of IGF-I as a survival factor for the mammary gland (Hadsell et al., 1996; Neuenschwander et al., 1996). Taken together these findings suggest that therapeutic approaches, which target inhibition of IGF action in the mammary gland, may prove successful, by

mimicking a natural, physiological process. This process may not be limited to the mammary gland since IGFBP-5 is also expressed in association with apoptosis in a variety of tissues and at various developmental stages (see Allan et al., 2001). We therefore propose that this represents a fundamental and generalised initiating event in the process of cell death and, as a consequence, control of IGF actions may have far reaching implications in cell biology.

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