High expression in involuting reproductive tissues of uterocalin/24p3, a lipocalin and acute phase protein

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During reproduction the mass and number of cells in the uterus and the mammary gland increase rapidly and then diminish more rapidly after their reproductive functions are completed. The diminishment of tissue mass, known as involution, involves an ordered series of events that includes apoptosis of resident cells, neutrophil invasion, the release of degradative enzymes and phagocytosis of cellular debris. Local signals are believed to regulate the progression of involution in each tissue. Here we show that the mammary gland and uterus express high levels of uterocalin, a protein that specifically induces apoptosis in neutrophils and other leucocytes. In the mammary gland, uterocalin expression is induced by weaning. In both tissues, uterocalin is expressed at extremely high levels such that it constitutes an average of 0.2–0.5% of the total extractable protein at its peak. Epithelial cells in the uterus and mammary gland produce uterocalin. In each case, the protein is secreted into the tissue lumen, with mammary-derived uterocalin being found in the milk. The period of highest uterocalin expression *in vivo* is consistent with the hypothesis that one of its physiological roles is to induce apoptosis of infiltrating neutrophils and thereby delay the entry of neutrophils into the tissue. It is proposed that the role of uterocalin during involution is to provide a window of time during which resident cells are protected from the degradative enzymes, free radicals and other secreted products of activated phagocytes to allow these cells to prepare to survive the processes of involution.

Key words: mammary gland, secreted protein, uterus.

INTRODUCTION

During pregnancy, the mouse mammary gland undergoes periods of rapid growth and development, followed by massive involution and tissue resorption [1,2]. These events are regulated by growth factors and hormones [3] and marked by large changes in the expression levels of certain genes [2–4]. The accumulation of milk along with the levels of circulating hormones such as prolactin and glucocorticoids are thought to regulate the process of mammary involution that involves extensive apoptosis [4–6].

Uterocalin was first identified as a superinducible protein 24000 (SIP24) secreted by quiescent Balb/c 3T3 cells after treatment with cycloheximide and growth factors [7]. The 24p3 gene was cloned from primary mouse kidney cells infected with simian virus 40 [8] and shown to encode SIP24 [9]. Because of its very high levels of expression in the post-partum uterus, this protein was named uterocalin [10]. Uterocalin is expressed in a variety of mouse tissues including the vagina, lung, spleen, uterus, epididymis, testis, liver and kidney of young mice [9,11–13]. The gene is also highly regulated *in vivo* where its expression in the uterus peaks immediately post-partum [9,11].

Uterocalin is a member of the lipocalin protein family, many members of which have characteristic functions of binding and transporting small hydrophobic molecules [14,15]. Uterocalin has been reported to bind oleic and retinoic acids [16]. The chicken equivalent of uterocalin, exFABP (extracellular fatty acid-binding protein), also binds retinoic acid and various fatty acids including oleic, linoleic, stearic and arachidonic acids [17].

Its expression as an acute phase protein suggests that uterocalin might play an anti-inflammatory role [9]. The observed expression of NGAL (neutrophil gelatinase-associated lipocalin), the human homologue of uterocalin, in epithelial tissues with a high probability of exposure to micro-organisms permits speculation that NGAL might also be part of an innate anti-microbial defence mechanism [18].

Tissue involution and the inflammatory response involve leucocyte infiltration and activation. In both instances the normal and healthy cells in the tissue are at risk of being destroyed by the ebullient neutrophil response. Mechanisms exist *in vivo* to moderate the inflammatory response that may also be used by involuting tissues to control leucocyte-induced damage. Uterocalin provides one means of suppressing leucocyte activity by its ability to induce apoptosis in neutrophils and other leucocytes [19]. Moderating the neutrophil numbers may also be a major function of uterocalin during the acute phase response of the liver when there is a rapid expansion of the neutrophil population.

Here we report that uterocalin expression is regulated in the mouse mammary gland through gestation and lactation. Extremely high levels of uterocalin expression were observed during involution in the mammary gland and uterus. Expression of the gene and protein peaks during the first stage of involution in the mammary gland after weaning and in the uterus after parturition.

While only very low levels are found in the mammary gland of the non-pregnant mouse, expression of uterocalin increases in the maternal mammary gland, starting at mid-gestation, to high levels at birth that are maintained throughout lactation. When the pups are weaned naturally, there is a peak of expression lasting approx. 4 days. At this time uterocalin protein makes up approx. 0.5% of the total protein extracted from the mammary gland. The high level of uterocalin expression during

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SGP2, sulphated glycoprotein-2; SIP24, superinducible protein 24000; PP, post-partum; PW, post-weaning.

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the first phase of involution suggests that this protein might prevent leucocyte accumulation during this period by inducing apoptosis of neutrophils that enter the tissue. A biological advantage of decreased neutrophil activity during the early phase of involution would be to allow time for cells that will survive the ensuing tissue regression to prepare and protect themselves from damage.

EXPERIMENTAL

Animals and plasmids

Virgin CF1 mice were mated, and the day of the plug designated day 0, the day of birth designated post-partum (PP) 1 and the day of weaning designated post-weaning (PW) 0. The mice were killed by cervical dislocation on the desired day, and the uterus, mammary gland and liver were removed and flash frozen in liquid nitrogen. For experiments in which natural weaning was allowed, the pups were removed between PP18 and PP21. For early involution, the pups were removed on PP8.

A 5′ segment of 24p3 cDNA was a gift from S. Hraba-Renevey (Institut d'Embryologie Moléculaire et Morphogénèse, Geneva, Switzerland). The full-length uterocalin cDNA was cloned by us using the 24p3 fragment for screening. The sulphated glycoprotein-2 (SGP2) plasmid [20] was from M. D. Griswold (Washington State University, Pullman, WA, U.S.A.). The stromelysin-1 plasmid [21] was from L. Matrisian (Vanderbilt University, Nashville, TN, U.S.A.). The rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was cloned by Fort et al. [22].

Cell culture

HC11 epithelial cells were obtained from B. Groner (Georg Speyer Haus Institute for Biomedical Research, Frankfurt, Germany) by way of L. G. Sheffield (University of Wisconsin, Madison, WI, U.S.A.) and grown until confluent in RPMI 1640 (Gibco-Life Technologies, Grand Island, NY, U.S.A.) with 0.5 % fetal calf serum, 10 units/ml each of penicillin and streptomycin, 10 ng/ml epidermal growth factor and 5 μg/ml insulin. The medium was then changed to a medium of the same content with the exception of epidermal growth factor. The cells were grown for another 2 days and then treatment with dexamethasone was initiated.

RNA isolation and Northern blotting

Tissues were frozen immediately in liquid nitrogen upon removal from the animal, then stored at -70 °C. Total RNA was isolated using Trizol Reagent (Gibco-Life Technologies) following the manufacturer's instructions. For Northern blots, 20 μg of total RNA was resolved by electrophoresis through a 1 % agarose/formaldehyde gel. The RNA was transferred by capillary action overnight to a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The blots were hybridized with the following ^{32}P-labelled probes: uterocalin (*Nco*I–*Nco*I fragment, 440 bp), rat GAPDH probe (*Xba*I–*Pst*I fragment, 1258 bp), SGP2 (*Eco*RI fragment from SGP2 plasmid) and stromelysin (*Bam*HI–*Kpn*I fragment from pG4MTRKX plasmid). Between hybridizations, the membranes were stripped by boiling two consecutive times in 0.1 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) with 0.1 % SDS for 3 min. Quantitative data were obtained by exposure to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The signal in each lane for uterocalin was normalized with the GAPDH signal, as well as with an internal standard for uterocalin (pooled mammary or uterine RNA containing uterocalin mRNA) that was included on each gel. Results from different Northern blots that were normalized to the same internal standard were compiled to provide an average of values over different experiments and from different animals.

Quantitative protein analysis and Western blots

Frozen tissues were homogenized at approx. 100 mg of tissue/ml of buffer (150 mM NaCl, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 0.15 units/μl aprotinin, 0.1 mM PMSF and 10.8 mM $NaPO_4$, pH 7.4). In a comparison study, this buffer extracted substantially more protein per mg of tissue than a standard hypotonic extraction buffer lacking detergents. The lysates were centrifuged at 16000 ***g*** for 15 min at 4 °C, and the supernatants were collected. Protein concentrations of the supernatants were determined by using the Bradford assay (Pierce Coomassie Plus; Pierce, Rockford, IL, U.S.A.), and 50 μg of total protein was resolved by SDS/PAGE through a 12.5 % gel. The proteins were transferred to a nitrocellulose membrane (Micron Separations, Westborough, MA, U.S.A.) and the blot hybridized with anti-uterocalin/SIP24 sera [23] at a 1:200 dilution. Protein A conjugated to horseradish peroxidase (Sigma, St. Louis, MO, U.S.A.) was used at a 1:10000 dilution for the secondary complex. This enabled detection by chemiluminescence with ECL reagents (Amersham, Arlington Heights, IL, U.S.A.) according to the manufacturer's instructions. To determine the amount of uterocalin in each sample, recombinant uterocalin produced from *Escherichia coli* was loaded in varying amounts to create a standard curve. The intensity of the standards and the samples was measured by densitometry, and the amount of uterocalin present was determined from the standard curve.

Milk protein fractionation

Milk was removed from mice in mid-lactation and post-weaning by first injecting 3 m-units/g of body weight of oxytocin and after 10 min stimulating the gland by massage until milk was ejected. The milk protein was fractionated by precipitation of casein at pH 4.6 and centrifugation [24]. The protein content of each fraction was determined by the Bradford assay and resolved by SDS/PAGE.

RESULTS

Uterocalin mRNA is expressed in the mouse mammary gland

The observation that uterocalin is expressed by epithelial tissues exposed to the external environment prompted examination of its expression in the mammary gland. The relative amounts of uterocalin mRNA in mammary and liver tissues were determined by Northern blot analysis of samples removed from these tissues at various times in gestation, lactation and post-weaning. Uterocalin was detected in the mammary glands at very low levels by day 5 and day 11 of gestation, and a peak of expression was seen around parturition (Figure 1). By 2 days post-partum, the average level of uterocalin mRNA expression was about 50-fold over the average level on day 5 of gestation. The uterocalin mRNA level then declined slightly and remained high during lactation. A second peak of uterocalin expression occurred on PW2, 2 days following weaning of the pups. At this time there was an average increase of approx. 100-fold over the average level on day 5 of gestation. This very high level of uterocalin mRNA expression was maintained for the first 4 days post-weaning, from PW1 to PW4. After PW4, the amount of uterocalin mRNA in the mammary gland declined to the levels present in the lactating gland and remained at this level through PW10. Uterocalin levels in the liver were very low at all time points. Thus, uterocalin

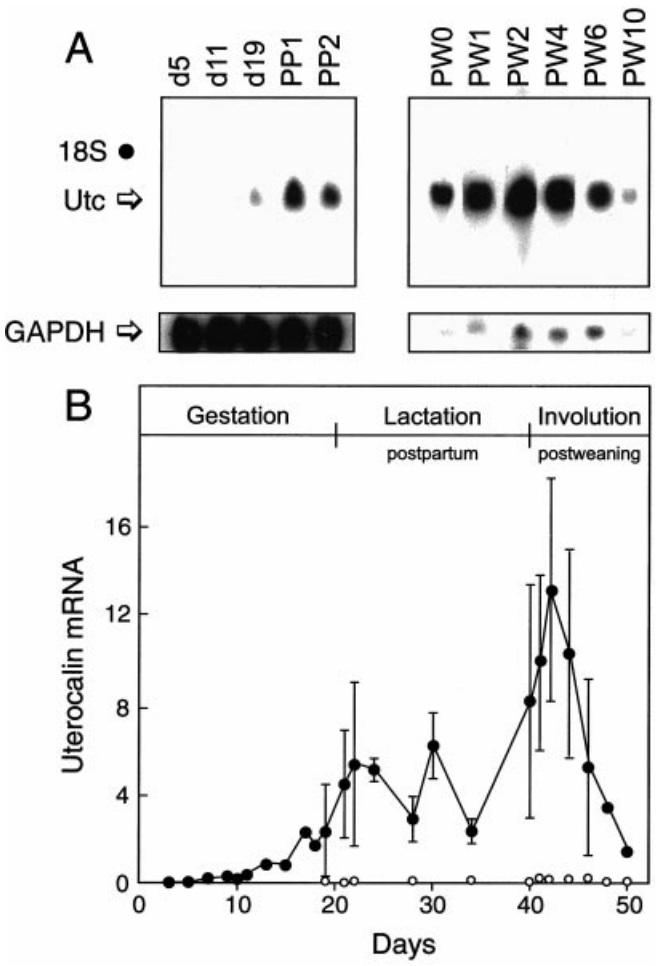

Figure 1 Expression of uterocalin during reproduction

Female CF1 mice were killed at various times during gestation, lactation and post-weaning. Mammary gland and liver tissues were collected, and total RNAs were isolated and analysed by Northern blot. The membranes were probed first with a ^{32}P-labelled uterocalin probe, and second with a ^{32}P-labelled rat GAPDH probe as a control for loading. (**A**) The top panels contain representative samples of uterocalin mRNA detected in the mammary gland at various time points in reproduction. Shown are samples from days during gestation (d5, d11, d19), post-partum (PP1, PP2), and post-weaning (PW0–PW10). The position of uterocalin (Utc) is marked. The GAPDH control probe is shown in the lower panel. Because of the large difference in expression of uterocalin during gestation and post-weaning, less RNA was loaded per channel in the samples from involuting tissue (right-hand panels). (**B**) Quantitative results in which the values for uterocalin RNA were normalized to those for GAPDH in the same sample. The amount of uterocalin present in the mammary gland and liver is plotted as a function of reproductive stage. For the mammary gland, the means of 2–5 values, each from different mice, are shown with the sample S.D. Most points are the average of 3 independent values. Exceptions are d11 (2 mice) and PW1 (5 mice). For the liver, all points are the average of two mice. Where error bars are not evident, the error is smaller than the size of the symbol. ●, Mammary gland; ○, liver.

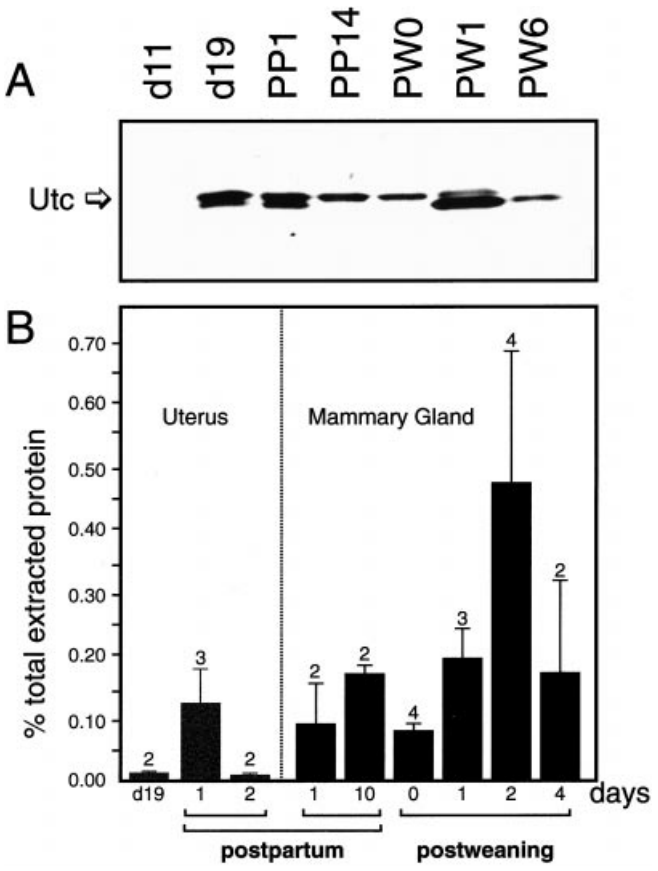

Figure 2 Levels of uterocalin protein in the mammary gland and uterus during reproduction

Mammary gland and uterine tissue homogenates were prepared and analysed by Western blot probed with anti-uterocalin serum as described in the Experimental section. (**A**) A Western blot showing that uterocalin (Utc) can be detected in the mammary gland from day 19 of gestation to 6 days post-weaning, but not on day 11 of gestation. Equal amounts of total protein were loaded in each lane. (**B**) A graph showing the percentage of the total protein contributed by uterocalin in the mammary gland and uterus. The amount of uterocalin in each band on the Western blot was determined quantitatively. Each value was obtained from an independently isolated sample taken from a different mouse. The numbers of animals used to obtain each average point are shown above each bar.

mRNA is selectively expressed in the mammary gland and not in the liver during reproduction. The highest level of uterocalin mRNA expression in the mammary gland occurs immediately post-weaning.

Uterocalin protein expression in mammary gland and uterus

To determine the level of uterocalin protein expression in the mammary gland at various reproductive stages, quantitative Western blots were performed on tissue homogenates. Uterocalin protein was first detectable in the mammary gland on day 19 of gestation and continued to be detected through 6 days post-weaning (Figure 2A). The largest amount of uterocalin was found in tissue samples removed around the time of weaning. By 2 days post-weaning, uterocalin made up approx. 0.5% of the total extracted mammary gland protein (Figure 2B). Uterine samples were also analysed by the same method for comparison. On PP1 uterocalin was on average 0.12% of the total extracted uterine protein, revealing a similar high level of expression in the uterus and mammary gland during their respective periods of involution.

Uterocalin expression after forced weaning

To determine if increased uterocalin expression in the mammary gland was a consequence of weaning, the pups were removed on day 8 post-partum. Mammary gland tissue was collected on 1, 2 or 4 days post-weaning, and the mRNA was analysed by Northern blot. A peak of uterocalin expression was seen 2 days post-weaning when there was an approx. 4-fold increase in expression over day 8 post-partum (Figure 3). The timing of the

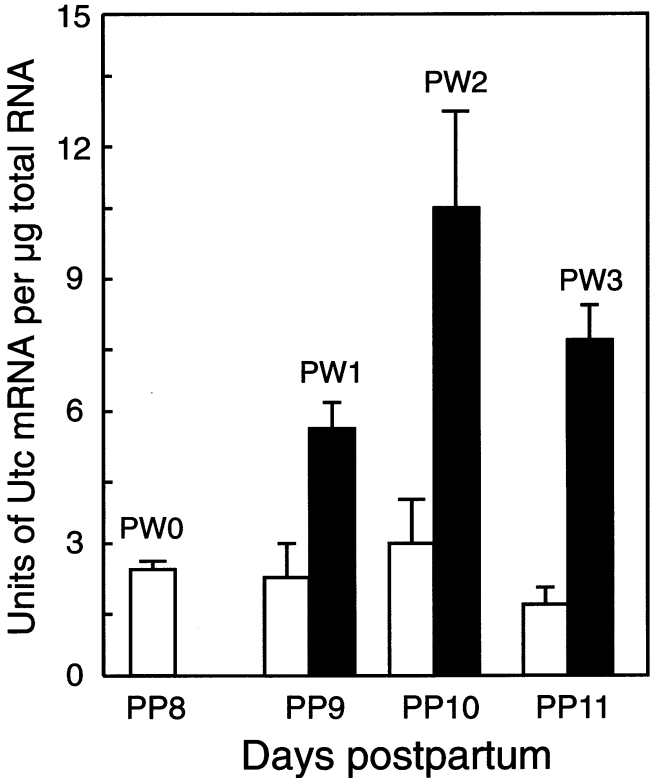

Figure 3 Expression of uterocalin mRNA after early weaning

Pups were removed from their mothers on day 8 post-partum, and the mammary gland tissue collected either 1, 2 or 3 days later (PW1–PW3). Total RNA was prepared from the tissue and analysed by Northern blot with probes to uterocalin and GAPDH. The samples were normalized to GAPDH and to a uterocalin standard. The averages of triplicate values are shown with their sample S.D. Each value was obtained from an independently isolated sample from a different mouse. Open bars, mothers that are suckling young; filled bars, mothers that have been weaned at PP8.

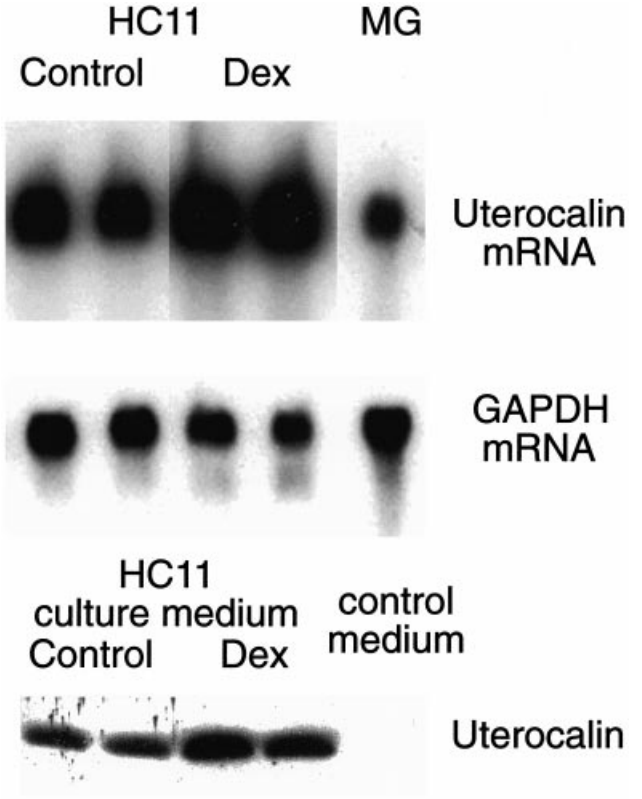

Figure 4 Expression of uterocalin by HC11 cells

Confluent HC11 cells were incubated in RPMI 1640 with 0.5% fetal calf serum, 10 units/ml each of penicillin and streptomycin and 5 μg/ml insulin for 2 days with or without 0.1 μM dexamethasone (Dex). For the top two panels, the cells were harvested and the RNA was analysed by Northern blot. The blots were probed for uterocalin and GAPDH. A sample of mammary gland (MG) mRNA is shown on the same blot. For the bottom panel and in a different experiment, the medium was removed from the cells, resolved by SDS/PAGE and uterocalin detected by Western blot. The control medium in which the cells had been cultured (but which had not been exposed to the cells) was also analysed. The uterocalin protein band is shown.

increase and the increment in mRNA expression levels were similar to that observed post-weaning in the mammary glands of mothers whose pups were weaned naturally (Figure 1B).

Uterocalin is a secreted product of mammary epithelial cells

Uterocalin is a product of epithelial cells of the uterus. To determine if mammary epithelial cells were also capable of making uterocalin, we tested the expression of uterocalin in the mouse mammary HC11 epithelial cell line that shows many functional features of differentiated mammary epithelium [25]. High levels of uterocalin mRNA were expressed in HC11 mammary epithelial cells and uterocalin mRNA expression by these cells was increased about 2-fold by dexamethasone (Figure 4).

The medium of HC11 cells treated with or without dexamethasone was also examined by Western blot to determine whether HC11 cells secrete uterocalin. The results in the bottom panel of Figure 4 show that uterocalin is secreted and that dexamethasone-treated cells secrete about twice as much as the control cells. No detectable protein band was detected by the antibody in samples of medium that had not been incubated with the cells. HC11 cells that were metabolically labelled with [^{35}S]methionine also secreted a protein with the molecular mass of uterocalin (24 kDa). Dexamethasone treatment increased the secretion of the metabolically labelled protein. Thus, HC11 mammary epithelial cells synthesize and secrete uterocalin.

Uterocalin is secreted into the milk

To determine whether uterocalin might be produced by the epithelium *in vivo*, we looked for the protein in the milk. Milk fractions, resolved by SDS/PAGE and analysed by Western blot revealed uterocalin in the supernatant of the pH 4.6 precipitation. Uterocalin was found only in the pH 4.6 supernatant (Figure 5). Milk samples were taken from females in mid-lactation and during involution and in both instances the protein was found in the pH 4.6 supernatant of the fractionated milk.

Uterocalin is expressed in the first phase of mammary gland involution

Mammary gland involution can be divided into at least two stages according to the expression of particular genes [4]. The SGP2 gene (which encodes clusterin, also called apolipoprotein J) marks the first stage and stromelysin-1 marks the second stage

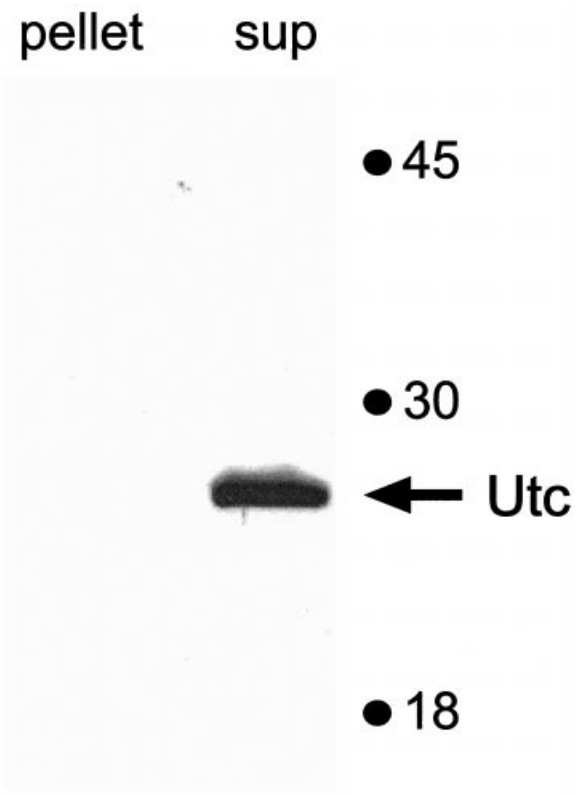

Figure 5 Presence of uterocalin in the milk

Milk from a lactating female 12 days after delivery was fractionated to separate the casein (pellet) from other proteins (sup). The pellet was resuspended in a volume of 0.1 M sodium phosphate buffer, pH 6.2, equal to the original sample prior to precipitation. Both samples were resolved by SDS/PAGE and analysed by Western blot. The position of uterocalin is shown by an arrow (Utc). The numbers and corresponding dots show the positions of three molecular-mass markers (ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; myoglobin, 18 kDa).

of involution. The levels of expression of these two genes and uterocalin were determined in the same samples (Figure 6). The period of expression of uterocalin corresponds to the first stage of mammary involution, which is identified by a peak of SGP2 gene expression. This is followed by involution stage 2, which is identified by a peak in stromelysin-1 gene expression.

Uterocalin is expressed in the initial phase of uterine involution

The uterus undergoes involution immediately post-partum. In the mouse, the uterus grows 4–5 times its weight during gestation and then drops to its non-pregnant weight within 3–4 days of birth ([26] and Figure 7). Immediately prior to this rapid drop in tissue mass, uterocalin and SGP2 are elevated in their expression. As for the mammary gland, a second phase of involution in the uterus was marked by an increase in stromelysin-1 expression. Unlike in the mammary gland where the periods of expression of uterocalin and SGP2 overlapped almost completely, the period of expression of SGP2 extended further in time than that of uterocalin (Figure 7). The decrease in uterocalin mRNA expression precisely paralleled the decrease in tissue mass which suggests that the cells expressing uterocalin are shed with the superfluous tissue.

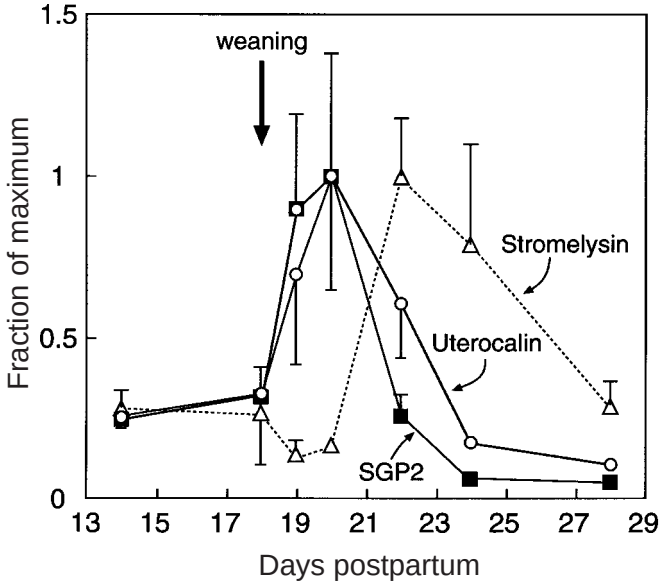

Figure 6 Expression of uterocalin, SGP2 and stromelysin-1 mRNA during involution of the mammary glands

Mammary glands were removed at various times over the period of weaning. The day of weaning was PP18. Each data point was normalized to the GAPDH mRNA in the same sample. The data points shown represent the averages of 3 (PP14, PP18, PP22, PP24) or 2 (PP20 and PP28) independent values, each taken from different mice. The resulting average values were normalized to the highest value in each set to determine fractional values of mRNA expression for each data set. The means ± S.D. are shown. Where there is no error bar, the error was smaller than the size of the symbol. ○, Uterocalin; ■, SGP2; △, stromelysin-1.

DISCUSSION

The consistent and extremely high expression, shown here, of uterocalin mRNA and protein in the mammary gland and the uterus during the first stage of involution suggests that uterocalin plays a role in tissue involution. Its identification as an acute phase protein [9] and its ability to induce apoptosis of neutrophils and other leucocytes [19] further suggest that uterocalin might moderate the immune response during involution. Also, this same function of uterocalin may be important during lactation for maternal contributions to immune regulation in the young.

The change in uterocalin expression in the mammary gland throughout lactation is a specific local response of the mammary gland and not a general response to the state of pregnancy. Although the liver expresses high levels of uterocalin mRNA during the acute phase response [9], low levels of uterocalin expression were measured in the liver at all times tested during gestation, lactation and post-weaning. The conclusion that uterocalin is regulated locally is consistent with the observation that the initiation of mammary gland involution is controlled by local factors [5].

Mammary gland involution is initiated by weaning. Here we have shown that weaning also induces uterocalin. The first 3 days of involution are characterized by massive apoptosis of the epithelial cells, and their sloughing into the alveolar lumen [1,2]. At this time, genes associated with apoptosis such as SGP2 and interleukin-1β-converting enzyme are expressed at higher levels by the mammary gland [2,4]. Beginning around day 4 of involution, the basement membrane begins to break down, and the alveolar structures disappear. Gene expression shifts again with a decrease in the apoptosis-associated genes and an increase

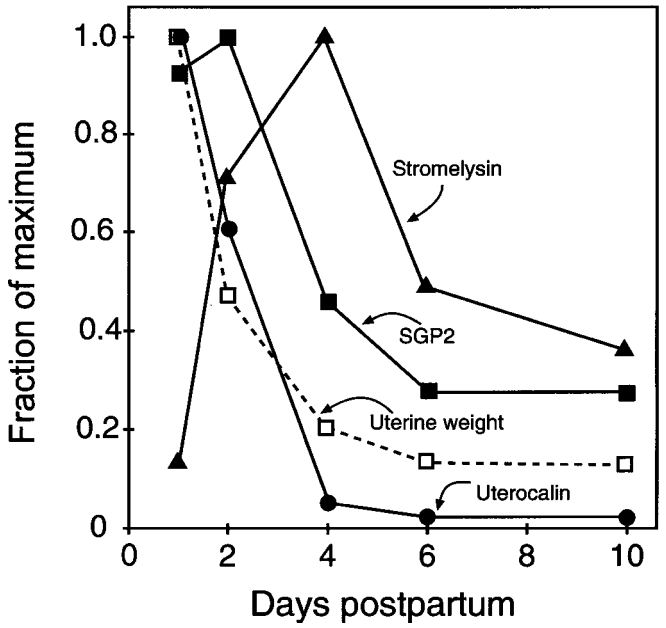

Figure 7 Expression of uterocalin, SGP2 and stromelysin-1 mRNA during involution of the uterus

Uteri were removed at various times after birth. Each dissected tissue was rinsed in balanced salts solution, the excess liquid was adsorbed to a Kimwipe and the tissue was weighed. RNA was extracted and the amount of uterocalin, SGP2 and stromelysin mRNA was determined by Northern blot analysis. Each data point was normalized to the GAPDH mRNA in the same sample. The data points shown represent the averages of 2 independent values, each taken from different mice. The resulting average values were normalized to the highest value in each set to determine fractional values of mRNA expression for each data set. ●, Uterocalin; ■, SGP2; ▲, stromelysin-1; □, uterine weight.

in the expression of proteolytic enzymes such as gelatinase A and stromelysin-1 [2,4]. Uterocalin expression parallels that of SGP2, which marks the first, apoptotic, stage of mammary gland involution.

In the uterus and the mammary glands, involution is aided by the sloughing of tissue into the epithelial lumen. Uterocalin appears exclusively in the uterine epithelium and after birth it is associated with the epithelial surface [10]. The parallel loss of uterocalin expression and uterine mass is consistent with these observations and suggests that the cells producing uterocalin are lost during involution. Uterine SGP2 follows a slightly delayed but similar time course of expression to uterocalin and the period of expression of these genes during mammary gland involution is even closer in time. In other studies, SGP2 expression has been shown associated with cells undergoing apoptosis and also with apparently viable cells in a renal regeneration model [27].

Regulation of uterocalin expression in the uterus and mammary gland likely involves several factors. The lactogenic hormones prolactin, insulin and glucocorticoids are elevated during lactation, and glucocorticoids increase the expression of uterocalin in cultured cells and *in vivo* [9,13]. The increase in uterocalin expression during involution, however, is likely to be caused by other factors because the levels of prolactin and glucocorticoids decrease at the time of weaning [28]. Uterocalin expression during involution of the uterus and mammary gland may be linked to the expression of genes associated with apoptosis such as tumour necrosis factor α, which stimulates uterocalin expression in cultured cells [9].

As in the uterus, epithelial cells are a source of uterocalin in the mammary gland. This is shown here by the fact that HC11 mammary epithelial cells express uterocalin at high levels that are comparable to the levels of uterocalin expression in the mammary gland. Also, uterocalin is secreted into the milk. Although lipocalins have been identified in the milk of many species, including in marsupials [29], the milk of different species seems to contain different lipocalin gene products. For example, β-lactoglobulin, a lipocalin and the major protein of bovine whey, is found in ungulate but not in human or rodent milk. Although different lipocalins are expressed by the mammary glands of different species, these proteins have a similar biochemical function of binding lipids. The widespread occurrence of lipocalins in milk suggests that their presence might be important to the young. However, although the interaction of β-lactoglobulin with long-chain fatty acids has been extensively studied over many years, the physiological function of β-lactoglobulin, the best studied of the milk lipocalins, is still unknown. Thus, a discussion at this time of the potential role of lipocalins in milk would be highly speculative.

The role of uterocalin in the mammary gland may be to suppress entry of phagocytic cells into the involuting tissue during the first, apoptotic, phase of involution. This is consistent with the ability of uterocalin to stimulate apoptosis of neutrophils [19]. It is interesting to note in this regard that hydrocortisone, which induces uterocalin, delays the onset of the second phase of involution [4]. The combined observations that high glucocorticoids inhibit mammary epithelial apoptosis and that the effect of uterocalin on apoptosis was specific for leucocytes among the cell types that were tested suggest that uterocalin does not induce apoptosis of mammary epithelial cells [19,28]. Regardless of the effect of uterocalin on mammary epithelial apoptosis, it is known that some mammary epithelial cells survive the involution process to become the post-involuted mammary gland. Macrophages are present in the mammary gland in low numbers during lactation and in the early stage of involution. Their numbers begin to increase on day 3 post-weaning and continue to increase up until PW10 [4]. We believe that the role of uterocalin is to protect mammary epithelial cells during the early phase of involution to allow those cells that do not undergo apoptosis to reorganize and survive the consequences of the phagocytic activity of leucocytes and macrophages that later enter the tissue to engulf cellular debris. Phagocyte activation involves the release of damaging free radicals and proteases from which cells destined to survive involution would need protection.

Clusterin, with which it is co-regulated, may work in concert with uterocalin to protect cells destined to survive involution. SGP2 encodes a subunit of clusterin (also called apolipoprotein J), which is expressed in involuting tissues. Clusterin protects cells against oxidative and chemical damage such as that which would be perpetrated by invading phagocytes [30,31]. This effect of clusterin may be achieved as a result of its activity as an extracellular chaperone [32]. During the initial apoptotic stage of tissue involution, the combination of clusterin and uterocalin expression is envisioned as having the synergistic actions of organizing a subset of cells for protection against oxidative and chemical damage (clusterin) and inducing apoptosis in invading neutrophils (uterocalin) to provide a grace period for completing the cellular protective stance before the phagocytic phase begins.

In summary, we have found that uterocalin, an acute phase protein that induces apoptosis in neutrophils and other leucocytes, is highly expressed during the first stage of involution of the mammary gland and the uterus, during which there is extensive apoptosis. The parallel expression of uterocalin in the uterus and mammary gland during the same early period of involution for each tissue suggests that uterocalin plays a role in

suppressing the inflammatory response during the first phase of involution.

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REFERENCES

1 Walker, N. I., Bennett, R. E. and Kerr, J. F. (1989) Cell death by apoptosis during involution of the lactating breast in mice and rats. Am. J. Anat. **185**, 19–32
2 Strange, R., Li, F., Saurer, S., Burkhardt, A. and Friis, R. R. (1992) Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development **115**, 49–58
3 Hennighausen, L. and Robinson, G. W. (1998) Think globally, act locally: the making of a mouse mammary gland. Genes Dev. **12**, 449–455
4 Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dano, K. and Werb, Z. (1996) Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. Development **122**, 181–193
5 Li, M., Liu, X., Robinson, G., Bar-Peled, U., Wagner, K. U., Young, W. S., Hennighausen, L. and Furth, P. A. (1997) Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. Proc. Natl. Acad. Sci. U.S.A. **94**, 3425–3430
6 Marti, A., Feng, Z., Altermatt, H. J. and Jaggi, R. (1997) Milk accumulation triggers apoptosis of mammary epithelial cells. Eur. J. Cell. Biol. **73**, 158–165
7 Nilsen-Hamilton, M., Hamilton, R. T. and Adams, G. A. (1982) Rapid selective stimulation by growth factors of the incorporation by BALB/C 3T3 cells of [^{35}S]methionine into a glycoprotein and five superinducible proteins. Biochem. Biophys. Res. Commun. **108**, 158–166
8 Hraba-Renevey, S., Turler, H., Kress, M., Salomon, C. and Weil, R. (1989) SV40-induced expression of mouse gene 24p3 involves a post-transcriptional mechanism. Oncogene **4**, 601–608
9 Liu, Q. and Nilsen-Hamilton, M. (1995) Identification of a new acute phase protein. J. Biol. Chem. **270**, 22565–22570
10 Liu, Q., Ryon, J. and Nilsen-Hamilton, M. (1997) Uterocalin: a mouse acute phase protein expressed in the uterus around birth. Mol. Reprod. Dev. **46**, 507–514
11 Kasik, J. W. and Rice, E. J. (1995) An increase in expression of the lipocalin 24p3 is found in mouse uterus coincident with birth. Am. J. Obstet. Gynecol. **173**, 613–617
12 Chu, S. T., Huang, H. L., Chen, J. M. and Chen, Y. H. (1996) Demonstration of a glycoprotein derived from the 24p3 gene in mouse uterine luminal fluid. Biochem. J. **316**, 545–550
13 Garay-Rojas, E., Harper, M., Hraba-Renevey, S. and Kress, M. (1996) An apparent autocrine mechanism amplifies the dexamethasone- and retinoic acid-induced expression of mouse lipocalin-encoding gene 24p3. Gene **170**, 173–180
14 Flower, D. R., North, A. C. and Attwood, T. K. (1991) Mouse oncogene protein 24p3 is a member of the lipocalin protein family. Biochem. Biophys. Res. Commun. **180**, 69–74
15 Flower, D. R. (1996) The lipocalin protein family: structure and function. Biochem. J. **318**, 1–14
16 Chu, S., Lin, H., Huang, H. and Chen, Y. (1998) The hydrophobic pocket of 24p3 protein from mouse uterine luminal fluid: fatty acid and retinol binding activity and predicted structural similarity to lipocalins. J. Pept. Res. **52**, 390–397
17 Cancedda, F. D., Malpeli, M., Gentili, C., Di Marzo, V., Bet, P., Carlevaro, M., Cermelli, S. and Cancedda, R. (1996) The developmentally regulated avian Ch21 lipocalin is an extracellular fatty acid-binding protein. J. Biol. Chem. **271**, 20163–20169
18 Cowland, J. B. and Borregaard, N. (1997) Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics **45**, 17–23
19 Devireddy, L. R., Teodoro, J. G., Richard, F. A. and Green, M. R. (2001) Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. Science **293**, 829–834
20 Collard, M. W. and Griswold, M. D. (1987) Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. Biochemistry **26**, 3297–3303
21 Matrisian, L. M., Bowden, G. T., Krieg, P., Furstenberger, G., Briand, J. P., Leroy, P. and Breathnach, R. (1986) The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. Proc. Natl. Acad. Sci. U.S.A. **83**, 9413–9417
22 Fort, P., Marty, L., Piechaczyk, M., el Sabrouty, S., Dani, C., Jeanteur, P. and Blanchard, J. M. (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res. **13**, 1431–1442
23 Davis, T. R., Tabatabai, L., Bruns, K., Hamilton, R. T. and Nilsen-Hamilton, M. (1991) Basic fibroblast growth factor induces 3T3 fibroblasts to synthesize and secrete a cyclophilin-like protein and beta 2-microglobulin. Biochim. Biophys. Acta **1095**, 145–152
24 Green, M. R. and Pastewka, J. V. (1976) Characterization of major milk proteins from BALB/c and C3H mice. J Dairy Sci. **59**, 207–215
25 Ball, R. K., Friis, R. R., Schoenenberger, C. A., Doppler, W. and Groner, B. (1988) Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. EMBO J. **7**, 2089–2095
26 Fang, Y., Lepont, P., Fassett, J., Ford, S. P., Mubaidin, A., Hamilton, R. T. and Nilsen-Hamilton, M. (1999) Signaling between the placenta and the uterus involving the mitogen-regulated protein/proliferins. Endocrinology **140**, 5239–5274
27 Gobe, G. C., Buttyan, R., Wyburn, K. R., Etheridge, M. R. and Smith, P. J. (1995) Clusterin expression and apoptosis in tissue remodeling associated with renal regeneration. Kidney Int. **47**, 411–420
28 Feng, Z., Marti, A., Jehn, B., Altermatt, H. J., Chicaiza, G. and Jaggi, R. (1995) Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. J. Cell Biol. **131**, 1095–1103
29 Piotte, C. P., Hunter, A. K., Marshall, C. J. and Grigor, M. R. (1998) Phylogenetic analysis of three lipocalin-like proteins present in the milk of *Trichosurus vulpecula* (Phalangeridae, Marsupialia). J. Mol. Evol. **46**, 361–369
30 Miyake, H., Nelson, C., Rennie, P. S. and Gleave, M. E. (2000) Acquisition of chemoresistant phenotype by overexpression of the antiapoptotic gene testosterone-repressed prostate message-2 in prostate cancer xenograft models. Cancer Res. **60**, 2547–2554
31 Viard, I., Wehrli, P., Jornot, L., Bullani, R., Vechietti, J. L., Schifferli, J. A., Tschopp, J. and French, L. E. (1999) Clusterin gene expression mediates resistance to apoptotic cell death induced by heat shock and oxidative stress. J. Invest. Dermatol. **112**, 290–296
32 Humphreys, D. T., Carver, J. A., Easterbrook-Smith, S. B. and Wilson, M. R. (1999) Clusterin has chaperone-like activity similar to that of small heat shock proteins. J. Biol. Chem. **274**, 6875–6881

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