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Concurrent Pregnancy Retards Mammary Involution: Effects on Apoptosis and Proliferation of the Mammary Epithelium after Forced Weaning of Mice[1]

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ABSTRACT

The effect of pregnancy on postweaning mammary gland involution was investigated in mice. On the third day after forced weaning at Lactation Day 10, the apoptotic index was 56% lower in mammary tissue of mice that were pregnant at the time of weaning than in nonpregnant mice. Conversely, the bromodeoxyuridine-labeling index was increased sevenfold in pregnant mice compared to nonpregnant controls (3.5% vs. 0.5%, respectively). Structure of mammary alveoli was largely maintained in postweaning pregnant mice. The effect of pregnancy on three specific mammary epithelial cell survival pathways was also examined. First, pregnancy blocked the loss of Stat5a phosphorylation during involution. Significantly, loss of Stat5a phosphorylation during involution was not correlated with loss of Stat5a nuclear localization. Second, pregnancy maintained nuclear-localized progesterone receptor during lactation. Third, pregnancy was associated with increased expression of bfl-1 during involution but had little effect on the expression of other bcl-2 family members. The data indicate that pregnancy inhibits mammary cell apoptosis after weaning while permitting proliferation of the mammary epithelium, and they support the hypothesis that Stat5a and progesterone-signaling pathways act in concert to mediate this effect.

apoptosis, lactation, mammary glands, progesterone receptor, prolactin

INTRODUCTION

Mammary glands of adult mammals undergo periods of proliferation, lactation, and involution as dictated by the reproductive status of the animal. During pregnancy, extensive growth and differentiation of the mammary epithelium results in the lobuloalveolar development and differentiation necessary for lactation. After the onset of lactation, continued milk synthesis and maintenance of the population of epithelial cells require the frequent removal of milk. Forced weaning, or the gradual cessation of milk removal that occurs during natural weaning, initiates the process of mammary gland involution. This process is characterized by the apoptotic death of mammary epithelial cells and their removal by phagocytes, both professional (macrophages) and amateur (epithelial cells) [1, 2]. Apoptosis is regulated by both local and endocrine factors, although local factors often appear to dominate [3]. Many features of mammary involution are common across species, but the rapidity and degree of involution varies. Furthermore, the kinetics of mammary involution may be influenced by the pregnancy status of the animal at the cessation of milk removal [4]. For example, maintenance of the mammary epithelium at the conclusion of lactation in dairy cows may be because cows are typically in the last trimester of pregnancy when milking is terminated.

The objective of this experiment was to test the hypothesis that pregnancy retards mammary involution and to evaluate the potential involvement of prolactin and progesterone in this process. Cell proliferation and apoptosis during mammary involution were assessed, as were transcript levels of apoptosis-related regulatory genes and expression and activation of Stat5a and progesterone receptor (PR).

MATERIALS AND METHODS

Experimental Design

Female mice of the NRMI strain were cohabited with males of the same strain throughout pregnancy and the first week of lactation. Litter size was adjusted at birth by cross-fostering pups of the same age to ensure that all litters contained at least six pups. On Day 10 of lactation, pups were removed to induce mammary involution. Three days postweaning, mice were injected i.p. with bromodeoxyuridine (BrdU; 30 μg/g body weight; cell proliferation labeling reagent; Amersham Life Science, Arlington Heights, IL) at 1000 h and at 1400 h. Additionally, lactating mice were injected with BrdU on Day 10 of lactation to serve as noninvolution controls. Mice were killed by cervical dislocation 2 h after the second injection, and the fourth mammary glands were removed and weighed. Approximately one-half of the left mammary gland was fixed and processed for light microscopy as subsequently described. The remaining mammary tissue was frozen in liquid nitrogen and stored at −80°C until biochemical analyses. Uteri were removed, and the pregnancy status of mice was determined at the time of death. Mice fell into four categories: 1) involution, nonpregnant; 2) involution, pregnant; 3) lactating, nonpregnant; and 4) lactating, pregnant.

Mice were used in accordance with the National Research Council guidelines for use of laboratory animals [5].

Immunohistochemistry

Mammary tissue was fixed overnight in 10% (v/v) neutral buffered formalin at 4°C. Tissues were then stored in 70% (v/v) ethanol until further processing. Subsequently, tissues were dehydrated and embedded in paraffin according to standard techniques. Tissues were sectioned (thickness, 5 μm) and stained with hematoxylin and eosin or processed for immunohistochemical detection of apoptotic cells or BrdU-labeled cells.

In situ detection of apoptotic cells utilized the ApopTag kit (Oncor,

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Gaithersburg, MD) for terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL). After deparaffinization and hydration, slides were incubated for 15 min at room temperature with proteinase-K (20 μg/ml PBS; Oncor), and subsequent labeling was in accordance with the manufacturer's recommended protocol. Sections were counterstained with methyl green and mounted with Permaslip (Alban Scientific Inc., St. Louis, MO).

The BrdU-labeled cells were detected using biotinylated monoclonal antibody against BrdU and streptavidin-peroxidase detection (Zymed Laboratories, Inc., San Francisco, CA). All incubations were at room temperature. Briefly, tissue sections were deparaffinized, then rehydrated in a graded series of alcohol and quenched with 3% H_2O_2 in absolute methanol for 10 min. Sections were then washed with PBS and tissue digested with proteinase-K (20 μg/ml PBS) for 15 min. The sections were washed in distilled H_2O and then denatured in 4 N HCl for 20 min. After washing with PBS and incubating with blocking solution for 10 min, the tissue sections were incubated with the anti-BrdU monoclonal antibody for 40 min. Slides were then washed in PBS, followed by incubation with streptavidin-peroxidase for 10 min. Sections were washed in PBS, followed by incubation with the chromogen, diaminobenzidine (DAB). After washing with distilled H_2O, tissues were counterstained with hematoxylin.

For immunohistochemical detection of PR, slides were deparaffinized in xylene and hydrated in a graded series of ethanol to PBS (pH 7.4). Tissue sections were quenched with 3% H_2O_2 in PBS for 10 min and then washed in PBS (three washes, 2 min each). Heat-induced antigen retrieval in citrate buffer was then used. Slides were heated in a microwave at high power (650 W) in 400 ml of 10 mM citrate buffer (pH 6.0) in a covered glass staining dish for 5 min, then left undisturbed for 5 min, and then microwaved for an additional 5 min. Slides remained in the buffer for a 30-min cooling period. They were then washed in PBS (3 washes, 2 min each) and blocked with 5% nonimmune goat serum in PBS (30 min) before overnight incubation at 4°C with rabbit polyclonal antibody against PR (PR C-19; used at 1:100 (v/v) dilution in PBS containing 1% normal goat serum; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Slides were then washed in PBS (3 washes, 5 min each). A 1:200 (v/v) dilution of goat anti-rabbit IgG (Sternberger Monoclonals, Inc., Lutherville, MD) was prepared in PBS containing 1% normal goat serum and 2% normal mouse serum and incubated for 30 min at room temperature. Slides were then incubated with the adsorbed second antibody for 30 min at room temperature. They were washed in PBS (three washes, 5 min each) and then incubated with mouse peroxidase anti-peroxidase (mouse clonoPAP; Sternberger Monoclonals) at a dilution of 1:200 (v/v) in PBS containing 1% normal goat serum for 30 min at room temperature. After washing in PBS (3 washes, 5 min each), sections were incubated with the chromogen, DAB. Slides were washed with distilled H_2O, and tissue sections were counterstained with hematoxylin, dehydrated, and mounted with Permaslip.

Cells expressing Stat5 were localized by immunohistochemistry. Tissue sections were deparaffinized, rehydrated, and then quenched with 3% H_2O_2 as described above. Antigens were unmasked by boiling the sections for 1 min in 10 mM citric acid. Immunohistological staining was performed with a 1:125 (v/v) dilution of Stat5a antibody (L-20; Santa Cruz Biotechnology) and the HistoMouse-SP Kit (Zymed Laboratories) according to the manufacturer's protocol.

Tissue sections were viewed by light microscopy, and apoptotic, BrdU-labeled, and nuclear Stat5a-labeled cells were quantified. Ten fields were quantified per slide (one slide/mouse). A field was selected under low power and slightly out of focus. The objective was then switched to 40×, and the cells within an 8 × 8 ocular grid were counted. At least 1000 cells were evaluated per mouse.

RNase Protection Assay

Total RNA was extracted from frozen mammary tissue according to the method of Chomczynski and Sacchi [6] and quantified by ultraviolet absorption. RNase protection assays for Bcl-2 family members were performed using the Riboquant Multiprobe RNase Assay System (PharMingen, San Diego, CA) and 10 μg of RNA. Purified samples were separated on precast, denaturing polyacrylamide DNA sequencing gels using the Quick Point Rapid DNA sequencing system (Novex, San Diego, CA). Gels were oven-dried and exposed to x-ray film at −70°C using an intensifier screen. Intensity of bands on the film was quantified using a Shimadzu scanner (CS-9000U; Shimadzu Corporation, Kyoto, Japan). For each tissue sample, the total density of RNase-protected bands that coincided to expression of cell survival genes (anti-apoptotic genes; bfl-1 + bcl-2 + bcl-w + bcl-x_L) was calculated, as was the total density of proapoptotic genes (bax + bak + bad). Data were expressed as ratios of cell survival to apoptotic genes.

TABLE 1. Effect of pregnancy on apoptosis and BrdU incorporation of mammary epithelial cells 3 days after weaning.[a]

	n	Mammary gland weight (g)[b]	% Apoptosis[c]	% BrdU-labeled[c]
Nonpregnant	7	0.42 ± 0.08	7.21 ± 0.08	0.50 ± 0.21
Pregnant	9	0.77 ± 0.14	3.20 ± 0.75	1.59 ± 1.17
P		0.06	0.006	0.008

[a] Values are means ± SEM.
[b] Mammary gland weight is the mean for the sum of fourth mammary glands (left + right) for each animal 3 days after pup removal.
[c] Expressed as the percentage of epithelial cells.

Stat5a Immunoprecipitation and Western Blot Analysis

Proteins were extracted from frozen mammary tissue (−70°C). One milliliter of lysis buffer was added to 0.1 g of tissue and homogenized using a Polytron Homogenizer. Antibodies used were Stat5a (gift from Dr. Lothar Hennighausen, National Institutes of Health, Bethesda, MD) and phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) monoclonal antibodies. Protein lysates were rocked for 1 h at 4°C and then centrifuged at 11 000 × *g* for 15 min. The supernatants (500 μl) were then incubated with 2 μl of Stat5a antisera for 30 min at 4°C on a vertical rotator. Protein A-Sepharose beads (Sigma Chemical Co., St Louis, MO) were added, and incubation was continued overnight. Samples were washed 3 times with lysis buffer, resuspended in 2× sample buffer (250 mM Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, 2% 2-mercaptoethanol, and 0.006% bromophenol blue), boiled for 3 min, centrifuged briefly at 11 000 × *g*, and electrophoretically fractionated on an SDS/8% polyacrylamide gel. Proteins were then transferred onto polyvinylidene difluoride membranes. For Western blot analysis, membranes were blocked overnight with TBST (10 mM Tris [pH 8.0], 150 mM NaCl, and 0.1% Tween 20) plus 2% BSA. Primary antibody (antiphosphotyrosine antibody) diluted to 0.2 μg/ml (1:5000) in TBST/2% BSA was incubated with blots for 1 h at room temperature. After three washes in TBST, horseradish peroxidase-conjugated goat anti-mouse IgG diluted to 1:5000 was added, and incubation was performed for another 1 h at room temperature. After 4 additional washes in TBST, proteins were detected using the enhanced chemiluminescence system. Blots were stripped by incubating in 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM 2-mercaptoethanol for 30 min with intermittent agitation at 65°C and then reprobed with antisera for Stat5a (1:5000).

Analysis of PR Expression by Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from tissues using a guanidinium thiocyanate-phenol-chloroform extraction method as described previously [6] and quantitated on a Beckman DU40 (Beckman Coulter, Inc., Fullerton, CA) spectrophotometer. Equal amounts of RNA template (1 μg) were reverse transcribed in a 20-μl reaction containing 500 ng of random primers, 500 μM deoxyribonucleoside triphosphates, 1 mM dithiothreitol, 1× first-strand buffer, and 200 U of SuperScript reverse transcriptase (Gibco BRL, Rockville, MD). Primer set 5′-CATGTCAGTGGACAGATGCT-3′ (forward) and 5′-ACTTCAGACATCATTTCCGG-3′ (reverse), spanning the ligand-binding domain of mouse PR, was utilized for the subsequent polymerase chain reaction (PCR) [7]. The 25-μl PCR reaction contained 2.5 μl (1:5 dilution) of first-strand synthesis, 1 μM primers, 250 μM dNTPs, 1× PCR buffer, and 0.05 U of Taq DNA polymerase (Sigma). The PCR protocol was 35 cycles of 94°C for 45 sec, 56°C for 1 min, and 72°C for 2 min. The amplified product visualized by ethidium bromide staining was 427 base pairs (bp). The β-actin primer set utilized was 5′-ATCGTGGGC-CGCCCTAGGCA-3′ (forward) and 5′-TGGCCTTAGGGTTCAGAGGG-3′ (reverse), yielding a 244-bp amplified product as previously described [7].

Statistical Analysis

Effect of pregnancy on mammary gland weight and percentage apoptotic cells, percentage BrdU-labeled cells, and percentage cells demonstrating nuclear-localized Stat5a were evaluated by Student *t*-test. Percentage data were arcsine-transformed before analysis, but untransformed

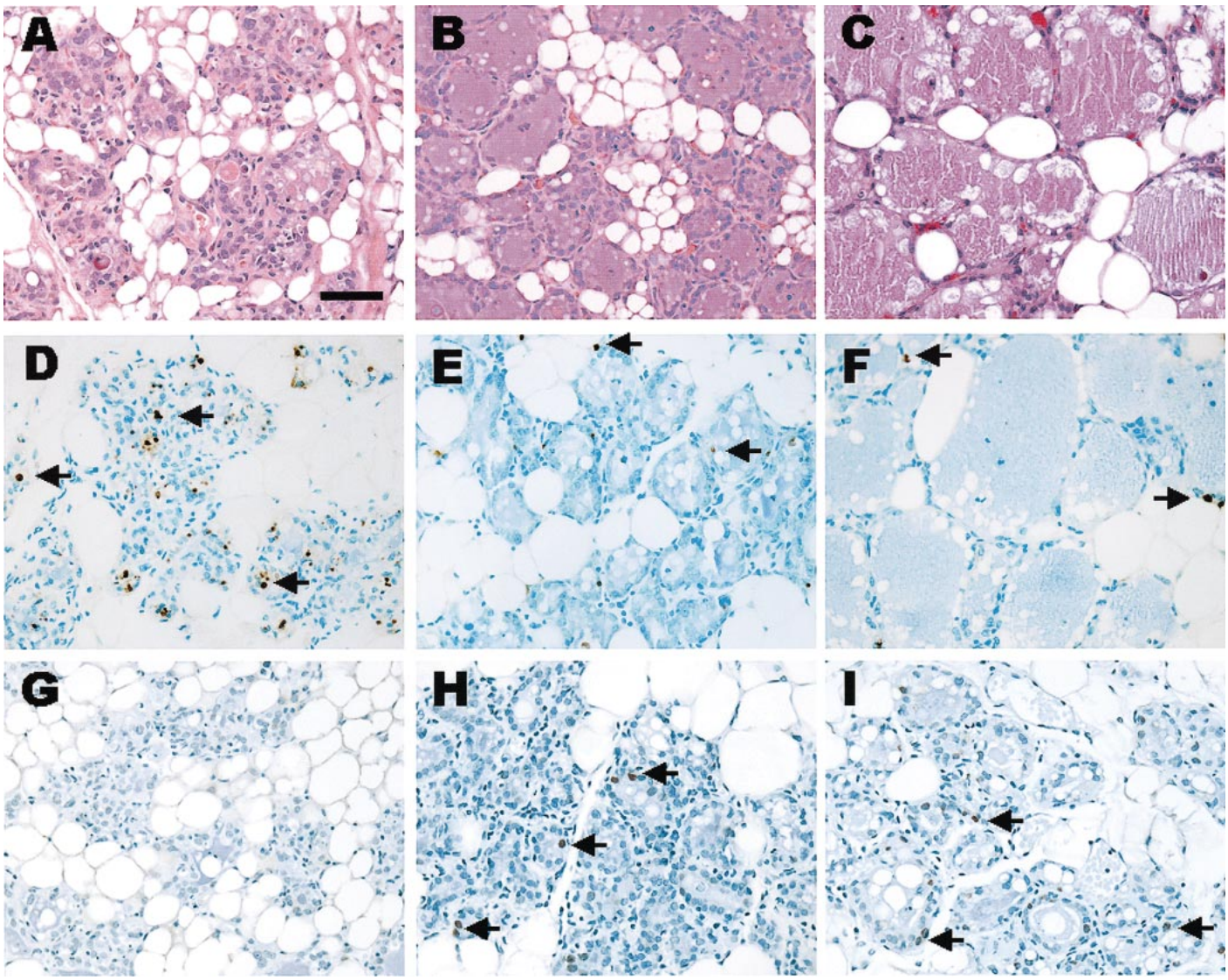

FIG. 1. Histological evaluation of pregnancy effects on mammary gland involution. Top row shows mammary tissue sections were stained with hematoxylin and eosin. Middle row shows detection of in situ apoptosis by TUNEL; apoptotic cells and cell fragments are labeled brown. Bottom row shows detection of BrdU-labeled cells by immunohistochemistry; labeled cells contain brown nuclei. **A**, **D**, and **G**) Tissues from nonpregnant mice 3 days after removing pups. **B**, **E**, and **H**) Tissues from mice during early pregnancy and 3 days after pup removal. **C**, **F**, and **I**) Tissues from mice during late pregnancy and 3 days after pup removal. Arrows indicate some of the apoptotic, TUNEL-positive cells (middle row) and BrdU-labeled cells (bottom row). Bar = 50 μm.

data are presented in Table 1. The RNase protection assay data were analyzed by one-way analysis of variance, and post-hoc comparisons of treatment means were made using the Bonferroni multiple comparison test. A statistical software package was employed (Prism, version 3.02; GraphPad Software, Inc., San Diego, CA).

RESULTS

Within 72 h of weaning pups on Day 10 of lactation, extensive mammary involution occurred in the glands of nonpregnant mice. Size of mammary alveoli had decreased markedly from that of the lactating gland, integrity of alveoli was frequently lost, and mammary epithelial cells were surrounded by an increasingly prominent stroma of adipocytes (Fig. 1A). However, when mice were pregnant at the time of weaning on Day 10 of lactation, alveolar size and integrity were largely maintained at evaluation on Day 3 postweaning (Fig. 1, B and C). Even so, increased prominence of adipocytes provided evidence of limited mammary involution in pregnant mice. The fourth mammary glands averaged 0.46 and 0.80 g for nonpregnant and pregnant mice, respectively ($P = 0.06$). The trend toward increased mammary gland mass provided further quantitative support for an inhibitory effect of pregnancy on mammary involution.

Apoptosis in the mammary epithelium on Day 3 postweaning was assessed by in situ labeling using the TUNEL method (Fig. 1, D–I). The apoptotic index was greater in nonpregnant than in pregnant mice and averaged 7.2% and 3.2%, respectively, of epithelial cells ($P = 0.006$) (Table 1). This is depicted in Figure 1, wherein tissue from a nonpregnant mouse shows extensive apoptosis 3 days after weaning (Fig. 1D), in contrast to the more limited apoptosis shown by mammary tissue from pregnant mice (Fig. 1, E and F). Conversely, the BrdU-labeling index of the mammary epithelium was 0.5% and 3.5% for nonpregnant and pregnant mice, respectively, at 3 days after weaning ($P = 0.008$) (Table 1). The BrdU-labeled mammary epithelial cells were infrequent in mammary tissues from nonpreg-

TABLE 2. Effect of pregnancy on the expression of bcl-2 family members during mammary gland involution.[a]

			Survival genes				Apoptotic genes		
	n	S/A[b]	bfl-1	bcl-2	bcl-x_L	bcl-w	bax	bak	bad
Lactating[c]	3	1.40[A]	0.048[A]	0.001[A]	0.233[A]	0.279[A]	0.251[A]	0.116[A]	0.304[A]
Involv-NP[d]	4	0.99[A]	0.052[A]	0.099[B]	0.271[A]	0.073[B]	0.360[A]	0.077[A]	0.273[A]
Involv-P[e]	5	1.12[A]	0.151[B]	0.109[B]	0.181[A]	0.073[B]	0.268[A]	0.118[A]	0.234[A]
SE[f]		0.24	0.027	0.022	0.035	0.036	0.042	0.013	0.019

[a] Gene expression was assessed by RNase protection assay. Data are expressed relative to the total of all genes in the bcl-2 family. Means without a common uppercase letter differ ($P < 0.05$).
[b] S/A, Ratio of survival to apoptotic genes ([bfl-1 + bcl-2 + bcl-x_L + bcl-w]/[bax + bak + bad]).
[c] Lactating mice were evaluated on Day 10 of lactation.
[f] Involv-NP, Involution in nonpregnant mice. Pups were removed from lactating, nonpregnant mice on Day 10 of lactation, and mammary tissue was evaluated on Day 3 of mammary involution.
[e] Involv-P, Involution in pregnant mice. Pups were removed from lactating, pregnant mice on Day 10 of lactation, and mammary tissue was evaluated on Day 3 of mammary involution.
[f] SE, Pooled standard error for n = 3.

nant mice (Fig. 1G) but were evident in mammary tissue from pregnant mice 3 days after weaning (Fig. 1, H and I).

The levels of mRNA expression for bcl-2 family members (bfl-1, bcl-2, bcl-w, bax, bak, bad, and bcl-x_L) were assessed by RNase protection assay (Table 2). The proportionate expression of antiapoptotic/proapoptotic transcripts was equivalent in mammary tissue of lactating mice and in tissues of postweaning mice regardless of pregnancy status ($P > 0.05$). The transcript level for the survival gene, bfl-1, was increased in the postweaning mammary tissue from pregnant mice as compared to lactating mice and postweaning, nonpregnant mice ($P < 0.05$). Other members of the bcl-2 family were not affected by pregnancy status during the postweaning period.

Stat5a protein was expressed in lactating and involuting mammary gland. Coincident pregnancy blocked the loss of activated, tyrosine-phosphorylated protein and maintained higher levels of Stat5a protein during involution, as demonstrated by immunoprecipitation and Western blot analysis (Fig. 2). Significantly, loss of tyrosine phosphorylation was not associated with loss of nuclear localization in nonpregnant mice (Fig. 3, A–C). The percentage of cells demonstrating nuclear-localized Stat5a was significantly higher in involuting mammary gland of nonpregnant (55 ± 9, SEM) as compared to pregnant (31 ± 5) mice ($P = 0.033$).

Progesterone receptor was evident in lactating mammary tissue of pregnant mice and during the postweaning period in both pregnant and nonpregnant mice. Evaluation of PR expression in lactating tissues by immunohistochemistry indicated that PR was expressed when mice were concomitantly pregnant but not when mice were nonpregnant (Fig. 3, D and E). This finding was confirmed by reverse transcription (RT)-PCR (Fig. 4). Transcripts for PR were evident in lactating tissues of pregnant mice and in involuting tissues of pregnant and nonpregnant mice, but transcripts were not detected in lactating tissue of nonpregnant mice. No statistical analyses of PR immunohistochemistry or RT-PCR were performed, because these comparisons involved demonstration of complete absence versus presence of PR expression.

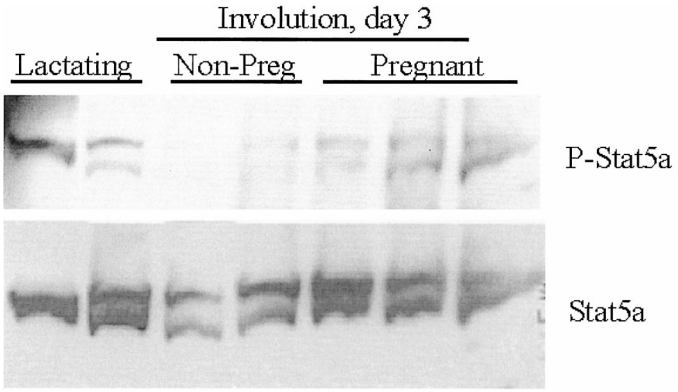

FIG. 2. Effect of pregnancy on levels of phosphorylated Stat5a. Upper panel shows phosphorylated Stat5a. Lower panel shows total Stat5a. Pregnancy increased expression of total Stat5a protein phosphorylated-Stat5a.

DISCUSSION

Mammary involution was impeded in mice that were pregnant at the time of weaning. Histologically, this was demonstrated by enhanced postweaning maintenance of alveolar structure in mammary tissue from pregnant mice compared with nonpregnant mice. Pregnancy inhibited mammary involution by decreasing the frequency of epithelial apoptosis, and it may have indirectly inhibited involution by increasing the proliferation of mammary epithelial cells. The latter conclusion is supported by the increased proportion of BrdU-labeled cells in mammary tissue of pregnant mice. These data are consistent with the concept that the mammogenic effects of pregnancy partially offset the local effects of milk stasis on mammary involution. The net result appears to be increased cell turnover of mammary epithelial cells after weaning in the face of a limited decline in epithelial cell number, which is analogous to data reported for dairy cows [8].

After weaning, injections of glucocorticoids, progesterone, and prolactin inhibit mammary involution in mice [9]. Similarly, local inhibition of involution has been observed following local implants of progesterone and deoxycorticosterone [10]. Conversely, reduction of circulating concentrations of prolactin by bromocriptine accelerates mammary involution [1], and Stat5a appears to be a locally acting molecule, the down-regulation of which initiates the process of epithelial regression and reorganization during involution [11]. However, when milk stasis was induced by sealing a nipple and permitting pups to suckle other glands, the absence of milk removal induced mammary apoptosis and regression in the gland. This was despite continued exposure of the involuting gland to the normal milieu of galactopoietic hormones [3], suggesting that local stimuli for apoptosis override systemic hormones. In the present experiment, the physiological stimulus of a concurrent pregnancy inhibited regression of the mammary gland after forced weaning.

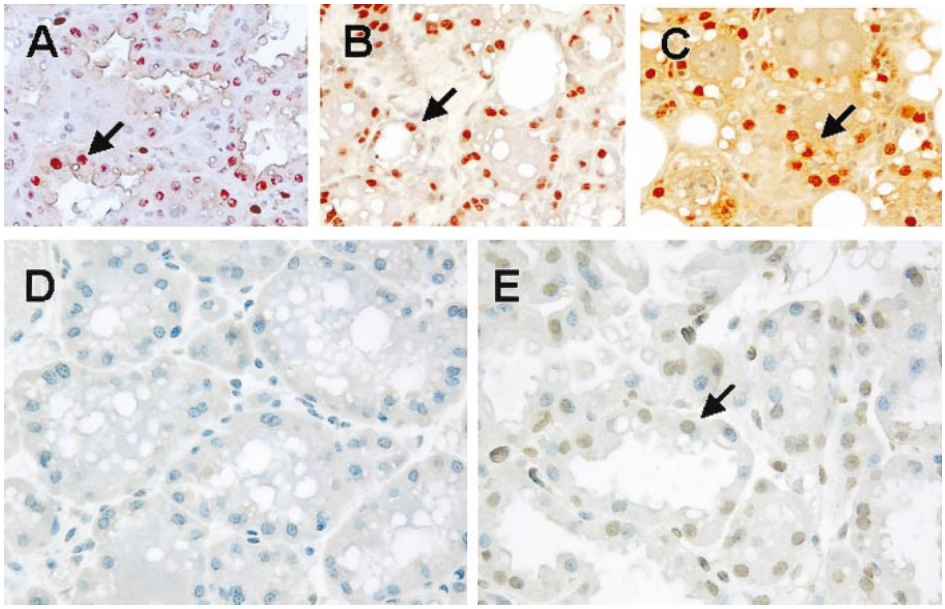

FIG. 3. Stat5a (**A–C**) and PR (**D** and **E**) staining in lactating and involuting mammary tissue. More Stat5a-positive nuclei were evident in involuting mammary gland from nonpregnant than from pregnant mice. **A**) Stat5a staining in tissue from a lactating, nonpregnant mouse. **B**) Tissue form nonpregnant mouse 3 days after removing pups. **C**) Tissue from pregnant mouse 3 days after removing pups. **D**) Nuclear staining of PR is not evident in mammary tissue of lactating, nonpregnant mice. **E**) Nuclear staining of PR is evident in mammary tissue of lactating, pregnant mice.

The hormones that mediate the effect of pregnancy on mammary involution remain to be identified. Because progesterone was shown to inhibit mammary involution after forced weaning [9, 10], this steroid hormone must be considered as a potential regulator. Although lactating mammary glands of rodents have been shown to lack PR [12, 13], expression of the receptor is influenced by the pregnancy status of the animal. In the present study, PR was expressed in the mammary tissue of pregnant, lactating mice. Thus, the lactating state per se may not preclude expression of PR, and mammary epithelial cells of pregnant, lactating mice contain the receptors to respond to a progestin signal. Progesterone receptor has also been characterized in the lactating mammary gland of cows and goats [14, 15].

The presence of activated, phosphorylated Stat5a in pregnant mice suggests that either prolactin or placental lactogen may mediate the effect of pregnancy on mammary involution. The presence of activated Stat5a is a survival signal for mammary epithelial cells [11]. Both placental lactogen and prolactin are able to induce Stat5a phosphorylation [16, 17]. The data point to a specific role for placental lactogen, because prolactin levels equivalent to those present during lactation were incapable of either maintaining Stat5a phosphorylation or of inhibiting apoptosis in nonpregnant mice [3].

A novel observation was that Stat5a retains nuclear localization in the involuting mammary gland even as it becomes dephosphorylated. These data suggest that Stat5a can be dephosphorylated in the nucleus, and that nuclear export of Stat5a can be disassociated from dephosphorylation. Recent work in tissue-culture cell model systems has demonstrated that nuclear export of the related family member Stat1 is dependent on the chromosome region maintenance 1 export receptor and follows nuclear dephosphorylation of Stat1 [18, 19]. Retention of unphosphorylated Stat1 in the nucleus reduced the transcriptional response to a stimulatory cytokine in these tissue-culture cell models [19]. Retention of unphosphorylated Stat5a during involution may be a factor that contributes to the observed down-regulation of milk protein gene expression following forced weaning [20].

Other hormones of pregnancy may also regulate mammary involution. Estrogen enhances mammary regression [21] during lactation, but its effects on induced involution are unknown. Although estrogen receptor is present in lactating mammary gland, it has been reported to be inactive because of the insensitivity to estrogenic induction of PR [22]. In lieu of the present results, this does not appear to be true for lactating tissues of pregnant mice. Recent evidence suggests that estrogen receptor β_2 acts as a dominant repressor of estrogen receptor α during lactation in rats [23]. Additional investigation regarding the effects of pregnancy on estrogen receptor variants in lactating mammary tissue may help to clarify interactions among steroid receptors and their variants.

The effect of pregnancy on mammary involution after forced weaning appears to differ from that of concomitant pregnancy on mammary regression during natural weaning in mice [24]. During natural weaning, pups gradually reduce their intake of milk, and autocrine regulation of milk secretion may prevent the extensive milk stasis that is observed with forced litter removal during peak lactation [25]. Pregnancy has been reported to increase the rate of mammary involution during the declining phase of lactation

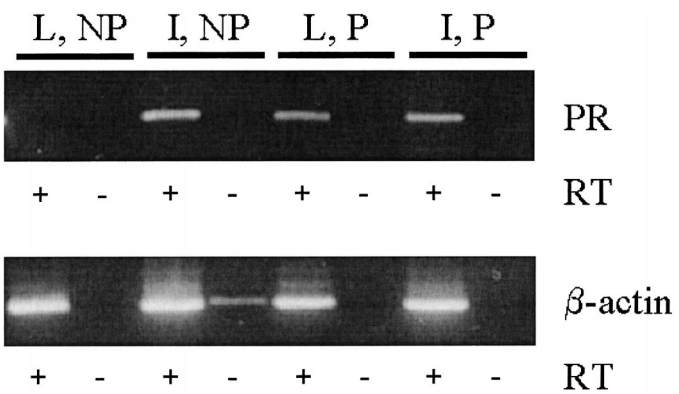

FIG. 4. Expression of PR transcripts in lactating and involuting mammary tissues of pregnant and nonpregnant mice. Upper panel shows RT-PCR of PR transcripts. Lower panel shows RT-PCR of β-actin transcripts. Lanes designated by + were reversed transcribed; lanes designated by − were not reverse transcribed before PCR. I, Involuting (postweaning); L, lactating; NP, nonpregnant; P, pregnant.

brought on by natural weaning [1]. The differences in the effect of pregnancy observed in the present study and in that of Wilde et al. [1] may be a function of both the stage of lactation and the frame of reference. Pregnancy hastened mammary regression during late lactation as compared to that in lactating, nonpregnant mice [1]. In the present study, effects of pregnancy were evaluated at the early phases of mammary involution after forced weaning during peak lactation. Concomitant pregnancy retarded mammary involution during the nonlactating period after forced weaning as compared to that in similarly treated, nonpregnant mice. In both situations (late lactation and postweaning), cell proliferation was promoted by concomitant pregnancy, and mammary involution occurred.

The bcl-2 family of genes encode for proteins that are key regulators of apoptosis [26] and possess either apoptotic- or antiapoptotic (survival)-promoting activity. The primary family members that promote survival are bcl-2, bcl-x_L, bfl-1, and bcl-w; factors that promote apoptosis include bax, bcl-x_S, bak, bad, and bid. The integration of death and survival signals determines the fate of a tissue or a cell. Milk stasis increases the expression of both survival and death proteins within the mammary gland. The presence of survival proteins during early mammary involution may be important for maintaining the integrity of alveoli and the reversibility of involution during the first 24–48 h. However, the prevailing expression of proapoptotic proteins likely pushes the tissue into irreversible involution. In mammary tissue, bax and bcl-x_S have been implicated as being important proteins in promoting involution of this tissue. When expression of pro- and antiapoptotic members of the bcl-2 family were evaluated in the present study, it was found that pregnancy had little effect on transcript levels for these proteins. However, it may be noteworthy that, in those mice (concomitantly pregnant) with reduced mammary involution, expression of bfl-1 transcripts was increased. In the presence of elevated bfl-1, cell survival is promoted, but unlike the case with other family members, cell proliferation may continue [27, 28].

This study shows that pregnancy retards, but does not prevent, mammary involution. Alveolar integrity was maintained for 72 h after forced weaning on Day 10 of lactation. Loss of mammary epithelial cells was reduced by the combined influence of decreased apoptosis and enhanced mammary cell proliferation. Thus, although alveolar structural integrity was maintained for the 72-h period of investigation, mammary cell turnover occurred. Increased expression of bfl-1 may be involved in mediating the effects of pregnancy on mammary involution. Steroids of the fetal-placental unit are hypothesized to be the hormonal signals promoting the effects of pregnancy. However, prolactin and placental lactogen cannot be ruled out as regulators.

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