Glucocorticoids Maintain the Extracellular Matrix of Differentiated Mammary Tissue During Explant and Whole Organ Culture (44518)

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Abstract. **Mouse mammary whole organ culture (WOC) and explant culture of lactating tissue were used to investigate the mechanism by which glucocorticoids maintain secretory epithelium following lobuloalveolar development. The relative number of mammary epithelial cells expressing glucocorticoid receptors did not change with the loss of secretory epithelium during involution as demonstrated with competitive binding assays and immunohistochemistry for the glucocorticoid receptor. Furthermore, glucocorticoids did not inhibit AP-1 binding activity. However, Northern analysis demonstrated that genes associated with the breakdown of the extracellular matrix were not expressed in tissues cultured with glucocorticoids, in contrast to their upregulation during involution of mammary tissue cultured with insulin alone. Tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA expression was lowest in tissue cultured in the presence of glucocorticoids and increased 2.3-, 3.4-, and 9-fold when tissues were involuted in the presence of insulin (Ins) alone, Ins and hydrocortisone (Hyd) with 0.005 mg/ml, or 0.01 mg/ml collagenase IV, respectively. These data indicate that glucocorticoids maintain mammary differentiation in part by inhibiting the turnover of basement membrane.** [P.S.E.B.M. 2000, Vol 224:76–86]

The mammary gland undergoes substantial morphological and ultrastructural changes during involution. Investigators have found that lactogens, in particular glucocorticoids, delay and/or inhibit the onset of mammary involution. Ossowski (1) demonstrated that injecting mice with Hyd at the time of weaning prevented a rise in urokinase (uPA) levels associated with the onset of mammary involution and tissue remodeling. Injection of Hyd at the time of weaning also partially maintained β-casein expression and prevented activation of the matrix metalloproteinase (MMP) gelatinase A and the increase in stromelysin-1 (MMP-3) mRNA associated with the onset of involution (2). Implantation of slow release glucocorticoid pellets into mammary glands at the time of weaning completely inhibited involution of the secretory epithelium partly by inhibiting the expression of stromelysin-1, c-*jun*, and sulfated glycoprotein-2 (SGP-2). However, glucocorticoids did not inhibit AP-1 DNA binding activity, protein kinase-A (PKA) activity, or the induction of c-*fos*, *jun*B, and *jun*D mRNA expression (3).

These findings have led to the hypothesis that glucocorticoids inhibit tissue remodeling associated with mammary involution, but not apoptosis (2, 4). To investigate the role of glucocorticoids in the maintenance of mouse mammary secretory epithelium, we used two defined *in vitro* systems: WOC and mammary explant culture. These culture systems maintain mammary epithelial cells in their natural environment including extracellular matrix (ECM) and cell-cell contacts, while permitting the addition and removal of known hormones and/or factors to the media (5). In a previous study, we determined that WOC mimicked *in vivo*

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mammary gland involution, as the secretory cells died *via* apoptosis and peaked 6 days following hormone withdrawal (6). In this study, mouse mammary WOC was used to determine that glucocorticoids were necessary for maintenance of secretory epithelium following lobuloalveolar development during WOC. We then examined the mechanism(s) of glucocorticoid maintenance of mammary differentiation by examining mammary morphology and expression of genes that regulate extracellular matrix and basement membrane turnover in the presence and absence of collagenase IV (gelatinase), using explant culture of lactating tissue. Culturing mammary tissue in the presence of the metalloproteinase, collagenase IV, allowed us to distinguish the effects of the basement membrane and those of glucocorticoids on maintenance of mammary differentiation.

Materials and Methods

Animals and *In Vitro* Culture of Mammary Tissue. Animal experimentation was conducted under the regulations of the Institutional Animal Care and Use Committee at the University of Vermont. For WOC, female BALB/c mice, 26–28 days old (Charles River Laboratories, Quebec, Canada) were treated with subcutaneous estrogen and progesterone (E/P) pellet implants (7, 8). Nine days later, abdominal mammary glands were removed and cultured at 37°C in a humidified chamber of 50% O_2 and 5% CO_2 for 5 days in Waymouth's medium 752/1 (Sigma Chemical Co, St. Louis, MO; penicillin, 100 U/ml; streptomycin, 100 μg/ml); gentamycin sulfate, 50 μg/ml; 20 m*M* Hepes supplemented with the lactogenic hormone complex: Ins (5.0 μg/ml), aldosterone (Ald; 0.10 μg/ml), Hyd (0.10 μg/ml), prolactin (PRL; 1.0 μg/ml), and epidermal growth factor (EGF; 0.060 μg/ml) to achieve lobuloalveolar differentiation (7, 9). In the first experiment, on Day 5 media were changed to Waymouth's medium supplemented with: Ins; Ins and Ald; Ins and Hyd; Ins and progesterone; Ins and PRL; Ins, Ald, and Hyd; or lactogenic hormone complex and cultured for 6 additional days to determine the effects of hormones on maintenance of mammary morphology. In the second experiment, on Day 5 the medium was changed to Waymouth's medium supplemented with: Ins; Ins and Hyd; Ins Hyd, and 0.005 or 0.01 mg/ml collagenase IV (Sigma Chemical Co., St. Louis, MO) and cultured for 6 additional days, to examine the effects of glucocorticoids and ECM on maintenance of mammary morphology.

For explant culture, eight BALB/c mice (Charles River, Canada) that had lactated 5–9 days were euthanized by CO_2 narcosis followed by cervical dislocation. All 10 mammary glands were removed and cut into explants (2 mm). Approximately 70 mg of tissue were floated on siliconized lens paper and cultured in Waymouth's medium supplemented with Ins (5.0 μg/ml); Ins and Hyd (0.10 μg/ml), Ins, Hyd and 0.005 mg/ml or 0.01 mg/ml collagenase IV for 6 days in a humidified chamber of 5% CO_2 and 50% O_2 with medium changes every day.

Hormones were purchased from Sigma Chemical Co. (St. Louis, MO) except PRL, which was obtained from NIH Hormone Distribution Program (NIDDK-oPRL-20; AFP-10677C), and EGF was obtained from Collaborative Biomedical Products (Bedford, MA).

Morphology. Following WOC, mammary whole mounts were prepared by defatting in acetone, staining with hematoxylin, dehydrating in alcohol, clearing with toluene, and mounting on slides (10). The degree of mammary differentiation was evaluated by counting the total number of lobuloalveolar structures per gland (n = 15) at ×250 on an Axiovert 35 microscope (Zeiss, West Germany). Analysis of variance followed by Duncan's multiple range test were used to determine statistical significance (SAS).

Tissues for histologic analysis and immunohistochemistry were prepared by fixing in methacarn (methanol-chloroform-glacial acetic acid, 6:3:1), washing in methanol followed by methyl-benzoate, clearing in xylene, and embedding in paraffin. Six-μm sections were prepared and stained with hematoxylin and eosin or left unstained for immunohistochemistry.

Isolation and Electrophoresis of DNA. DNA was isolated according to the method described in Casey *et al.* (6). Twenty μg of DNA were electrophoresed on a 1.4% agarose gel. Gels were stained with ethidium bromide, and bands were visualized with a UV transilluminator and photographed.

Northern Blot Analysis. Following culture, tissues were submerged in liquid N_2, and stored at −80°C for future analysis. Total RNA was isolated from tissues as described by Chomczynski and Sacchi (11). Twenty-five μg of RNA were electrophoresed on a 1% agarose formaldehyde gel for 5 hr at 50 V (12). RNA was transferred overnight onto Gene Screen Plus (Dupont-NEN, Boston, MA), and membranes were dried for 2 hr at 80°C.

Mouse β-casein (a gift from J Rosen at Baylor College of Medicine, Houston, TX); mouse WAP (a gift from L Hennighausen at the National Institute of Health, Bethesda, MD); mouse laminin (ATCC 63177); mouse urokinase (uPA; ATCC 63256); mouse tissue inhibitor of metalloproteinase 1 (TIMP-1; ATCC 63324); stromelysin-1 (matrix metalloproteinase 3, MMP-3; ATCC 63083) cDNAs were labeled with ^{32}P dCTP(3000 Ci/mmol) by random priming (Boehringer Mannheim, Indianapolis, IN). Blots were hybridized with labeled probes (10^6 cpm/ml) overnight at 42°C in a Hybaid Oven (Hybaid Ltd, Middlesex, UK) and washed. Autoradiographs were exposed for 24 hr to 3 days at −80°C. Blots were washed and reprobed with 18S RNA labeled by nick translation (Gibco BRL, Gaithersburg, MD).

Competitive-Glucocorticoid-Binding Assay. Competitive binding of ^3H-hydrocortisone to mammary tissue was examined by modification of Gorewit *et al.* (13) procedure. Mammary glands were cut into explants (≈ 2-mm pieces) and washed in PBS. One hundred mg of explants were incubated for 2 hr at 37°C with ^3H-hydrocortisone (0.138 μCi; Du Pont, New England Nuclear

Products, Boston, MA) in PBS and with one of the following: 0.001 μg/ml hydrocortisone or 0.01 μg/ml aldosterone, 0.01 μg/ml estradiol 17-β or 0.01 μg/ml progesterone. Tissue was transferred to 1225 Sampling Manifold vacuum filter (Millipore Corp., Burlington, MA, Type: SM, Pore size: 5 μ*M*) and rinsed extensively with PBS. Explant tissue and filter paper were incubated at 50°C overnight in Solvable Solution (Du Pont, Boston, MA). Bio-Safe II scintillation fluid (Research Products International Corp., Mount Prospect, IL) was added, and samples were counted in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Analysis of variance was calculated and evaluated with Duncan's multiple range test (SAS).

Glucocorticoid Receptor (GR) Immunohistochemistry. Tissue sections were deparaffinized in xylene and rehydrated. Sections were incubated in 0.1% hydrogen peroxide for 15 min, to quench the endogenous peroxidase activity. After incubating sections with 1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO) and blocking with 10% goat serum, sections were incubated with 2.0 μg/ml polyclonal rabbit antiglucocorticoid receptor antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C overnight. Negative control sections were treated with 20 μg/ml control peptide (GR E-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to block the binding of the glucocorticoid receptor primary antibody. Sections were washed in PBS and incubated with 6 μg/ml biotinylated goat-antirabbit IgG (Vector laboratories, Burlingame, CA) for 30 min at room temperature. After sections were washed in PBS, they were incubated with streptavidin peroxidase enzyme conjugate (Zymed Laboratories Inc., South San Francisco, CA) mounted and photographed at ×320. Results were analyzed by counting epithelial cell nuclei that were stained positive (red) for GR. For each treatment 1200 cells were counted (4 glands per treatment, 300 epithelial cells per gland), and analysis of variance was calculated and evaluated for significance using Duncan's multiple range test (SAS).

Nuclear Extraction and AP-1 Gel Shift Assay. Nuclei were isolated from glands developed for 5 days in the lactogenic hormone complex and maintained in this complex for an additional 2, 4, or 6 days or involuted in Ins alone for 2, 4, or 6 days. Nuclear extracts were prepared from tissue immediately upon removal from culture (14). Two hundred mg of tissue were homogenized with a Tekmar Tissumizer (Cincinnati, OH) in 10 m*M* Hepes-potassium hydroxide, pH 7.9, 1.5 m*M* β-mercaptoethanol, 1 m*M* DTT, 0.2 m*M* PMSF, and incubated on ice for 10 min. Nuclei were collected by centrifugation for 10 min at 4°C at 3000*g* in a Beckman CPR centrifuge. Nuclear pellets were resuspended in 100 μl of 20 m*M* HEPES-potassium hydroxide, pH 7.9, 25% glycerol, 420 m*M* sodium chloride, 1.5 m*M* magnesium chloride, 0.2 m*M* EDTA, 1 m*M* β-mercaptoethanol, 1 m*M* DTT, 0.2 m*M* PMSF, and incubated for 10 min on ice, followed by centrifugation at 11,000*g*. Supernatant was collected, and extracts were stored at −80°C. Protein concentration was determined with Bio-Rad microassay (Bio-Rad Laboratories, Hercules, CA).

AP-1 oligonucleotides (5′ AAGCATGAGTCAGACAC 3′) were chemically synthesized (Integrated DNA Technologies, Inc., Coralville, IA) and radiolabeled with γ-^{32}P ATP (4500 Ci/mmol) using T4 polynucleotide kinase (5 U/μl; Gibco BRL, Gaithersburg, MD) for 30 min at 37°C. Nuclear extracts containing 5 μg protein were incubated for 10 min on ice in 10 m*M* HEPES-NaOH, pH 7.4, 50 m*M* KCl, 1 m*M* EDTA, 5 m*M* magnesium chloride, 1 m*M* DTT, 10% glycerol, and 2 μg polyd(IC). Radiolabeled AP-1 oligonucleotide was added and incubation was continued for 20 min. Protein/DNA complexes were resolved on a 6% polyacrylamide gel at 4°C. Positive controls were run with nuclear extracts from phorbol-ester (TPA)–treated Hela cells, and negative controls were run with no protein.

Zymography. Zymography was performed for gelatin-degrading enzymes as described by Talhouk *et al.* (22). Briefly, mammary tissue was frozen immediately in liquid N_2 and stored at 80°C. Tissue was homogenized with a Tekmar Tissumizer 1:1 (wt/vol) in extraction buffer (1% Triton X-100 in 500 m*M* Tris-HCl buffer, pH 7.6, 200 m*M* NaCl, 10 m*M* $CaCl_2$). The suspension was freeze-thawed four times in ethanol and dry ice and microcentrifuged (12,000*g* for 30 min at 4°C). Supernatant was collected and stored at −80°C until zymography. Protein concentration was determined with BioRad Microassay (Bio-Rad Technologies, Inc., Hercules, CA). Forty μg of protein was electrophoresed on 7% SDS-polyacrylamide gel prepared with 1 mg/ml gelatin (Sigma Chemical Co., St. Louis, MO). After electrophoresis, gels were washed two times for 30 min in 2.5% Triton X-100 at room temperature, and then incubated at 37°C for 24 hr in 50 m*M* Tris-HCl, pH 8.0, 5 m*M* $CaCl_2$, and 0.02% NaN_3. Gels were stained with Coomassie Blue R250 for 1 hr and destained overnight in water. Gelatin-degrading enzymes were visualized as clear bands, indicating proteolysis of the substrate protein in the gel.

Densitometry. Densitometric analysis of autoradiographs was performed on a Macintosh 7100166 computer using the public domain NIH Image program (developed at the U.S. National Institute of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Results

Glucocorticoids Maintain Secretory Epithelium. Lobuloalveolar development induced during WOC was maintained in media supplemented with the lactogenic hormone complex, Ins and Hyd, or Ins, Ald, and Hyd as demonstrated by mammary whole mounts and histologic analysis (Figs. 1A (a, b, and c) and 1B (a, b, and c)). Lobuloalveoli of mammary glands from all other hormone combinations in culture underwent degeneration and incomplete regression (Figs. 1A (d, e, and f) and 1B (d, e, and f)), whereas glands maintained in Ins alone were almost completely involuted (Figs. 1A (g) and 1B (g)).

The total number of lobuloalveolar structures in mam-

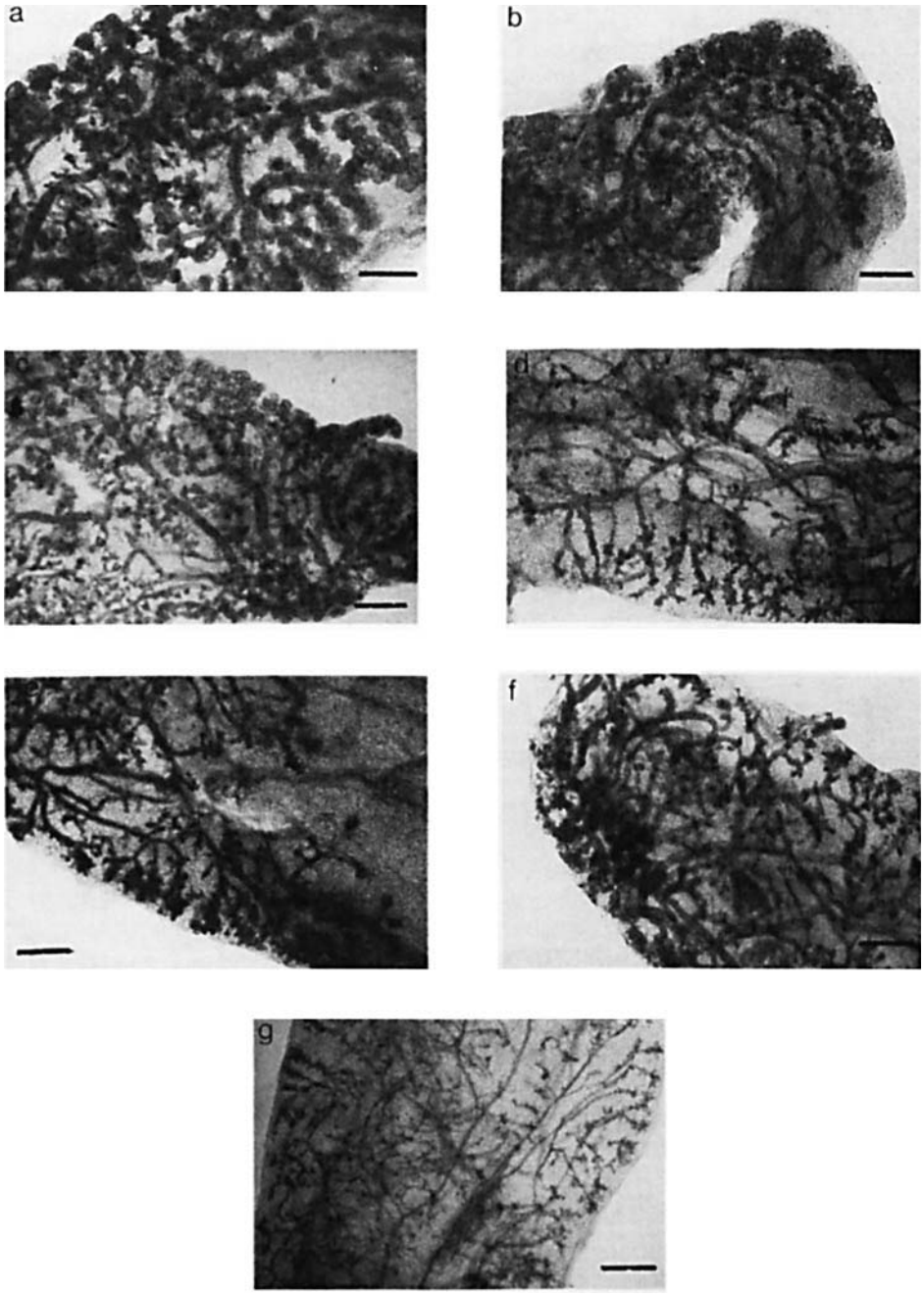

Figure 1. (A) The effect of hormone treatment on the maintenance of mammary morphological differentiation was examined in hematoxylin-stained whole mounts. Glands developed for 5 days in WOC with the lactogenic hormone complex and then cultured for 6 additional days in (a) lactogenic hormone complex, (b) Ins and Hyd, or (c) Ins, Ald, and Hyd appeared to maintain lobuloalveolar development completely. Alveolar degeneration and incomplete regression of lobuloalveolar structures were apparent in glands maintained in (d) Ins and PRL, (e) Ins and Ald, or (f) Ins and Prog. Lobuloalveoli were almost completely regressed in glands maintained in (g) Ins alone. Bar is equal to 0.55 mm.

mary whole mounts was counted to determine differences in varying hormone combinations. Glands cultured with Ins and Hyd or Ins, Ald, and Hyd following initial induction of lobuloalveolar development were able to maintain the greatest number of lobuloalveoli, followed by glands maintained in the lactogenic hormone complex (Fig. 2).

Electrophoretic analysis of DNA isolated from mammary glands after *in vivo* E/P treatment revealed intact high-molecular-weight DNA as previously shown (6). Oligonucleosomal DNA fragmentation was observed in all other treatments indicating that cell turnover *via* apoptosis occurs during WOC regardless of the hormone treatment (data not shown).

Glucocorticoid Receptor Binding and Expression Remains Relatively Unchanged in the Transition from Differentiation to Regression in Mammary Tissue Developed During WOC. Glucocorticoid regulated maintenance of mammary differentiation was in-

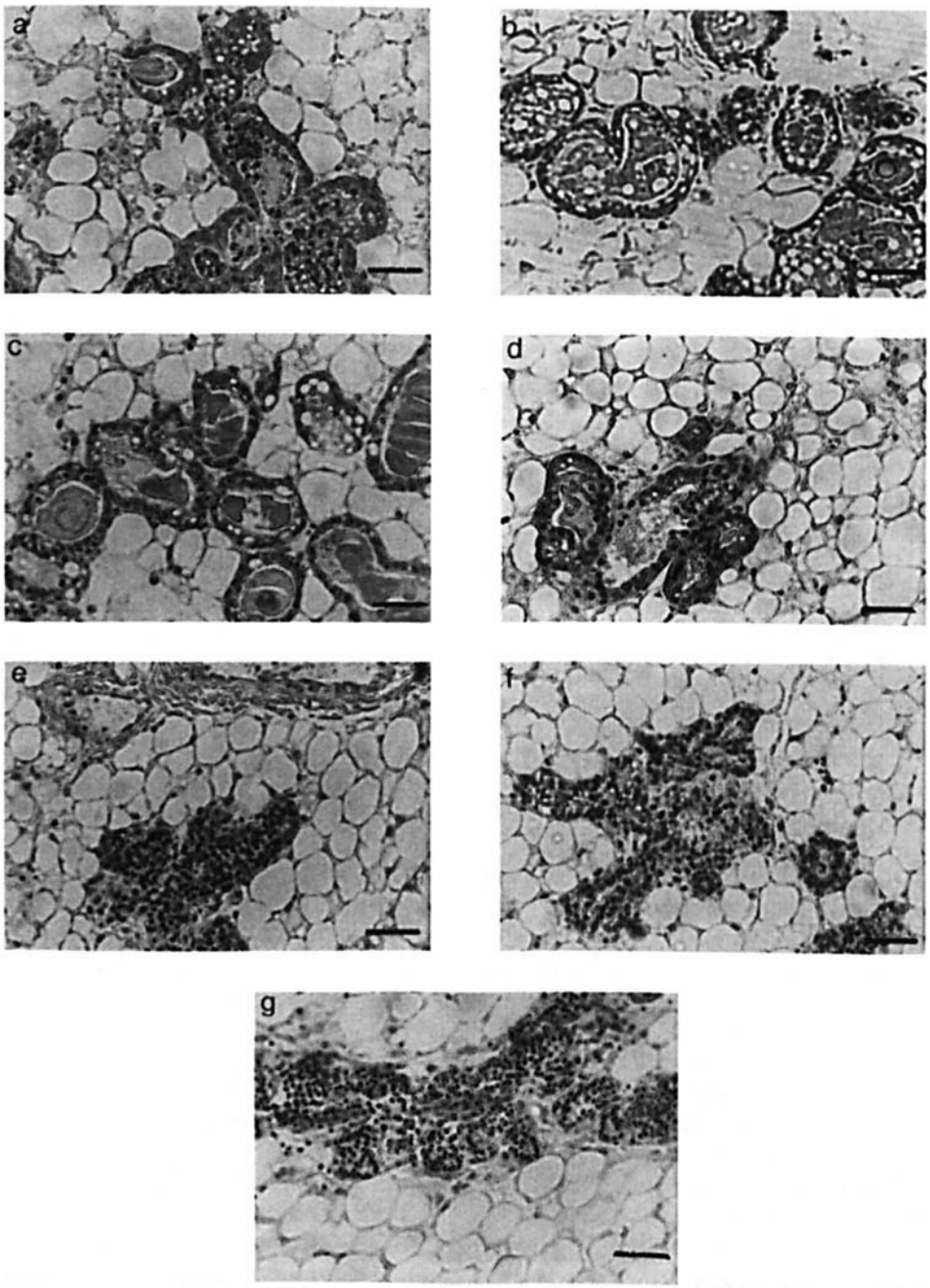

Figure 1 continued. (B) The effect of hormone treatment on the maintenance of alveolar structures was examined in hematoxylin- and eosin-stained, 6-μm sections of mammary tissue from glands developed for 5 days in WOC with the lactogenic hormone complex and then cultured for 6 additional days in (a) lactogenic hormone complex, (b) Ins and Hyd, or (c) Ins, Ald, and Hyd appeared to maintain lobuloalveolar development completely. Alveolar degeneration and incomplete regression of lobuloalveolar structures were apparent in glands maintained in (d) Ins and PRL, (e) Ins and Ald, or (f) Ins and Prog. Lobuloalveoli were almost completely regressed in glands maintained in (g) Ins alone. Bar is equal to 0.082 mm.

vestigated using competitive binding assays for ^{3}H-hydrocortisone and immunohistochemistry to determine if glucocorticoid binding varied when tissues were cultured in various hormone combinations. Glands maintained in the lactogenic hormone complex specifically bound the greatest amount of ^{3}H-hydrocortisone (0.409 pmol/100 mg tissue) followed by Ins and PRL (0.323 pmol/100 mg tissue) and Ins, Ald, and Hyd (0.248 pmol/100 mg tissue), with the least amount of binding in tissue maintained in Ins alone (0.038 pmol/100 mg tissue). Competition for the glucocorticoid receptor by progesterone, estrogen, and aldosterone with ^{3}H-hydrocortisone was also investigated. The same trend was observed when progesterone was used to compete with ^{3}H-hydrocortisone, although progesterone competition for the glucocorticoid receptor was approximately half of that observed with hydrocortisone. Competition for the gluco-

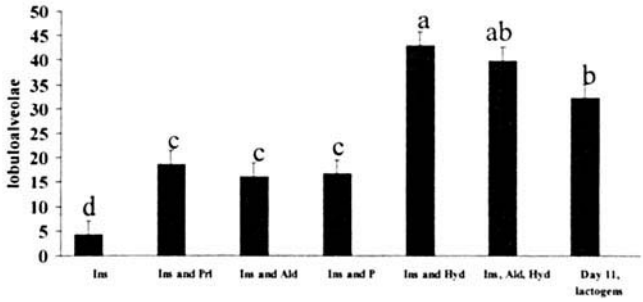

Figure 2. Maintenance of morphological differentiation was evaluated by counting the total number of lobuloalveolar structures in 15 glands from each treatment. Analysis of variance followed by Duncan's multiple range test was used to determine statistical significance. The lowercase letters above each bar indicate significant difference between groups if the letters differ at $P < 0.05$, SE = 2.8.

corticoid receptor was not detected when estradiol or aldosterone was used to assess competitive binding.

Since the differences observed in ^{3}H-hydrocortisone binding may be due to differences in the number of cells per gland, and since a fair amount of variability was evident in the binding data, immunohistochemistry was used to determine the percentage of cells expressing glucocorticoid receptors. Red-stained nuclei demonstrated that glucocorticoid receptors were present in all hormone treatments (Fig. 3), regardless of whether lobuloalveolar structures were maintained (lactogenic hormone complex; Ins,Ald,Hyd; or Ins and Hyd), partially maintained (Ins and PRL; Ins and Ald; Ins and progesterone) or regressed (Ins alone). Tissues maintained in the lactogenic hormone complex or in Ins and Hyd had the greatest percentage of epithelial cells expressing glucocorticoid receptors; however, there was no statistical difference observed among any of the treatments (Fig. 4).

Glucocorticoids Do Not Inhibit AP-1 Binding in Differentiated Mammary Tissue. To determine if glucocorticoids maintain lobuloalveolar development by inhibiting AP-1 binding, nuclear extracts from glands were incubated with radiolabeled AP-1 oligonucleotides. AP-1 binding activity was observed in both involuting (glands cultured in Ins alone for 2, 4, or 6 days) and differentiated tissue (glands maintained for 2, 4, or 6 days in the lactogenic hormone complex following lobuloalveolar development) and was not different in any treatments following densitometric analysis of autoradiographs (Fig. 5).

Maintenance of Lobuloalveolar Structures Is Dependent on Presence of Basement Membrane. Collagenase IV was added to mammary WOC media following lobuloalveolar differentiation to determine the morphological effect of disruption of the basement membrane. Morphology of the glands varied dramatically depending upon the concentration of collagenase IV used in culture. Integrity of lobuloalveolar structures was lost as collagenase IV concentration increased. Glands cultured in 1 mg/ml collagenase IV were completely digested within 48 hr of culture (data not shown). Glands cultured in 0.1 mg/ml collagenase IV were partially digested, and epithelial tissue,

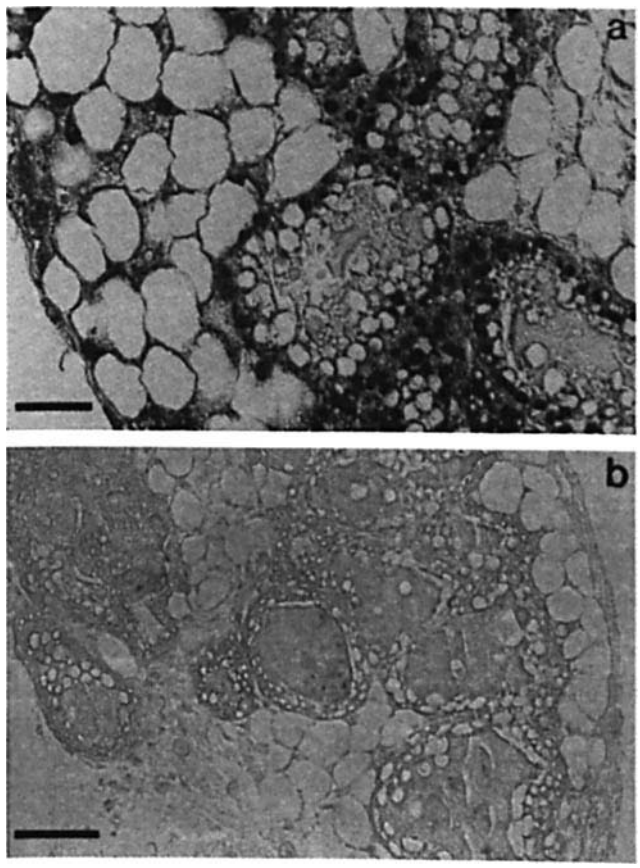

Figure 3. Immunohistochemistry for the glucocorticoid receptor demonstrated the nuclear localization of the receptor in mammary epithelial cells in 6-μm sections from glands maintained in the lactogenic hormone complex incubated with the (a) GR primary antibody and the (b) control peptide indicates the specificity of the antibody for the receptor. Bar is equal to 0.082 mm.

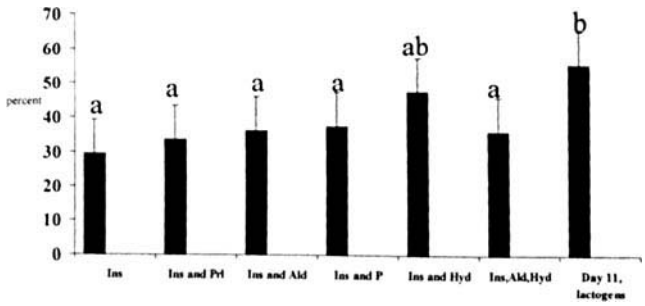

Figure 4. The effect of hormone treatment on the percentage of mammary epithelial cells expressing the glucocorticoid receptor was evaluated by counting the number of cells expressing the receptor as indicated by immunohistochemistry. Analysis of variance followed by Duncan's *t* test for multiple comparison was used to determine statistical significance. $P < 0.05$, SE = 5.9.

although amorphous, was identifiable upon examination of whole mounts (Fig. 6a). Glands cultured in 0.01 mg/ml collagenase IV maintained lobuloalveolar-like structures, although they lacked the grapelike structures observed in healthy lobuloalveoli (Fig. 6b). By contrast, glands cultured in the presence of 0.005 mg/ml collagenase IV maintained lobuloalveolar structures (Fig. 6c) and were virtually indis-

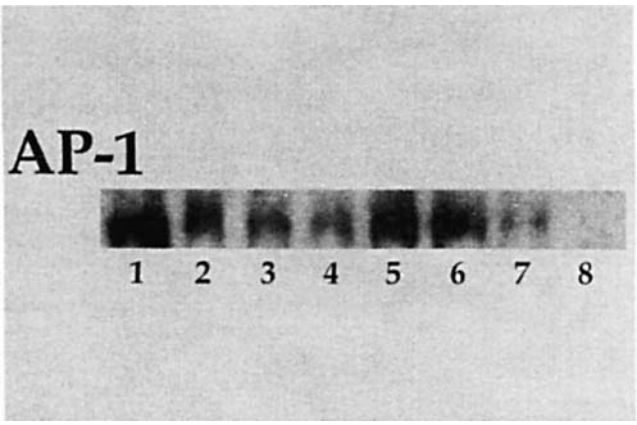

Figure 5. AP-1 gel shift assay with nuclear extracts isolated from glands cultured in the presence and absence of glucocorticoids demonstrates that glucocorticoids do not inhibit AP-1 binding activity. Radiolabeled AP-1 oligonucleotides were incubated with nuclear extracts containing 5 μg of protein from (1) TPA treated Hela cells (positive control); glands maintained in Ins for (2) 2 days; (3) 4 days; (4) 6 days; or lactogenic hormone complex for (5) 2 days; (6) 4 days; (7) 6 days; or (8) no nuclear extract (negative control).

tinguishable from glands cultured in lactogenic hormones alone (Fig. 6d).

For the remainder of these studies, mouse mammary explant culture was used to reduce the number of animals needed for isolation of RNA and Northern Blot analysis. Explant tissue isolated from lactating mice responded to culture conditions in a manner similar to mammary tissue that was isolated from E/P-treated virgin mice and induced to develop lobuloalveolar structures during WOC. Examination of mammary histology from tissue maintained in explant culture demonstrated that digestion of the basement membrane with collagenase IV resulted in an involution-like regression even though Hyd was present in the medium (Fig. 7). Explant tissue cultured for 6 days in Waymouth's medium supplemented with Ins and Hyd maintained healthy, active alveolar structures with many lipid droplets (Fig. 7a). Tissue cultured with Ins alone appeared to be undergoing involution with collapsing alveoli and adipose tissue repopulating the stromal area (Fig. 7b). Tissue cultured in Ins, Hyd, and 0.005 or 0.01 mg/ml collagenase IV appeared to be involuting, but at a slower rate than tissue incubated with Ins alone (Figs. 7c and 7d).

Glucocorticoid and Extracellular Matrix Regulate Expression of Genes Associated with Mammary Differentiation. To further evaluate the individual effects of Hyd and basement membrane on mammary differentiation, milk protein gene expression in explant tissue from lactating mice was examined by Northern analysis. β-Casein mRNA expression was maintained at very similar levels among all treatments (Table I). WAP mRNA expression was not maintained in any treatment during explant culture (data not shown).

To evaluate the effects of Hyd on genes associated with basement membrane turnover, Northern analysis was used to examine the expression of uPA, MMP-3, TIMP-1, and laminin mRNA. Expression of the basement membrane proteases, uPA and MMP-3, was greatest in tissue cultured in Ins alone, and there was little to no expression of MMP-3 and uPA in tissue cultured with Hyd, regardless of the presence of collagenase IV (Table I). TIMP-1 mRNA expression was upregulated in tissue cultured with Ins alone by 2.3-fold, with Ins, Hyd, and 0.005 mg/ml collagenase IV by 3.4-fold, and 9-fold in tissue cultured with Ins, Hyd, and 0.01 mg/ml collagenase IV, relative to tissue cultured with Ins and Hyd alone. Laminin mRNA was expressed in tissue cultured with Ins alone and Ins, Hyd, and 0.01 mg/ml collagenase IV, but was not expressed in other treatments (Table I).

Exogenous Collagenase IV Activity Is Detectable in Presence of Glucorticoids. Gelatin substrate zymography was used to determine the activity of gelatin proteases in the explant culture system. The active 62-kDa form of collagenase IV was present in the explant tissue cultured with 0.01 and 0.005 mg/ml collagenase IV (Fig. 8).

Discussion

Glucocorticoids in the presence of Ins were able to maintain both morphological and some functional mammary differentiation during WOC. Glucocorticoids are the main player in maintenance of differentiation, as Ins is an absolute requirement for maintenance of mammary epithelium *in vitro*. However, secretory epithelium is only maintained in the presence of cortisol (15). Although PRL is needed for induction of mammary differentiation and milk protein gene expression (5, 9), we found that it is not necessary for maintenance of lobuloalveolar structures.

The differential expression of milk protein mRNA was used as a marker of mammary differentiation. In general, β-casein expression was detected by Day 8 of pregnancy, and WAP expression was detected by Day 14 of pregnancy in mice (16). The state of differentiation that was maintained during explant culture was equivalent to the end of the first trimester in a mouse, as β-casein expression was maintained whereas WAP expression was lost. These data indicate that although Hyd is able to maintain mammary morphology and β-casein expression, other local and/or systemic factors, most probably PRL, must be present to maintain full mammary differentiation. Interestingly, when Northern blot analysis was used to determine if WAP mRNA was expressed in tissue developed and maintained during WOC, no expression of WAP mRNA was detected after 5 days of incubation in the lactogenic hormones. However, when tissue was cultured for 11 days in the presence of the lactogenic hormone complex, WAP mRNA expression was detected (data not shown). These data further support the need for PRL to induce WAP expression, and indicate, as *in vivo*, that WAP mRNA expression is induced during a later period of mammary development.

Maintenance of mammary differentiation through regulation of the number of glucocorticoid receptors is unlikely as competitive binding assays with ^{3}H-hydrocortisone and

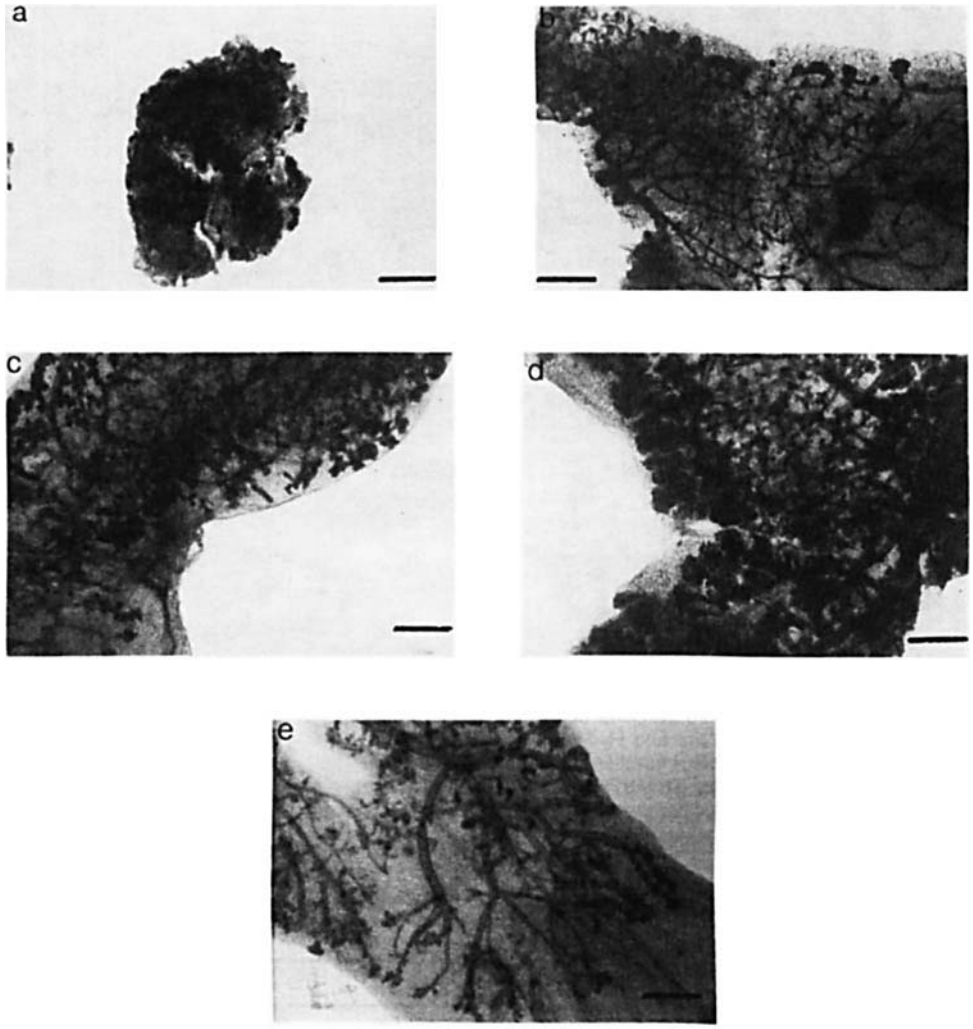

Figure 6. Whole mounts stained with hematoxylin from glands induced to develop lobuloalveoli during whole organ culture and then maintained in lactogenic medium for 6 days supplemented with: (a) 0.1 mg/ml, (b) 0.01 mg/ml, (c) 0.005 mg/ml collagenase IV, (d) lactogenic media alone, or (e) Ins alone, demonstrate the morphological effects of the loss of basement membrane on lobuloalveolar development. Bar is equal to 0.55 mm.

immunohistochemistry indicated that there was no difference in binding or number of cells expressing the receptor in differentiated or involuting tissue. We found that glucocorticoids do not inhibit AP-1 binding activity that supports a similar observation by Feng *et al.* (3). Furthermore, the observation that progesterone implants locally maintain mammary differentiation (3) may be due to progesterone binding to, and acting through, the glucocorticoid receptor. Our data and that of Shyamala (17) both demonstrate progesterone competing with hydrocortisone for the glucocorticoid receptor. Furthermore, mammary epithelial cells do not express progesterone receptors during lactation (18).

The ECM acts as a survival factor for cells. The greater the concentration of collagenase IV in culture, the greater the rate of involution of glandular epithelium as evidenced by morphological and histologic changes, regardless of the presence of Hyd. Culturing with collagenase IV at greater concentrations resulted in collapse of lobuloalveolar structures and sloughing of cells into the lumen of alveoli. Goberdham *et al.* (19) reported that apoptosis of primary mammary epithelial cells from pregnant mice cultured on collagen I in the presence of the lactogenic hormones Ins, Hyd, and PRL was suppressed in a dose-dependent manner when an overlay of basement membrane (EHS) was added. Furthermore, cells plated on EHS became detached from the ECM upon addition of antiβ1 integrin antibody and underwent apoptosis (20, 21). Thus, survival of the glandular epithelium is dependent upon both the hormonal stimulus of Hyd and an intact basement membrane.

In vivo expression of protease inhibitors TIMP-1 and PAI-1 mRNA precede the onset of ECM-degrading protease expression and activity, and remain high until Day 6 involution (22). Our data indicate that TIMP-1 expression is upregulated in involuting tissue in culture. Additionally, the presence of active collagenase IV protein greatly upregulated the expression of TIMP-1 suggesting that high levels of collagenase IV activate the expression of their inhibitors to maintain a balance in basement membrane turnover.

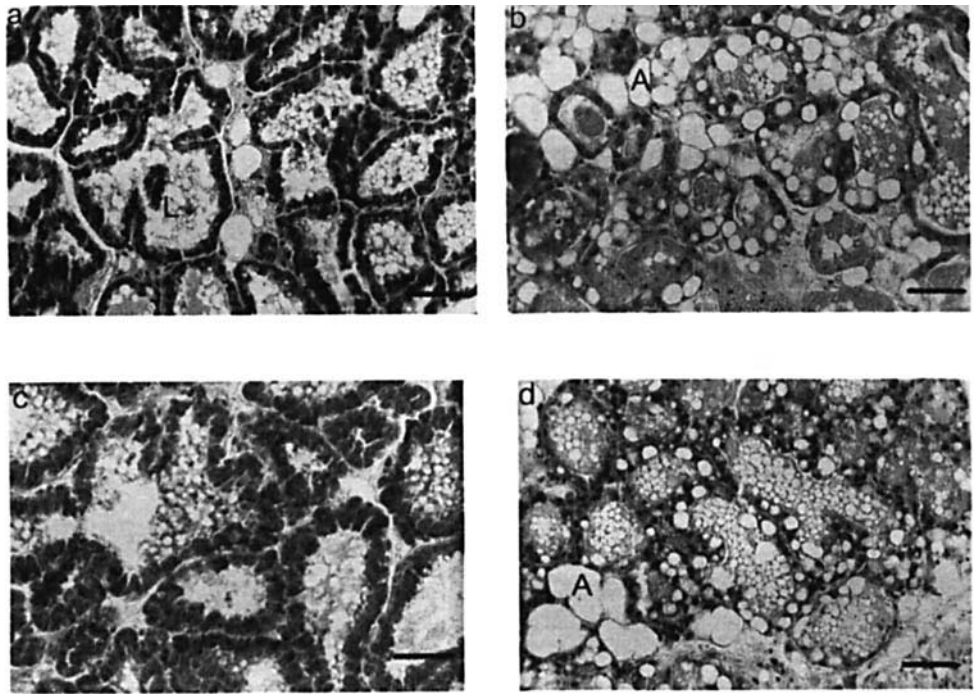

Figure 7. Hematoxylin and eosin stained 6-μm sections of explant tissue from 5- to 9-day lactating glands were cultured for 6 days in Waymouth's medium supplemented with: (A) Ins and Hyd (maintained healthy active alveolar structures with many lipid droplets (L) and secretion) or (B) Ins alone (exhibited alveolar degeneration as glandular epithelium was replaced with adipose tissue (A)). Tissue cultured in (C) Ins, Hyd and 0.005 mg/ml collagenase IV or (D) Ins, Hyd, and 0.01 mg/ml collagenase IV appeared to be involuting, but at a slower rate than tissue incubate with Ins alone, with collapsing alveoli (CA) and glandular epithelium being replaced by adipose tissue (A). Bar is equal to 0.227 mm.

When tissue was cultured with Ins alone, mRNA expression of the ECM proteases MMP-3 and uPA was upregulated. However, in the presence of Hyd, uPA and MMP-3 expression was suppressed, even when the tissue was involuting due to exogenous collagenase IV. These data are supported by the studies of other investigators showing that proteins and proteases associated with basement membrane are regulated by glucocorticoids (1, 23). This further demonstrates that glucocorticoids maintain a healthy and differentiated basement membrane by suppression of ECM protease expression.

Laminin was expressed in tissue cultured with Ins alone or with Ins and Hyd with 0.01 mg/ml collagenase IV. These culture conditions induced tissue degradation and possibly extracellular matrix turnover, as evidenced by the change in tissue morphology and histology. The high levels of laminin expression in these two treatments may indicate that the signal for ECM synthesis is not regulated by glucocorticoids but possibly by proteases or tissue degradation products resulting from involution.

The most prominent ECM-degrading protease detected in involuting tissue by substrate gel zymography is the 72-kDa gelatinase A (otherwise known as collagenase IV). The 72-kDa gelatinase, while low during lactation, is abundant as early as Day 1 of involution. The active form of gelatinase A, a 62-kDa band, appears on Day 3 of involution (22). This band was apparent in the collagenase IV-treated tissue in this study, but was not apparent in other treatments. It

Table I. Levels[a] of mRNA Expression Adjusted to 18S Expression

	Ins & Hyd[b]	Ins[c]	0.005[d]	0.01[e]
β-casein	9,668	10,111	12,928	10,162
uPA	1	9,379	1	1
MMP-3	3,404	8,764	3,505	2,547
TIMP-1	7,014	16,200	23,805	62,791
Laminin	1	6,987	1	8,043

[a] Arbitrary units. Tissue from eight pooled animals at 5–9 days lactation was chopped into explants and cultured for 6 days in the presence of [b]Ins and Hyd; [c]Ins alone; [d]Ins, Hyd, and 0.005 mg/ml; or [e]0.01 mg/ml collagenase IV. RNA was prepared from these cultures, electrophoresed on a 1% agarose-formaldehyde gel, and Northern transferred. Blots were hybridized with ^{32}P-dCTP-labeled probes and exposed to film. Densitometric analysis of autoradiographs was done using NIH image and adjusted to 18S expression.

was expected that a 62-kDa band would be detected in tissue incubated in Ins alone. This discrepancy may be due to the time point we chose to examine, in that by Day 6 of culture the active form of collagenase IV is not expressed, and may be apparent at an earlier time point.

Interestingly, electrophoretic analysis of DNA isolated from tissue indicated that mammary cells were undergoing apoptosis when cultured in the presence of glucocorticoids. We suggested in a previous paper (6), that this cell turnover may be due to the lack of a factor needed to maintain cell viability during WOC culture. However, these data may also lend support to the hypothesis of Lund *et al.* (2) and Li

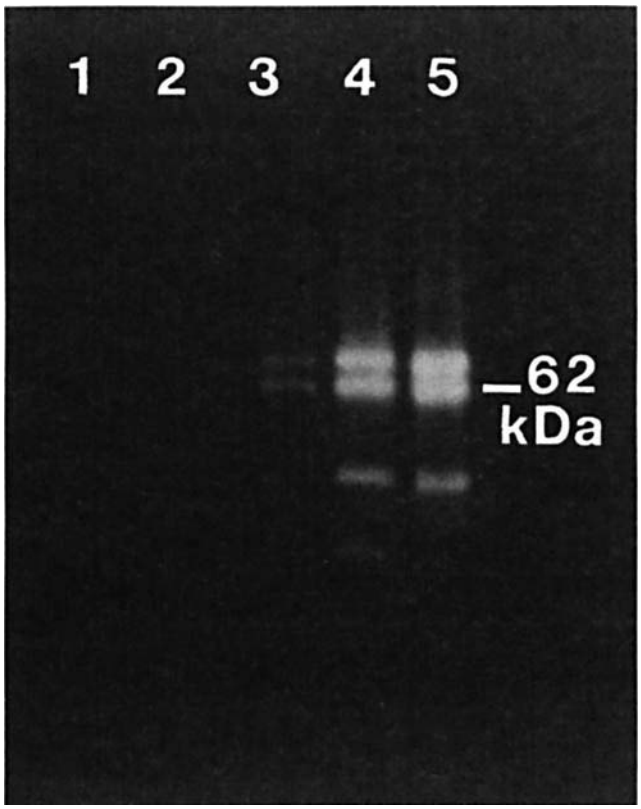

Figure 8. The cleared areas in the 1 mg/ml gelatin substrate zymogram demonstrated the activity of gelatin proteases that were present in explant tissue from 5- to 9-day lactating mice cultured for 6 days in (Lane 1) Ins and Hyd; (Lane 2) Ins; (Lane 3) Ins, Hyd, and 0.005 mg/ml collagenase IV; and (Lane 4) Ins, Hyd, and 0.01 mg/ml collagenase IV. Lane 5 is the positive control, 0.005 mg/ml collagenase IV alone. The 62-kDa band indicates the active form of collagenase IV.

et al. (4), which states that there are two phases of involution. Briefly, the first phase is marked by apoptosis and is initiated within 24 hr of cessation of the suckling stimulus. The second phase is marked by an increased expression of tissue remodeling enzymes and a decreased expression of their inhibitors. Progression to and through the second phase of involution may be inhibited if the suckling stimulus is resumed and/or if the animal is treated with glucocorticoids (2, 4). Similarly, our data suggest that glucocorticoids play a role in the second phase by inhibiting mammary tissue remodeling.

By using defined *in vitro* systems, the complications of *in vivo* systemic factors were eliminated including the animal's biological response to the presence of foreign proteins or overexpression of transgenes. These defined systems allowed for the evaluation of the individual effects of both hormones and the ECM protease collagenase IV on mammary differentiation. Whole mounts from glands developed during WOC were used to observe the morphological effects and glucocorticoid receptor expression, whereas effects of gene expression were evaluated in lactating tissue incubated in explant cultures. These data further demonstrate the role glucocorticoids play in inhibition of basement membrane turnover to maintain mammary differentiation. Furthermore, the data also indicate that ECM metabolites may regulate basement membrane deposition and turnover, as TIMP-1 and laminin expression were greatest in treatments with the greatest degree of tissue remodeling.

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