

Uteroglobin Gene Expression in the Rabbit Uterus throughout Gestation and in the Fetal Lung

Relationship between Uteroglobin and Eicosanoid Levels in the Developing Fetal Lung

Alessandro Peri,* Norman H. Dubin,[†] Ramasubbareddy Dhanireddy,[§] and Anil B. Mukherjee*

*Section on Developmental Genetics, Human Genetics Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-1830; [†]Department of Obstetrics and Gynecology, Union Memorial Hospital, Baltimore, Maryland 21218; and [§]Georgetown University Medical Center, Department of Pediatrics, Division of Neonatology, Washington, D.C. 20007

Abstract

Uteroglobin (UG) gene encodes a cytokine-like, multifunctional, antiinflammatory protein, with potent phospholipase A₂-inhibitory activity. It has been suggested that during implantation this protein protects the embryos from maternal immunological assault, facilitates the maintenance of quiescence in the uterus throughout pregnancy, prevents the onset of premature labor, and helps maintain an inflammation-free respiratory organ. This latter function of UG is suggested to be accomplished by preventing hydrolysis of surfactant phospholipids by a lung-specific phospholipase A₂. Using reverse transcription polymerase chain reaction, in situ hybridization, immunofluorescence, and radioimmunoassay, we studied UG gene expression in the rabbit uterus throughout gestation and in the fetal lung. Here, we report that: (a) contrary to previous reports, UG gene expression in the rabbit uterus occurs throughout gestation with a precipitous decline just before parturition; (b) this gene expression is dramatically increased in the fetal lung with increasing gestational age; and (c) while there is an inverse relationship between the levels of UG, PGE₂, and PGF_{2α}, a positive correlation was found in that of UG and leukotriene C₄ in the fetal lung. Our results raise the possibility that dysregulation of UG gene expression, at least in part, may contribute to the onset of premature labor and the development of inflammatory lung disease in premature neonates. (*J. Clin. Invest.* 1995. 96:343–353.) **Key words:** Clara cell 10-kD protein gene • inflammation • prostaglandins • leukotrienes • lung development

Introduction

During the past decade dramatic progress has been made in understanding the molecular mechanisms of early mammalian development. However, several aspects of preimplantation embryonic growth, differentiation and maturation of the fetus, and the mechanism of parturition remain poorly understood. Several critical questions are still unanswered. For example, it is not clear: (a) what role(s) the ovarian steroid-dependent endome-

trial secretory proteins play in maintaining a quiescent, immunologically privileged environment for the developing fetus; (b) how the motility and invasiveness of the trophoblasts, a highly invasive cell type which has the potential for transforming into choriocarcinoma, are regulated; (c) what initiates contractility of the uterine smooth muscles to facilitate birth of the neonate; and (d) how the surfactant, a phospholipid-protein complex, essential for respiration, is protected from hydrolysis by a specific group I phospholipase A₂ (PLA₂)¹ in the developing fetal lung. Undoubtedly, these are complex questions and a single unified mechanism to explain all facets of these physiological phenomena may not exist. However, there is compelling evidence to suggest that factors endogenous to the uterus and to the lung, at least in part, may play critical role(s) in regulating many of these vital physiological processes. Because progesterone is essential for the maintenance of pregnancy in almost all mammalian species, we focused our investigation on the expression of a steroid-regulated gene, uteroglobin (UG), in the rabbit to answer some of these questions.

UG, (1) or blastokinin, (2) is a low molecular weight (15.8 kD), homodimeric, multifunctional, cytokine-like, secretory protein. UG was first detected in the rabbit uterine fluid during early pregnancy (for a review see references 3 and 4). Subsequently, it was found in organs other than the uterus and it is now clear that this protein is constitutively expressed at a high level in the epithelia of the respiratory system. In addition, UG is present in the gastrointestinal and genitourinary systems of both males and females and a low level of this protein is detectable in the blood which increases during pregnancy (5–11). Specific steroid and nonsteroid hormones regulate UG gene expression in individual organs. For example, UG gene expression is stimulated threefold in the lung by corticosteroid treatment of the rabbit and in the uterus it is dramatically elevated by progesterone treatment (for a review see reference 3). Prolactin has been demonstrated to augment progesterone-dependent UG gene expression (12).

Several physical and biological properties of UG have been established. These include binding of progesterone (13) and polychlorinated biphenyls (14), masking of antigenicity of preimplantation embryos (15–17) and epididymal spermatozoa (18) in vitro, the inhibition of monocyte and neutrophil chemotaxis and phagocytosis (19–21), and the inhibition of ADP- and thrombin-induced (but not arachidonic acid-induced) platelet

Address correspondence to Anil B. Mukherjee, Building 10, Room 9S242, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-1830. Phone: 301-496-7213; FAX: 301-402-0234.

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1. Abbreviations used in this paper: DEPC, diethylpyrocarbonate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hUG, human uteroglobin; LTC₄, leukotriene C₄; PLA₂, phospholipase A₂; RT-PCR, reverse transcriptase PCR; rUG, rabbit uteroglobin; T_m, melting temperature; UG, uteroglobin.

aggregation (22). Some of these properties may, at least in part, be due to the potent inhibitory effect of UG on PLA₂ activity (23–25). It should be noted that while some lipocortins (26), a family of proteins, functionally similar to, but genetically different from UG, also inhibit PLA₂ activity by substrate depletion, the exact mechanism of PLA₂ inhibition by UGs is not yet clear.

Recently, the cDNA (27) and the 5' region (28) of the gene encoding human uteroglobin (hUG), a counterpart of rabbit UG (rUG), have been characterized. Human UG or Clara cell 10-kD protein has 61.5% amino acid sequence identity with rUG (27), 54.2% similarity with rat UG (29), and 52.8% with mouse UG (30, 31). Although this protein was originally discovered in the alveolar Clara cells (32) it is detectable in many extrapulmonary tissues similar to the ones in which rUG is expressed (33). We have recently demonstrated that hUG gene is expressed in the human endometrium (34) and this expression is induced by progesterone. It appears that some of the biological properties of hUG are virtually identical to rUG (35).

It has been reported that UG in the rabbit uterine fluid is first detectable on day 3 of pregnancy, and peak level is reached on day 5 (for a review see reference 3). UG, by inhibiting PLA₂ activity, may down-regulate the production of proinflammatory lipid mediators, which promote contraction and motility of the uterine smooth muscle. Therefore, it is suggested that UG facilitates the maintenance of myometrial quiescence during gestation. Moreover, UG may protect surfactant phospholipids from hydrolysis by a lung-specific extracellular PLA₂. This may cause not only a decreased half-life of the surfactant but also release of arachidonic acid, a chemotactic agent for inflammatory cells. Taken together, these events may contribute to: (a) the onset of premature labor and (b) the initiation and propagation of inflammation in the lung of premature neonates with respiratory distress syndrome (36). However, previous studies have reported that after day 9 of gestation endometrial UG gene expression was virtually undetectable (for a review see reference 3). This is inconsistent with the proposed role of UG during pregnancy. Additionally, a systematic study on the expression of UG gene in the fetal lung and its relationship to eicosanoid levels in this organ have not been critically evaluated. By using reverse transcriptase (RT) PCR, *in situ* hybridization, radioimmunoassay, and immunofluorescence, we established, for the first time, that both UG mRNA and immunoreactive UG protein are readily detectable in the rabbit uterus throughout the entire period of gestation. In addition, we also found that: (a) a relatively low level of UG gene expression is first detectable in the fetal lung at or around day 22 of gestation and reaches a peak immediately before parturition; (b) there is an inverse relationship between the levels of UG and prostaglandins E₂ and F_{2α} in the fetal lung; (c) there is a direct correlation between the levels of UG and those of leukotriene C₄ (LTC₄) which is known to stimulate surfactant secretion (37, 38). These results suggest that expression of UG gene in the uterus throughout gestation and in the fetal lung may play physiologically important functions.

Methods

Animals. Dated pregnant New Zealand white rabbits were obtained from a commercial breeder (Hazleton Research Products, Inc., Denver, PA) after a protocol for animal use was approved by institutional Animal Care and Use Committee. Animals were housed singly with 12-h light and 12-h dark cycles and food and water were provided *ad lib*. Rabbits

were killed at day 18, 22, 24, 26, 28, 29, 30, and 31 of pregnancy by intravenous administration of pentobarbital (60 mg/kg). Uterine and fetal lung samples were aseptically collected and they were either frozen in liquid nitrogen for RNA extraction, or embedded in OCT compound (American Histolabs, Inc., Gaithersburg, MD) and frozen in dry ice/ethanol (for *in situ* hybridization), or fixed in 4% paraformaldehyde dissolved in PBS, pH 7.4 (for immunofluorescence). Portions of the fetal lungs were homogenized for RIA of UG, prostaglandin E₂, F_{2α}, and LTC₄. Total protein was estimated by the method of Lowry et al. (39).

Uteroglobin antibody. UG, from the uteri of rabbits primed with human chorionic gonadotropin, was purified to homogeneity according to previously published method (16). A monospecific, polyclonal antibody (1:70,000 titer) against this purified rabbit UG was generated by immunizing a goat with pure antigen complexed with complete Freund's adjuvant. Subsequently the animal received four booster shots with pure uteroglobin solution in PBS (1 mg/booster) at monthly intervals. The antiserum was collected, titer checked, and specificity determined. Affinity-purified IgG from the high titer (1:70,000) antiserum was used for immunofluorescence and total antiserum at 1:10,000 dilution was used for RIA. This antibody is immunoreactive to rabbit, rat, mouse, and human UG but not any nonspecific protein tested so far including albumin, myoglobin, or carbonic anhydrase.

RNA extraction. Total RNA was extracted from the uterine specimens using the RNazol method (40) (Cinna/Biotech, Friendswood, TX), according to the instructions of the manufacturer. RNA concentrations were determined spectrophotometrically by absorption at 260 nm.

Oligonucleotide primers and probes. Purified oligonucleotide primers and probe for amplification and detection, respectively, of UG cDNA were synthesized by Synthecell Corp. (Rockville, MD). The sequence of the sense primer (RUG-L) was 5'-CCATTCTGCCACCATGAAGC-3' melting temperature [T_m] = 74°C, nucleotides 35–54 of UG mRNA). The sequence of the antisense primer (RUG-R) was: 5'-GGC-TTCTCAAGTCCGCAGGGA-3' (T_m = 75°C, nucleotides 360–341). The sequence of the oligonucleotide probe (RUG-P), used for hybridization of PCR products, was: 5'-GGATGTCAGATGAAGAAGGTG-3' (T_m = 68°C, nucleotides 223–242). The probe was labeled at the 3' end with digoxigenin-11-ddUTP using the Oligonucleotide 3' End Labeling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN), following the manufacturer's instructions. Oligonucleotide primers and probe for amplification and detection of the cDNA of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, were also obtained from Synthecell Corp. The sequence of the sense primer (GAPDH-L) was: 5'-CCATGGAGAAGGCTGGGG-3' (T_m = 74°C, nucleotides 388–405 of GAPDH mRNA), and the sequence of the antisense primer (GAPDH-R) was: 5'-CAAAGTTGTCATGGATGACC-3' (T_m = 66°C, nucleotides 582–563). The sequence of the probe (GAPDH-P) was: 5'-CTAAGCAGTTGGTGGTGCA-3' (T_m = 68°C, nucleotides 549–531). The use of these oligonucleotides has been previously described by Dveksler et al. (41). The T_ms of primers and probes were calculated by means of the program PRIMER DESIGNER (Scientific & Educational Software, State Line, PA).

For *in situ* hybridization experiments, two 48-mer oligonucleotide probes were obtained from Synthecell. The sequence of the antisense probe (RUG-P4) was: 5'-CTCTGGTCTCTGGGGCAGGGAGTCCAAACACCTTCTTCATCTGCATCC-3' (nucleotides 270–223 of UG mRNA). The sequence of the sense probe (RUG-PS) was complementary to that of RUG-P and had the opposite orientation; this probe was used as negative control. Both probes were 3' end labeled with digoxigenin-11-ddUTP using the Oligonucleotide Tailing Kit (Boehringer Mannheim Biochemicals).

Other materials. The Gene Amp Thermostable rTth Reverse Transcriptase RNA PCR kit was purchased from Perkin-Elmer Cetus Corp. (Norwalk, CT). Lumi-Phos 530, antidigoxigenin-alkaline phosphatase-conjugated antibody, blocking reagent, DNA molecular weight marker standard VI (digoxigenin labeled), random primer pd(N)₆, 4-Nitro blue tetrazolium chloride, 5-Bromo-4-chloro-3-indolyl-phosphate, and rabbit anti-goat antibody labeled with fluorescein isothiocyanate were from Boehringer Mannheim Biochemicals. "Perfect Match," used to destabi-

lize mismatched primer-template complexes in PCR, was purchased from Stratagene Inc. (La Jolla, CA). Diethylpyrocarbonate (DEPC)-treated ultra pure DNase-RNase free water, 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH = 7), hybridization denaturing solution (1.5 M NaCl, 0.5 M NaOH), and hybridization neutralization solution (1.5 M NaCl, 1.0 M Tris) were obtained from 5 Prime—3 Prime Inc. (Boulder, CO). Mineral oil, acetic anhydride, triethanolamine, sodium pyrophosphate, sodium heparin, dextran sulfate, levamisole, normal sheep serum, and mounting medium were from Sigma Chemical Co., (St. Louis, MO). NuSieve agarose was purchased from FMC Corp. BioProducts (Rockland, ME). Ultra pure agarose and ethidium bromide were from BRL Life Technologies (Gaithersburg, MD). Nytran membranes were obtained from Schleicher & Schuell, Inc. (Keene, NH). *N*-lauroylsarcosine (Na salt) was purchased from ICN Nutritional Biochemicals (Cleveland, OH) and SDS from Bio Rad Laboratories (Richmond, CA). Xylene was from Mallinckrodt Inc. (Paris, KY), paraformaldehyde from Eastman Kodak Co. (Rochester, NY) and formamide was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). Triton X-100 was obtained from Research Products International Corp. (Mount Prospect, IL).

Reverse transcription and PCR amplification. Reverse transcription of total RNAs from uterine samples and cDNA amplification were performed using the DNA Thermal Cycler 480 (Perkin-Elmer Cetus Corp.). The Gene Amp Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin-Elmer Cetus Corp.) was used to prepare the reaction mixture. The mixture for reverse transcription (20 μ l/tube) contained: 1 × reverse transcription buffer, 1 mM MnCl₂, 200 μ M dATP, 200 μ M dCTP, 200 μ M dGTP, 200 μ M dTTP, 5 U/20 μ l rTth DNA polymerase, and 25 μ M random primer pd(N)₆. The reagents were diluted using DEPC-treated ultra pure water. RNAs from uterine specimens were heated for 10 min at 95°C and then kept on ice before addition to the reaction tubes. After addition of the RNAs, the tubes were incubated at room temperature for 10 min to allow the extension of the exameric primers by reverse transcriptase, before raising the reaction temperature. In all the experiments a control tube contained all the reagents except for the RNA and the final volume was obtained by adding DEPC-treated ultra pure water. The RNAs were reverse transcribed in the thermal cycler at 70°C for 30 min to generate the first strand cDNA. The tubes were then removed from the thermal cycler and kept on ice. The mixture for amplification of the cDNAs contained: 1 × chelating buffer, 3 mM MgCl₂, 300 nM RUG-R primer, 300 nM RUG-L primer, 1 U/100 μ l Perfect Match. 80 μ l of this mixture was added to each tube and the tubes were covered with 50 μ l of mineral oil to avoid evaporation during PCR. The tubes were heated for 2 min at 95°C in the thermal cycler. The cDNAs were then amplified for 40 cycles, using the following program: 1 min at 95°C (denaturation) and 1.5 min at 69°C (annealing-extension). A final annealing-extension segment (10 min at 69°C) followed the last cycle. In PCR experiments with GAPDH primers reaction mixtures and reverse transcription conditions were as described above. Reverse transcription for subsequent GAPDH or UG amplification was performed in the thermal cycler at the same time to avoid interexperimental variations. After reverse transcription the samples that were not immediately used for amplification were kept at 4°C. For GAPDH cDNA amplification, after heating the samples for 2 min at 95°C, the following program was used: 1 min at 95°C (denaturation); 1 min at 61°C (annealing); 1 min at 72°C (extension). 30 amplification cycles were performed. After the last cycle the amplification was prolonged for an additional 10 min at 72°C. To avoid contamination separate sets of pipettes were used to dispense the reagents and the RNAs into the tubes. Another set of pipettes was used for PCR products. RNAs and products were handled in separate areas. All pipettes and tubes were irradiated for 1 h with shortwave ultraviolet (UV) light before use. Aerosol-resistant tips were used in all procedures.

Detection and characterization of PCR products. 10 μ l of each PCR product was electrophoresed using 2% NuSieve (FMC Corp.), 1% Ultra Pure agarose (BRL Life Technologies) gels. The gels were incubated for 45 min in denaturing solution (5 Prime—3 Prime, Inc.) and for 30 min in neutralization solution (5 Prime—3 Prime, Inc.). After 30 min this solution was replaced with fresh neutralization solution for an additional 15 min. PCR products were transferred onto nylon membranes

by conventional capillary blotting overnight and then cross-linked to the membranes by UV irradiation for 3 min in a UV Stratalinker-1800 (Stratagene Inc.). The membranes were then incubated for 2 min in 5 × SSC and for 1.5 h at 63°C in prehybridization solution (5 × SSC, 0.1% *N*-lauroylsarcosine Na salt, 0.02% SDS, 1% Boehringer blocking reagent). After this incubation, PCR products were hybridized with 5 nM digoxigenin-labeled RUG-P or 5 nM digoxigenin-labeled GAPDH-P for 4 h at 63°C. The probes were heated for 10 min at 95°C before addition to the prehybridization solution. Posthybridization washes were performed as follows: 2 × 5 min at 37°C in 2 × SSC, 0.1% SDS; 1 × 5 min at 63°C in 0.1 × SSC, 0.1% SDS. For detection of hybridized probes an immunochromiluminescent method was used, according to the manufacturer's instructions (Boehringer Mannheim Biochemicals). Briefly, the membranes were incubated in the presence of an antidigoxigenin-alkaline phosphatase-conjugated antibody (Boehringer Mannheim Biochemicals) and then passed through Lumi-Phos 530 (Boehringer Mannheim Biochemicals). The membranes were exposed to x-ray films (Kodak X-OMAT-AR; Eastman Kodak Co.).

In situ hybridization. Fresh frozen tissue specimens were cut and placed on clean glass slides (American Histolabs, Inc.). One slide for each specimen was stained with hematoxylin and eosin for histological assessment. The slides were kept at -70°C until the day of the experiment. The slides were initially placed at room temperature for 10 min and then fixed in 4% paraformaldehyde in PBS (pH = 7.4) for 5 min at room temperature. The slides were then rinsed 2 × 5 min in PBS and placed into 0.25% acetic anhydride, 0.1 M triethanolamine (pH = 8) for 10 min at room temperature. The slides were passed through increasing concentrations of ethanol (70–100%, 1 min each), through 100% chloroform for 5 min, and then through 100% and 95% ethanol, 1 min each. The slides were placed upright to dry. 50 μ l of prehybridization solution (50% formamide, 600 mM NaCl, 80 mM Tris, 4 mM EDTA, 0.1% sodium pyrophosphate, 0.2% SDS, 0.2 mg/ml sodium heparin, 10% dextran sulfate [pH = 7.5]) was applied to each slide and the sections were covered with parafilm. The slides were placed in a humidified chamber and incubated for 2 h at 42°C. After that, excess solution was shaken off and 50 μ l prehybridization solution containing 1 ng/ μ l (68 nM) of digoxigenin-labeled RUG-P4 or RUG-PS was placed on the sections. The slides were covered with parafilm and incubated in a humidified chamber at 42°C overnight (20 h). Posthybridization washes were performed as follows: after dipping several times rapidly into Copling jars containing 1 × SSC and for 30 s into 2 × SSC/50% formamide, the slides were washed 4 × 15 min each in 2 × SSC/50% formamide at 45°C and for 30 min in 1 × SSC at room temperature. After these posthybridization washes the slides were passed 2 × 5 min each through buffer 1 (100 mM Tris, 150 mM NaCl, pH = 7.5) and incubated for 30 min in buffer 1 containing 3% normal sheep serum, 0.3% Triton X-100, to decrease the background. After this incubation, the sections were covered with 50 μ l of the blocking solution containing antidigoxigenin-alkaline phosphatase-conjugated antibody (1:500) (Boehringer Mannheim Biochemicals) and incubated in a humidified chamber at room temperature for 5 h under a parafilm coverslip. The slides were then washed 2 × 10 min in buffer 1 and 1 × 5 min in buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM MgCl₂, pH = 9.5). The slides were incubated overnight in the dark in buffer 3 with 0.34 mg/ml nitroblue tetrazolium salt and 0.18 mg/ml 5-bromo-4-chloro-3-indoyl phosphate toluidinium salt (Boehringer Mannheim Biochemicals) in the presence of 1 mM levamisole to block peripheral endogenous alkaline phosphatase. After this incubation the slides were rinsed 4 × 30 min in 1 × SSC, dried, mounted in mounting medium (Sigma Chemical Co.), and sealed with nail polish. The slides were examined with a photomicroscope (Axhiomat; Carl Zeiss, Inc., Thornwood, NY) at a magnification of 400.

Immunofluorescence. After fixation in 4% paraformaldehyde, rabbit tissue specimens were embedded in paraffin, 0.025-mm sections were cut, placed on clean glass slides (American Histolabs, Inc.), and kept at 4°C. One slide for each specimen was stained with hematoxylin and eosin for histological assessment and the counterpart was used for immunofluorescence as previously described (16). Briefly, paraffin was removed with xylene (Mallinckrodt Inc.) and the slides were passed

through a series of decreasing concentrations (100–70%) of ethanol and then immersed in PBS (Biofluids Inc., Rockville, MD). PBS was shaken off and the slides were soaked in 3% albumin for 30 min. Next, 50 μ l of goat anti-rabbit UG antibody (1:10 in PBS) was added to each section. The slides were incubated for 1 h at room temperature in a humidified chamber. In negative controls the incubation with the first antibody was replaced by one of the following conditions: (a) PBS only; (b) normal goat serum; (c) anti-rabbit UG antibody, preadsorbed in solution with UG (75 μ M). After this incubation the slides were washed for 30 min in PBS with constant agitation. 50 μ l of rabbit FITC-labeled anti-goat antibody (1:10 in PBS) (Boehringer Mannheim Biochemicals) was added to each tissue section and the slides were incubated for 45 min in a humidified chamber at room temperature. The slides were then washed 3 \times 15 min each with PBS with constant agitation, and rinsed once with 50 mM sodium bicarbonate buffer (pH = 9.5). Excess buffer was shaken off and the slides were mounted in 50% glycerol/sodium bicarbonate buffer, coverslipped, sealed with nail polish, and examined with a Zeiss Axhioimat photomicroscope with epifluorescence (\times 400).

RIAs

Uteroglobin. RIA for UG was performed by using the procedure described by Mayol and Longenecker with minor modifications (42). Briefly, fetal lungs from timed pregnancy rabbits were homogenized in a protease inhibitor buffer. Samples or standards were diluted in PBS, pH 7.4, containing 0.1% BSA, 0.1% NaN_3 , 0.1 M EDTA, and 0.5% normal goat serum to a final vol of 1 ml. Goat anti-UG antibody was added to a final dilution of 1×10^4 . After overnight incubation at 4°C, ^{125}I -UG prepared by the Chloramine-T method was added (15×10^3 cpm/tube). These mixtures were incubated at 37°C for 2 h followed by an incubation at 4°C for 4 h. The reaction was stopped by adding 200 μ l of antigoat serum raised in a burro. These mixtures were then further incubated at 4°C for 12 h. Samples were centrifuged at 1,500 g for 30 min at 4°C and the supernatants were aspirated and discarded. The pellet containing the bound fraction was counted in a Micromedic gamma counter equipped with a logit data reduction program. The sensitivity of the assay was 15 pg/tube with an ED_{50} of 160 pg/tube. This assay has been validated using uterine flushings from progesterone-treated rabbits, tracheal washings from dexamethasone-treated rabbits, plasma, and serum. Liver and skeletal muscle extracts served as negative controls. This assay was also standardized with pure UG. Proteins with similar molecular weights to UG, including α -fetoprotein, myosin light chain, SP1 protein, human atrial natriuretic peptide, and β_2 -microglobulin, did not cross-react in this assay. Intraassay variations were < 5% and interassay variations were 6–8%.

Prostaglandins E_2 and $\text{PGF}_{2\alpha}$. PGE_2 and $\text{F}_{2\alpha}$ were assayed by RIA as validated and described in detail previously (43). Sensitivity for each assay was 12 pg/tube and the intraassay coefficient of variation was < 10%.

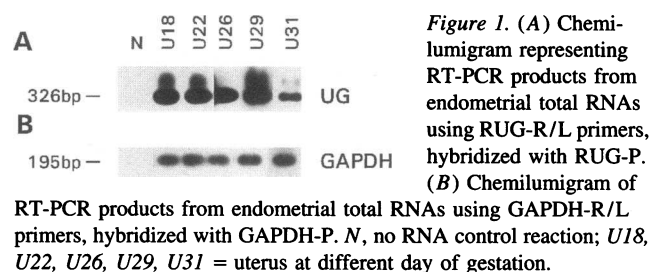
Leukotriene C_4 . LTC_4 was assayed by RIA using a commercial kit (Amersham Corp., Arlington Heights, IL) after partial purification on a Sep-pak phenyl column (Waters Associates, Milford, MA). Sensitivity, as determined in our laboratory, was 8 pg and the coefficient of variation was 12%.

Statistical analyses. The data derived from radioimmunoassays of uteroglobin and eicosanoids were assessed by analysis of variance. Regression analysis of the data was used to determine the existence of any correlation among UG, PGE_2 , $\text{PGF}_{2\alpha}$, and LTC_4 levels.

Results

Detection of UG gene transcription in the rabbit uterus throughout gestation

Detection by RT-PCR. Total RNAs from endometria of pregnant rabbits were analyzed by RT-PCR to detect UG mRNA. Specific bands indicating the presence of UG mRNA in the endometrium throughout pregnancy (day 18, 22, 26, 29, and



31) were detected and are represented in Fig. 1 A. The intensity of the signal is similar in samples obtained from rabbits at different stages of pregnancy, although a marked decrease was observed on day 31. The quality of the RNAs was tested by RT-PCR detection of GAPDH transcripts. Fig. 1 B shows GAPDH-specific bands obtained from the same endometrial total RNAs as shown in Fig. 1 A. No marked differences in the intensity of the signals were observed, indicating that the quality of the different RNA samples used was virtually the same.

Detection of UG mRNA by in situ hybridization. To determine the cellular localization of UG mRNA in the endometrium, in situ hybridization experiments were performed. Endometrial sections from pregnant rabbits were incubated in the presence of a UG-specific antisense probe (RUG-P4) or in the presence of the sense strand (RUG-PS). Brightfield photomicrographs of in situ hybridization of rabbit endometrial tissues are shown in Fig. 2. Fig. 2, A–E represent endometria at day 18, 22, 26, 29, and 31 of pregnancy, respectively. Signal for UG mRNA is readily detectable at all stages of pregnancy and is localized at the luminal and glandular epithelium of the endometrium. The intensity of the signal decreased toward the end of pregnancy (day 29 and 31). No UG mRNA was detected in the stromal cells. In Fig. 2 F, a photomicrograph of an endometrial tissue section hybridized with the sense probe (RUG-PS) is shown and no signal is appreciable. Other negative controls were also performed omitting the antisense probe from the hybridization solution, or omitting the antisense probe and incubation with the antidigoxigenin-alkaline phosphatase-conjugated antibody (data not shown).

Detection of UG in the uterus throughout gestation by immunofluorescence.

The presence of UG in the endometrium of pregnant rabbits was assessed by indirect immunofluorescence with an antibody to rabbit UG on paraformaldehyde-fixed sections. The results obtained are shown in Fig. 3. The intensity of immunofluorescence varied in the endometrial samples obtained from rabbits at different stages of pregnancy (day 18, 22, 26, 29, and 31). The epithelial cells of the endometrium consistently showed bright immunofluorescence. The positivity was slightly decreased in the section corresponding to day 31 of pregnancy (Fig. 3 E). The specificity of the immunoreactivity was demonstrated by the absence of immunofluorescence in sections incubated with (a) PBS (Fig. 3 F); (b) anti-rabbit UG serum preadsorbed with rabbit UG (not shown); and (c) normal goat serum (not shown).

Detection of UG mRNA in fetal rabbit lung at different stages of development

Detection by RT-PCR. UG-specific transcripts in total RNAs from fetal lungs were detected using RT-PCR. UG mRNA was detected in the lung of fetuses at day 22, 26, 29, and 31 of

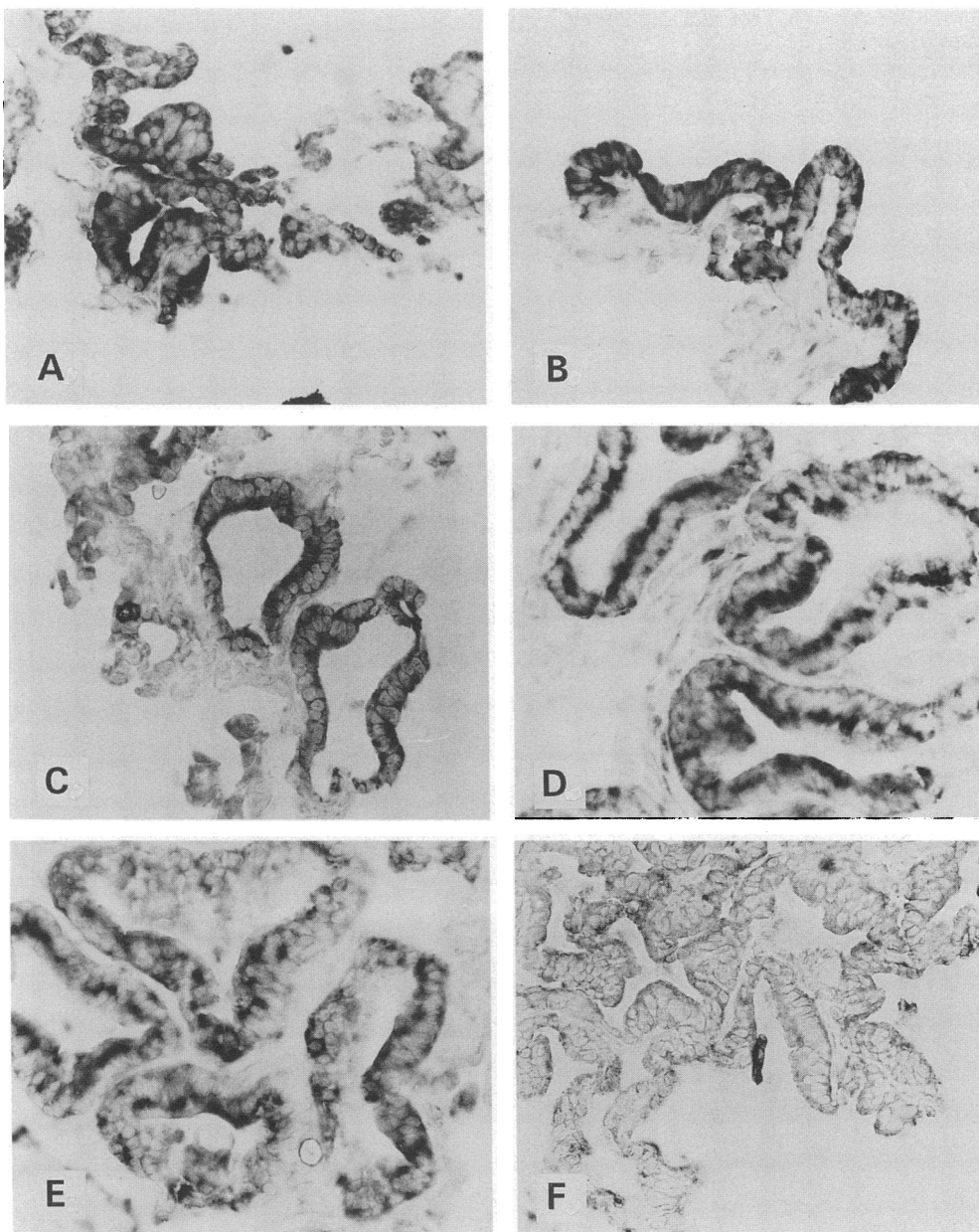


Figure 2. Brightfield photomicrographs of sections of rabbit endometrium at different days of gestation (*A* = 18, *B* = 22, *C* = 26, *D* = 29, *E* = 31) hybridized with RUG-P4 or with RUG-PS, as a negative control (*F*). The specific labeling is present in either the surface and the glandular epithelium, but not in the stroma. $\times 400$.

pregnancy (Fig. 4 *A*). The signal appeared more intense at the end of pregnancy, indicating that the expression of UG gene is related to the degree of maturity of the fetal lung. A comparison of relative intensities of UG-specific transcriptional signals in RNA samples obtained from lungs of fetuses of different gestational ages were possible because the signal for GAPDH mRNA was virtually identical in all the samples tested (Fig. 4 *B*). The identical GAPDH signal in all the samples indicates that the quality of the RNAs subjected to RT-PCR was the same.

Detection by *in situ* hybridization. The presence of UG mRNA in the fetal lung was also assessed using *in situ* hybridization. An antisense probe (RUG-P4) was used for detection of UG-specific transcript, whereas a sense probe (RUG-PS) served as a negative control. The results obtained are shown in Fig. 5 *A*, *C*, and *E*. Some cells with staining for UG mRNA were present in the lung of a fetus at day 22 (Fig. 5 *A*) of pregnancy. Comparable results were obtained from the lung of fetuses at day 26 of pregnancy (data not shown). The positive

staining for UG transcript dramatically increased in fetal lungs at day 29 (data not shown) or day 31 of pregnancy (Fig. 5 *C*). Conversely, no positivity was observed when the lung sections were incubated with the sense probe (Fig. 5 *E*). Similarly, no staining was observed when the antisense probe was omitted from the hybridization solution or when the antisense probe and the antidigoxigenin-alkaline phosphatase-conjugated antibody incubations were both omitted (data not shown).

UG immunofluorescence in rabbit fetal lung

Immunofluorescence was performed to detect UG in lung sections of fetal rabbits. As shown in Fig. 5 *B*, a specific signal was observed in fetal lung at day 22 of pregnancy. A similar signal was also detected on day 26 of gestation (data not shown). A relatively brighter immunofluorescence was detected in the lung of fetuses at day 29 (not shown) and 31 (Fig. 5 *D*) of pregnancy. Controls to assess the specificity of immunoreactivity were performed by omitting the first antibody and incubat-

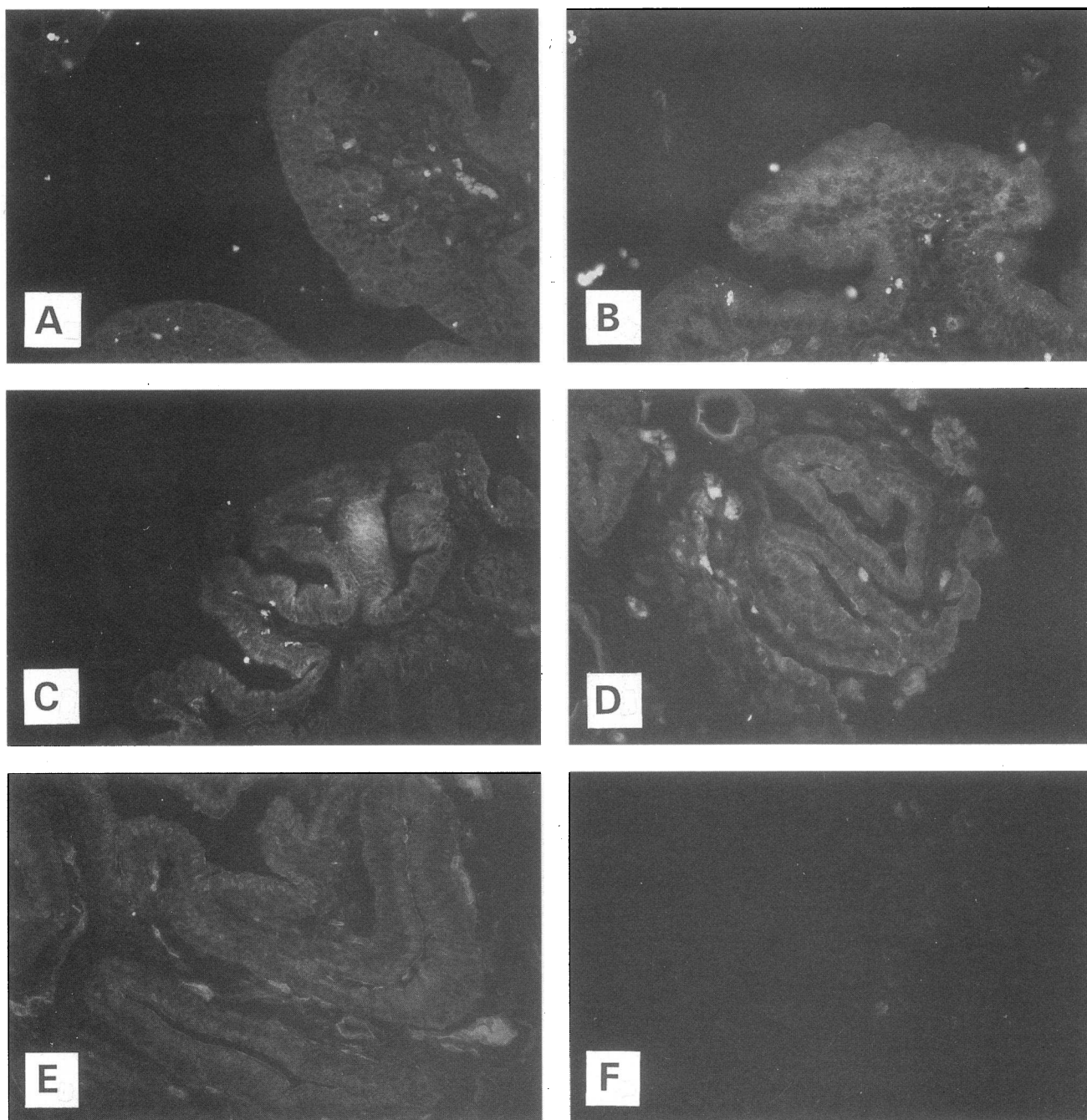


Figure 3. Indirect immunofluorescence of rabbit endometrial sections at different days (*A* = 18, *B* = 22, *C* = 26, *D* = 29, *E* = 31) of pregnancy. A monospecific, polyclonal goat anti-UG IgG (see Methods) was used for these experiments. FITC-conjugated rabbit anti-goat IgG was used as the second antibody. Immunofluorescence is consistently detectable in the epithelial cells. Specific labeling is absent in the section (*F*) in which the first antibody was replaced by PBS. $\times 400$.

ing the sections with PBS (Fig. 5 *F*), anti-rabbit UG antibody preadsorbed with rabbit UG (not shown), and normal goat serum (not shown).

Quantitation of UG and eicosanoid levels in fetal lung homogenates by RIA

Radioimmunoassays were performed using homogenates of fetal lung tissues at different gestational ages for the detection of UG, LTC₄, PGE₂, and PGF_{2 α} . The measurements were obtained from fetuses at day 24, 26, 28, 29, and 30 of pregnancy. The levels of LTC₄ (Fig. 6 *A*) were ~ 20 ng/mg protein until day 28 of preg-

nancy; these levels markedly increased in specimens collected on days 29 and 30, when the levels were above 30 ng of UG/mg protein. Specific UG immunoreactivity was barely detectable until day 26 of pregnancy, but a substantial and steady increase was noted during the last 3 d of gestation. On day 30, as much as 800 ng of UG/mg protein was measured in the fetal lungs. Conversely, the levels of PGE₂ and PGF_{2 α} during the terminal part of gestation showed a completely different pattern. Both PGE₂ (Fig. 6 *C*) and PGF_{2 α} (Fig. 6 *D*) levels decreased and the lowest values were measured on day 30 of pregnancy. A regression curve was generated to determine any correlation among the levels of PGE₂,

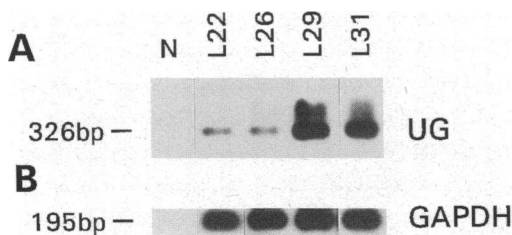


Figure 4. (A) Chemilumigram representing RT-PCR products from fetal lung total RNAs using RUG-R/L as primers. RUG-P served as the probe. (B) Chemilumigram of RT-PCR products from fetal lung total RNAs using GAPDH-R/L primers, hybridized with GAPDH-P. N, control reaction without RNA; L22, L26, L29, L31 = lung tissues at different days of fetal maturation.

PGF_{2α}, LTC₄, and UG (Fig. 7). While an inverse relationship was observed between the levels of UG and those of PGE₂ and PGF_{2α} (Fig. 7 A), a positive correlation was apparent between UG and LTC₄ levels (Fig. 7 B).

Discussion

A striking finding of our present investigation is that UG gene is expressed in the rabbit uterus throughout pregnancy. In contrast to previous studies, we not only detected UG mRNA by RT-PCR in the uterus at specified times (days 18, 22, 26, 29, and 31) throughout gestation, but also found a virtually constant level of gene expression during these days, except for an apparent precipitous decline on day 31 of pregnancy (term 32 d). As previously reported, the results of *in situ* hybridization experiments suggest that this gene expression occurs primarily in the luminal and glandular epithelium of the endometrium. The validity of these results was further confirmed by the detection of UG protein by immunofluorescence.

It has been suggested that progesterone is required for the maintenance of pregnancy in almost all mammals (44). The highest plasma progesterone level (~18 ng/ml) has been reported towards the end of the second week of gestation in the rabbit (45). Afterwards, progesterone levels decline slightly and a level of 12–15 ng/ml is maintained during the third and fourth week of pregnancy. This level of progesterone is dramatically reduced (6 ng/ml) on day 30 of pregnancy and to 2 ng/ml immediately after parturition. Since UG gene is regulated in the rabbit uterus by progesterone (for a review see reference 3), it is not surprising that its expression is detectable throughout gestation. Our observation that UG gene expression declines immediately before the end of gestation is consonant with the reduced plasma progesterone levels before parturition. In fact, this decline in the level of UG gene expression may signal the uterus to prepare for impending parturition.

The results of our present investigation differ from those of the previous studies, which reported the presence of UG in the uterus only during early pregnancy. Krishnan and Daniel (2) detected UG in uterine flushings by gel filtration chromatography. Using this method of assay the protein was virtually undetectable by day 9 of pregnancy. Subsequently, an RIA for uteroglobin was developed (42); however, even with this method, UG in the pregnant uterus was barely detectable on day 12 of pregnancy. The presence of UG mRNA, assessed by *in vitro* translation of a poly A-rich fraction of endometrial RNA, also declined after the first week of pregnancy (46). However, using

this method a systematic determination of UG gene transcription throughout gestation was not carried out. There are several other studies in which detection of UG and/or UG mRNA in the endometrium during early pregnancy have been reported (46–50). However, these experiments were performed using endometrial specimens during the preimplantation to implantation phase (days 0–6) and no data from rabbits after the first week of gestation were available. The apparent discrepancy between the results of previous investigations and those of our present study may be due to the fact that: (a) RT-PCR and *in situ* hybridization techniques using digoxigenin-labeled probes have a higher sensitivity than Northern blotting of total RNA used in previous studies and (b) the antibody used for immunofluorescence and RIA in the present investigation is monospecific and of a very high titer and affinity (data not shown). Nonetheless, the levels of UG expression is physiologically significant as it has demonstrated that nanomolar concentration of UG can effectively inhibit both group I and II PLA₂ activities (3, 4, 23–25, 35).

The presence of UG in the rabbit endometrium during early pregnancy has been suggested to provide immunological protection to the implanting embryos (15–17) and spermatozoa (18) in the female genital tract after coitus. On the basis of the results of our present investigation, we speculate that persistent presence of UG in the rabbit endometrium throughout pregnancy may not only extend this protection but also facilitate maintenance of quiescence in the gravid uterus while a decline in the level of this protein may signal parturition. This hypothesis is supported, in part, by several lines of evidence. UG, is a potent inhibitor of PLA₂ (23), a family of key enzymes which generate free fatty acids (e.g., arachidonic acid) from cellular phospholipids. Arachidonic acid is metabolized via the arachidonic acid cascade generating proinflammatory lipid mediators (e.g., PGE₂, PGF_{2α}, etc.). These mediators induce uterine smooth muscle contractility; in fact, PGE₂ and PGF_{2α} are clinically used to terminate pregnancy (for a review see references 51, 52). Furthermore, eicosanoids are potent chemoattractants for the immune cells (53) (e.g., neutrophils, monocytes, and macrophages). Thus, an inadvertent activation of PLA₂ during gestation may lead to interruption of pregnancy. Interestingly, Cordella-Miele et al. (54) have demonstrated that an elevated level of transcription of a PLA₂ gene in the rat uterus is clearly detectable just before parturition. It is important to note that in the human uterus an inverse relationship between uteroglobin and eicosanoid levels has been reported (55). These findings may lend support to the hypothesis that UG is a second messenger of progesterone action in the rabbit uterus, at least from the standpoint of maintaining quiescence in this organ during pregnancy.

It is not as yet clear as to how UG may transduce its signal to target cells of various organs to manifest its biological effects. However, it has been recently demonstrated that rabbit UG is transported through the trophoblast layer (56) to the blastocoele of the embryo via a specific active transport process. Kundu et al. have demonstrated that human UG binds to the surface of several cell types including the trophoblasts via a high affinity binding protein (Kundu, G., G. Mantile, L. Miele, E. Cordella-Miele, and A. B. Mulcherjee, manuscript in preparation). More significantly, the putative receptor of hUG has some similarity to the recently characterized rabbit (57) and bovine (58) PLA₂ receptor, in terms of its ligand specificity. In contrast to the PLA₂ receptor, the putative UG receptor appears to be a nonglycosylated protein. These novel findings are more significant

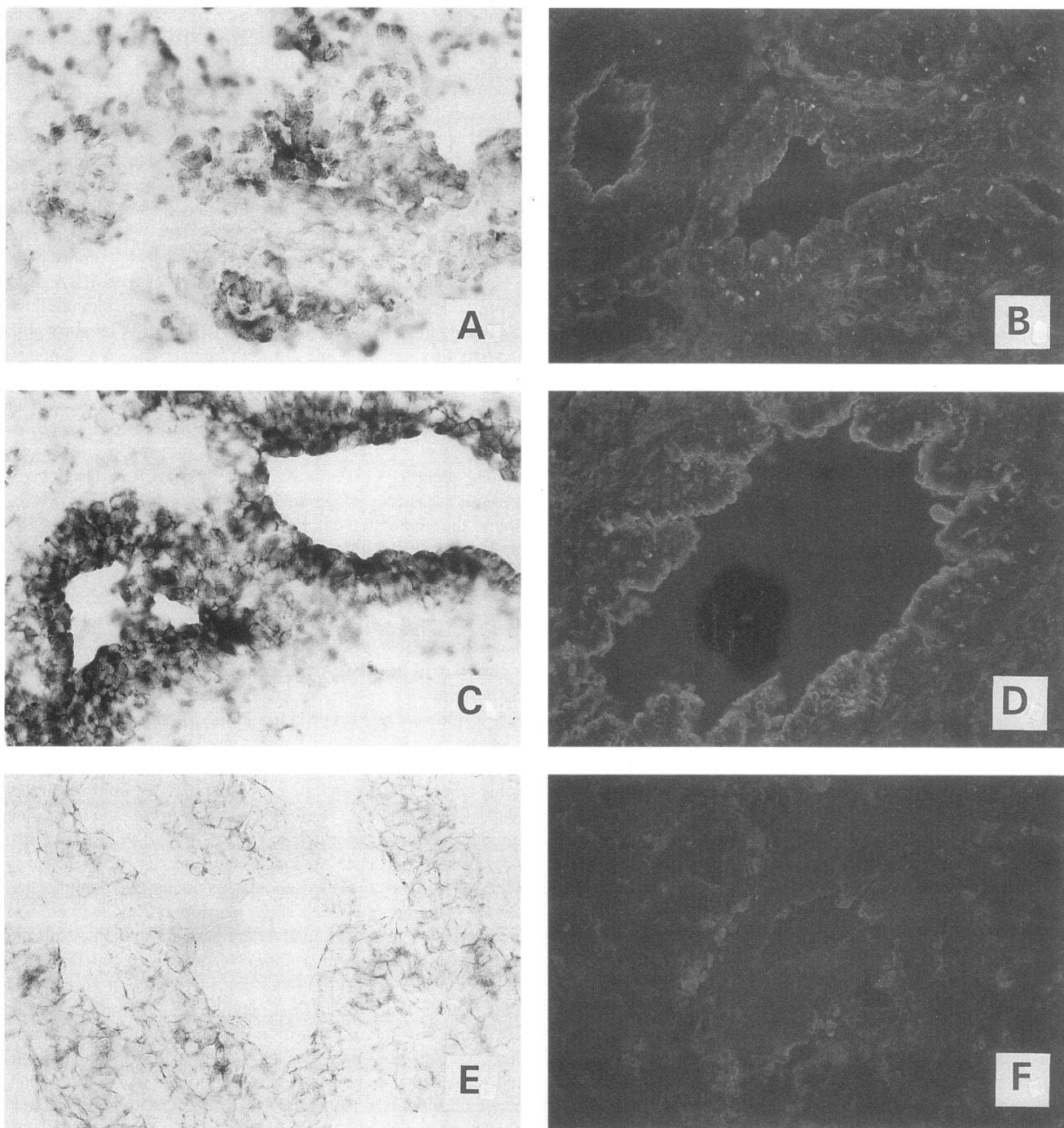


Figure 5. Brightfield photomicrographs of rabbit fetal lung sections at different days of gestation ($A = 22$, $C = 31$), hybridized with RUG-P4. No specific labeling was observed in a fetal lung section hybridized with RUG-PS (E). The corresponding immunofluorescence is shown in panels B (day 22) and D (day 31). No immunofluorescence was detectable when PBS was used instead of anti-UG antibody (F). $\times 400$.

when considered together with the facts that an extracellular PLA_2 cDNA from human placenta (59) has been cloned and characterized and structural similarity between UG and an extracellular PLA_2 (60) has been reported. Molecular characterization of UG and PLA_2 receptors and their regulation of expression in the uterine cells during pregnancy may yield vital information regarding the physiological regulatory role(s) of these two important classes of proteins.

Another fundamental aspect of the physiology of pregnancy is the development of the fetal lung. At parturition, a mammalian fetus is compelled to make a transition from a literally

aquatic environment to a terrestrial air-breathing mode of existence. Thus, maturity of the fetal lung is essential to facilitate this transition. The fetal lung at term synthesizes and secretes surfactant, a phospholipid-protein complex. A deficiency of surfactant in the lung of a premature newborn causes neonatal respiratory distress syndrome, a disease of considerable mortality and morbidity (36). Although much progress has been made in the successful treatment of this disease, especially by endotracheal administration of surfactant (61–63), a high percentage of these treated neonates develop a chronic inflammatory lung disease known as bronchopulmonary dysplasia. Although the

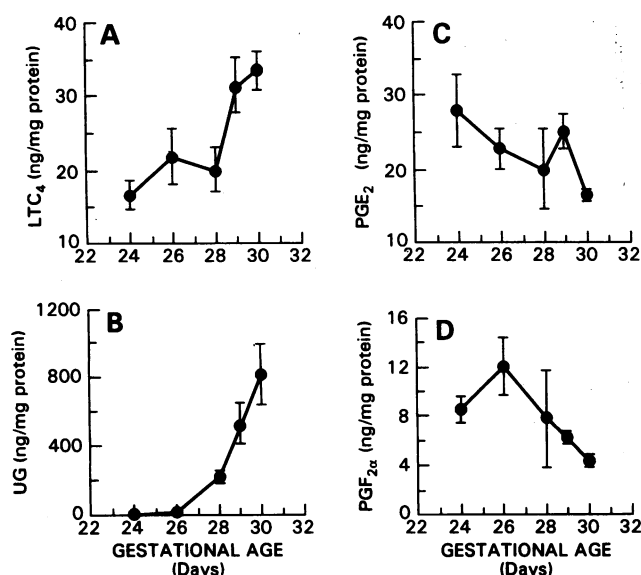


Figure 6. Levels of LTC₄ (A), UG (B), PGE₂ (C), PGF_{2α} (D) in fetal lung as measured by RIA. Note the increase in the levels of LTC₄ and UG before parturition and the concomitant decrease of PGE₂ and PGF_{2α} levels.

mechanism of pathogenesis of bronchopulmonary dysplasia is not clearly understood, it is suggested that lipid mediators of inflammation play a role. A possible mechanism may involve activation of a lung-specific PLA₂ initiating the arachidonic acid cascade generating such inflammatory mediators as PGs, LTs and platelet activating factor. UG gene is constitutively expressed in the lung and inducible by corticosteroid (for a review see reference 3) which also enhances synthesis and secretion of surfactant. It is conceivable that under normal circumstances of gestation the fetal lung at parturition is fully protected by the presence of UG. However, in a premature lung lacking UG, a rise in PLA₂ activity would hydrolyze cell membrane phospholipids which would initiate and propagate inflammation. Thus, the results of our present investigation are significant in that they establish the pattern of UG gene expression during development of the fetal rabbit lung and its relationship to the level of proinflammatory eicosanoids in this organ.

Our results indicate that (a) UG mRNA is detectable in fetal lung from day 22 of pregnancy and the highest levels were observed before parturition, (b) LTC₄ levels are maintained at a constant level until day 28 of pregnancy and thereafter increase by ~ 70% above the base line from day 29 and (c) PGE₂ and PGF_{2α} levels decrease with increased UG levels, correlating with increased gestational age of the fetus. While these eicosanoids are known to be proinflammatory, it has also been demonstrated that LTs stimulate pulmonary surfactant secretion in cultured type II cells (37, 38). Use of lipoxygenase inhibitors confirmed that the enhanced surfactant secretion is mediated by products of the lipoxygenase pathway (37). Thus, while UG level is inversely related to those of PGE₂ and PGF_{2α}, its level appears to directly correlate with that of LTC₄ in the fetal lung. An alternative explanation of the positive correlation between UG and LTC₄ levels observed in the present study may be because LTs are potent mediators of inflammation and bronchoconstriction (64–70) and the presence of UG is to counteract these effects of LTs. The discrepancy between the decrease in PGs and increase in LTs in the presence of UG expres-

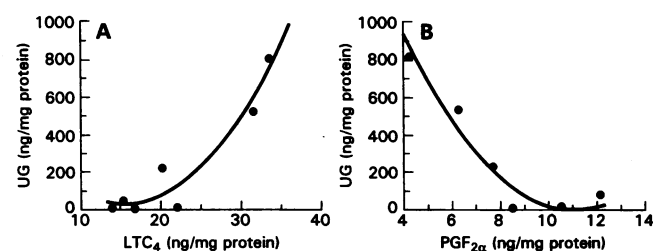


Figure 7. Regression curves to demonstrate the correlation between UG and LTC₄ levels (A) and UG and PGF_{2α} levels (B) in rabbit fetal lungs.

sion is not yet clearly understood. However, cyclooxygenase, an enzyme which catalyzes the production of prostaglandins from arachidonic acid, is induced by group I PLA₂ (unpublished observations), reported to be expressed in the lung (71). Most likely, this regulation is accomplished via the group I PLA₂ receptor-mediated pathway. Since UG and PLA₂ receptors are similar in terms of their ligand specificity it is possible that UG, by competing with the PLA₂ receptor, may cause down-regulation of cyclooxygenase gene expression (consequently, decreased level of PGs), leaving lipoxygenase pathway fully operational and therefore LT levels remaining unaltered.

Recently, we demonstrated that surfactant-producing alveolar type II cells also express UG (72) which may prevent surfactant degradation by inhibition of a lung-specific group I PLA₂. This may suggest a surfactant-protective role for UG in the lung. In conclusion, UG gene may play important physiological roles during pregnancy, perhaps by preventing the onset of premature labor and by protecting the fetal lung from the development of respiratory distress syndrome and bronchopulmonary dysplasia possibly by down regulating the production of lipid mediators of inflammation.

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