

# Interacting Influences of Pregnancy and Corneal Injury on Rabbit Lacrimal Gland Immunoarchitecture and Function

Chuanqing Ding,<sup>1</sup> Natalie Chang,<sup>1</sup> Yi-Chiang Fong,<sup>2</sup> Yanru Wang,<sup>3</sup> Melvin D. Trousdale,<sup>2</sup> Austin K. Mircheff,<sup>3</sup> and Joel E. Schechter<sup>1</sup>

**PURPOSE.** Previous reports indicated that pregnancy and corneal injury (CI) trigger alterations of lacrimal gland (LG) growth factor expression and redistributions of lymphocytes from periductal foci to acini. The purpose of this study was to test our hypothesis that pregnancy would exacerbate the changes induced by CI.

**METHODS.** Corneas were injured with scalpel blades, and, 2 weeks later, LGs were collected for immunocytochemistry and Western blot analysis. Lacrimal fluid was collected under basal and pilocarpine-stimulated conditions for protein determination and Western blot analyses.

**RESULTS.** There were significant increases of immunoreactivity for prolactin, TGF- $\beta$ 1, and EGF in duct cells during pregnancy and after CI, most prominent in pregnant animals with CI. Pregnancy decreased baseline lacrimal fluid secretion, whereas CI did not have a noticeable effect; pregnancy and CI combined resulted in increased fluid production. Pregnancy and CI each increased pilocarpine-induced lacrimal fluid production, whereas protein concentrations were decreased. Prolactin, TGF- $\beta$ 1, and EGF were detected in LG by Western blot analysis but were minimally detectable in lacrimal fluid. RTLA<sup>+</sup> and CD18<sup>+</sup> cells were redistributed from periductal to interacinar sites during pregnancy and after CI, most prominent in pregnant animals with CI.

**CONCLUSIONS.** Like pregnancy, CI is associated with redistribution of immune cells from periductal to interacinar sites and enhanced immunoreactivity of prolactin, TGF- $\beta$ 1, and EGF in ductal cells. Although baseline lacrimal fluid secretion varied, the glands of all three experimental groups produced significant amounts of fluid in response to pilocarpine, but protein concentrations were decreased. (*Invest Ophthalmol Vis Sci*. 2006;47:1368-1375) DOI:10.1167/iov.05-1034

There is a general clinical impression that pregnant women more frequently report symptoms of dry eye disease than nonpregnant women. Although there is still no peer-reviewed

literature that we can find to support this hypothesis, our preliminary epidemiologic study suggests that a subpopulation of women indeed experience increased symptoms of dry eye during the third trimester of their pregnancies.<sup>1</sup>

Prolactin and several other paracrine factors have been documented to be present in the lacrimal glands, and it has been proposed that they play a role in regulating lacrimal secretion.<sup>2-7</sup> Growth factors have also been detected in the lacrimal fluid,<sup>2,3,8-10</sup> although, apart from supporting corneal wound healing, their roles in ocular surface function are still unclear.

Much previous work has suggested that sex hormones, prolactin, and growth factors all contribute to changes in the lacrimal gland and the onset and progression of dry eye.<sup>11-13</sup> Because there is a significant change of the hormonal milieu during pregnancy, pregnancy would seem to serve as an excellent model for studying the influences of normal, physiological endocrine changes on lacrimal function. In our earlier studies, we found that as pregnancy progresses in New Zealand White rabbits, immunolabeling of prolactin, basic fibroblast growth factor (FGF)-2, epidermal growth factor (EGF), and transforming growth factor (TGF)- $\beta$ 1, becomes more intense within intra- and interlobular ducts. We also found that the number of immune cells in periductal foci decrease, whereas the number of immune cells at interacinar sites in the gland increase, suggesting that these cells are drawn away from the periductal foci and toward the interacinar interstitium.<sup>14,15</sup>

Acute changes in lacrimal fluid secretion rates and longer-term changes in gene expression occur within the lacrimal gland in response to corneal injury (CI).<sup>15-17</sup> The changes in growth factor expression are accompanied by immunoarchitectural changes reminiscent of those that occur during pregnancy. Because the occurrence of such changes in pregnancy seemed to us consistent with an overall heightened state of immune readiness,<sup>14</sup> we hypothesized that (1) pregnancy would enhance the changes of immunoarchitecture and growth factor expression induced by CI, (2) lacrimal secretory function would change during both pregnancy and after CI, and (3) pregnancy would exacerbate the functional changes associated with CI. The results reveal a more complex pattern of interactions between the influences of pregnancy and corneal insult.

## MATERIALS AND METHODS

### Animals, Experimental Groups, and Tissue Preparation

New Zealand White adult female rabbits (Irish Farms, Norco, CA) weighing approximately 4.0 kg were used throughout our studies. Pregnant rabbits were time dated with day 0 corresponding to the date of coitus. Normal gestation in the rabbit is 31 days. Animals were anesthetized with a mixture of ketamine (40 mg/mL) and xylazine (10 mg/mL) and given an overdose of pentobarbital (80 mg/kg) for euthanasia.

From the Departments of <sup>1</sup>Cell and Neurobiology, <sup>3</sup>Physiology and Biophysics, and the <sup>2</sup>Doheny Eye Institute, Keck School of Medicine, University of Southern California, Los Angeles, California.

Supported by National Eye Institute Grants EY10550 (JES), EY05801, and EY13720 (AKM), EY12689 (MDT), and EY03040 (Doheny Core) and a grant from the Sjögren's Syndrome Foundation (CD).

Submitted for publication August 5, 2005; revised November 11 and December 16, 2005; accepted February 9, 2006.

Disclosure: C. Ding, None; N. Chang, None; Y.-C. Fong, None; Y. Wang, None; M.D. Trousdale, None; A.K. Mircheff, None; J.E. Schechter, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Chuanqing Ding, Department of Cell and Neurobiology, University of Southern California, Keck School of Medicine, 1333 San Pablo Street, BMT 304, Los Angeles, CA 90089-9112; cding@usc.edu.

Nonpregnant animals without CI were considered the control group. The experimental groups were: pregnant without CI, nonpregnant with CI, and pregnant with CI.

CI was performed on the 15th day of pregnancy. A scalpel blade was used to penetrate the corneal stroma in a five-point dice pattern, whereas the animals were under general anesthesia. The procedure has been detailed previously.<sup>18</sup> Pregnant rabbits were killed on the 29th day of pregnancy, and rabbits in the nonpregnant with CI group were killed 14 days after CI.

Inferior lacrimal glands were removed, and some tissue fragments were placed in optimal cutting temperature (OCT) compound (Miles, Elkhart, IN) and rapidly frozen with liquid nitrogen. Some fragments were fixed and processed for epoxy embedding for light and electron microscopy, as previously described.<sup>19</sup> Additional fragments were used for determination of protein concentration and for Western blot analysis. All studies conformed to the standards and procedures for the proper care and use of animals, as described in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

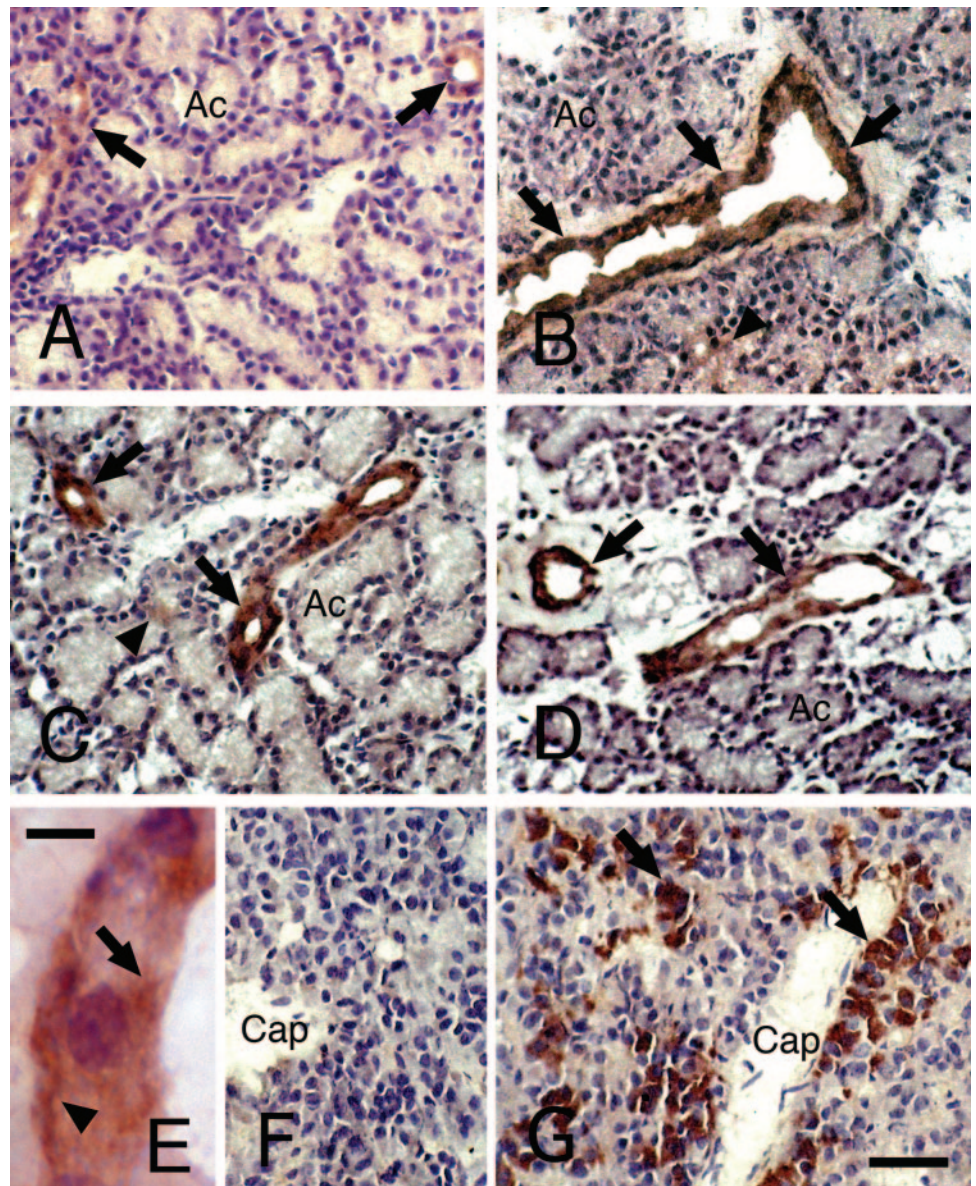
Animals used in these studies were: 5 nonpregnant without CI, 16 pregnant without CI, 6 nonpregnant with CI, and 11 pregnant with CI.

### Immunohistochemistry

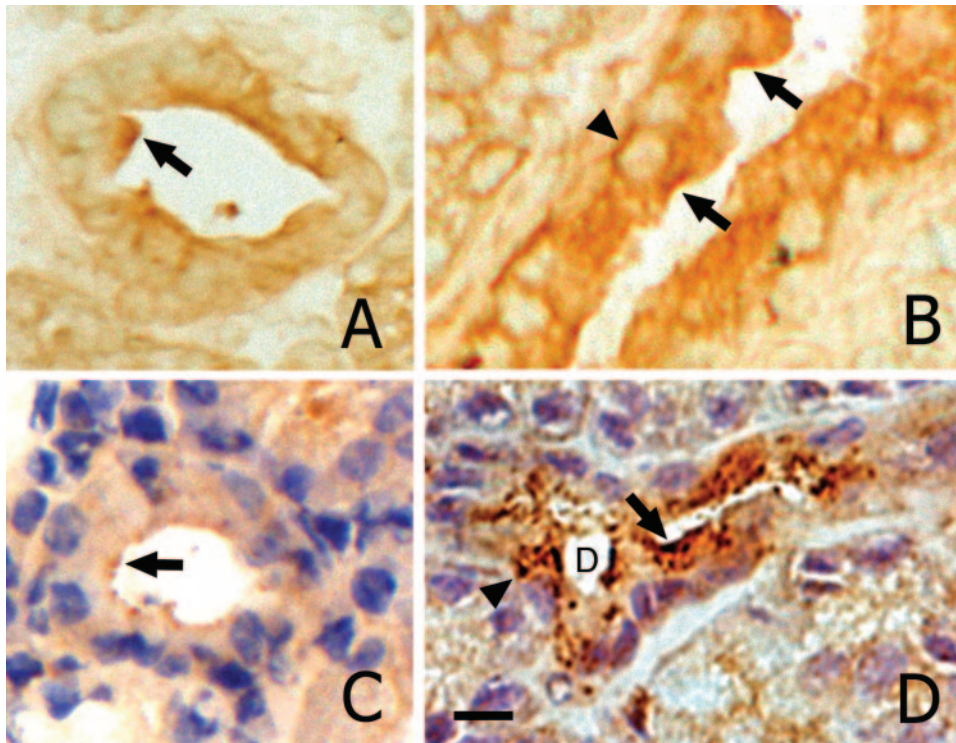
Tissue fragments were cryosectioned at 6 to 8  $\mu\text{m}$  in thickness and affixed to polyfrost glass slides (Fisher Scientific, Hampton, NH). Sections were fixed briefly with acetone or 4% paraformaldehyde and processed using the standard immunohistochemistry protocol.

Protocols for immunohistochemical staining were run in parallel for sections from the control and experimental groups. Two primary antibodies were used to label immune cells: goat anti-rabbit T lymphocyte antigen (RTLA; Cedarlane, Ontario, Canada), and mouse anti-rabbit CD18 (Spring Valley, Woodbine, MD). RTLA marks thymus-derived immune cells in rabbits and CD18 labels bone-marrow-derived immune cells. Other antibodies used were: guinea pig anti-rabbit prolactin (obtained from Alfred F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK] Pituitary Hormones and Antisera Center), chicken anti-human TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN), and mouse anti-human EGF (CalBiochem, Cambridge, MA).

Secondary antibodies used for immunohistochemistry were goat anti-mouse IgG for EGF and CD18, donkey anti-goat IgG for RTLA, goat anti-guinea pig IgG for prolactin, and goat anti-chicken IgG for TGF- $\beta$ 1. Slides were treated with ABC reagent (Vector Laboratories, Burlingame, CA) and diaminobenzidine and mounted (Immunount; Shan-



**FIGURE 1.** Immunohistochemistry of prolactin in lacrimal (A-E) and (F, G) pituitary glands. (A) Nonpregnant control without CI. Prolactin immunoreactivity (IR) was primarily detected in epithelial cells of intralobular and small interlobular ducts (arrows) and minimal prolactin-IR was detected in the acinar areas. Ac, acinus. (B) Pregnant without CI. Prolactin-IR in duct cells was significantly increased in pregnant animals (arrows). Some IR was also observed in the acinar areas (arrowhead). (C) Nonpregnant with CI. Like pregnancy, CI also increased prolactin-IR significantly in duct cells (arrows), with some also detectable in the acinar cells (arrowhead). (D) Pregnant with CI. The combined effects of pregnancy and CI appeared to increase prolactin-IR significantly in duct cells (arrows). (E) The prolactin-IR was aggregated in apical (arrow) cytoplasm but occasionally was evident in the basal (arrowhead) cytoplasm. This phenomenon was also observed in the other two experimental groups. (F) Negative control of pituitary gland showed no prolactin-IR, whereas that with the primary antibody (G) showed strong IR in mammothrophs in the pituitary gland (arrows). Cap, capillary. Bar: (A-D, F, G) 100  $\mu\text{m}$ ; (E) 20  $\mu\text{m}$ .



**FIGURE 2.** Immunohistochemistry of TGF- $\beta$ 1 and EGF. (A, B) TGF- $\beta$ 1, without counterstain. (C, D) EGF with counterstain. (A) Nonpregnant control without CI. (A) Interlobular duct with faint TGF- $\beta$ 1-IR, mostly in the apical cytoplasm (arrow) of the cells. (B) Nonpregnant with CI. Intralobular and interlobular ducts were highly immunoreactive to TGF- $\beta$ 1, located in the apical (arrows) and occasionally basal sides (arrow-head) of the cytoplasm, although the latter was not observed uniformly in all slides and all animals. (C) Nonpregnant control without CI. Clumps of EGF-IR are evident in this intralobular duct, mostly in the apical sides of the duct cells (arrow). (D) Pregnant without CI. Like CI, pregnancy also increased EGF-IR, although less than the combined effect of pregnancy with CI. An intralobular duct is evident with extensive EGF-IR. EGF-IR was observed in the apical (arrow) cytoplasm and occasionally in the basal (arrow-head) cytoplasm of the duct cells. D, duct lumen. Bar, 20  $\mu$ m.

don/Lipshaw, Pittsburgh, PA). Negative controls specimens consisted of exposure to secondary antibody and ABC reagent without exposure to primary antibody. Sections of rabbit anterior pituitary gland served as the positive control for prolactin. Slides were counterstained with methylene blue.

### Image Analysis

Immunohistochemical data for RTLA and CD18 were quantified using a computer-assisted, digital image-analysis system. The detailed procedure has been described previously.<sup>15</sup>

The objective of the image analysis was to determine whether redistribution of immune cells from periductal sites to interstitial sites occurs in all the experimental groups.

### Lacrimal Gland Fluid Collection

Lacrimal ducts were cannulated with small-bore polyethylene tubing (inner diameter [ID]: 0.28 mm, outer diameter [OD]: 0.61 mm; BD Bioscience, Sparks, MD) that had been pretreated with protease inhibitor. Basal lacrimal secretion was collected for 15 minutes, followed by intravenous administration of pilocarpine (1 mL, 1.2 mg/mL) and three additional collections for 10 minutes each. Collecting tubes with the fluids were weighed and the volume determined from the weight differential. These volumes divided by the duration of the collection period gave the flow rate (in microliters/minute).

### Protein Determination, SDS-PAGE, and Western Blot Analysis

Protein concentrations in lacrimal gland lysates and lacrimal fluid were determined according to the Bradford protein protocol on a microplate reader (SOFTmax software; E-max microplate reader; Molecular Devices, Sunnyvale, CA).

Samples for polyacrylamide gels (SDS-PAGE) were prepared so that the same amount of protein could be loaded into each lane on the gel. Polyacrylamide gels at 12% were run according to standard protocols.

Protein loads were 100  $\mu$ g per lane for analyses of TGF- $\beta$ 1 and EGF and 50  $\mu$ g per lane for analyses of prolactin. Proteins were transferred onto nitrocellulose membranes and exposed to primary antibody overnight at 4°C (1:1000, guinea pig anti-rabbit prolactin, obtained from

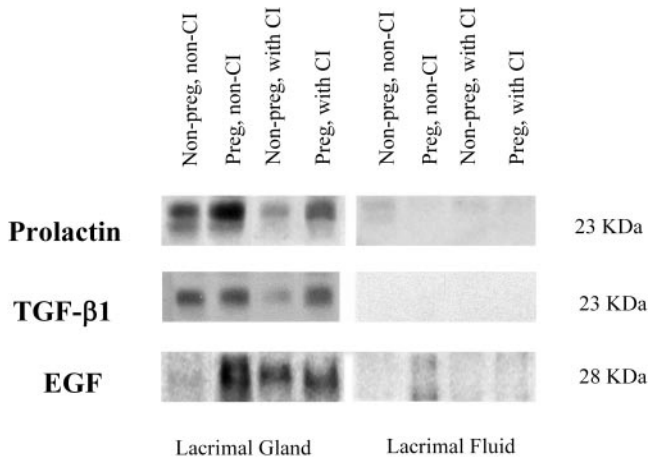
Alfred F. Parlow, NIDDK; 1:500, mouse anti-human TGF- $\beta$ 1; Chemicon Inc., Temecula, CA; 1:500, goat anti-human EGF; R&D Systems Inc.). Membranes were then exposed to the appropriate dye-conjugated secondary antibodies (IRDye 800; Rockland, Gilbertsville, PA); goat anti-guinea pig IgG (1:3000) for prolactin; goat anti-mouse IgG (1:2000) for TGF- $\beta$ 1; and donkey anti-goat IgG (1:2000) for epidermal growth factor [EGF]. Reference samples were: sheep prolactin, 22.5 kDa (Sigma-Aldrich, St. Louis, MO), and human EGF, 6.35 kDa (Oncogene, Cambridge, MA). Near-infrared fluorophores (IRDye-800; Rockland) were determined (Odyssey Infrared Fluorescence Imaging System; LiCor Instruments, Lincoln, NB), and densitometry was conducted on computer (Labworks 4.0; Ultra-Violet Products, Inc., Upland, CA).

## RESULTS

### Immunohistochemistry Observations

**Prolactin.** As reported previously, staining of prolactin in lacrimal glands of control animals was minimal in the acinar and intra- and small interlobular ductal epithelial cells (Fig. 1). Overall, immunoreactivity of prolactin was least in control animals and increased during pregnancy and after CI. The overall increases in the experimental groups were associated primarily with the intra- and small interlobular duct cells, whereas acinar cell staining was lightly immunoreactive. In the pregnant and CI rabbits, immunoreactivity for prolactin appeared to be predominantly within the apical cytoplasm of the ductal epithelial cells, but occasionally could be seen in the basal side of the cytoplasm (Fig. 1E). Nonpregnant and non-CI rabbits did not show this basal localization pattern. Immunoreactivity of prolactin in mammothrophs in the pituitary gland confirmed the specificity of the staining.

**TGF- $\beta$ 1 and EGF.** In general, the staining patterns of TGF- $\beta$ 1 and EGF were similar to that of prolactin, primarily localizing within the ductal epithelial cells—that is, least in control animals and increased during pregnancy and after CI. Therefore, we only included images of nonpregnant with CI for TGF- $\beta$ 1, and pregnant without CI for EGF (Fig. 2). In most



**FIGURE 3.** Western blot analysis of prolactin, TGF-β1, EGF in lacrimal gland lysates and lacrimal fluid. Prolactin: A major band of ~23 kDa was detected in the lacrimal gland lysates; its intensity was increased by pregnancy but decreased by CI. In lacrimal fluid, a band of 23 kDa was minimally detected. TGF-β1: In lacrimal gland lysates, a major band of ~23 kDa was detected in nonpregnant control rabbits. The intensity of this band was increased by 10% in pregnant animals, but decreased by 80% by CI. However, the combination of pregnancy and CI appeared to have no effect on its intensity. TGF-β1 was minimally detected in lacrimal fluid, although densitometric analysis showed some changes. EGF: In nonpregnant control rabbits, a major band of ~28 kDa EGF corresponding to the membrane-bound precursor, was minimally detectable, whereas either pregnancy or CI increased its intensity dramatically in the lacrimal gland lysates, with the strongest intensity observed in pregnant rabbits without CI. EGF was only minimally detectable in lacrimal fluid samples; its intensity was increased by either pregnancy or CI, whereas the intensity was decreased slightly by the combined effects of these two.

specimens, it appeared that the immunoreactivity of TGF-β1 and EGF was strongest in the pregnant animals with CI.

**Western Blot Analysis**

**Prolactin.** Probing Western blot analysis for prolactin revealed a major band at approximately 23 kDa in lacrimal gland lysates from the control and all three experimental groups (Fig. 3). Densitometric analysis revealed that pregnancy increased the band’s intensity significantly within the gland, whereas CI decreased it. In pregnant rabbits with CI, pregnancy appeared to mitigate partially the decrease associated with CI in the gland (Table 1).

The presence of 23-kDa prolactin in the lacrimal fluid samples was minimally detectable in nonpregnant without CI rabbits and was reduced in all three experimental groups, with the strongest band detected in nonpregnant with CI animals. Compared with the changes within the lacrimal gland, it appears

that prolactin in lacrimal fluid varied inversely with that seen within gland lysates. However, it should be noted that the intensity within the lacrimal fluid of all groups was generally very faint, and care should be taken in interpreting these data. Similar care should also be taken in evaluating the results of TGF-β1 and EGF.

**Transforming Growth Factor-β1.** A major band was demonstrated at approximately 23 kDa in lysates of lacrimal gland from the control and all three experimental groups. Pregnancy increased its intensity by 10%, whereas CI decreased it by 80%. However, in pregnant rabbits with CI, there was virtually no change in intensity.

As for prolactin, TGF-β1 was minimally detectable within lacrimal fluid. However, densitometric analysis demonstrated that the density in the three experimental groups was increased between 1.8- and 3.3-fold. The highest density was detected in nonpregnant animals with CI, which had the lowest density within the gland lysates.

**Epidermal Growth Factor.** A major band at ~28 kDa, corresponding to the membrane-bound precursor of EGF, was detectable in lacrimal gland lysates from control and all experimental groups. It was minimally detectable in the lacrimal glands of nonpregnant animals without CI, and increased dramatically by either pregnancy or CI, with the strongest being observed in pregnant animals without CI, a 24-fold increase. However, the influences of pregnancy and CI appear to be antagonistic, suggested by the finding that the intensity of EGF in pregnant animals with CI being intermediate between pregnant without CI and nonpregnant with CI groups.

EGF was minimally detectable within the lacrimal fluid, with pregnancy and CI appearing to increase its intensity, a 4-fold increase for pregnancy and 1.5-fold increase for CI. However, its intensity was slightly lower when the effects of pregnancy and CI were combined (i.e., in comparison to nonpregnant animals without CI).

**Immune Cell Redistribution**

**RTLA<sup>+</sup> Staining.** RTLA<sup>+</sup> cells were observed in the lacrimal glands of all four groups, in both interacinar and periductal areas (Table 2).

**Interacinar Cells.** Interacinar RTLA<sup>+</sup> cells were increased over control in all three experimental groups (*P* < 0.01). Both pregnancy and CI alone significantly increased the number of interacinar RTLA<sup>+</sup> cells, and the effect of CI was greater than that of pregnancy (*P* < 0.01). Pregnancy did not alter the effect of CI on interacinar RTLA<sup>+</sup> cells (*P* > 0.05) in pregnant animals with CI.

**Periductal Cells.** Pregnancy decreased the number of periductal RTLA<sup>+</sup> cells (*P* < 0.05). Whereas CI also decreased periductal RTLA<sup>+</sup> cells in nonpregnant animals, the effect was only half as much as pregnancy alone (*P* < 0.01). CI did not

**TABLE 1.** Densitometric Analysis of Total Immunoreactivity Expressed in Arbitrary Units

	LG			LG Fluid		
	Prolactin	TGF-β1	EGF	Prolactin	TGF-β1	EGF
Nonpregnant, without CI	1.0	1.0	1.0	1.0	1.0	1.0
Pregnant, without CI	3.0	1.1	25.0	0.2	3.4	5.0
Nonpregnant, with CI	0.2	0.2	8.6	0.7	4.3	2.5
Pregnant with CI	0.9	1.0	10.4	0.3	2.8	0.7

Data are presented in arbitrary units, with the intensity of nonpregnant without CI equal to 1.0. LG, lacrimal gland lysates.

TABLE 2. Image Analysis of RTLA Immunoreactivity in Rabbit Lacrimal Gland

	Nonpregnant, without CI	Pregnant, without CI	Nonpregnant, with CI	Pregnant, with CI
Interacinar	1,625 ± 117 (30)	4,746 ± 211 (30)	5,929 ± 202 (30)	5,994 ± 298 (30)
Periductal	9,479 ± 1,071 (13)	3,498 ± 483 (13)	6,270 ± 621 (9)	2,780 ± 238 (21)

Data are presented as mean square micrometers ± SEM. Numbers in parentheses are the number of replicates.

significantly alter the number of periductal RTLA<sup>+</sup> cells in pregnant rabbits ( $P > 0.05$ ).

**CD18<sup>+</sup> Staining.** CD18<sup>+</sup> cells were observed in all four groups, both interacinar and periductal (Table 3). *Interacinar Cells.* Both pregnancy and CI alone increased interacinar CD18<sup>+</sup> cells, and the effect of CI was greater than that of pregnancy. Moreover, the effect of CI on CD18<sup>+</sup> cells was additive with the effect of pregnancy. The differences among all four groups were significant ( $P < 0.01$ ).

*Periductal Cells.* Pregnancy alone decreased the number of periductal CD18<sup>+</sup> cells ( $P < 0.01$ ), with a similar effect observed during CI. However, the two conditions did not have any synergistic effect on the number of periductal CD18<sup>+</sup> cells.

### Lacrimal Secretory Functions

**Fluid Production.** In control animals, lacrimal fluid was secreted at a basal rate of 0.35  $\mu\text{L}/\text{min}$  (Table 4). In pregnant rabbits, the secretion rate decreased to 0.21  $\mu\text{L}/\text{min}$  ( $P < 0.05$ ). CI had no significant effect on the basal fluid secretion rate in nonpregnant animals, but it increased the basal rate nearly twofold in pregnant animals ( $P < 0.05$ ).

The cholinergic agonist, pilocarpine, elicited a significant increase of lacrimal fluid secretion in all four groups. Although pregnancy decreased the basal fluid flow rate, it increased the gland's capacity to secrete fluid in response to pilocarpine stimulation by 118% (Table 4 ratio of 11.40:5.23). CI also significantly increased the gland's capacity to secrete fluid in response to pilocarpine, but only by 42% (Table 4 ratio of 7.44:5.23)—that is, only approximately one third as much as pregnancy ( $P < 0.05$ ).

Moreover, CI partially reversed the effect of pregnancy on the pilocarpine-induced fluid secretion rate, so that the rates in pregnant animals with CI fell between those of pregnant without CI and nonpregnant with CI. Although no significant difference of stimulated lacrimal fluid secretion was observed between the two injured groups ( $P = 0.21$ ), there were differences between the fluid secretion rates among all other groups ( $P < 0.05$ ).

**Protein Secretion.** Protein secretion in pilocarpine-stimulated lacrimal fluids from rabbits of each group is shown in Table 5. Whereas pregnancy more than doubled the pilocarpine-induced lacrimal fluid flow rate, it decreased the concentration of protein in the secreted fluid by 38% (Table 5; ratio of 22.23:13.75). However, taking into consideration that there was an increased volume of lacrimal fluid in response to pilocarpine in pregnant animals without CI, the total amount of protein secreted increased by 35% (Table 5; ratio of 22.23:29.97).

CI also decreased stimulated lacrimal fluid protein concentration by 53% (Table 5; ratio of 22.23:10.38). However, taking into consideration the increased lacrimal fluid volume, the total amount of protein secreted in nonpregnant animals with CI was only 34% (Table 5; ratio of 22.23:14.78) less than in control animals.

Like the changes in fluid secretion, CI partially reversed the effect of pregnancy in increasing the amount of protein secretion; therefore, the combined effect of pregnancy and CI increased the amount of protein secreted by 19.4% (Table 5; ratio of 22.23:26.57).

### DISCUSSION

The results of the present study confirm the surprising finding that two different physiological states—pregnancy and CI—have qualitatively similar effects on lacrimal gland growth factor expression and immunoarchitecture. Both states cause lacrimal gland ductal epithelial cells to upregulate their contents of prolactin, TGF- $\beta$ 1, and EGF and are accompanied by redistribution of lymphocytes from periductal foci to a diffuse interacinar distribution.

Despite the overall similarity between the influences of pregnancy and CI on lacrimal immunoarchitecture, there are certain differences between these two states. Pregnancy is associated with greater decrease in the number of periductal RTLA<sup>+</sup> cells, and the influence of pregnancy appears to override the influence of CI. On the other hand, CI is associated with greater increase in the number of interacinar RTLA<sup>+</sup> cells, and the influence of CI on interacinar RTLA<sup>+</sup> cells appears to override the influence of pregnancy. Pregnancy and CI are associated with similar decreases in the number of periductal CD18<sup>+</sup> cells, but CI is associated with a greater increase in the number of interacinar CD18<sup>+</sup> cells. Moreover, the influences of the two states on the number of interacinar CD18<sup>+</sup> cells appear to be additive.

Pregnancy is associated with a significant decrease in basal lacrimal fluid flow rate but a 118% increase in the rate the gland produces fluid in response to pilocarpine stimulation. CI is associated with no change in basal lacrimal fluid production and only a 42% increase of pilocarpine-induced fluid production, but in the setting of pregnancy, CI significantly increases the basal rate of fluid production. While pregnancy increases pilocarpine-induced protein secretion, CI decreases it. Therefore, the combined effects of pregnancy and CI appear to increase pilocarpine-induced protein secretion to a lesser de-

TABLE 3. Image Analysis of CD18 Immunoreactivity in Rabbit Lacrimal Gland

	Nonpregnant, without CI	Pregnant, without CI	Nonpregnant, with CI	Pregnant, with CI
Interacinar	1,485 ± 60 (30)	2,362 ± 155 (30)	3,313 ± 110 (30)	4,294 ± 128 (30)
Periductal	6,282 ± 724 (14)	2,967 ± 346 (20)	3,197 ± 548 (17)	3,174 ± 331 (16)

Data presented as mean square micrometers ± SEM. Numbers in parentheses are the number of replicate measurements.

TABLE 4. Basal and Pilocarpine-Stimulated Lacrimal Fluid Production

	Nonpregnant, without CI	Pregnant, without CI	Nonpregnant, with CI	Pregnant, with CI
Basal	0.35 ± 0.04 (8)	0.21 ± 0.05 (6)	0.32 ± 0.11 (4)	0.99 ± 0.14 (6)
Stimulated	5.23 ± 0.40 (35)	11.40 ± 0.56 (38)	7.44 ± 0.65 (14)	8.69 ± 0.82 (18)

Data are presented as mean microliters/minute ± SEM. Number in parentheses are measurement replicates.

gree than pregnancy alone, suggesting that CI partially reversed the effect of pregnancy.

Our immunohistochemistry data showed that either pregnancy or CI, and the combination of these two states, result in increased immunoreactivity of prolactin, TGF- $\beta$ 1, and EGF within the ductal epithelial cells. The origin of these elevated amounts of peptides is unclear. In pregnant animals, as in nonpregnant ones, the overall staining for prolactin, TGF- $\beta$ 1, and EGF was minimal in the acinar cells. However, because the number of lacrimal acinar cells far outnumber ductal epithelial cells, the cumulative release of these peptides may still be significant, despite the minimal amounts detected by immunocytochemistry. Although these three peptides were only minimally detectable in the lacrimal fluids within the detectability of our instrument and the time parameters of our studies, we cannot rule out the significance of their presence in the fluids and potential important influences on the ocular surface, in both physiological and pathologic conditions, such as CI. We plan continued assays in the future to further clarify these results. We also cannot distinguish between the alternatives that ductal epithelial cells themselves may produce these peptides, or alternatively may be reabsorbing and transporting these peptides from the acinar effluent within the ductal lumen. In either case, the ductal epithelial cells must be viewed as a key component in regulation of the primary lacrimal fluid.

The specific signaling mechanism(s) underlying the redistribution of immune cells from periductal foci to interacinar areas during pregnancy and after CI remains to be clarified, as well as the significance of this event in altering lacrimal function. It is known that the acinar effluent is modified as it passes across the ducts, with exchanges of water, electrolytes, and proteins ultimately resulting in the final fluid exiting the gland.<sup>20-22</sup> It is our working hypothesis that there is interaction between the acinar and ductal elements within the gland (i.e., the ductal epithelial cells serve in monitoring and/or modulating the primary lacrimal fluid secreted from the acinar cells that traverses the duct system). Our studies suggest that prolactin, TGF- $\beta$ 1, and EGF within the ductal epithelial cells are likely to function in signaling periductal immune cells (Schechter JE, unpublished data, 2000), and the occasional immunolocalization of these peptides within the basal cytoplasm of the ductal epithelial cells appears to support that hypothesis.

Among the signals most salient for the present discussion are prolactin and TGF- $\beta$ 1, paracrine mediators expressed by the lacrimal epithelium. The information that has emerged

from studies of other tissues of the mucosal immune system, including intestines and the mammary gland, suggest that prolactin and TGF- $\beta$ 1 likely support maturation of IgA<sup>+</sup> plasmablasts and survival of IgG<sup>+</sup> plasmacytes, while providing autocrine support for expression of the polymeric immunoglobulin receptors (pIgR) that mediate transcytosis of dimeric IgA through the epithelium.<sup>23-26</sup> Reciprocally, the immune effector and regulatory tissues secrete mediators that influence epithelial cell functions. Dimeric IgA itself is known to be an important signaling molecule in other systems. In Sjögren's syndrome, agonistic autoantibodies to M<sub>3</sub> muscarinic acetylcholine receptors are thought to induce a state of functional quiescence in the epithelium,<sup>27</sup> whereas interleukin (IL)-1 and tumor necrosis factor (TNF)- $\alpha$  are thought to impair both secretomotor neurotransmission and epithelial responses.<sup>28,29</sup>

Finally, the body's endocrine environment exerts direct and indirect influences on the effector tissues and likely on the control tissues as well. It is well established that androgens support expression of pIgR by epithelial cells in the lacrimal gland,<sup>30,31</sup> as in the prostate.<sup>32</sup> The results of the present study demonstrate that the hormonal milieu of pregnancy modulates expression of paracrine mediators by the lacrimal epithelium. Dissecting the interacting signaling relationships will require substantial effort, but several parameters seem clear. The prevailing endocrine signals in pregnancy are elevated levels of estrogen and progesterone, as well as an estrogen-induced increase of pituitary prolactin production. Also of importance, bioavailable testosterone levels decrease, as a consequence of estrogen-stimulated hepatic sex hormone binding globulin production. Our preliminary studies appear to confirm that estrogen and progesterone support the patterns of lacrimal immunoreactivity and paracrine mediator expression seen in pregnancy (Ding C, unpublished data, 2004). Estrogen, progesterone, and prolactin interact to support pIgR expression and IgA<sup>+</sup> plasmacyte homing and survival in the mammary gland.<sup>33-35</sup> The diffuse, interacinar distribution of immune cells of the lacrimal gland during pregnancy is strongly reminiscent of the immunoreactivity of the mammary gland before parturition and during lactation, so we infer that an analogous underlying physiological rationale may direct the pregnancy-associated changes we have documented in lacrimal gland immunoreactivity.

Other workers have previously shown that lacrimal epithelial cells upregulate their expression of TGF- $\beta$ 1 and EGF within minutes after CI.<sup>19</sup> It seems likely, although not established definitively, that activation of sensory nerves in the cornea

TABLE 5. Protein Secretion in Pilocarpine-Stimulated Lacrimal Fluid

	Nonpregnant, without CI (13)	Pregnant, without CI (14)	Nonpregnant, with CI (4)	Pregnant, with CI (7)
Concentrations ( $\mu$ g/ $\mu$ L)	22.23 ± 1.2	13.75 ± 0.77	10.38 ± 0.88	15.99 ± 0.73
Total amount ( $\mu$ g)	22.23	29.97	14.78	26.57

Data are presented as the mean ± SEM. Numbers in parentheses are replicate measurements. Concentration is the measured protein concentration in lacrimal fluid, and total amount is the total protein content in the lacrimal fluid adjusted for the increased volume.

initiates, and autonomic secretomotor nerves mediate, this response.<sup>26</sup> It has been presumed that lacrimal TGF- $\beta$ 1 and EGF interact with local mediators to support wound healing,<sup>36</sup> although the demonstration that EGF receptor expression also is upregulated suggests that local, autocrine, and paracrine responses also occur. Our data, as presented herein, demonstrated that rabbit lacrimal gland also increases its expression of prolactin after CI.

Whatever the signals—systemic hormonal changes or neural signals triggered by ocular surface injury that initiate upregulation of lacrimal epithelial prolactin, TGF- $\beta$ 1, and EGF—it appears that once expressed, these paracrine mediators elicit a stereotypical response, with specific manifestations particular to the underlying physiological state. One of the specific manifestations this study shows to be associated with pregnancy is a decrease in basal lacrimal gland fluid secretion accompanied by an increased capacity to produce fluid in response to pilocarpine stimulation. It is possible that the decrease in basal fluid secretion results from a decrease in corneal sensitivity, rather than from any of the intrinsic signals associated with this physiological state. Then, it is reasonable to hypothesize that either decreased basal fluid production rates, or decreased concentrations of protein in fluid elicited by reflex signals from the ocular surface, are responsible for the ocular surface disease that we documented in our rabbit model<sup>37</sup> and for the increased frequency of dry eye complaints among women who are pregnant.

### Acknowledgments

The authors thank Michael Wallace, Michael Pidgeon, Kaijin Wu, Tamako Nakamura, and Bobbi Platler for assistance in these studies.

### References

- Wong J, Ding C, Yiu S, Smith R, Goodwin T, Schechter JE. An epidemiological study of pregnancy and dry eye. *Ocul Surf*. 2004;3:S127.
- Frey WH, Nelson JD, Frick ML, Elde RP. Prolactin immunoreactivity in human tears and lacrimal gland: possible implications for tear production. In: Holly FJ, ed. *The Preocular Tear Film in Health, Disease, and Contact Lens Wear*. Lubbock, TX: The Dry Eye Institute, 1986;798–807.
- Van Setten GB, Tervo T, Virtanen I, Trakkanen A, Tervo T. Immunohistochemical demonstration of epidermal growth factor in the lacrimal gland and submandibular glands of rats. *Acta Ophthalmol*. 1990;68:477–480.
- Wilson SE, Lloyd SA, Kennedy RH. Basic fibroblast growth factor (FGF) and epidermal growth factor (EGF) receptor messenger RNA production in human lacrimal gland. *Invest Ophthalmol Vis Sci*. 1991;32:2816–2820.
- Mircheff AK, Warren DW, Wood RL, Tortoriello PJ, Kaswan RL. Prolactin localization, binding, and effects on peroxidase release in rat exorbital lacrimal gland. *Invest Ophthalmol Vis Sci*. 1992;33:641–650.
- Wood RL, Park KH, Gierow JP, Mircheff AK. Immunogold localization of prolactin in acinar cells of lacrimal gland. *Adv Exp Med Biol*. 1994;350:75–77.
- Li Q, Weng J, Mohan RJ, et al. Hepatocyte growth factor (HGF) and HGF receptor in lacrimal gland tears and cornea. *Invest Ophthalmol Vis Sci*. 1996;37:727–739.
- Tsutsumi O, Tsutsumi A, Oka T. Epidermal growth factor-like corneal wound healing substance in mouse tears. *J Clin Invest*. 1988;81:1067–1071.
- Ohashi Y, Motokura M, Kinoshita Y, et al. Presence of epidermal growth factor in tears. *Invest Ophthalmol Vis Sci*. 1989;30:1879–1882.
- Yoshino K, Garg R, Monroy D, Ji Z, Pflugfelder SC. Production and secretion of transforming growth factor beta (TGF- $\beta$ ) by the human lacrimal gland. *Curr Eye Res*. 1996;15:615–624.
- Azzarolo AM, Bjerrum K, Maves CA, et al. Hypophysectomy-induced regression of female rat lacrimal glands: partial restoration and maintenance by dihydrotestosterone and prolactin. *Invest Ophthalmol Vis Sci*. 1995;36:216–226.
- Mircheff AK, Warren DW, Wood RL. Hormonal support of lacrimal function, primary lacrimal deficiency, autoimmunity, and peripheral tolerance in the lacrimal gland. *Ocular Immunol Inflamm*. 1996;3:145–172.
- Sullivan DA, Wickham LA, Rocha EM, Kelleher RS, da Silveira LA, Toda I. Influence of gender, sex steroid hormones, and the hypothalamic-pituitary axis on the structure and function of the lacrimal gland. *Adv Exp Med Biol*. 1998;438:11–42.
- Schechter J, Wallace M, Carey J, Chang N, Trousdale M, Wood R. Corneal insult affects the production and distribution of FGF-2 within the lacrimal gland. *Exp Eye Res*. 2000;70:777–784.
- Schechter J, Carey J, Wallace M, Wood R. Distribution of growth factors and immune cells are altered in the lacrimal gland during pregnancy and lactation. *Exp Eye Res*. 2000;71:129–142.
- Wilson SE, Liang Q, Kim WJ. Lacrimal gland HGF, KGF, and EGF mRNA levels increase after corneal epithelial wounding. *Invest Ophthalmol Vis Sci*. 1999;40:2185–2190.
- Fang Y, Choi D, Searles RP, Mathers WD. A time course microarray study of gene expression in the mouse lacrimal gland after acute corneal trauma. *Invest Ophthalmol Vis Sci*. 2005;46:461–469.
- Trousdale MD, Nobrega R, Stevenson D, et al. Role of adenovirus type 5 early region 3 in the pathogenesis of ocular disease and cell culture infection. *Cornea*. 1995;14:280–289.
- Schechter J, Weiner R. Changes in basic fibroblast growth factor coincident with estradiol-induced hyperplasia of the anterior pituitaries of Fischer 344 and Sprague-Dawley rats. *Endocrinology*. 1991;129:2400–2408.
- Mircheff AK, Lambert RW, Maves CA, Gierow JP, Wood RL. Subcellular organization of ion transporters in lacrimal acinar cells: secretagogue-induced dynamics. *Adv Exp Med Biol*. 1994;350:79–86.
- Walcott B. The lacrimal gland and its veil of tears. *News Physiol Sci*. 1998;13:97–103.
- Dartt DA. Regulation of lacrimal gland secretion by neurotransmitters and the EGF family of growth factors. *Exp Eye Res*. 2001;73:741–752.
- McGee DW, Aicher WK, Eldridge JH, Peppard JV, Mestecky J, McGhee JR. Transforming growth factor-beta enhances secretory component and major histocompatibility complex class I antigen expression on rat IEC-6 intestinal epithelial cells. *Cytokine*. 1991;3:543–550.
- Rafferty DE, Montgomery PJ. Effects of transforming growth factor  $\beta$  on immunoglobulin production in cultured rat lacrimal gland tissue fragments. *Reg Immunol*. 1993;5:312–316.
- Rafferty DE, Montgomery PC. The effects of transforming growth factor-beta and interleukins 2, 5 and 6 on immunoglobulin production in cultured rat salivary gland tissues. *Oral Microbiol Immunol*. 1995;10:81–86.
- Beuerman RW, Mircheff AK, Pflugfelder SC, Stern ME. The lacrimal functional unit. In: Pflugfelder SC, Beuerman RW, Stern ME, eds. *Dry Eye and Ocular Surface Disorders*. New York: Marcel Dekker; 2004:11–41.
- Qian L, Wang Y, Xie J, et al. Biochemical changes contributing to functional quiescence in lacrimal gland acinar cells after chronic ex vivo exposure to a muscarinic agonist. *Scand J Immunol*. 2003;58:550–565.
- Zoukhri D, Kublin CL. Impaired neurotransmitter release from lacrimal and salivary gland nerves of a murine model for Sjögren's syndrome. *Invest Ophthalmol Vis Sci*. 2001;42:925–932.
- Zoukhri D, Hodges RR, Byon D et al. Role of proinflammatory cytokines in the impaired lacrimation associated with autoimmune xerophthalmia. *Invest Ophthalmol Vis Sci*. 2002;43:1429–1436.
- Sullivan DA, Hann LE. Hormonal influence on the secretory immune system of the eye: endocrine impact on the lacrimal gland accumulation and secretion of IgG and IgA. *J Steroid Biochem*. 1989;34:253–262.
- Gao J, Lambert RW, Wickham LA, Banting G, Sullivan DA. Androgen control of secretory component mRNA levels in the rat lacrimal gland. *J Steroid Biochem*. 1995;52:239–249.
- Parr MA, Ren HP, Russell LD, Prins GS, Parr ED. Urethral glands in the male mouse contain secretory component and immunoglobulin

- lin A plasma cells and are targets of testosterone. *Biol Report*. 1992;47:1031-1039.
33. Weisz-Carrington P, Roux M, McWilliams M, Phillips-Quagliata JM, Lamm ME. Hormonal influence on the secretory immune system in the mammary gland. *Proc Natl Acad Sci USA*. 1978;75:2928-2932.
  34. Rincheval-Arnold A, Belair L, Djiane J. Developmental expression of plgR gene in sheep mammary gland and hormonal regulation. *J Dairy Res*. 2002;69:13-26.
  35. Rosato R, Jammes H, Belair L, Puissant C, Kraehenbuhl JP, Djiane J. Polymeric-Ig receptor gene expression in rabbit mammary gland during pregnancy and lactation: evolution and hormonal regulation. *Mol Cell Endocrinol*. 1995;110:81-87.
  36. Imanishi J, Kamiyama K, Iguchi I, Kita M, Sotozono C, Kinoshita S. Growth factors: importance in wound healing and maintenance of transparency of the cornea. *Prog Retin Eye Res*. 2000;19:113-129.
  37. Zhu Z, Stevenson D, Schechter JE, Mircheff AK, Atkinson R, Trousdale MD. Lacrimal histopathology and ocular surface disease in a rabbit model of autoimmune dacryoadenitis. *Cornea*. 2003;22:25-32.