Investigation of the Role of Prolactin in the Development and Function of the Lacrimal and Harderian Glands Using Genetically Modified Mice

Kathleen A. McClellan,1,2 Fiona G. Robertson,3 Jon Kindblom,4 Håkan Wennbo,4 Jan Törnell,1 Brigitte Bouchard,5 Paul A. Kelly,5 and Christopher J. Ormandy5

PURPOSE. To determine whether prolactin receptor is essential for normal development and function of the lacrimal gland and whether hyperprolactinemia can alter lacrimal development.

METHODS. Lacrimal gland morphology and function were examined in two genetic mouse models of prolactin action: a prolactin receptor knockout model that is devoid of prolactin action and a transgenic model of hyperprolactinemia.

RESULTS. Image analysis of lacrimal and Harderian gland sections was used to quantify glandular morphology. In females, lacrimal acinar area decreased by 30% and acinar cell density increased by 25% over control subjects in prolactin transgenic animals, but prolactin receptor knockout mice showed no changes. In males, transgenic animals showed no changes, but prolactin receptor knockout mice showed a 5% reduction in acinar area and an 11% increase in acinar cell density, which was lost after castration. The morphology of the Harderian glands underwent parallel changes but to a lesser degree. A complete loss of porphyrin accretions was seen in the Harderian glands of male and female knockout animals. No differences in tear protein levels were seen in knockout animals by two-dimensional gels. Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis showed that the level of secretory component and IgA in knockout mouse tears remained unchanged. There was no change in the predisposition of the 129 mouse strain to conjunctivitis in the knockout animals.

CONCLUSIONS. Prolactin plays a small role in establishing the sexual dimorphism of male lacrimal glands. In females, hyperprolactinemia causes a hyperfemale morphology, suggesting a role in dry eye syndromes. Prolactin is required for porphyrin secretion by the Harderian gland but plays no essential role in the secretory immune function of the lacrimal gland. (Invest Ophthal Vis Sci. 2001;42:23–30)

In women, dry eye syndromes occur mostly during alteration in the endocrine environment caused by pregnancy, lactation, oral contraceptive use, or menopause, with consequent changing levels of several pituitary factors: hyperprolactinemia to persistent pain and corneal damage leading to blindness.1,2 The cause of the disease lies in the disruption of the stability of the tear film, causing poor lubrication between eyelid and globe, resulting in mechanical and inflammatory damage to the corneal epithelium. Disruption of the tear film can be caused by changes in tear composition, supply, drainage, or evaporation and results from deficiencies in the external adnexa including low or excessive tear or tear protein production by the lacrimal gland, poor production of oils by the meibomian gland, low mucus production by the conjunctival goblet cells, and/or abnormal drainage through the tear duct. Adverse environmental conditions such as low humidity, high temperature or high dust levels can exacerbate these deficiencies.3 Current routine treatment relies on artificial tear supplementation or surgical intervention to reduce tear drainage through the canaliculi.

Tear deficiency due to declining lacrimal gland function is the major cause of tear film instability and dry eye.4 It carries the additional problem of reduced secretory immunity, because the lacrimal gland is the main source of IgA in tears.5 Two major causes have been identified: primary tear deficiency, the most prevalent cause of dry eye, which results from lacrimal gland destruction by a round cell infiltrate,6 and Sjögren’s syndrome, an autoimmune disease resulting in lymphocytic invasion and destruction of the epithelium of the lacrimal gland.7 The cause of primary tear deficiency remains unknown, but age-related endocrine changes have been hypothesized as a factor involved in the onset of dry eye.7 Endocrine regulation of the lacrimal gland8 is apparent from its sexually dimorphic morphology and function: women experience dry eye problems, and especially Sjögren’s dry eye, more frequently than men. In male rodents, the glands are larger, contain larger acini, and show lower acinar cell density.9 Functionally, male glands secrete higher levels of IgA and secretory component.10,11 Castration of males results in the loss of sexual dimorphism. Glands assume a more female morphology, and the levels of IgA and secretory component are reduced. Treatment of castrated animals with androgens reestablishes male morphology and increases IgA and secretory component output.1,12–15 Of note, in mouse models of autoimmune disease, androgen treatment can suppress the immunopathologic lesions of the lacrimal glands.16–18 Topical androgen application has been suggested for treatment of both Sjögren’s and non-Sjögren’s dry eye syndrome.19

Androgens do not act alone on the lacrimal gland. Mice without androgen receptors do not have a deficit in lacrimation.19 Hypophysectomy or pituitary transplant can prevent androgen-induced reduction of tear volume, IgA, and secretory component levels after castration.1,12–15 The identity of this pituitary factor is unknown, but because transplanted pituitaries secrete high levels of prolactin, and prolactin treat-
ment of dwarf mice has trophic effects on the lacrimal gland,\textsuperscript{8} it has been hypothesized to be a second hormone influencing lacrimal morphology and function. Short-term treatment with prolactin can restore the lacrimal expression of a number of genes that are altered after hypophysectomy, and can prevent dihydrotestosterone restoration of the levels of other genes, suggesting that prolactin modulates the lacrimal gland, both alone and in combination with androgens.\textsuperscript{1} These findings suggest that physiological levels of prolactin are required for the trophic actions of androgens on the lacrimal gland and that both hyper- and hypoprolactinemia may prevent this action. This hypothesis is consistent with the hormonal states in which dry eye is most common, but there is no convincing evidence in its favor. Because androgen receptors\textsuperscript{20} and prolactin and its receptor\textsuperscript{2} are expressed by the acinar cells of the lacrimal gland, this interaction may occur directly within the lacrimal gland.

We have used two genetic mouse models of prolactin action: a transgenic mouse (PRLtg), hyperprolactinemic because of overexpression of rat prolactin,\textsuperscript{22} and a prolactin receptor knockout mouse (PRLR\textsuperscript{+/−}) that has no prolactin receptors,\textsuperscript{25} to examine the hypothesis that prolactin is the pituitary factor involved in the function and maintenance of the lacrimal gland. These models allow two fundamental questions to be investigated: using animals exposed to altered prolactin function from the early embryonic stage: Is prolactin essential for normal lacrimal development and function? Can increased levels of prolactin modulate lacrimal development?

**Materials and Methods**

**Mice**

The prolactin receptor knockout mouse (PRLR\textsuperscript{+/−}) was generated by replacement of exon 5 of the PRLR gene, which encodes cysteine residues essential for ligand binding and receptor activation, with the NEO cassette.\textsuperscript{23} PRLR\textsuperscript{+/−} mice used in these experiments were derived from chimera mice made using E14 embryonic stem cells (129/OlaHsd) bred to 129/Sv Pas mice. Genetically similar wild-type control animals (PRLR\textsuperscript{+/+}) that have no prolactin receptors,\textsuperscript{23} to examine the hypothesis that prolactin is the putative factor involved in the function and maintenance of the lacrimal gland. These models allow two fundamental questions to be investigated: using animals exposed to altered prolactin function from the early embryonic stage: Is prolactin essential for normal lacrimal development and function? Can increased levels of prolactin modulate lacrimal development?

**Histology and Morphometric Analysis**

Individual lacrimal and Harderian glands were excised, laid flat onto filter paper (4 M; Whatman, Clifton, NJ) to maintain morphology during fixation, and fixed overnight in 10% neutral buffered formalin. Specimens were paraffin embedded, sectioned at 5 μm, and stained with hematoxylin-eosin. Specimens were photographed using a microscope (DMRB; Leica, Heidelberg, Germany) fitted with a CCD video camera (model 3; Sony, Tokyo, Japan) coupled to an image analysis program (Q500MC; Leica) running on a desktop computer. Acinar areas were measured using images captured at ×10 magnification. Epithelial cells were counted in images captured at ×20 magnification using the image analysis software.

**2-D Gel Analysis of Tear Proteins**

Mouse tears were collected from the eye with a 3-mm\textsuperscript{2} piece of filter paper by insertion between the orbit and lower eyelid of anesthetized mice. The portion of tear-soaked paper was added to 125 μl of a solution containing 5 M urea, 2 M thiourea, 1% (3-cholamidopropyl)dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPS), 2% sulfobetaine 3-10, 0.5% 3/10 Phospholipase (Amersham Pharmacia Biotech, Amersham, UK), 40 mM Tris, and 0.001% bromophenol blue for 1 hour, after which the solution was vortexed. Seven- centimeter pH 3-10 immobilised pH gradient strips (IPGs; Amersham Pharmacia Biotech) were rehydrated with solution for 6 hours. Isoelectric focusing was conducted using a 2-dimensional gel electrophoresis apparatus (Multiphor II; Amersham Pharmacia Biotech) at 20°C and was maintained for 14,000 Vh. Two-dimensional (2-D) separation was conducted using 12.5% polyacrylamide gels in which the immobilized pH gradient strips were embedded with 1% (wt/vol) agarose and then run at 10 mA/gel. The gels were fixed and silver stained, and protein maps were constructed.

**Enzyme-Linked Immunosorbent Assay**

To determine IgA levels in tears, an isotype-specific sandwich enzyme-linked immunosorbent assay (ELISA) was performed, using a direct plate binding assay. Plates were coated with a goat anti-mouse IgA capture antibody (PharMingen, San Diego, CA), and bound immunoglobulin was revealed by a secondary biotinylated goat anti-mouse IgA (Southern Biotechnology, Birmingham, AL) followed by streptavidin-alkaline phosphatase and specific substrate (p-nitrophenylphosphate [pNPP]). Plates were read using an ELISA plate reader at 405 nm. Quantification was performed by comparison with a standard curve established using purified mouse IgA (PharMingen).

**Western Blot Analysis**

The sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% acrylamide), transferred to membranes (Immobilon P; Amersham) using a semidry transfer apparatus (Bio-Rad; Herts, UK) and probed with either a polyclonal rabbit anti- serum against rat secretory component, cross-reacting with the murine secretory component (courtesy of Jean Paul Vaerman), or a biotin conjugated rabbit polyclonal anti-murine IgA (Zymed Laboratories, South San Francisco, CA). For the IgA blots, membranes were incubated directly with streptavidin-horse radish peroxidase (Amersham), and revealed using enhanced chemiluminescence (ECL; Amersham). For the secretory component blots, membranes were incubated with a biotin-conjugated anti-rabbit antibody (Vector, Peterborough, UK), before streptavidin-horse radish peroxidase and ECL assays. All incubations and washes were in phosphate-buffered saline (PBS) with 0.05% Tween.

**Statistical Analysis**

Cell density, acinar area, and porphyrin secretions of lacrimal and Harderian glands were compared using an unpaired, two-tailed Student’s t-test (Statview 4.0; Abacus, Berkeley, CA). Incidence of conjunctivitis was analyzed using Kaplan–Meier survival analysis (Statview 4.0) and probabilities were calculated using the Mantle–Cox log rank method.

**Results**

**Lacrimal Gland Development**

The role of prolactin in the development of the sexually dimorphic morphology of the lacrimal glands was examined in mature animals (12-10 weeks) by comparison of PRLR\textsuperscript{+/−} with PRLR\textsuperscript{+/−} and PRLtg with PRLwt. The lacrimal is a tubuloalveolar gland composed of ducts (acinii) without a clearly distinguished lumen. The ducts are surrounded by a fibrous
basement membrane and contain two major cell types: the secretory epithelial cells (acinar cells), identified by large round nuclei located at the basement membrane surface of a cytoplasm replete with secretory vesicles, and less frequent myoepithelial cells closely associated with the basement membrane and displaying elongated nuclei. A mainly acellular stroma fills the intraductal space. These glands showed typical sexual dimorphism; female glands (Figs. 1A, 1B, 1C) showed smaller acini and increased acinar cell density when compared with male glands (Figs. 1D, 1E, 1F).

The degree of sexual dimorphism was quantified by measurement of lacrimal acinar area using image analysis software, and acinar and acinar cell density by direct counting per field. Results were expressed as a percentage of female control levels, using elements per field, which correlated very well with direct area measurements. This allows comparison between the different mouse strains used. The sexual dimorphism of the lacrimal gland was easily detected by this technique. Male control animals (PRLR<sup>+/+</sup>, PRL<sup>wt</sup>) had approximately 75% of the female control number of acini per field because of an increase in average acinar area. Acinar cell density decreased to 75% of female levels (Fig. 2).

In PRLtg females, hyperprolactinemia caused the lacrimal glands to assume a hyperfemale state with average acini per field increasing to 130% (P < 0.0016) of control because of a decline in average acinar area associated with a similar increase (25%, P = 0.0087) in acinar cells per field. The morphology of male PRLtg glands remained unchanged (P = 0.07). These changes increased the degree of sexual dimorphism seen in these animals.

In female PRLR<sup>−/−</sup> animals, loss of the prolactin receptor had no effect on the morphology of the lacrimal gland. In PRLR<sup>−/−</sup> males there was a small (5%) but significant (P < 0.0001) increase in acini per field, caused by a decrease in acinar area associated with an 11% (P = 0.0034) increase in acinar cell density. Thus, in this model we also saw the acinar area decline and cell density increase, but in males not females, indicating a decrease in the degree of sexual dimorphism (Fig. 2). The small magnitude of this effect may not be physiologically relevant.

To determine whether we had missed a transient effect of hypoprolactinemia in the PRLR<sup>−/−</sup> animals during the onset of sexual dimorphism at puberty, we examined the lacrimal glands of animals at 4, 6, and 8 weeks of age (Fig. 3). The onset of sexual dimorphism was seen in females as a slight decrease in acinar area and slight increase in acinar cell number and in males as an increase in acinar area and dramatic decrease in acinar cell number, indicating that it is the male gland that most alters its morphology during puberty, consistent with the hypothesized trophic role of androgens. There was no difference in the rate of onset of sexual dimorphism between PRLR<sup>++/+</sup> and PRLR<sup>−/−</sup> animals of either gender.

**FIGURE 1.** Lacrimal histology from mature animals (12–16 weeks of age), stained with hematoxylin-eosin: (A, B, and C) females; (D, E, and F) males. (A, D) Histology in PRLR<sup>++/+</sup> animals was very similar to PRL<sup>wt</sup> animals (not shown). (B, E) PRLR<sup>−/−</sup> animals and (C, F) PRLtg animals. Original magnification, ×20.

**FIGURE 2.** Lacrimal morphometric analysis from mature animals. The number of acini and acinar cells were counted in 10 random microscope fields per animal, three to five animals per group, and statistically analyzed. Results are expressed as a percentage of female PRLR<sup>++/+</sup> for the knockout model and as a percentage of female PRL<sup>wt</sup> values for the transgenic model.
We examined the role of prolactin in the maintenance of sexual dimorphism by castrating PRLR<sup>1</sup>/<sup>1</sup> and PRLR<sup>2</sup>/<sup>2</sup> males and examining lacrimal morphology 21 days later (Fig. 4). In PRLR<sup>1</sup>/<sup>1</sup> animals, castration resulted in a 10% decrease in acinar area and a 12% decrease in acinar cell density. PRLR<sup>2</sup>/<sup>2</sup> animals underwent much the same change in acinar area, resulting in a 7% (<i>P</i> = 0.0001) difference. Acinar cell density showed a greater proportional change, so that no significant difference (<i>P</i> = 0.43) between genotypes was then seen in acinar cell density. These results indicate that the small reduction in acinar area resulting from a loss of the prolactin receptor is independent of androgen action, but that acinar cell number may be influenced by an interaction between prolactin and androgen. Again, however, the small magnitude of this effect calls into question its physiological relevance.

**Harderian Gland Development**

Histologic investigation (Fig. 5) showed that the Harderian glands of the mouse strains used in this study had larger acini with a defined lumen and more acinar cells than the lacrimal glands. They also exhibited sexually dimorphic characteristics similar to the lacrimal glands. Male glands had larger acini and fewer acinar cells than females. Quantification, as used for the lacrimal glands, showed smaller but similar effects of hyper- or hypoprolactinemia on acinar size or cell number in adults (Fig. 6).

Female PRLtg glands showed an 8% decrease in acinar area that failed to reach statistical significance (<i>P</i> = 0.15) and a 10% (<i>P</i> = 0.05) increase in acinar cell density. Male PRLtg animals showed no changes. Female PRLR<sup>-/-</sup> morphology remained unchanged, but male glands showed a 2% decrease in area that failed to reach statistical significance (<i>P</i> = 0.09) and a 10% (<i>P</i> = 0.007) increase in cell density. These changes exactly mirror those seen in the lacrimal gland, but their small magnitude places them close to the level of detection for the quantification technique that was used.

Female acini, and male acini to a lesser extent, contained solid accretions of porphyrin in the 129 mouse strain used to make the prolactin receptor knockout (Figs. 5A 5D). Hypo-
prolactinemia resulted in a complete loss of these solid porphyrin accretions in both males and females (Figs. 4B, 4E), quantified by counting accretions per field (Fig. 7). In males a 97% loss was seen ($P < 0.0026$), and in females an 83% ($P < 0.0001$) reduction occurred. The mouse strain used for construction of the transgenic model shows virtually no porphyrin accretions, making this analysis difficult. In males a 33% ($P = 0.096$) increase was seen and in females a 266% ($P = 0.16$) increase was seen, but the overall small number of accretions found prevented reliable statistical analysis of this effect.

Lacrimal and Harderian Function

To determine whether hypoprolactinemia alters the function of the lacrimal or Harderian glands, we took a number of approaches. Tear proteins were analyzed by 2-D gels, IgA and secretory component levels were analyzed by ELISA and Western blot, and alteration to the genetic susceptibility of the 129 mouse strain to conjunctivitis was searched for during an aging study.

Silver staining of 2-D gels of tears from $PRLR^{+/+}$ and $PRLR^{-/-}$ animals and comparison to consensus gels of mouse serum identified many spots specific to the tear film. Repeated tear sampling and 2-D analysis (10 replicates) indicated that...
none of these spots showed reproducibly altered patterns of expression (data not shown), leading to the conclusion that synthesis and secretion of the major tear proteins was unchanged. Individual spots could, however, show different relative levels in a single experiment, underlining the need for multiple replicates for reliable results using this technique.

The secretory immune function of the eye is maintained through the concentration of IgA in tears. IgA is synthesized by the acinar cells of the lacrimal gland and is transferred to tears in association with the polymeric IgA receptor, secretory component. IgAs protect the cornea and conjunctiva from inflammatory and infectious disease. To determine whether the levels of IgA and secretory component in tears is dependent on prolactin, we measured these species by Western blot and ELISA (Fig. 8). In tears from \( \text{PRLR}^{-/-} \) animals, Western blot analysis showed IgA and secretory component levels were identical with levels found in tears from \( \text{PRLR}^{+/+} \) animals. This was confirmed by ELISA for IgA. These experiments discount any essential role for prolactin in the maintenance of the major molecular species involved in the secretory immune system of the eye.

We used a genetic characteristic of the 129 strain to test for reduced ocular immune function. The 129 mouse strain is susceptible to conjunctivitis, which begins as mild suppurative palpebral conjunctivitis and progresses to the mucocutaneous junction at the exit of the meibomian duct, where a suppurrative process develops within and adjacent to the duct, associated with the formation of small ulcers over the conjunctiva. A genetic deficiency in secretory immunity appears to be the cause. As the disease advances, the eyelid becomes swollen and the surrounding hair becomes matted, and in our facility, mice at this stage are culled. To determine whether the secretory immune system of the eye was further compromised in \( \text{PRLR}^{-/-} \) mice, we aged a group of \( \text{PRLR}^{+/+} \) and \( \text{PRLR}^{-/-} \) animals and compared their survival. Animals that died or were culled for other causes were removed from the study. By 18 months of age the overall culling rate due to conjunctivitis was, for females, \( \text{PRLR}^{+/+} \) of 10 (40%) and \( \text{PRLR}^{-/-} \) of 9 (36%), and for males, \( \text{PRLR}^{+/+} \) of 8 (44%) and \( \text{PRLR}^{-/-} \) of 3 of 14 (21%). These data were analyzed further by Kaplan–Meier survival analysis (Fig. 9). Calculation of probabilities indicated no significant difference between genotypes when analyzed without reference to gender \((P = 0.16 \text{ Mantle–Cox log rank})\) or when analyzed separately by gender \((P = 0.82,\) females \( P = 0.98, \) Mantle–Cox log rank). These experiments detected no essential role for prolactin in the function of the secretory immune system of the eyes of animals predisposed to conjunctivitis.

### DISCUSSION

The results demonstrate that in females, prolactin plays no essential role in the development or maintenance of the morphological or function of the lacrimal glands, but that prolactin in excess can alter lacrimal gland morphology. Application of these findings to the previous investigation of the endocrine control of the lacrimal gland revealed unseen difficulties in the interpretation of results. In experiments using hypophysectomy in females or female pituitary dwarf mice, it is now clear that loss of prolactin was not the cause of the alterations in lacrimal gland morphology and function. Prolactin is not the pituitary factor lost in these models that influences the lacrimal gland, nor is it the pituitary factor that modulates androgen action in these experimental paradigms. These experiments also show that the effects in females of pituitary transplant or prolactin injection were due to the hyperprolactinemic result of these manipulations and did not reveal an effect of normal prolactin levels on the lacrimal glands. Thus, when prolactin treatment or pituitary transplant are combined with hypophysectomy, the effects of hyperprolactinemia are overlaid on the independent effects of hypophysectomy. Effects previously attributed to a physiological role of prolactin are in fact due to superphysiological levels of the hormone, which causes female lacrimal glands to assume a hyperfemale morphology. Translating this result to humans suggests hyperprolactinemia may
The gland also contains porphyrins, which are thought to be involved in sensing day length. Neonatal rat pups with undeveloped eyes or blind moles with vestigial eyes continue to respond to changed photoperiod when their eyes are removed, but not when their eyes and Harderian glands are removed. A number of these photoperiod responses involve the pineal gland, and the Harderian gland synthesizes melatonin and contains melatonin receptors, suggesting that it may have endocrine activity. The Harderian gland is sexually dimorphic and sensitive to steroid and pituitary hormones including prolactin. Our results indicate that the Harderian gland responds to prolactin in the same way as the lacrimal gland but that it is less sensitive, resulting in effects at the level of detection of our techniques. An essential role for prolactin was found in the formation of porphyrin accretions by the Harderian glands of male and female mice. Because testosterone levels in male PRLR−/− animals are normal, this observation establishes prolactin as a major and essential hormone controlling porphyrin accumulation in mice, as hypothesized from hypophysectomy and prolactin-bromocriptine treatment studies in rodents. Why prolactin should control accumulation of porphyrins in the Harderian gland remains an open question, but given prolactin’s diverse reproductive actions and the photo period sensing and signaling ability of the Harderian gland, it is tempting to speculate that it may have a role in the control of seasonal breeding.

It is important to distinguish between the endocrine state produced by an absence of prolactin action and that produced by hyperprolactinemia. These conditions can be considered to be separate endocrine states and demonstrate that the failure to show an essential role for a hormone in a process does not indicate that an excess of that hormone will similarly be without effect. This is the case with prolactin in the female lacrimal gland. Although not essential for normal development, hyperprolactinemia produces a hyperfemale morphology that may predispose to dry eye.

References


