Vitamin A Deficiency Alters the Expression of Mucin Genes by the Rat Ocular Surface Epithelium

Mamoru Tei, Sandra J. Spurr-Michaud, Ann S. Tisdale, and Ilene K. Gipson

PURPOSE. To study effects of depletion of retinoic acid on expression of the mucins ASGP (rMuc4), rMuc5AC, and rMuc1, by the corneal and conjunctival epithelia of the rat.

METHODS. Nineteen-day-old Sprague–Dawley male rats were fed a casein-based vitamin A–deficient diet or casein-based diet with vitamin A as control. Rats from both groups were killed at 1, 3, 5, 13, 15, 18, and 20 weeks after initiation of feeding. Expression of the three mucin genes by the ocular surface epithelium was assayed by reverse transcription–polymerase chain reaction (RT-PCR) and in situ hybridization.

RESULTS. In vitamin A–deficient rats, ASGP mRNA was not detected by RT-PCR after 15 weeks of feeding. rMuc5AC mRNA was detected by RT-PCR at 15 weeks, but by 18 and 20 weeks was no longer detectable. By in situ hybridization, ASGP mRNA was localized in the entire ocular surface epithelium after 1 week of feeding, was diminished but detectable above background by 13 weeks, and was not detectable at 20 weeks. rMuc5AC mRNA was detected in the goblet cells of vitamin A–deficient rats by in situ hybridization at 13 weeks, but was lost by 20 weeks, as were identifiable goblet cells. rMuc1 mRNA were detected by RT-PCR through all time points of 1 to 20 weeks in both vitamin A–deficient and control rats, indicating no significant change in rMuc1 mRNA expression with vitamin A deficiency.

CONCLUSIONS. Both the membrane-spanning mucin ASGP (rMuc4) and the secretory mucin rMuc5AC are directly or indirectly regulated by vitamin A in the ocular surface epithelium, whereas the membrane-spanning mucin rMuc1 is not. (Invest Ophthalmol Vis Sci. 2000;41:82–88)
ing the gelled mucus layer of the tear film at the air-ocular surface epithelium interface. To establish animal models for the study of goblet cell differentiation and regulation and function of this gel-forming mucin, our laboratory has cloned a rat homologue of the human MUC5AC gene.\textsuperscript{21} It is designated rMuc5AC, and a 3783-bp region of the 5′ end of the molecule, including a cysteine-rich domain, was sequenced. Previous in situ hybridization studies from our laboratory demonstrated that this rat homologue of MUC5AC is expressed in the goblet cells of the rat conjunctiva.\textsuperscript{21}

Previous in vitro studies have indicated that vitamin A derivatives are required for maintaining mucosal cell differentiation, mucin production, and mucin gene expression.\textsuperscript{22–26} Recent studies using cultured tracheobronchial epithelium showed that MUC5AC mRNA as assayed by either northern blot analysis\textsuperscript{22} or by quantitative reverse transcription–polymerase chain reaction (RT-PCR)\textsuperscript{27} is increased when cells were cultured in the presence of vitamin A. These in vitro results indicate the need for assessment of the role of retinoids in the regulation of mucin gene expression in vivo. It has long been known that keratinization occurs and that goblet cells disappear in the conjunctiva of vitamin A–deficient humans and in animal models using rats. Little is known, however, of how decreased vitamin A affects conjunctival or corneal epithelium at the mucin gene expression level. The purpose of our experiments was thus to use the vitamin A–deficient rat model to determine the effects of retinoic acid depletion on expression of the membrane-spanning mucins rMuc1 and ASGP and on the goblet cell–specific mucin rMuc5AC.

### MATERIALS AND METHODS

#### Animals and Tissues

All procedures used in these studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Nineteen-day-old Sprague–Dawley male rats were fed a casein-based vitamin A–deficient diet (Teklad, Madison, WI).\textsuperscript{29} Age-matched control male rats had vitamin A included in the casein-based diet. Body weight and ocular surface and skin characteristics were monitored for signs of vitamin A deficiency twice a week. At 1, 3, 5, 13, and 20 weeks after initiation of feeding, two rats from each group were killed. In addition, two rats from the vitamin A–deficient group were killed because of poor general condition, one at 15 and one at 18 weeks after initiation of feeding. Conjunctivas for RNA isolation were frozen in liquid nitrogen immediately after removal and stored at −80°C until RNA isolation. Anterior segments of the eyes with conjunctiva and lids included were fixed immediately after removal in 4% paraformaldehyde in 0.1 M phosphate buffer and processed through paraffin for in situ hybridization and histochemical staining.

#### RNA Isolation and RT-PCR of rMuc1, ASGP, and rMuc5AC

Total RNA was isolated from tissues of Sprague–Dawley rats using TRIzol reagent (Gibco, Grand Island, NY), according to the protocol provided by the manufacturer.

Recently, our laboratory successfully used semiquantitative RT-PCR to correlate the expression of endocervical mucin mRNA to blood hormone levels.\textsuperscript{29} We used the same approach to study rMuc5AC, ASGP, and rMuc1 mRNA expression by the rat ocular surface during progression from mild to severe vitamin A deficiency. In this study, rat β-actin was used as the internal control.\textsuperscript{30} Total RNA from rat conjunctiva (1 µg/sample) was subjected to reverse transcription using random hexamer primers as previously described.\textsuperscript{15} A 1-µl aliquot (one twentieth of total volume) of the same RT products per sample was used in the PCR amplification reaction for each of the genes investigated.

Oligonucleotide primers to the nontandem repeat region of rMuc1, ASGP, and rMuc5AC were designed from published or GenBank sequences (Table 1). PCR amplifications were performed as previously described.\textsuperscript{15} The sequence of each PCR product was confirmed by sequencing using the dideoxy-mediated chain termination method.

PCR conditions were optimized for all genes studied using RT product from adult rat conjunctival total RNA. The linear range of the amplification reaction for β-actin, rMuc5AC, ASGP, and rMuc1 was determined by checking amplification after each cycle from cycles 21 to 35 for β-actin, rMuc5AC, and ASGP, and from cycles 29 to 41 for rMuc1. These determinations showed 27 cycles to be in the midlinear phase for β-actin, rMuc5AC, and ASGP and 35 cycles to be in the midlinear phase.

| Table 1. PCR Primers Used for Semiquantitative Analysis |
|-----------------|-----------------|-----------------|
| ![Table](#) | ![Table](#) | ![Table](#) |
| **PCR Primers** | **Size** | **Reference** |
| rMuc1 | 5′-TCGACAGCCAATGGCACTG-3′ (154–173) | 286 bp | GenBank accession no. AF007554 |
| | 5′-CTCTGAGCCACCATGCA-3′ (439–420) | | |
| ASGP | 5′-CGTCTAGACAGAGCTGCAT-3′ (4761–4785) | 638 bp | Wu et al.\textsuperscript{19} |
| | 5′-GGTGGAGCTGTCAGTTAG-3′ (5398–5378) | | |
| rMuc5AC | 5′-TGTAGAGTGGCAGCTGCTTT-3′ (726–745) | 470 bp | Inatomi et al.\textsuperscript{21} |
| | 5′-CAGTGGGGCTAAAGAG-3′ (1195–1176) | | |
| β-Actin | 5′-TTGTAACCAACTGGAGCATGG-3′ (1552–1575) | 764 bp | | |
| | 5′-GATCTTGATCTTTGATTCTTAGG-3′ (2991–2844) | | |
| In situ hybridization probes | | | |
| ASGP | 638 bp | Wu et al.\textsuperscript{19} |
| rMuc5AC | 228 bp | Inatomi et al.\textsuperscript{21} |
for rMuc1. All PCR amplifications started with denaturation at 94°C for 5 minutes and ended with a final elongation at 72°C for 7 minutes. The parameters for PCR amplifications were as follows: rMuc5AC and β-actin: 27 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute; ASGP: 27 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute; rMuc1: 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute.

The PCR products were run on a 1.5% agarose gel stained with ethidium bromide and photographed with positive-negative film (Type 665; Polaroid, Cambridge, MA). The amount of amplified product was quantified for each sample from the negatives using a computing densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA) and software (Image Quant, ver. 2.0; Molecular Dynamics). The final amount of PCR product was expressed as the ratio of mucin gene amplified to that of β-actin to account for any differences in starting amounts of RNA.

In Situ Hybridization and Histologic Analysis
To demonstrate when and where ASGP and rMuc5AC expression is altered, in situ hybridization was performed,16 by using radiolabeled RNA probes to the 3′ end of ASGP and radiolabeled RNA probes to the tandem repeat of rMuc5AC. The cDNA used to generate the ASGP riboprobes was generated by RT-PCR using adult rat endocervix RNA and ASGP-specific primers (Table 1) and was subcloned into pGEM-T Easy vector (Promega, Madison, WI). The cDNA used for rMuc5AC has been described previously.16 Hybridization was performed overnight at 52°C using 1 × 10⁶ cpm/ml of 35S-UTP-labeled sense or antisense riboprobe transcribed from plasmid Bluescript SK(−) using T3 or T7 RNA polymerase (Boehringer-Mannheim, Indianapolis, IN) for rMuc5AC and from pGEM-T Easy vector using T7 or SP6 RNA polymerase for ASGP. Sections were counterstained with hematoxylin and eosin. Six-micrometer sections were stained with periodic acid-Schiff (PAS) reagent by conventional techniques to determine goblet cell presence and distribution.
RESULTS

A change in the average weight of vitamin A–deficient male rats relative to that of control rats was first observed at 6 weeks after initiation of feeding of the vitamin A–deficient diet. The average weight of the vitamin A–deficient males remained constant for an additional 7 weeks, and additional weight loss began at 13 weeks after initiation of feeding. Severe general manifestations (hair loss, bleeding from nose) and severe ocular manifestations (accumulation of reddish secretions at the eyelid margin, loss of corneal clarity, corneal vascularization) appeared between 13 and 20 weeks after initiation of feeding.

Histologic Characteristics of Vitamin A–Deficient Rats

Of the time points examined (1, 3, 5, 13, 15, 18, and 20 weeks) the first difference between conjunctival epithelial or goblet cell morphology of vitamin A–deficient rats compared with control rats was observed at 13 weeks (Figs. 1, 2). At 13 weeks, one rat was more severely affected than others (compare Figs. 2C and 2E). In the mildly affected eye, the conjunctival epithelium was partially keratinized with goblet cells present within regions of keratinization, and the conjunctival epithelium appeared to have more cell layers (Figs. 2C, 2E). Inflammatory cells were seen in the adherent mucus, and lymphocytes were observed in the conjunctival epithelium. In the severely affected eye, the degree of keratinization appeared to be greater, and the presence of morphologically distinct goblet cells was less, as was the thickness of the epithelium (Fig. 2E). At 20 weeks after initiation of feeding, the entire conjunctival epithelium of the vitamin A–deficient rat eyes was keratinized, and goblet cells were not evident (Figs. 1K, 2G).

PAS staining of goblet cells was detected from 1 to 13 weeks of feeding (Fig. 3). At 13 weeks, the partially keratinized conjunctival epithelium still had goblet cells intercalated within it (Figs. 3B, 3C). In severely affected eyes at 13 weeks (Fig. 3C), PAS staining was evident, but the shape of the goblet cells was not round. No PAS-positive cells were seen at 20 weeks in the completely keratinized conjunctival epithelium of vitamin A–deficient rat conjunctiva (Fig. 3D).

Semiquantitative RT-PCR Analysis of Message Levels of ASGP and rMuc5AC

To compare the mRNA expression of ASGP and rMuc5AC within experimental groups, the linear phase of amplification of each cDNA was demonstrated so that gene expression could be semiquantitatively compared. For ASGP, rMuc5AC, and the control housekeeping gene β-actin the linear phase of amplifi-
cation was established at 27 cycles. In control rats, ASGP, rMuc5AC, and β-actin mRNA was detected from 1 to 20 weeks after initiation of feeding. ASGP mRNA was not detected in ocular surface tissue at 15, 18, and 20 weeks after initiation of feeding (Fig. 4). In the deficient rats, rMuc5AC was present until 15 weeks, but the amount of mRNA was low. By 18 and 20 weeks, the vitamin A–deficient rats no longer expressed detectable amounts of rMuc5AC (Fig. 4).

Densitometric comparisons of both ASGP and rMuc5AC mRNA to that of β-actin were obtained in each of these groups (Fig. 5). The ratios of ASGP/β-actin mRNA and rMuc5AC/β-actin were higher than 1 until 3 weeks after initiation of feeding, when they both gradually decreased. There were no dramatic ratio changes between 3 and 13 weeks after initiation of feeding. After 13 and 15 weeks, ASGP mRNA and rMuc5AC, respectively, were not detected (Fig. 4), even with 40 cycles of PCR amplification (data not shown).

rMuc1 RT-PCR
rMuc1 mRNA did not amplify in either the experimental or control group at 27 cycles, but all groups showed amplification at 35 cycles. rMuc1 mRNA was detected from 1 to 20 weeks after initiation of feeding in both vitamin A–deficient and control rats (Fig. 4C). There was no apparent change in rMuc1 mRNA expression in either control or vitamin A–deficient rats through the 20 weeks of feeding.

In Situ Hybridization of ASGP
In situ hybridization using 35S-labeled riboprobes for the 3′ domain of ASGP resulted in detection of expression of ASGP in the cells of both the corneal and conjunctival epithelia in control rats and rats taken at 1 week after initiation of feeding the vitamin A–deficient diet (Figs. 1B, 1D). The epithelium of the ocular surface labeled predominantly in the apical cell layers. Label was not obvious within goblet cells; however, at the level of the light microscope, using 35S-labeled probes, label on the stratified cells cannot be distinguished from label at the base of the goblet cell where the nucleus and endoplasmic reticulum are packed tightly against the basal cell surface by the mucin packets. The labeling of ASGP mRNA in the conjunctival epithelium was weaker than the labeling detected in the corneal epithelium at 13 weeks after initiation of feeding in mildly affected deficient rats (Figs. 1F, 1H), whereas in severely affected rats, PAS staining was still evident, but goblet cell morphology was aberrant. In rats fed the deficient diet for 20 weeks, PAS staining and goblet cells were not evident in the conjunctival epithelium (D). In 20-week control animals, goblet cells with intense PAS staining were evident (E). All micrographs are of the same magnification; bar, 50 μm.

In Situ Hybridization of rMuc5AC
In situ hybridization using 35S-labeled riboprobes for the tandem repeat region of rMuc5AC resulted in detection of expression of rMuc5AC in the goblet cells of control-fed rats at all time points examined (Fig. 2J). The expression of rMuc5AC was detected in both cornea and conjunctiva (Figs. 2F, 2H). The sense sequence of the same region of ASGP, used as a negative control probe, did not show any binding in control or vitamin A–deficient groups (Figs. 1T, 1V).
2B, 2D). In severely affected rats fed for 13 weeks, some conjunctival epithelial cells showed diffuse binding in areas along the apical region of the epithelium where the goblet cells were no longer morphologically identifiable (Figs. 2E, 2F). However, these regions did stain with PAS (Fig. 3C). At 20 weeks after initiation of feeding, expression of rMuc5AC was not detected in the keratinized conjunctival epithelium (Fig. 2H). The sense sequence of the same region of rMuc5AC, used as a negative control probe, did not show any binding in control or vitamin A–deficient groups (Fig. 2L).

**DISCUSSION**

A major conclusion of the present study is that both the membrane-spanning mucin ASGP and the gel-forming mucin rMuc5AC were downregulated by vitamin A deficiency. The second membrane-spanning mucin, Muc1, was not affected by the absence of vitamin A, even after severe keratinization. These data suggest that the membrane mucin ASGP (rMuc4) and the secretory mucin rMuc5AC are regulated by retinoic acid, whereas, the membrane-spanning mucin rMuc1 is not. This potential regulation of ASGP and rMuc5AC by retinoic acid may be at the transcriptional level with direct retinoic acid receptor interaction with the regulatory regions of the mucin genes. To date, the regulatory regions of neither ASGP nor rMuc5AC have been characterized, but data from cultured human tracheal cells show induction of MUC5AC mRNA by retinoids. These latter data suggest that there is potential direct transcriptional regulation of MUC5AC by retinoic acid.27

Retinoic acid receptors are members of the superfamily of nuclear receptors activated by lipophilic ligands. These receptors are of two categories: retinoic receptors (RARs) and retinoid X receptors (RXRs). Recently, Bossenbroek et al.5 have demonstrated the presence of mRNA for all subtypes of RARs and RXRs in rabbit corneal epithelium and corneal and conjunctival fibroblasts. These data suggest that appropriate receptors are available within ocular surface tissues that would allow direct regulation of the mucin genes by vitamin A. Proof of retinoic acid regulation of the mucin genes within ocular surface epithelium awaits characterization of the regulatory region of each mucin gene and development of culture systems of ocular surface epithelia that would be useful for experimental analysis of mucin gene regulation.

A second major conclusion of this study is that mucin gene expression was downregulated as keratinization of the epithelium occurred, and on complete keratinization, expression of mucin genes ASGP and rMuc5AC was entirely lost. These data suggest a second potential method of regulation of the expression of the mucin genes. Their expression may be regulated indirectly or secondarily because of the alteration of the differentiation pathway of the epithelia. If the differentiation of a nonkeratinizing epithelium is switched to a keratinizing epithelial differentiation mode, as is the case in vitamin A deficiency, a whole set of genes is turned on or off. These genes may indirectly affect mucin gene expression. In support of this hypothesis is the demonstration that retinoids through RARs and RXRs inhibit mRNA expression of a group of squamous differentiation markers including transglutaminase type I, involucrin, keratin 5, and keratin 13 by cultured human bronchial epithelium.31

Our data regarding the effect of vitamin A deficiency on rMuc5AC gene expression are compatible with previous reports that retinol deficiency is associated with a reduction in the goblet cell population11 and other studies that show ab-

![Figure 4](image-url)  
**FIGURE 4.** Assay of ASGP (rMuc4), rMuc5AC, and Muc1 by RT-PCR in vitamin A–deficient and control rats. β-actin was used as control. Numbers at bottom indicate weeks consuming a vitamin A–deficient (−) or –replete (+) diet; M, 100-bp DNA marker. Note that ASGP mRNA did not amplify after 13 weeks of a deficient diet. A faint rMuc5AC PCR product was still present at 15 weeks, but was not present after 18 or 20 weeks of a deficient diet.

![Figure 5](image-url)  
**FIGURE 5.** Semiquantitative RT-PCR analysis of ASGP and rMuc5AC mRNA in vitamin A–deficient rats compared with control rats. The ratio is the level of ASGP or rMuc5AC RT-PCR product divided by the level of β-actin PCR product, all from mRNA from vitamin A–deficient ocular surface tissue, which is, in turn, divided by the same ratio obtained for control-fed animals. The ratio was higher than 1 at and between 1 and 3 weeks after initiation of feeding. There was no apparent change of the ratio between 3 and 13 weeks. After 13 weeks, the ratio decreased rapidly.
sence of mucin expression in retinoid-deficient tracheal cultures that are characterized by squamous differentiation. The data also demonstrate that the alteration in glycoconjugate expression—for example, mucin expression—is at the transcriptional level rather than at the level of glycosylation of the mucin, because mucin mRNA is lost.

Data from this study show that mucin gene expression is altered not only in goblet cells but in the stratified epithelium of cornea and conjunctiva as well. In the deficient rats of this study, the ASGP mucin mRNA of the stratified epithelium disappeared before the goblet cell mucin rMuc5AC mRNA. These findings may relate to the observation in humans that corneal xerophthalmia responds more rapidly to vitamin A deficiency than does goblet cell return and response. Perhaps the stratified epithelial cells respond more rapidly to vitamin A than does the goblet cell population. Alternatively, the goblet cell stem cell population may require considerably more time to differentiate than does the stratified cell population.

In summary, we show that the expression of ocular surface mucins was downregulated in the vitamin A-deficient rat. We demonstrated that mRNA for rMuc5AC and ASGP were not detected after the appearance of severe keratinization, whereas rMuc1 mRNA expression was not affected by vitamin A deficiency. The expression of the membrane-spanning mucin ASGP and the gel-forming mucin rMuc5AC may be regulated by retinoic acid.

References