Post-transcriptional and Transcriptional Control of Collagen Gene Expression in Normal and Modulated Rabbit Corneal Endothelial Cells

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In a previous report, collagen synthesis did not correlate with steady-state collagen RNA levels; substantial amounts of type I collagen RNAs in endothelial cells were not translated into the respective protein. The current investigation was extended to study the level of the control mechanism in collagen gene expression in normal corneal endothelial cells or those modulated by corneal endothelium modulation factor released by polymorphonuclear leukocytes. Northern-blot analysis using cloned rabbit types I and IV cDNA probes (same species as RNA sources) demonstrated specific mRNA transcripts for collagen types I and IV in the endothelial cells, although the steady-state level of these mRNAs in modulated endothelial cells was low. The turnover rate of collagen RNAs was determined; normal cells contain very stable α2(I) and α2(IV) mRNAs whose half-lives exceed 24 hr. The same messages decayed rapidly in the modulated cells, where they had an apparent half-life of approximately 8 hr. Using nuclear run-off transcription, the rate of transcription in normal cells was found to be slightly lower than that in modulated cells. When the relative rate of collagen gene transcription was compared, that of α2(I) was the lowest and of α2(IV), the highest in both cells. The relative transcriptional rates of individual collagen chains did not account for the steady-state levels, suggesting that transcriptional regulation in corneal endothelial cells was less than was translational regulation. On the other hand, during early stages of corneal endothelial cell modulation induced by factors released by polymorphonuclear leukocytes, there was a differential effect on both transcriptional and the steady-state level of collagen RNAs. Preferential enhancement of type I collagen RNA was observed, suggesting that there were at least temporal transcriptional changes during the early stage of modulation.


Corneal endothelium in vivo responds to diverse types of pathology by converting to fibroblast-like cells.1-3 These morphologically modulated cells, in turn, produce fibrillar collagens, among which type I collagen is the predominant species, and deposit an abnormal fibrillar extracellular matrix (retrocorneal fibrous membrane, RCFM) between Descemet’s membrane and the endothelium monolayer. This clinically observed phenomenon is one of the many instances where less organized and less specific connective tissue replaces a previously functional and carefully constructed matrix. The characteristic collagen phenotypes were determined in the experimentally induced RCFM in rabbit corneas and compared with the collagen phenotypes synthesized by rabbit corneal endothelial cells.4-6 Normal endothelial cells in culture synthesize predominantly type IV collagen; type I collagen is the major collagensous protein in RCFM in vivo, and it is synthesized by the RCFM cells in culture. Thus, corneal endothelium modulation involves phenotypic changes in collagen gene expression.

To elucidate the mechanism of corneal endothelium modulation, we established an in vitro model in which polymorphonuclear leukocytes modulate type IV collagen-synthesizing endothelial cells to type I collagen-synthesizing cells.7-10 Such modulated endothelial cells share phenotypic characteristics with the cells that constitute retrocorneal fibrous membranes. The major collagen phenotype is type I, which can
form interstitial extracellular matrices between multiple layers of the modulated cells.7

Regulation of collagen gene expression takes place at many levels, as is the case for other eukaryotic gene products.11-14 Interplay between positive and negative elements present in the α1(I) collagen gene promoter region and in the first intron has been proposed to be the normal regulator of gene transcription.15,16 However, our previous studies demonstrated that the amounts of collagen mRNAs do not account for the amount of translated proteins in corneal endothelial cells, suggesting that there is, at least in part, posttranscriptional control of types I and IV collagen expression in rabbit corneal endothelial cells.

In the current investigation, we extended our studies of the control mechanism of collagen gene expression in both normal and modulated corneal endothelial cells. Some of the unique features we observed were that the stable and abundant α2(IV) collagen RNA was actively translated, while the other equally stable and abundant α2(I) collagen RNA in the normal cells was not. However, the modulated endothelial cells that contain low steady-state types I and IV collagen RNA levels had a markedly increased turnover rate of the messages, suggesting that corneal endothelium modulation was associated with stability of these collagen RNAs. Transcriptional control was observed during the early stage of corneal endothelium modulation, however.

Materials and Methods

Cell Cultures

Isolation and establishment in culture of primary rabbit corneal endothelial cells were as previously described.8 Briefly, Descemet’s membrane–corneal endothelial complex was treated with 0.2% collagenase and 0.5% hyaluronidase for 90 min at 37°C. Cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 50 μg/ml of gentamicin in a humidified atmosphere of 5.0% CO2 in air. Modulated endothelial cells (often referred to as fibroblastic corneal endothelial cells, FCEC) were established by polymorphonuclear leukocyte (PMN)-conditioned medium.8 The PMNs were obtained from the peritoneal cavity of New Zealand rabbits after glycogen stimulation, and PMN-conditioned medium was used for initiation of modulation and maintenance of all modulated endothelial cells in culture until they reached irreversible metaplasia. For these studies, FCEC from the 90th passage were used; these cells had collagen phenotypes identical to those of earlier passage cultures. The PMN-conditioned medium was purified further as previously described.17 The four-step purifica-

Preparation of RNA

Cytoplasmic RNA was isolated from normal or modulated endothelial cells by a method previously described, with modification.19 Briefly, the cells were homogenized in buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Total RNA was isolated by cesium chloride density-gradient centrifugation using a 5.7 M CsCl cushion in an SW 28.1 rotor (Beckman, Irvine, CA) for 17 hr at 28,000 rpm. The pellet containing total RNA was dissolved in 10 mM Tris HCl, pH 7.4, 5 mM ethylenediaminetetraacetate acid, and 1% sodium dodecyl sulfate, and extracted with a 4:1 mixture of chloroform and 1-butanol. The aqueous phase was precipitated with 2.2 volume of ethanol containing 0.3 M sodium acetate, pH 5.2, at −20°C for 24 hr; the precipitate was collected by centrifugation at 12,000 × g for 30 min at 4°C. The total RNA in the final pellet was dissolved in sterile water, and the RNA concentration measured by absorbance at 260–280 nm. Poly(A)RNA was obtained by oligo(dT)-cellulose chromatography.

Hybridization Analysis

For northern blot analysis, poly(A)RNA or cytoplasmic RNA was denatured by treatment with formaldehyde-formamide, separated by electrophoresis on a 0.8% agarose gel, and blotted to a nitrocellulose filter. The RNA was prehybridized, hybridized, and washed as described previously.10 Rabbit α2(I) and α2(IV) cDNA clones were isolated and characterized by Dr. Yoshifumi Ninomiya (Harvard Medical School, Boston, MA). Rat α1(I) and α2(I) cDNA clones were gifts from Dr. David Rowe (University of Connecticut, Farmington, CT).

Nuclear Runoff Transcription Assay

Nuclei were isolated from the normal and modulated cells, and the transcription reaction was done by incubating freshly prepared nuclei for 5, 10, 15, and 30 min at 30°C in a 200-μl reaction mixture containing 10 mM Tris HCl, pH 7.5, 3.6 mM MnCl2, 200 mM (NH4)2SO4, 3.6 mM dithiothreitol, 0.6 mM each
of adenosine, guanosine, and cytidine triphosphates, and 100 μCi of (α-32P)uridine triphosphate (700 Ci/ mmol; ICN, NEN, Boston, MA). The 32P-labeled RNA was freed of DNA and protein, precipitated with ethanol, and finally resuspended in sterile water. For filter hybridization, plasmids were linearized by boiling, and the DNA was denatured by incubation with 0.5 M NaOH for 20 min at 22°C, followed by neutralization with five volumes of fifteen times concentrated SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The DNA was immobilized on nitrocellulose filters, prehybridized, and hybridized with 32P-labeled RNA at 42°C for 2 days. To establish the optimal incubation time for each cell culture, the reaction mixture from 5-, 10-, 15-, 20-, or 30-min incubations was loaded on HAFT filter (Millipore, Bedford, MA), and the RNA was precipitated with cold 10% trichloroacetic acid followed by extensive washing. Then the filters were quantified as to their radioactivity. In a given experiment, the same amount of radioactivity was used for all hybridizations; these were done in duplicate. After hybridization, the filters were washed twice for 30 min each at 55°C in threefold concentrated SSC containing 0.5% sarkosyl and twice for 30 min each in single-concentrated SSC, also at 55°C.

Results

Collagen mRNA Levels

Our previous studies showed that the amounts of types I and IV collagen RNAs did not reflect the amount of translated proteins in either normal or fully modulated endothelial cells. Since we used rabbit α2(I) cDNA, human α1(I), and human α1(IV) cDNA probes in the previous studies, the efficiency of hybridization of these RNAs might have been different due to the differential degree of homology between mRNAs and cDNAs. To eliminate variation in the experimental conditions, we used recently cloned rabbit cDNA probes to confirm the previous findings. Figure 1 shows the profile of the steady-state levels of collagen RNAs isolated from normal and from fully modulated endothelial cells (FCEC). In these cells, α2(I) RNA was present as characteristic doublets, but the relative amounts of the messages differed greatly, being markedly decreased in the fully modulated cells, in which the lower transcript was more abundant. The two forms of rabbit α2(I) collagen RNA, with nominal sizes of 5.6 and 5.0 kilobases (kb), corresponded closely to the major rat α2(I) mRNAs, and presumably resulted from differential use of polyadenylation attachment sites. However, bovine aortic endothelial cells demonstrated doublets smaller than rabbit and rat α2(I) mRNAs. Hybridization with α2(IV) collagen cDNA revealed a 6.9-kb mRNA as a major transcript, with a minor high molecular weight transcript in the normal cells. Human aortic endothelial cells contain a single α2(IV) RNA species estimated to be 6.4 kb; F9 embryonal carcinoma cells had profiles similar to those of rabbit corneal endothelial α2(IV) transcripts. Expression of α2(IV) RNA was detected in the fully modulated cells, albeit at a very low level. Very similar observations were made using mouse α1(IV) collagen cDNA probe (data not shown; rabbit cDNA probe is not currently available). The mouse α1(IV) clone in normal cells hybridized to a single RNA transcript estimated to be 7.0 kb; the same-size message was barely detectable in the modulated cells. Northern hybridization of the actin cDNA to the total RNAs or poly(A+)RNAs indicated no degradation, as evidenced by the presence of a major band at 2.0 kb (data not shown). Based on these findings, we confirmed our previous results with respect to the apparent presence of α2(I) RNAs in normal endothelial cells and of α2(IV) RNA in the modulated cells, despite the distinctly different steady-state levels.

Stability of Collagen mRNAs

The presence of high steady-state levels of α2(I) RNA in normal endothelial cells, and the apparent presence of α2(IV) RNA as intact molecules in modulated cells (FCEC), raises a question of how (or if) the stability of these transcripts played a role in controlling levels of these mRNAs. The rates of turnover of α2(I) and α2(IV) collagen RNAs were determined by
inhibition of RNA synthesis with 60 μM DRB followed by quantitative northern blot hybridization analysis of these mRNAs as a function of time. Normal endothelial cells contained very stable α2(I) and α2(IV) mRNAs whose half-lives exceeded 24 hr (perhaps one complete cell cycle, Fig. 2), so the cells maintained high levels of α2(I) and α2(IV) mRNAs. However, these messages in the modulated cells decayed with an apparent half-life of approximately 8 hr, suggesting that α2(I) and α2(IV) mRNAs were degraded at an accelerated rate; therefore, a significant reduction in the levels of these mRNAs would be expected in the modulated cells (Fig. 2). In normal cells, α1(I) mRNA decayed with an apparent half-life of approximately 12 hr (data not shown). This compared well with the value of 10 hr in mouse 3T3 cells\(^7\) and agreed also with the findings in skin fibroblasts, in which α2(I) mRNA had an approximate twofold greater half-life than did α1(I) mRNA.\(^2\) The same message in modulated cells had a half-life of 8–12 hr (data not shown), suggesting that α1(I) mRNA maintained an unchanged, albeit short, half-life after modulation, unlike α2(I) mRNA.

Analysis of Collagen Transcription

The nuclear runoff transcription assay was used to measure the level of transcription of collagen genes in these cells. In this assay, nuclei were transcribed in vitro to allow the in vivo-initiated nascent RNA transcripts to elongate in the presence of \(^{32}\)P-uridine triphosphate, and labeled nuclear collagen mRNAs were quantified by slot-blot hybridization, yielding the relative levels of transcription in these cells. We first determined the optimal incubation time for maximum transcription of the isolated nuclei because normal and modulated cells differed greatly in their growth potentials and collagen phenotypes under similar conditions of culture.\(^6\) The monolayer of endothelial cells reached the highest incorporation of \(^{32}\)P-uridine triphosphate into nuclear RNAs at 15 min; the multilayered 7-day-old modulated cells (FCEC) reached highest transcription at 5 min (data not shown). Accordingly, these conditions were used throughout the in vitro transcription experiments. When overall transcriptional rates were determined, normal endothelial cells showed a rate 37% lower than that of the modulated cells (Table 1). The relative rate of collagen gene transcription was deter-

### Table 1. Incorporation of \(^{32}\)P-UTP into nuclear RNAs

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Total nuclei (×10(^5))</th>
<th>Total incorporation (cpm × 10(^5))</th>
<th>Transcriptional rate (cpm/nucleus)</th>
</tr>
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<tbody>
<tr>
<td>Normal cells</td>
<td>7.72</td>
<td>3.3</td>
<td>0.43</td>
</tr>
<tr>
<td>Modulated cells</td>
<td>3.73</td>
<td>2.3</td>
<td>0.50</td>
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Isolated nuclei from monolayer endothelial cells or modulated cells were transcribed in the presence of \(^{32}\)P-UTP, and the \(^{32}\)P-RNAs were precipitated with TCA.
gene transcription was compared among the chains these three collagen chains was slightly higher in normal cDNAs (as an internal standard), and to pBR322 mined by hybridizing 32P-labeled RNA to immobilized cDNAs, to actin cDNAs (as an internal standard), and to pBR322 (for the background hybridization, Table 2). The amount of cloned cDNAs on filters exceeded that required to hybridize all of the mRNAs. Unlike the overall transcriptional rate, the transcription rate of these three collagen chains was slightly higher in normal cells than in the modulated cells, varying from 11-75% greater. When the relative rate of collagen gene transcription was compared among the chains, analyzed, α2(I) transcription was the lowest, and α2(IV) was transcribed at the highest rate in both cells. The transcriptional rate of α2(IV) collagen RNA in the modulated cells did not account for the RNA level, which was very insignificant, thus suggesting that the reduced level of α2(IV) collagen RNA was determined primarily by how fast it was degraded, not by how it was transcribed. The α2(I) collagen RNA in the normal cells, whose relative transcription rate was much lower than that of α2(IV) message, was at a surprisingly high level, due mainly to the presence of the stable transcript, which obviously overcame the low transcriptional rate. The level of α2(I) collagen RNA in the modulated cell also probably was determined by the stability of the message.

Collagen Gene Expression During Early Stages of Modulation

Since the modulation process requires a number of passages (approximately ten) in the presence of corneal endothelium modulation factor (CEMF) released by PMNs, the early stage during modulation was studied to explore the mechanism of initial action of CEMF on collagen gene expression. Our previous studies demonstrated that the corneal endothelial cells, after exposure to PMNs, assumed morphologically altered cell shapes and concomitant induction of type I collagen synthesis. The expression of collagen RNAs was studied after two passages under the following conditions. Control cultures were maintained in DMEM supplemented with 10% fetal calf serum (DMEM-10). Partially modulated cells were maintained in the CEMF-containing DMEM-10. Reverted cells were maintained initially in CEMF-containing DMEM-10 for two passages, during which time mixed cultures of responding and nonresponding cells were established, and CEMF was then removed and the culture maintained for an additional 5 days. The reverted cells assumed the characteristic morphology of subcultured endothelial cells. They were enlarged and attenuated, while the partially modulated cultures contained an admixture of polygonal (nonresponding) and elongated spindle-shaped (modulated) endothelial cells (data not shown). A CEMF, prepared by a four-step purification, was used for this study.17 Northern blot analyses were done under moderately stringent conditions (45°C hybridization; the final wash was done in high-strength SSC at 55°C). The control endothelial cells contained the highest amount of α2(IV) RNA; the partially modulated cells contained the greatest amount of α2(I) RNA. The reverted cells contained the least amount of both collagen messages (Fig. 3). There was a marked difference in the steady-state levels of α2(I) and α2(IV) messages between the fully (Fig. 1) and partially modulated (Fig. 3) cells. Nuclear runoff transcription assays showed that there was selective activation of type I collagen transcription during the early stage of corneal endothelium modulation (Fig. 4). After exposure to the partially purified CEMF, the cells immediately displayed enhanced transcription of type I collagen mRNAs, probably resulting in the high steady-state levels of the messages. However, the high steady-state level of α2(IV) messages was probably due to the combination of relatively high transcription and stability.

Table 2. Incorporation of [32P]uridine into types I and IV collagen nuclear RNA sequences

<table>
<thead>
<tr>
<th>Cell cultures</th>
<th>α1(I)</th>
<th>α2(I)</th>
<th>α2(IV)</th>
<th>α2(I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cells (cpm)</td>
<td>161</td>
<td>61</td>
<td>289</td>
<td>2.64</td>
</tr>
<tr>
<td>Modulated cells (FCEC) (cpm)</td>
<td>92</td>
<td>47</td>
<td>266</td>
<td>1.96</td>
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All hybridization data were corrected for the respective lengths of the cDNA probes. Rat α1(I) cDNA is 1600 bp; rat α2(I) cDNA is 1500 bp; rabbit α2(IV) cDNA is 1400 bp. Due to the instability of rabbit α2(IV) cDNA at present, type I collagen cDNAs were used. Actin cDNAs (gifts from Dr. Bjorn R. Olsen, Harvard Medical School, Boston, MA) served as internal standard and the value of hybridization of pBR322 was subtracted. The RNA that was hybridized was determined by cpm of the filters hybridized to the respective cDNA probes. Arrows indicate 28s rRNA.

Probes Rb α2(I) Rb α2(IV)

Fig. 3. Northern-blot analysis of cellular RNAs (15 μg per lane) isolated from normal endothelial cells (1), partially modulated cells (2), and reverted cells (3). Culture conditions were described in detail in the text, and RNAs were electrophoresed under the conditions described in Figure 1. The blots were hybridized to the respective cDNA probes. Arrows indicate 28s rRNA.
in vitro Transcription

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<tr>
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<th>DNA</th>
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<tbody>
<tr>
<td></td>
<td>rat α1(I)</td>
<td></td>
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<tr>
<td></td>
<td>rat α2(I)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rb α2(IV)</td>
<td></td>
</tr>
<tr>
<td>μg</td>
<td>1</td>
<td>2</td>
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Fig. 4. Nuclear run-off transcriptional analysis of collagen mRNAs of endothelial cells in the presence or absence of corneal endothelium modulation factor (CEMF). Nuclei were transcribed in vitro to allow the in vivo-initiated nascent RNA transcripts to elongate in the presence of 32P-UTP. The DNAs were blotted onto a nitrocellulose filter and hybridized to the respective 32P-labeled nuclear RNAs.

Discussion

The mechanism by which corneal endothelium modulation takes place has been shown to involve a phenotypic switch in collagen gene expression.7-10 Our previous studies demonstrated that the amounts of collagen mRNAs did not account for the amount of translated proteins in endothelial cells, suggesting that there was, at least in part, posttranscriptional control of collagen gene expression in corneal endothelial cells. The steady-state levels of functional messenger RNA species can be regulated at many levels in eukaryotic cells, among which the changes in rate of transcription, or changes in the stability of the transcripts, account for much of the substantial change in the steady-state level of mRNAs. When the transcription was determined, α2(I) collagen transcription was found to be lower, and α2(IV) collagen was transcribed at a higher rate. Both messages were very stable when half-life was analyzed. Obviously, the mechanisms responsible for type I collagen expression cannot be identical to those responsible for type IV collagen expression. A differential regulation of interstitial (I and III) and basement membrane (IV) collagen gene expression has been shown to be mediated by glucocorticoid in developing rat intestine.11 There is a decreased rate of synthesis of types I and III collagen mRNAs and an increased rate of synthesis of type IV collagen mRNAs after dexamethasone treatment. The relatively high transcriptional rate and the stability of α2(IV) collagen RNA result in a high steady-state level of the message; the surprisingly high level of α2(I) collagen RNA was due mainly to the presence of the stable transcript, which obviously overcomes the low transcriptional rate in corneal endothelial cells. However, the modulated endothelial cells (FCEC) that contain low steady-state α2(I) and α2(IV) collagen mRNA levels had slightly reduced transcriptional activities of these collagen genes when compared with those of normal endothelial cells, and a markedly increased turnover rate of the mRNAs. In this case, the reduced levels of these collagen mRNAs were determined primarily by how fast they were degraded, not by how fast they were transcribed. Thus, corneal endothelium modulation was associated with rapid turnover rates of these collagen mRNAs.

To understand how collagen gene expression is regulated during corneal endothelium modulation that leads to production of a RCFM (which can be classified as a collagen disorder), it is necessary to understand how collagen gene expression is regulated in normal endothelial cells in which two unique features of collagen expression are observed; one stable and abundant collagen mRNA (type IV) is actively translated, while the other equally stable and abundant collagen mRNA (type I) is not translated. The deficiencies in protein synthesis are not responsible for the translational repression of α2(I) collagen mRNA; the same cells use the same system for their physiologic collagens (IV and VIII).6,24 Therefore, it is important to understand the mechanisms for translational regulation of collagen expression in endothelial cells. The regulation of collagen gene expression takes place at many levels, as is the case for other eukaryotic gene products.11-16,25,26 To a first approximation, general transcription control is considered to be mediated by trans-acting DNA-binding proteins that stimulate transcription by binding to the cis-acting sequences. However, as evidenced in our studies, the impact of transcriptional regulation was far less than was the control at the translational level in corneal endothelial cells. Although the mechanisms that regulate the translation of these collagen RNAs are yet to be elucidated, translation and stability of collagen RNAs appear to play important roles in corneal endothelial cells and their modulation. It has recently become apparent that mRNA turnover is important to gene regulation, cell growth, and cell differentiation.27 The importance of the poly(A) tail as a determinant of mRNA stability, either due to the binding of poly(A)
binding protein to poly(A) or due to its intrinsic, structural properties, is currently being studied.

Since the modulation process took a number of passages to reach the stage of irreversible metaplasia in the presence of CEMF, there appeared to be at least two stages of modulation: the reversible (early) stage and the irreversible (FCEC) stage. The fully modulated cells (FCEC) contain very unstable messages, regardless of their translational efficiency. However, during early stages of modulation, there is a differential effect on both transcription rate and the steady-state levels of collagen RNAs. Preferential enhancement of type I collagen RNAs was observed within two passages in the presence of CEMF. This suggests that there are at least temporal transcriptional changes during the early stage of modulation. It appears that type I collagen expression was “turned on” during the initial stage of endothelium modulation while the characteristic endothelial gene expression (types IV and VIII) was retained. After treatment with the CEMF, the transcription of type I collagen genes was active, yielding to translationally active type I collagen RNAs. The mechanism of this action is unknown; furthermore, how CEMF is involved in such a mechanism is yet to be determined.

Key words: collagen gene expression, mRNA stability, corneal endothelial cells, translational regulation, transcriptional regulation

References