Posttranslational Regulation of Type I Collagen in Corneal Endothelial Cells

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Purpose. Type I collagen synthesis in corneal endothelial cells does not correlate with steady state collagen RNA levels; although substantial amounts of α2(I) collagen RNA are present in these cells, type I collagen is not detected. This allowed the authors to investigate the possibility of posttranscriptional control of type I collagen in corneal endothelial cells.

Methods. The α2(I) collagen RNA structures of normal and modulated corneal endothelial cells were analyzed by S1 nuclease protection analysis, whereas the nucleotide sequences were obtained by rapid amplification of cDNA ends technique. In situ hybridization of type I collagen was demonstrated with immunofluorescence; synthesis and degradation of the molecule were analyzed by pulse-chase experiments and then by immunoprecipitation with antiprocollagen I antibody.

Results. The cDNA covering the 5'-untranslated region (UTR) of α2(I) collagen RNA obtained from normal corneal endothelial cells and from modulated corneal endothelial cells that predominantly produce type I collagen demonstrate identical sequences in their 5' untranslated and coding sequences. In both mRNA, the length of the 5'-untranslated segment is 127 nucleotides. There were also two AUG codons; the second AUG codon, which is 17 nucleotides upstream from the translation initiation codon, is conserved, as observed in human and chicken α2(I) mRNA. When the sequence covering the 3'-UTR of corneal endothelial α2(I) mRNA was compared with that of α2(I) mRNA obtained from the modulated cells, there were differences in only two nucleotides. The length of the 3'-untranslated segment of each mRNA is 297 nucleotides up to the consensus polyadenylation recognition site (AAUAAAAUAAA), which both cells use. Immunofluorescent staining of corneal tissue in vivo demonstrated that the corneal endothelium stains with anti-type I collagen antibodies, but there is no staining in the underlying Descemet's membrane. In pulse-chase experiments, the newly synthesized type I procollagen, composed of proα1(I) and proα2(I) chains as determined by V8 protease peptide mapping, reached the highest intracellular level at 45 minutes, after which its detection decreased. Cells chased for 120 minutes demonstrated no trace of type I procollagen in the cell layer; medium fractions showed no detectable type I procollagen during the entire 120-minute chase.

Conclusions. These results suggest that type I collagen is synthesized in corneal endothelial cells and that such undesired expression is regulated at the posttranslational level, perhaps by intracellular degradation. Invest Ophthalmol Vis Sci. 1996;37:11–19.

The corneal endothelium is a delicate monolayer of terminally differentiated cells located in the most posterior portion of the cornea, and it is essential for maintaining corneal transparency. 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 they deposit an abnormal fibrillar extracellular matrix (retrocorneal fibrous membrane [RCFM]) between Descemet's membrane and the endothelium monolayer. The presence of RCFM, which is comprised of irregular fibrils, blocks light transmission, resulting in a loss of vision. Collagen phenotypes have been characterized in experimentally in-
duced RCFMs in rabbit corneas and compared with the collagen phenotypes synthesized by normal rabbit corneal endothelial cells. Normal corneal endothelial cells in culture synthesize predominantly types IV and VIII collagens, whereas type I collagen is the major collagenous protein in RCFM in vivo and is synthesized also by RCFM cells in culture. Thus, corneal endothelial modulation involves phenotypic changes in collagen gene expression.

To elucidate the mechanism of corneal endothelial modulation, we established an in vitro model in which a soluble factor (corneal endothelium modulation factor) released by polymorphonuclear leukocytes modulates type IV-synthesizing corneal endothelial cells to type I-synthesizing cells. These modulated endothelial cells share phenotypic characteristics with the cells that constitute RCFM. The major collagen, type I, forms interstitial extracellular matrices between multiple layers of the modulated cells. The phenotypically modulated corneal endothelial cells contain unstable but highly translatable a2(I) mRNA. Interestingly, the steady state level of a2(I) mRNA in the modulated cells is lower than that in normal corneal endothelial cells. Thus, the amount of collagen RNA does not account for the amount of translated proteins, suggesting that there is posttranscriptional control of type I collagen expression in rabbit corneal endothelial cells.

Several instances have been described in which the amount of type I collagen was less than expected from the corresponding mRNA levels. Furthermore, intrinsic structural properties of mRNA have been known to play important roles in translational efficiency. Recently, analysis has shown that there are two distinct a1(XIV) mRNA with distinct sequences in their 5'-untranslated regions (UTRs). At the same time, the 3'-UTR is known to be specialized in posttranscriptional regulation of mRNA metabolism: The 3'-UTR has been described as a repository for cis-acting sequences that regulate RNA localization, stability, cytoplasmic polyadenylation, and translation.

The intrinsic structure of mRNA has a role in translational regulation. We sought to determine whether there is any difference between the untranslated regions of the corneal endothelial a2(I) mRNA and that of the translated a2(I) mRNA. In this article, we report the nucleotide sequences of the 5'-UTR and the 3'-UTR of a2(I) mRNA of corneal endothelial cells and the comparison of these sequences with the corresponding sequences of a2(I) mRNA obtained from modulated corneal endothelial cells, in which type I collagen is the predominant species. Our finding on the structural identity of both a2(I) mRNA led us to address a unique question concerning whether type I collagen is produced by corneal endothelial cells. Immunofluorescent staining of cornea with anti-type I collagen antibodies and pulse-chase experiments suggests that the molecule is indeed synthesized but not secreted into the extracellular spaces. The regulation of type I collagen expression, therefore, may reside at the level of posttranslational, perhaps by intracellular degradation in corneal endothelial cells.

METHODS

Cell Cultures

Isolation and establishment in culture of primary rabbit corneal endothelial cells were as previously described. Briefly, Descemet's membrane–corneal endothelium complex was treated with 0.2% collagenase and 0.5% hyaluronidase for 90 minutes at 37°C. Cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 µg/ml of gentamicin in a humidified atmosphere of 5.0% CO2 in air. Modulated corneal endothelial cells were established by corneal endothelium modulation factor released by polymorphonuclear leukocytes. This factor has been characterized previously. Animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Collagen Synthesis and Immunoprecipitation

Cells were labeled for 15 minutes with Dulbecco's modified Eagle's medium containing 100 µCi L-[2,3,4,5-3H]proline (101 Ci/mmol (Amersham Life Science, Arlington Heights, IL) and 50 µg/ml ascorbate. Incubation was continued in the presence of 1 mM proline for as long as 120 minutes. Labeling was stopped at 30-, 45-, 60-, 90-, and 120-minute chase by washing the cells with ice-cold phosphate buffered saline (PBS). Proteins from the medium fraction were precipitated with ammonium sulfate to a 45% concentration, followed by dialysis in buffer I (0.15 M NaCl, 0.05 M Tris, pH 7.4, 0.1% Triton-X-100), and the cell layer was homogenized in 1.0 M NaCl, 0.05 M Tris, pH 7.4, and 0.1% Triton-X-100 and was dialyzed against buffer I. Aliquots of the samples were incubated with 5 ml of anti-procollagen I antibody (gift from Dr. Nirmala Sundaraj, University of Pittsburgh, PA) and 50 µl of protein G (Sigma, St. Louis, MO) for 1 hour on ice. The reaction mixtures were centrifuged at 10,000 rpm for 2 minutes at room temperature. After extensive washing with PBS containing proteinase inhibitors (paramethyisulfonyl fluoride and ethylenediaminetetraacetic acid [EDTA]), the pellets were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).
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SDS-PAGE

Polypeptides were electrophoresed under the conditions described by Laemmli, and fluorograms were developed as described previously.

Southern Blot Analysis

DNA was electrophoresed on a 1.2% agarose gel, transferred to a nitrocellulose filter, and hybridized to the α2(I) probe. Rabbit α2(I) was labeled by the random primed DNA labeling method.

S1 Nuclease Protection Analysis

The probe used was 920-base pairs (bp) long and spanned the entire 3'-untranslated region (3'-UTR) of the analysis α2(I) collagen RNA. Double-stranded cDNA was labeled with [α-32P]dATP using Klenow fragment. Total cellular RNA was dissolved in 30 μl of hybridization buffer (80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA) that contained 5000 cpm of radioactive DNA. Samples were heated for 10 minutes at 80°C and then hybridized overnight at 42°C. S1 nuclease reaction was carried out for 30 minutes at 22°C in 300 μl of cold S1 nuclease buffer (30 mM sodium acetate, pH 5.0, 250 mM NaCl, 1 mM ZnCl₂, 5% glycerol). Protected DNA fragments were purified and dissolved in 4 μl of 70% formamide, 10 mM NaOH, 30% glycerol, 0.025% xylene cyanol, and 0.025% bromophenol blue, and then boiled for 5 minutes and subjected to electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

Conditions for Polymerase Chain Reaction

A Perkin-Elmer 9600 (Perkin-Elmer, Norwalk, CT) was used for most of the polymerase chain reactions. First, the template was denatured at 95°C for 2.5 minutes, cooled to 55°C, and then heated to 72°C for 10 minutes in the presence of Taq polymerase. This was followed by 40 cycles of a three-temperature program: 95°C for 50 seconds; 56°C for 1 minute, 25 seconds; and 72°C for 2 minutes, 30 seconds. Finally, a 72°C extension cycle was performed for 10 minutes.

cDNA Cloning and Nucleotide Sequencing

To obtain a cDNA that corresponds to the 5'-most end of α2(I) mRNA, corneal endothelial mRNA was used as a template for 5'-rapid amplification of cDNA ends (RACE). The RACE technique involves reverse transcription, double-stranded cDNA synthesis, and amplification of specific cDNA by polymerase chain reaction (PCR) using a gene-specific primer. To ensure amplification of only the α2(I) cDNA, two gene-specific primers were synthesized from exon 8 (5'-AGTTTGCACAGCTACACCGCCGTCG-3') and exon 5 (5'-ATCTCTACATCTGCCTGGGAGG-3'). The 5'-RACE products were subcloned into pBluescript vector and sequenced with the Sequenase system. To obtain a cDNA that corresponds to the 3' untranslated region of α2(I) mRNA, the cellular and polyA+ RNA of normal and of modulated corneal endothelial cells were used as templates for reverse transcription reaction. The dT₇-adapter primer, 5'-GACTCGAGTTCACTCATTTTTTTTTTTTTTTT-3', and the gene-specific primer, 5'-ATGGCCCAAGCTCTTGCTAACTGA-3' (the last 24mer of the coding sequence in exon 52), were used.

Immunofluorescence

Human corneal tissue was obtained from the Lions Doheny Eye Bank (Los Angeles, CA). Eyes were collected within 15 hours of death and placed immediately in Dextran (Chiron, Irvine, CA) at 4°C. The sections (8 μm) were first overlaid with 10% normal goat serum and incubated in a moist chamber for 40 minutes, followed by incubation for 90 minutes with polyclonal anti-type I collagen antibodies (Chemicon, Temecula, CA) diluted 1:100 in PBS. After washing, the tissues were incubated for 45 minutes with fluorescein isothiocyanate-conjugated secondary antibodies at a 1:20 dilution. As a negative control to confirm the specificity of the anti-type I collagen antibody, bovine cartilage was sectioned and stained and compared with the corneal tissue. Simultaneously, the cartilage sections were stained with anti-type II collagen antibodies. Type II collagen obtained from rabbit cartilage (gift from Dr. Paul Benya, University of Southern California, Los Angeles) was used to make goat anti-type II collagen antibody.

Peptide Mapping by Staphylococcus aureus V8 Protease Digestion

Protein bands separated by electrophoresis and localized by fluorograph were swelled with buffer A (0.125 M Tris, pH 6.8, 0.1% SDS, 1 mM EDTA) and then placed onto 3% stacking gel slots. A 10- or 20-μg amount of the protease in buffer A containing 20% glycerol was overlaid, and electrophoresis was carried out on 10% nonreduced gel.

RESULTS

Sequence Analysis of the 3'-Untranslated Regions of α2(I) Collagen RNA

We demonstrated previously that α2(I) mRNA of rabbit corneal endothelial cells are present as characteristic doublets of 5.6 and 5.0 kilobases (kb). The appearance of these doublets results presumably from differential use of polyadenylation attachment sites. In spite of the abundant steady state levels of α2(I) mRNA in rabbit corneal endothelial cells, type I collagen is not synthesized by these cells in vitro.
FIGURE 1. Identification of polyadenylation signals in \( \alpha_2(1) \) collagen mRNA. Total cellular RNA (5 to 20 \( \mu \)g) was hybridized with a \( ^{32} \)P-labeled double-stranded EcoRI-PstI fragment from pCE499, a plasmid containing cDNA that spans the entire 3'-untranslated region of corneal endothelial \( \alpha_2(1) \) mRNA. Unhybridized nucleic acids were digested with SI nuclease, and the resultant protected DNA fragments were subjected to electrophoresis on a 6% polyacrylamide gel containing 8 M urea. Fragment sizes were determined by comparison of their mobilities with a 125-bp DNA ladder. 1 = 20 \( \mu \)g of cellular RNA from normal corneal endothelial (CE) cells; 2 = 20 \( \mu \)g of cellular RNA from modulated cells. The schematic diagram shows the EcoRI (E)-PstI (P) fragment used as a hybridization probe. (arrows) Potential polyadenylation signals in chicken \( \alpha_2(1) \) mRNA. *The \(^{32} \)P label at the EcoRI site. The 360-, 460-, and 750-bp fragments at the bottom of the diagram are the fragments protected by SI nuclease in normal and in modulated corneal endothelial cells.

mine if the translational repression of \( \alpha_2(1) \) mRNA in corneal endothelial cells is related to alternate polyadenylation sites, SI nuclease protection analyses were performed on normal corneal endothelial cells for comparison with the modulated cells, in which type I collagen is the major collagen species. Normal and modulated corneal endothelial \( \alpha_2(1) \) mRNA were hybridized with end-labeled EcoRI-PstI DNA fragment (920 bp), shown in Figure 1. This probe contains an EcoRI-PstI fragment of the \( \alpha_2(1) \) collagen gene (bases 4188 to 5108 relative to the major transcription start site) that spans all four of the previously identified consensus polyadenylation attachment sites in the chicken \( \alpha_2(1) \) collagen gene (AATAAATAAAA and two AATAAA sites).\(^{20} \) Normal and modulated corneal endothelial \( \alpha_2(1) \) mRNA protect radioactive DNA fragments of approximately 380, 460, and 750 bases. Use of the polyadenylation signal in chicken \( \alpha_2(1) \) mRNA should result in protected fragments of 329, 334, 783, and 806 bases (assuming that polyA+ addition takes place approximately 20 bases downstream from the consensus hexanucleotide signal). Among these protected DNA fragments, we cannot explain the presence of 460-bp fragments in rabbit \( \alpha_2(1) \) mRNA, when compared with the chicken counterparts. Nevertheless, our results show that the corneal endothelial \( \alpha_2(1) \) mRNA is polyadenylated at the same sites as in the modulated cells.

To confirm these findings, the sequences of the 3'-untranslated region of both messages were determined by the RACE procedure. A gene-specific primer (the last 24 nucleotides of the coding sequence in exon 52) was used in the PCR to amplify specifically the cDNA of the 3'-untranslated region of \( \alpha_2(1) \) mRNA. There were two PCR products in both normal and modulated cells: a major band of 410 bp and a minor product of 720 bp. Southern blot analyses of the products demonstrated that the 410-bp and 720-bp bands are \( \alpha_2(1) \) collagen DNA fragments (Fig. 2). As the endothelial cells became modulated, the lower transcript of the \( \alpha_2(1) \) mRNA became predominant, indicating that the lower transcript is active more translationally than is the upper transcript.\(^{21} \) We cloned the 410-bp bands from normal and modulated corneal endothelial cells and then sequenced and compared the clones. Only two nucleotides were different in these two mRNA, suggesting that normal corneal endothelial \( \alpha_2(1) \) mRNA has sequences that are almost identical to those of the translated \( \alpha_2(1) \) mRNA obtained from the modulated endothelial cells (Fig. 3). In both mRNA, the polyA track was added 17 nucleotides after the AAUAAAAA sequence. When we compared the results obtained from the SI nuclease protection assay and the 3'-RACE, the 360-bp fragment of the 3'-UTR obtained from SI nuclease digestion appeared to be the 410-bp cDNA fragment obtained from the 3'-RACE experiment. The 410-bp DNA fragment is composed of 297 nucleotides in the untranslated region, 18 nucleotides from primer to TGA codon in coding sequences, 17 nucleotides between polyA+ attachment site and the polyA track, and 80 adenylate residues. The 360-bp fragment is composed of 297 nucleotides of the 3'-UTR, 39 nucleotides (EcoRI to TGA) in coding sequences, and 17 (before polyA+ track) nucleotides. When the 3'-untranslated region of rabbit \( \alpha_2(1) \) mRNA was compared with chicken \( \alpha_2(1) \) mRNA, there was poor homology, except for one identical stretch of 22 nucleotides just before the consensus polyadenylation attachment site. If the mRNA uses the more 5'-sited polyadenylation sites as in the modulated cells.

Sequence Analysis of 5'-Untranslated Region of \( \alpha_2(1) \) Collagen RNA

Two sizes (0.3 and 1.0 kb) of PCR product were obtained from normal and the modulated corneal endo-
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FIGURE 2. Use of rapid amplification of cDNA ends with a gene-specific primer from exon 52 of α2(I) collagen gene. Rapid amplification of the 3′ end of α2(I) collagen DNA was described in Methods. (A) Polymerase chain reaction (PCR) products separated on a 1.2% agarose gel. 1 = PCR control, no RNA; 2 = 5 μg of cellular RNA from normal corneal endothelial (CE) cells; 3 = 2 μg of polyA+ RNA from normal corneal endothelial cells; 4 = 5 μg of cellular RNA from modulated corneal endothelial cells; 5 = 2 μg of polyA+ RNA from RNA modulated corneal endothelial cells. (B) Southern blot analysis. DNA was transferred to a nitrocellulose filter and hybridized to the ^32P-labelled α2(I) collagen probe. Hybridization and washing were performed under stringency conditions (65°C and 0.5 X SSC). (C) First nucleic acid of gene-specific primer (24mer): 4202 bases from the major transcription start site. The EcoRI-PstI fragment used for the S1 nuclease reaction is 14 bases longer at the 5′ end than is the gene-specific primer used for 5′-rapid amplification of cDNA ends (4188 bases versus 4202 bases). (arrows) Potential polyadenylation signals in chicken α2(I) mRNA. (arrowheads) Two PCR products of 410-bp and 720-bp that hybridized to the α2(I) cDNA probe.

In Vivo Localization of Type I Collagen in Corneal Endothelium

Because no difference was detected at the level of the mRNA structures, we turned our attention to the protein level, addressing whether type I collagen is synthesized by corneal endothelial cells but is not secreted into the extracellular space because of some unknown mechanism. We failed earlier to detect...
type I collagen in corneal endothelial cells by either immunoblot analysis of cell, medium and extracellular layers, or biosynthetic labeling. Therefore, we used an indirect immunofluorescent technique to determine the location of type I collagen in cornea in vivo. Positive staining for type I collagen was observed in corneal endothelium, whereas the underlying basement membrane (Descemet's membrane) demonstrated no staining (Fig. 5). To confirm the specificity of the antibody, cartilage sections were stained simultaneously with the antibody. Cartilage showed no staining with anti-type I collagen antibodies, but anti-type II collagen antibodies yielded positive staining.

Synthesis of Type I Procollagen

The in situ location of type I collagen suggests that it may be synthesized by corneal endothelial cells. Rapid intracellular degradation has been documented for collagen synthesized by tendon fibroblasts, and other cell lines; therefore, the possibility of intracellular degradation of type I collagen was tested by pulse-chase experiments followed by immunoprecipitation. Corneal endothelial cells were pulse labeled for 15 minutes and then incubated under chase conditions (1 mM proline) for as long as 120 minutes (Fig. 6). Subsequent immunoprecipitation with anti-procollagen I antibodies demonstrated that type I procollagen synthesis reached the highest level after a 45-minute chase, after which synthesis of the molecule decreased significantly. The newly synthesized type I procollagen appears to be composed of a 2:1 ratio of α1(I) and α2(I) chains. Because the synthesis and secretion of type I collagen requires the assembly of two proα1(I) and one proα2(I) chains, type I procollagen synthesized during a 45-minute chase was characterized further by limited proteolysis with Staphylococcus aureus V8 protease to determine whether the molecule is indeed a heteropolymeric type I procollagen (Fig. 6, lanes 6 and 7). The peptide maps of the upper chain, (proα1(I)), and the lower chain, proα2(I), are different, indicating that they are distinct chains. Secretion of the molecule was not detected during the entire 120-minute chase.

FIGURE 4. Nucleotide and amino acid sequences of the 5'-untranslated and coding regions of α2(I) collagen RNA of normal corneal endothelial cells. Chicken α2(I) nucleotide sequences were taken from reference 20 and human sequences from reference 22. Sequences were aligned so that the translation initiation codons were at the same place. AUG codons preceding the initiation codon are underlined; the initiation AUG codon is boxed. Underlined bases correspond to the primers used to synthesize cDNA. E5 = exon 5.

FIGURE 5. Immunofluorescent staining of type I collagen in human cornea (70-year-old man) and in bovine cartilage. Fresh frozen cornea (A) and bovine cartilage (B) were stained with polyclonal anti-type I collagen antibodies. Cartilage also was stained with polyclonal anti-type II collagen antibodies (C). DM = Descemet's membrane; CE = corneal endothelium. Magnification, ×700.
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![Diagram: Separation of cellular and medium \[H\]proline-labeled procollagen I. Corneal endothelial cells were labeled for 15 minutes, and then the incubation was continued in the presence of 1 mM proline for as long as 120 minutes. Cellular and medium samples were prepared as described in Methods and immunoprecipitated with anti-procollagen I antibody. To normalize the conditions, equal proportions of samples were used for immunoprecipitation, and one half of each immunoprecipitate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis.](image)

**FIGURE 6.** Separation of cellular and medium \[H\]proline-labeled procollagen I. Corneal endothelial cells were labeled for 15 minutes, and then the incubation was continued in the presence of 1 mM proline for as long as 120 minutes. Cellular and medium samples were prepared as described in Methods and immunoprecipitated with anti-procollagen I antibody. To normalize the conditions, equal proportions of samples were used for immunoprecipitation, and one half of each immunoprecipitate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The protein bands separated and localized were analyzed for limited prolylolytic cleavage by Staphylococcus aureus V8 protease, as described in Methods. 1 = procollagen marker obtained from keratocytes; 2 = 45-minute chase; 3 = 60-minute chase; 4 = 90-minute chase; 5 = 120-minute chase; 6 = peptide profile of proa2(I) in lane 2; 7 = peptide profile of proa2(I) in lane 2.

**DISCUSSION**

In previous studies, we demonstrated that there are unique features of collagen expression in corneal endothelial cells: The cells contain extremely high levels of stable a2(I) mRNA, even though they direct no synthesis of the protein. These findings suggest that there is, at least in part, translational control of collagen gene expression in these cells. Although the basis for translational regulation remains unknown in some cases, in principle this regulation can occur at the level of translation initiation or during elongation of the polypeptide chain, or it can be caused by intrinsic properties of the untranslated region of the transcript. Chondrocyte a2(I) mRNA has two of these regulatory mechanisms. The message has a severely reduced translation elongation rate, and a2(I) mRNA from chondrocytes in suspension cultures is 120 bases shorter at the 5' end than are those from other cells. Recent analysis has shown that there are two distinct a1(XIV) mRNA with distinct sequences in their 5'-untranslated regions. In other cases, the repression of translation can be explained by the binding of ribonucleoprotein to a specific cis-acting RNA sequence in the 5'-untranslated region of the mRNA. In spite of the production of ribonucleoprotein particles that alter the affinity for the translational machinery, such interaction is mediated through cis-acting elements of mRNA. Thus, it is highly probable that the principal mechanism of translational regulation results from intrinsic structural properties. Interestingly, corneal endothelial cells contain a high level of type I collagen mRNA: a1(I) mRNA reveals that there are neither qualitative nor quantitative differences in normal corneal endothelial cells when compared to modulated corneal endothelial cells (data not published). On the other hand, the steady state level of corneal endothelial a2(I) mRNA is much higher than that of modulated cells. We compared the 5'- and 3'-untranslated regions of a2(I) mRNA obtained from normal corneal endothelial cells with those of a2(I) mRNA that are actively translated. To our surprise, the cDNA covering the 5'-UTR of a2(I) mRNA obtained from normal and from modulated cells had identical sequences in their 5'-untranslated and coding sequences. In both mRNA, the length of the 5'-untranslated segment is 127 nucleotides, which is similar to that of chicken mRNA (133 nucleotides). There are two more AUG codons upstream to the translation initiation codon, one of which is conserved, as observed in chicken and in human a2(I) mRNA. Similarly, there are only two nucleotide differences in the 3'-untranslated region of these two messages, which is an acceptable range of PCR mistake caused by Taq polymerase. Furthermore, these differences appear not to be significant because there are vast differences in the sequence of this region of 3'-UTR between chicken and rabbit endothelial a2(I) mRNA. The difference may be derived from cloning artifacts. The length of the 3'-UTR is 297 nucleotides up to the consensus polyadenylation recognition sites (AAUAAAUTAAA), which both cells use. In both mRNA, 17 nucleotides were added to the polyA track after the AAUAAAUTAAA sequences. We eliminated the possibility of translational regulation, as well as transcripational regulation, of type I collagen expression in corneal endothelial cells.

Posttranslational regulation is an obvious choice to explain this unique feature of type I collagen expression. Because tissue culture systems do not always show phenotypes identical to those observed in vivo, the presence of type I collagen was determined by immunofluorescent techniques. Anti-type I collagen antibodies, which did not stain cartilage, did stain corneal endothelium but did not stain Descemet's membrane. The intracellular presence of type I collagen suggests that synthesis of this molecule takes place in corneal endothelial cells. However, the absence of type I collagen in Descemet's membrane indicates that the molecule is not secreted into the extracellular space. Further evidence was obtained from pulse-chase experiments followed by immunoprecipitation and peptide mapping: the identification of heteropolymers of type I procollagen in Descemet's membrane was ensured by immunoprecipitation of the newly synthesized proteins with...
anti-type I procollagen antibody and by distinct proteolytic peptide mapping analysis derived from each heteropolymERIC molecule. Synthesis of radioactively labeled type I procollagen reached peak levels after a 45-minute chase, after which synthesis of the molecule decreased. After a 120-minute chase, the cells demonstrated complete absence of the newly synthesized intracellular molecule; secretion of the molecule into the medium was not detected during the entire 120-minute chase, suggesting that the newly synthesized type I procollagen may undergo intracellular degradation. In our previous studies,10 we proposed the absence of type I procollagen synthesis in cell-free translation products directed by RNA from corneal endothelial cells. We also stated that the identification of each translated chain was not proven definitively. Because of the current findings, it is logical to assume that the band identified as proala(I) is the proala(I) chain and that the band immediately below is the proala(I) chain.10

Intracellular degradation of newly synthesized collagen has been described in a variety of collagen-producing cells and tissues23–26; 10% to 20% of all newly synthesized collagen is degraded by the same cells that are actively involved in its synthesis, and this degradation occurs before secretion. Although it is unclear why type I collagen is synthesized if it is destined for intracellular degradation, corneal endothelial cells maintain tight control over their physiologic phenotypes when exposed to various modulating factors.8 On the other hand, when modulation does occur in corneal endothelial cells, such as under the conditions that accompany inflammation, wounding, and subsequent atypical repair, type I collagen becomes the major collagen type and leads to production of an abnormal fibrillar matrix in the basement membrane.I4

The presence of this abnormal tissue blocks light transmission and can lead to blindness. To our surprise, in developing chick cornea, corneal endothelium secretes types I and II collagen during the formation of the primary corneal stroma and for several days after the invasion of fibroblasts, suggesting that type I collagen synthesis is a normal process in corneal endothelial cells at specific times during development.35 However, the same molecule is potentially hazardous in fully developed corneal endothelium. Therefore, type I procollagen must undergo intracellular degradation under physiological conditions in fully developed corneal endothelium. Recently discovered chaperone proteins have been proposed to perform numerous functions, which include stabilizing newly synthesized or unfolding polypeptides, facilitating translocation of nascent chains across the membrane, mediating assembly or disassembly of multimeric protein complexes, and targeting proteins for degradation within lysosomes.36,37 Among molecular chaperones, it is known that Hsp 47, an endoplasmic reticulum resident protein, is an important chaperone for type I procollagen synthesis.36,37 It is highly probable that Hsp 47 (or other chaperones with specificity) is involved not only in type I collagen synthesis but in degradation of corneal endothelial cells. Alternatively, ubiquitin may be involved in this selective degradation of type I collagen in corneal endothelial cells.38,39 Ubiquitin system has been shown to be the ATP-dependent major pathway for selective protein degradation in eukaryotes. Although it is unknown by which mechanism type I procollagen is degraded in these cells, these types of regulation suggest that our body uses multiple regulatory mechanisms to maintain the delicate balance of individual molecules and their roles.

Key Words

corneal endothelial cells, posttranslational regulation, type I collagen, untranslated region

References

10. Kay EP, He YG. Posttranscriptional and transcriptional
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