Extracellular Matrix Gene Alternative Splicing by Trabecular Meshwork Cells in Response to Mechanical Stretching

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PURPOSE. Elevated intraocular pressure (IOP), sensed as mechanical stretching by trabecular meshwork (TM) cells, triggers extracellular matrix (ECM) remodeling. In addition to changes in gene expression, alternative mRNA splicing may alter ECM protein isoforms. Changes in mRNA expression and alternative splicing of four ECM molecules in response to mechanical stretching of TM cells were investigated.

METHODS. Porcine TM cells were mechanically stretched for 12, 24, or 48 hours. RNA was isolated, and RT-PCR was performed with primers that flanked alternatively spliced domains. PCR products were identified by DNA sequencing. Quantitative RT-PCR (qRT-PCR) was performed with primers positioned within nonspliced and spliced regions of the genes.

RESULTS. Total levels of tenasin C, collagen type XII, and CD44 mRNA were increased, whereas versican mRNA levels were decreased in response to the mechanical stretch. In addition, each of these genes expressed alternate mRNA isoforms. Transcripts containing the fibronectin type III domain D of tenasin C, the long NC3 isoform of collagen type XII, the V1 isoform of versican, and exons v7 and v8 of CD44 all increased in response to mechanical stretching. A novel isoform of collagen type XII was observed that resulted in deletion of two exons, a frameshift, and a premature stop codon. This isoform was expressed only by stretched TM cells.

CONCLUSIONS. These alternative splicing events led to the modulation of potential GAG attachment sites and other ECM-binding motifs. These changes should affect TM cell-ECM and/or protein–protein interactions during the ECM remodeling that occurs coincident with homeostatic restoration of IOP to normal. (Invest Ophthalmol Vis Sci. 2007;48:1164–1172) DOI:10.1167/iovs.06-0875

The trabecular meshwork (TM) is a specialized tissue in the anterior portion of the eye that is composed of a series of fenestrated sheets and beams through which aqueous humor flows.1 The bulk of this filtering corneoscleral meshwork is composed of fibrillar-like lamellae covered by basement membrane.2,3 Highly differentiated, endothelial TM cells cover the outer surface of the beams and are responsible for the synthesis of extracellular matrix (ECM) components that form the structural basis of each beam. Proteoglycans are important ECM components composed of a peptide backbone to which glycosaminoglycan (GAG) side chains are covalently attached.4 GAGs are polysaccharide chains with repeating disaccharide units and include heparan, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and hyaluronic acid (HA).5,6 Because they include carboxyl and sulfate groups, GAGs are negatively charged at physiological pH. Other ECM components of trabecular beams include collagens, elastic fibers, cell-surface receptors and other glycoproteins. The structural organization of the ECM is complex with interactions existing between many of the components. A highly organized meshwork is created to which TM cells are attached and from which they respond.

Aqueous humor flows between the TM beams and drains into Schlemm’s canal at a rate of approximately 2.75 μL/min.6 Intraocular pressure (IOP) within the eye is primarily regulated by the resistance to aqueous outflow. The source of resistance is unclear, but the GAGs and/or other ECM components within the deepest portion of the TM, the juxtacanalicular region adjacent to Schlemm’s canal, may be involved. When IOP elevations occur, the ECM and cells of the juxtacanalicular region become mechanically stretched. TM and Schlemm’s lining cells probably sense this stretching via integrins and other cell surface receptors and modulate synthesis of a large number of extracellular and intracellular genes in response.7,8 Concomitantly, proteolytic enzyme activities are increased, such as matrix metalloproteinases-2 and -14, and levels of their inhibitors are decreased (e.g., TIMP-2).9 Normal IOP may be restored by selective ECM turnover controlled by these cells. Thus, mechanical stretching of TM cells may serve as a sensing mechanism for increased IOP, a signal that triggers ECM remodeling and a cue to activate synthesis of replacement matrix components.

Since mechanical stretching of TM cells initiates synthesis of new ECM components, we hypothesize that the replacement molecules may not be identical with those that they replace. TM cells may modulate protein diversity within the ECM by alternative mRNA splicing. This is a complex mechanism whereby different mRNAs are generated from a single gene. These in turn encode functionally diverse protein isoforms.10,11 Expression of splice variants is regulated developmentally, spatially, and/or in response to external stimuli. We have shown that mechanical stretching of TM cells affects alternative mRNA splicing of the IIICS region of the fibronectin gene.12 In the present study, alternative splicing of other extracellular genes, namely tenasin C, collagen type XII, versican, and CD44, is investigated. Some of the splice variants normally expressed by porcine TM cells in culture are reported, as well as changes that occur in response to a mechanical stretch. The potential impact of these modifications on GAG binding, protein–protein and cell–ECM interactions is evaluated, and the possible effects on outflow resistance in IOP homeostasis are discussed.
MATERIALS AND METHODS

Porcine eyes were acquired from Carlton Packing (Carlton, OR); Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin-amphotericin B, reverse transcriptase (RT; Superscript III), and a dsDNA quantitation kit (PicoGreen) were obtained from Invitrogen (Carlsbad, CA); fetal bovine serum from HyClone (Logan, UT); Falcon cell culture inserts (PET track-etched 3-μm pore membranes in six-well format) from BD Biosciences (Franklin Lakes, NJ); an RNA extraction kit (Cells-to-cDNA II) and DNase I (RNase-free) from Ambion (Austin, TX); a SYBR green qPCR kit (Finnzymes DynAmo HS) from New England Biolabs (Ipswich, MA); and a real-time PCR detector (Chromo4) and a thermal cycler (DNA engine) from Bio-Rad Laboratories (Hercules, CA).

Cell Culture

The trabecular meshwork was dissected from porcine eyes, and TM cells were cultured as previously described.7,8,12,13 By passages 3 to 5, cells were plated at 90% confluence onto the 25-mm diameter membranes of cell culture inserts placed in six-well plates.7,9,14 After a further 3 to 5 days, cells were placed in serum-free medium for 24 hours. The cells plated on the insert membranes were mechanically stretched over a glass bead head for 12, 24, and 48 hours, as described previously.7,8 Briefly, a 5.25-mm glass bead was placed in the dish, beneath the insert membrane, and a weight was applied to the lid of the plate. This forced the insert lip downward, causing an upward distortion and stretching of the insert membrane, which produced mechanical stretching of the cells and their ECMs.

mRNA and RT-PCR

Total RNA was isolated (Cells-to-cDNA II kit; Ambion). Briefly, the cells were washed in ice-cold phosphate-buffered saline (PBS) and then lysed with 100 μL of cell lysis buffer. After the cells were heated to 75°C for 10 minutes to destroy RNAses, genomic DNA was degraded by addition of DNase I for 15 minutes at 37°C. Finally, the DNase I was inactivated by heating to 75°C for 5 minutes. Five microliters of cell lysate containing the RNA was then reverse-transcribed to cDNA in a 20-μL reaction of reverse transcriptase (Superscript III; Invitrogen), according to the manufacturer’s instructions. For RT-PCR experiments, 3 to 5 μL of cDNA was used per PCR reaction. Positions of primer sets are denoted in each figure. The identity of amplified bands was confirmed by DNA sequencing (DNA sequencing core facility; VA Hospital, Portland, OR).

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was used to investigate changes in total mRNA expression and to quantify changes in proportions of specific splice variants. Positions of primer sets are denoted in each figure. Briefly, 2 μL of cDNA was used in a 20 μL reaction with Sybr Green qPCR mix (DynAmo HS; New England Biolabs). Products were amplified on a thermal cycler (DNA Engine; Bio-Rad) equipped with a detector (Chromo4; Bio-Rad), in 28 to 40 cycles (94°C for 30 seconds, 50°C to 60°C [primer dependent] for 30 seconds, and 72°C for 30 seconds). Fluorescence measurements were read after the completion of each cycle, and a melting curve was generated immediately after the final amplification cycle. Where multiple peaks were observed, fluorescence was acquired at a temperature higher than that of the non-specific peak melting temperature, so that data from non-specific peaks was eliminated. After qRT-PCR, products were analyzed on agarose gels to verify band sizes and purity. In parallel reactions, actin and GAPDH primers were used to ensure that stretched and nonstretched samples were loaded equivalently. DNA analysis (PicoGreen; Invitrogen) of some samples was used to estimate cell density of stretched and nonstretched cells.

Fluorescence data were analyzed (Opticon Monitor 2 software; Bio-Rad) with a seven-point dilution standard curve. A baseline was subtracted by using the average-over-cycle range method (2–20 cycles) and the threshold was adjusted to give $r^2 = 0.95–1.0$ (as close to 1.0 as possible). Relative template concentrations of each unknown sample were determined in triplicate, averaged, and plotted as relative fluorescence units (RFUs). For the investigation of changes in mRNA levels of stretched cells, RFUs were expressed as change multiples (stretched/nonstretched = x-fold change). Mean results (±SEM) were recorded and are shown along with the exact number of replicates in each figure legend. Changes over 1.5- and under 0.5-fold were considered significant.7 Relative amounts of spliced domains were calculated in an identical manner. In some instances, when mechanical stretching was found to affect total mRNA expression significantly, the change in the spliced domain was divided by total mRNA expression to give a normalized value.

**Figure 1.** (A) One of the six chains of tenascin C, which is composed of repeating EGF and fibronectin type III domains. Up to nine additional type III repeats (A1, A2, A3, A4, B, ad1, ad2, C, and D) can be included between domains 5 and 6 by alternative splicing. Hexameric assembly occurs by association of the N-terminal ends. (B) By using primers in domains 5 and 6, RT-PCR and ethidium bromide staining of an agarose gel showed the presence of two bands. DNA sequencing determined that the major isoforms of tenascin C expressed in pig TM cells were FnIII 5-D4 and FnIII 5 to 6. These variants were expressed in approximately equal proportions. (C) Other isoforms were also detected when primers were placed between FnIII A1 and FnIII B, and FnIII B and FnIII 6. DNA sequencing showed that these were FnIII A1B and FnIII B-D6, respectively. No other variants were detected with these primer sets. Arrows: placement of primers. Left: base pair markers.
RESULTS

Tenascin C

Tenascin C (TnC) is a large, hexameric glycoprotein that is involved in numerous cell functions, including adhesion, migration, developmental pattern formation, and tumor metastasis. Each of its six chains is composed of repeating EGF and fibronectin type III repeat domains (FnIII; Fig. 1A). In humans, alternative splicing leads to the insertion of up to nine extra FnIII domains between constitutively expressed domains FnIII 5 and 6. All combinations of these domains exist, but splicing is highly regulated, and splice forms are expressed in a tissue and cell-specific manner. TnC binds various ECM molecules including fibronectin, syndecan, heparin, and integrins. Analysis of the porcine genome showed sequences of TnC with homology to human FnIII repeats A1, B, C, and D (data not shown). To investigate which TnC isoforms are expressed by cultured TM cells, we designed primers to flank the alternate domains. Two major splice forms were detected by RT-PCR, one that lacked all alternate domains and one that contained domain FnIII D (Fig. 1B). These were expressed in an approximately 1:1 ratio. However, when primers were used between domains FnIII A1 and B or domains FnIII B and 6, other isoforms were also detected. Sequencing of these bands showed that they corresponded to the FnIII A1-B and FnIII B-D-6 isoforms, respectively (Fig. 1C). The FnIII C domain and all other domains with homology to A2, A3, A4, ad1, or ad2 were not detected in porcine TM cells.

To investigate whether mechanical stretching of TM cells affects splicing of domain FnIII D, primers were designed to amplify this domain (Fig. 2A). Quantitative RT-PCR showed a significant increase of FnIII D at 24 and 48 hours compared with a nonstretched control. However, total TnC mRNA levels were also increased with the use of primers in each exon encoding FnIII 6 (Fig. 2B). Therefore, the increase of FnIII D was normalized for total TnC mRNA at each time point. The resultant graph showed an approximately twofold increase in domain FnIII D at 48 hours when TM cells were stretched (Fig. 2C).

Collagen type XII

Collagen type XII is a homotrimer of α1(XII) chains that are composed of two collagen triple-helical regions flanked by three noncollagenous (NC) domains (Fig. 3A). The NC3 domain is extremely large, comprising approximately 85% of the molecule and is composed of repeating FnIII repeats and von Willebrand Factor A (vWFA) domains. The C-terminal NC1 domain and collagenous region contain a heparin binding site. Analysis of mRNA transcripts revealed four alternate isoforms with long or short variations of the NC3 and NC1 domains (Fig. 4A). The long version of the NC3 domain has an additional heparin-binding site and a potential chondroitin sulfate GAG chain attachment site compared with the short NC3 variant.

To investigate collagen type XII expression in response to mechanical stretching, qRT-PCR was performed with primers situated within the nonscollagenous, collagenous region. There was an approximately 1.5-fold increase in mRNA expression at all three time points (Fig. 3B). To determine which alternative splice forms were expressed by TM cells, we used sets of primers located in the long and short regions of the NC3 and NC1 domains (Fig. 4A). Each primer set contained a common 5′ forward primer paired with different reverse primer. Comparison of RFUs showed that the predominant isoforms expressed by porcine TM cells were the short variants of both the NC3 and NC1 domains (Figs. 4B, 4D). Alternative splicing in response to mechanical stretching was then analyzed. The long NC1 domain was slightly increased at 24 hours, but there was no significant alteration in splicing of the short NC1 isoform (Fig 4E). However, expression of the short NC3 isoform was decreased at 12 hours, whereas the long NC3 isoform showed a more than threefold increase at 24 hours and 48 hours (Fig. 4C). This increase was observed by agarose gel and ethidium bromide staining.

A novel splice form of collagen XII was also identified in mechanically stretched TM cells (Fig. 5). Two exons, 29 and 30, were spliced out in stretched cells over time and by 48 hours, ~50% of transcripts did not contain these two exons. This resulted in a loss of 271 bp and caused a frameshift that resulted in a premature termination codon in exon 31.
Versican

Versican belongs to a family of large, hyaluronan-binding proteoglycans that are termed the hyalectins. It is present in a number of tissues where it interacts with a variety of ECM components and influences cell adhesion, migration, proliferation, and ECM assembly. Versican is composed of four domains (Fig. 6A). Unique N- and C-terminal domains, G1 and G2, flank two central chondroitin sulfate GAG attachment domains: oGAG and βGAG. These central domains are encoded by two large exons (7 and 8) and are alternatively spliced. Four alternate transcripts are expressed: V0, V1, V2, and V3. These transcripts may contain both, either, or neither of the exons (Fig. 6). Consequently, the number of chondroitin sulfate GAG attachment sites varies with each isoform: from 17 to 23 for V0, 12 to 15 for V1, 5 to 8 for V2, and 0 for V3. It has recently been shown that the V1 and V0 isoforms are the predominant isoforms expressed by human TM.

To investigate whether total versican mRNA levels were changed in response to mechanical stretch, we designed primers to amplify a product within a nonspliced region of versican (Fig. 6B). The amount of total versican mRNA expressed by porcine TM cells decreased over the 48-hour time period. This suggests that in response to sustained stretching, TM cells produce less versican. Versican mRNA isoforms expressed in response to mechanical stretch were also investigated. The V1 isoform was present at relatively high levels, whereas the V2 and V0 isoforms were present at much lower levels, and the V3 isoform was not detected. Mechanical stretching increased the abundance of the V1 isoform at 48 hours nearly fourfold (Fig. 6C), but there was not a significant change in the amounts of V2 and V0 variants (Figs. 6D, 6E).

CD44

The CD44 family of cell surface adhesion molecules are transmembrane glycoproteins involved in cell–cell and cell–matrix interactions. All CD44 members are encoded by a single gene but can have an additional 10 exons included (v1–v10) in the juxtamembrane region (Fig. 7A). The amino terminal extracellular domain contains at least three HA-binding motifs. Analysis of CD44 isoforms in human trabecular meshwork cells revealed three isoforms: the CD44s variant, which contains no variable exons; CD44v-1, which contains v3, v8, v9, and v10; and CD44v-III, which contains v8 and v9. Total CD44 mRNA expression was quantitated by using primers placed in exons 4 and 5 (Fig. 7B) and, although not significant according to our criteria, there was a modest increase in expression of CD44 when TM cells were subjected to stretching. Porcine TM cells were found to express variable exons v3, v7, and v8 (data not shown). When TM cells were stretched, there was an increase in the proportion of the v7 and v8 domains (Fig. 7C). This was highest (~3-fold) at 12 hours and decreased to ~1.5-fold by 48 hours.

DISCUSSION

Altered expression of specific ECM genes has long been known to be an adaptive response of various cell types to mechanical stress. Two ECM molecules known to be upregulated in response to mechanical load are TnC and collagen type XII. This response has been shown in stretched fibroblasts in cell culture in vitro, as well as in mechanical load studies of rat ulnae (TnC) and in tooth-movement studies of the periodontal ligament of rats (collagen XII).

Cell culture studies indicate a rapid but reversible change in expression of both molecules, suggesting that this is a direct rather than secondary effect of mechanical stress. Consistent with these observations, we show an increase in mRNA expression of both tenascin C and collagen type XII when TM cells were subjected to mechanical stretching (Figs. 2, 3).

In addition, we found that mechanical stretching caused a significant increase in the amount of domain FnIII D incorporated into TnC mRNA transcripts (Fig. 2A). These results are similar to those of a previous study, in which an increase in FnIII D was observed when neonatal rat ventricular myocytes were subject to mechanical strain. Although the function of domain FnIII D is unknown, several recent studies have elucidated both its molecular roles and a correlation with disease. The amino acid sequence VFDDNFVLIK from human FnIII D was found to promote neurite outgrowth from cerebellar granule neurons in culture via interactions with α7β1 integrin. Also, the FnIII B-D isoform, but not FnIII D-6, promoted neuronal outgrowth via the F3/contactin cell adhesion molecule. We detected low levels of the B-D isoform in pig TM cells (Fig. 1C). Thus, inclusion of domain FnIII D most likely affects TM cell adhesion, potentially via α7β1 integrin and/or F3/contactin. Mutations in domain FnIII D have been found to cause disease. A coding single nucleotide polymorphism (SNP,
44,513 A/T in exon 17; Leu1677Ile) from domain FnIII D was found to associate strongly with adult bronchial asthma in a Japanese population. However, the role of the FnIII D domain in TM cells has not been determined.

The short NC3 and NC1 domains of collagen type XII predominate in normal porcine TM cells (Figs. 4B, 4D). The short NC3 domain is the principal isoform expressed by adult tissues, whereas the long NC3 species is primarily expressed in

**Figure 4.** (A) The long and short isoforms of the NC3 and NC1 domains, along with the positions of primers. The left side of the illustration corresponds to NC3 splicing, and the right side shows NC1 splicing. NC3 splicing: (B) The short NC3 isoform was more abundant than the long form in TM cells when relative levels of each splice variant were compared. (C) When TM cells were mechanically stretched, the short NC3 isoform was initially decreased, whereas there was a large increase in the NC3 long variant over time. An etidium bromide-stained gel shows the increase in the NC3 long isoform in stretched (S) cells, compared with nonstretched, control (C) cells, especially at the 48-hour time point. NC1 splicing: (D) In TM cells, the short NC1 isoform was more abundant than the long NC1 isoform. (E) When TM cells were stretched, there was no significant change in the NC1 splice variants expressed. The data represent the mean of results in four independent experiments (± SEM).

**Figure 5.** A novel collagen type XII splice variant was detected that skipped exons 29 and 30, and 271 bp were spliced out. This deletion caused a frameshift that led to an early termination codon in exon 31 (*). This skipping was only detected in the stretched TM cell samples and became more abundant with time. Arrows: the primer positions. Stretched (S) and nonstretched, control (C) samples.
In response to stretching, it was found that the long NC3 isoform increased in TM cells (Fig. 4C), whereas the short form was initially decreased. The increase of the long NC3 isoform provides additional binding sites for collagen type XII to heparin and chondroitin sulfate that are not found in the short form, which suggests that ECM remodeling by TM cells in response to stretching not only results in expression of an isoform usually reserved for embryonic development, but provides additional binding sites for extracellular ligands.

Concurrent with an increase in the long NC3 domain, a novel alternative spliced form of collagen type XII was observed (Fig. 5). It results in a premature stop codon located in the region that encodes the C-terminal vWFA domain. Of note, this isoform was only detected in stretched TM cells and levels increased over time. This splicing leads to the loss of a hyaluronan binding site (-B-X₇-B-) in the vWFA domain, a thrombospondin type I domain and the C-terminal collagenous region. The latter region contains a cell-binding RGD site and a site that interacts with two small leucine-rich proteoglycans: decorin and fibromodulin. Deletion of the collagenous region eliminates assembly of this truncated molecule into a collagen XII trimer and also abrogates the site responsible for interaction with collagen type I fibrils. It is thought that collagen XII is bound to a fibril via its collagenous domain, whereas the NC3 domains project into the extracellular milieu and mediate interactions between adjacent collagen fibrils, promoting their lateral alignment or stabilizing such an organization. Because this isoform lacks the region necessary to anchor it to a fibril, it is conceivable that truncated NC3 domains may disrupt fibril bundle organization and promote ECM disarray. Also, because it is not anchored to a fibril, the shortened collagen XII chain may be degraded or transported via Schlemm's canal. This could also lead to the removal of other molecules that bind the motifs in the NC3 domain.
Versican binds many other ECM components including hyaluronan, link protein, tenascin C, and fibronectin. It is also the main CS-bearing proteoglycan of TM; and, overall, this type of GAG chain contributes approximately 35% of total TM GAGs. CS chains have a high level of negative charge and, in addition to their structural role, are capable of binding water, CD44, chemokines, cytokines, and growth factors. In agreement with a previous report, the V1 isoform of versican was found to be the most abundant splice variant expressed by cultured TM cells. The low abundance of the V2 isoform and nondetectable V3 isoform may be artificially low due to a downregulation of these isoforms by TM cells in culture. Total versican mRNA levels decreased, and the proportion of the V1 isoform increased over time with mechanical stretching, thereby reducing the total amount of CS chain attachment sites. The reduction of versican and CS chain attachment sites when TM cells are stretched would affect interactions with other ECM components, thereby compromising ECM structural organization and potentially leading to dysregulation of signaling systems in the TM.

Although the consequences of versican splicing in TM are not known, recent studies have revealed the importance of its splicing in vitreous humor. Mutations within the acceptor site preceding exon 8, which encodes the βGAG domain, have been found to cause Wagner syndrome, a hereditary vitreoretinopathy. The intronic mutations cause skipping of the V1 exon and a concomitant increase in the ratio of the V2 and V3 isoforms. This imbalance of isoform ratios causes ultrastructural disorganization within the vitreous and the classic “empty” vitreous cavity.

Previous analysis of CD44 splice variants in human TM cells detected the presence of variable exons v3, v8, v9, and v10. Our results detected exons v3, v7, and v8 in porcine TM cells. We did not detect exons v9 or v10 in porcine cells, which is most likely a species difference but may also be due to the routine FGF treatment of TM cells that was used in their study. Mechanical stretching was found to increase exons v7 and v8 when TM cells were stretched. The inclusion of variant exon v7 into CD44 expands the repertoire of GAGs that are potentially bound, as the v7 domain binds heparin and chondroitin sulfate. In addition, it has been suggested that glycosylation of included variable exons modifies CD44 binding to hyaluronan, which may in turn affect CD44 receptor clustering on the cell surface. Because CD44 is intricately
involved in many aspects of cell function, including growth, survival, and differentiation, it is easy to visualize how the inclusion of variable exons v7 and v8 may modify TM cellular behavior.

The overall physiologic relevance of these changes in mRNA levels and splice variants has not been clearly established. However, it seems relatively clear that the TM and/or Schlemm’s lining cells can sense elevations in IOP and make homeostatic corrections in the outflow resistance.9,50,51 Furthermore, stretching produces responses similar to those produced by IOP elevation.7,9,50,51 Mechanical stretching of TM cells triggers a complex program of ECM remodeling.7,9,14 However, it should be noted that a direct relationship between ECM and outflow resistance has not been demonstrated. The hypothesis that TM cells sense and respond to elevated IOP by remodeling their ECMs to restore outflow resistance is compatible with the observations reported herein. Each of the four molecules that we studied and fibronectin,7 showed changes in alternative splice patterns when TM cells were stretched. The newly expressed variants displayed differences in binding motifs (e.g., the addition/removal of GAG chains and integrin binding sites). Alternative splicing of ECM transcripts is one mechanism by which TM cells can modulate ECM organization and cell-ECM interactions. This could be involved in adjusting the outflow resistance in response to variations in IOP.

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References


