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Peek, R; van Gelderen, BE; Bruinenberg, M; Kijlstra, A

Published in:
Investigative ophthalmology & visual science

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1998

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Molecular Cloning of a New Angiopoietinlike Factor from the Human Cornea

Ron Peek,1 B. Elske van Gelderen,1 Marcel Bruinenberg,2 and Aize Kijlstra1,3

PURPOSE. To isolate tissue-specific gene products that contribute to corneal integrity.

METHODS. A cDNA library was constructed and differentially hybridized. Cornea-specific clones were purified and further characterized.

RESULTS. In this study cornea-specific gene products were isolated by differential cDNA hybridization. In addition to known cornea-specific gene products, a transcript was isolated coding for a protein homologous to the angiopoietins, a recently described family of (anti)angiogenic factors. Subsequently, the full cDNA was sequenced, and the identified open reading frame was named cornea-derived transcript 6 (CDT6). Similar to the angiopoietins, CDT6 contains a hydrophobic NH2-terminal sequence, a coiled-coil domain, and a COOH-terminal fibrinogenlike domain. Expression of CDT6 could be detected only in the cornea and not in several other adult human tissues. Within the cornea, expression of CDT6 is confined to the stromal layer.

CONCLUSIONS. The human cornea shows high-level expression of a gene product homologous to the (anti)angiogenic factors, the angiopoietins. This homology, together with stromal-specific expression, suggests that this factor may contribute to the avascularity of the human cornea. (Invest Ophthalmol Vis Sci. 1998;39:1782-1788)

The human cornea consists of three clearly separated cellular components: the epithelium, the stroma, and the endothelium. The epithelium is a self-renewing tissue consisting of five to seven cell layers that are constantly replaced by proliferation and differentiation of the corneal limbal stem cells. The stroma is formed by fibroblastlike cells, the corneal keratocytes, which are embedded between ordered layers of collagen fibrils surrounded by proteoglycans.1,2 Although the keratocytes produce this stromal layer, which constitutes approximately 90% of the corneal thickness, little is known about keratocyte-specific gene expression and turnover. The inner cellular layer, the corneal endothelium, consists of a single layer of hexagonal cells and is essentially mitotic.3 The human cornea is densely innervated with nerve fibers of sensory origin from the trigeminal nerve.4,5 The most striking properties of the cornea—avascularity, transparency, and light refraction—are shared with the eye lens only.

Detailed information is available about the tissue- and developmental stage-specific expression of the ubiquitous structural proteins in the cornea and in the lens, which are thought to contribute to the optical properties of these tissues. However, relatively little is known about the factors involved in such processes as wound healing, tissue renewal, resistance to neoplasia, osmotic balance, and maintenance of avascularity. As a first step toward the identification of cornea-specific gene products involved in these processes, a cDNA library of human cornea was constructed and differentially hybridized. Putative cornea-specific gene products were obtained and further characterized. From this analysis a new human gene product was identified that showed tissue-specific expression and appeared to be similar to the recently identified family of angiopoietins, which are involved in signaling of vascular morphogenesis and maintenance.6,7

MATERIALS AND METHODS

RNA Isolation

For the human research, the tenets of the Declaration of Helsinki were followed and institutional human experimentation committee approval was granted. Human corneal total RNA was obtained from donor corneas (12-20 hours after death) that had been rejected for transplantation purposes because of suboptimal endothelial cell count. From frozen corneas the adhering sclera and limbus were carefully removed with a razor blade. From the remaining central corneal tissue 10-μm cryosections were prepared. The corneal slices were solubilized in RNaZol B (Cinna/Biotech Laboratories, Houston, TX) and processed according to the manufacturer’s protocol. To remove excess protein, the RNA was further extracted with phenol-chloroform (1:1) and chloroform. RNA was alcohol precipitated and dissolved in water. For the isolation of RNA from the three separate layers of the cornea (12 hours after death), 200 μl RNaZol B was pipetted in to the corneal cup and removed after 1 minute to obtain the endothelial RNA. The epithelial RNA was isolated by scraping off the epithelial layers with a scalpel and isolation with RNaZol B. The remaining stromal fraction was cut into approximately 15 small pieces...
human cornea and cultured skin fibroblasts, produced by reversed transcription of 1 μg total RNA in a volume of 20 μl 50 mM Tris-HCl (pH 8.5), 8 mM MgCl₂, 30 mM KCl, 1 mM dithiothreitol, 20 μCi [α-32P]dCTP (3000Ci/mMol), 1 mM each deoxythymidine triphosphate, deoxyguanosine triphosphate, deoxyadenosine triphosphate, and 5 units avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) at 37°C for 1 hour. Hybridization was performed overnight at 65°C in 6X SSC, 5X Denhardt's solution, and 100 μg/ml sheared denatured herring sperm DNA. After hybridization, filters were washed twice at 65°C in 0.2X SSC-0.1% sodium dodecyl sulfate. Filters were exposed to x-ray film at −70°C overnight. Plaques that hybridized with the corneal cDNA probe only were picked and subjected to a second round of purification.

Reverse Northern Blot Analysis

From the plaques of interest, plasmids were generated using the in vivo excision protocol supplied by the manufacturer. Approximately 5 μg plasmid DNA was (partially) digested with EcoRI and XhoI, size-fractionated by agarose gel electrophoresis, and stained with ethidium-bromide. (A) The blot of this gel was subsequently hybridized with first-strand cDNA probes from total RNA of several human tissues and cells: cornea (B), total retina (C), cultured B cells (D), cultured fibroblasts (E), and cultured human umbilical vein endothelial cells (HUVEC, F).

Figure 1. Reversed northern blot analysis of putative cornea-specific cDNA clones. Cornea-derived transcripts (CDT1-CDT7) and a cDNA clone encoding β-actin were (partially) digested with EcoRI and XhoI, size-fractionated by agarose gel electrophoresis, and stained with ethidium-bromide. (A) The blot of this gel was subsequently hybridized with first-strand cDNA probes from total RNA of several human tissues and cells: cornea (B), total retina (C), cultured B cells (D), cultured fibroblasts (E), and cultured human umbilical vein endothelial cells (HUVEC, F).

followed by RNAzol B extraction. The RNA of other human cadaveric tissues (6-12 hours) was obtained by lysis of the tissue in guanidinium thiocyanate followed by centrifugation in CsCl₂ to purify cellular RNA. RNA from cultured human skin fibroblasts and Epstein-Barr virus-transformed human B cells was isolated by washing the cells with phosphate-buffered saline and solubilization in RNAzol B. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins by collagenase treatment according to a modification of the technique described by Jaffe et al.² RNA from cultured HUVEC was isolated as described for fibroblasts and B cells.

cDNA Library Construction and Differential Plaque Hybridization

Sixty micrograms pooled total RNA isolated from six human corneas was used to construct a cDNA library (Lambda ZAP; Stratagene, La Jolla, CA) with a complexity of approximately 200,000 clones. Plaques were seeded at a density of 70 plaques/cm² and transferred to nitrocellulose membranes using standard techniques. Nitrocellulose replica's were hybridized with radiolabeled first-strand cDNA probes of RNA from

Sequence Analysis

The sequence of cDNA inserts was partially determined manually by the dideoxy method and partially by automatic sequencing. The coding sequence of CDT6 was read from both strands. The sequence from 1 to 58 was obtained by performing a standard 5' rapid amplification of cDNA ends (5'RACE). Briefly, the CDT6 transcript was reversed transcribed using a CDT6-specific primer 5'CCATGCGCTTGCTGTTGCTC3' on corneal RNA. cDNA was tailed with deoxyguanosine triphosphate and amplified by polymerase chain reaction using the C₅₈₀D and a nested CDT6-specific primer 5'GACCCAGTCCC3'. The amplified RACE products were ligated into pGEMTeasy (Promega, Madison, WI) and used to transform Escherichia coli JM109. Transformants were selected by hybridization with a CDT6-specific probe. Four hybridizing clones were sequenced from both strands and contained an identical extra 5' sequence of 58 bp with an ATG in the expected reading frame. The nucleotide sequence data reported in this study will appear in the European Molecular Biology Laboratory (EMBL), GenBank and DNA Data Bank of Japan (DDBJ) Nucleotide Sequence database under the accession number Y16132.
FIGURE 2. Sequences and structure of CDT6. (A) Nucleotide sequence of the CDT6 cDNA and deduced amino acid sequence. The putative polyadenylation signals are underlined, consensus N-glycosylation sites are indicated by asterisks. (B) Alignment of the fibrinogenlike domain of CDT6 with angiopoietins 1 and 2. Double dots indicate identical amino acids, single dots indicate similar amino acids, and dashes indicate a gap inserted to optimize alignment. Conserved cysteines are indicated by triangles. (C) Kyte-Doolittle hydrophobicity analysis of the CDT6 protein. (D) Analysis of the CDT6 protein for coiled-coil structures.

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Computer Analysis

The similarity of the CDT6 nucleotide and deduced amino acid sequences to known sequences were analyzed by performing BLAST searches on EMBL, GenBank, and DDBJ database sequences.10 The search for regions of hydrophobicity,11 and coiled-coil and conserved motifs, such as glycosylation and phosphorylation sites, was performed using the protein analysis software of the Computer Assisted Organic Synthesis-Computer Assisted Molecular Modelling (CAOS-CAMM) center of the University of Nijmegen, The Netherlands.

RESULTS

In search of cornea-specific gene products a cDNA library of total human cornea was constructed and hybridized under high-stringency conditions with first-strand cDNA probes of RNA isolated from total human cornea or from human skin fibroblasts. Plaques were detected that showed hybridization with the corneal cDNA probe but not with the skin fibroblast cDNA probe. Seven of these plaques (cornea-derived transcripts CDT1-CDT7) were selected and purified during a second round of hybridization.

To confirm the corneal-specific expression of CDT1 to CDT7 a technique, referred to as "reverse northern" was used.12 This technique allowed us to analyze the relative level of expression for a number of gene products simultaneously, using a cDNA probe generated from a small amount of total cellular RNA. A Southern blot containing the cloned CDT1-CDT7 cDNA fragments and a control β-actin cDNA fragment was hybridized with first-strand cDNA probes of RNA from cornea and several control cell types. As expected, reverse northern allowed us to detect tissue- or cell type-specific differences in messenger RNA levels (Fig. 1A). Hybridization with a cornea-specific cDNA probe showed strong hybridization to the β-actin control and the cornea-derived transcripts CDT1, CDT3, and CDT5 to CDT7. Hybridization to CDT2 and CDT4 was very weak and detectable only after prolonged exposure (not shown). These two gene products were therefore excluded from further analysis (Fig. 1B). Probes from total human retina (Fig. 1C), human B cells (Fig. 1D), human fibroblasts (Fig. 1E), and human umbilical vein endothelial cells (HUVEC; Fig. 1F) all hybridized strongly to the β-actin control and to CDT5, and the retina probe also hybridized to CDT7. The cDNA clones CDT1, CDT3, and CDT6 hybridized only with the cornea-specific cDNA probe and not with any of the control cDNA probes, even after extreme overexposure (not shown). These results indicate that the clones CDT1, CDT3, and CDT6 code for tissue-specific gene products highly expressed in the human cornea. Sequence analysis revealed that CDT1 coded for keratin K12, a tissue-specific transcript expressed in corneal epithelial cells,13 and CDT3 coded for aldehyde dehydrogenase, an enzyme that is highly overexpressed in the human cornea and is thought to fulfill a structural and an enzymatic role in this tissue.14 15 CDT7 coded for apolipoprotein J, which is known to be highly expressed in the corneal epithelial cells and is suggested to contribute to the nonkeratinizing nature of the epithelial cell layer.16

CDT6 appeared to be a cDNA with an approximate size of 2 kb. A database search to find nucleic acid sequence similarities between CDT6 and known sequences showed that CDT6 was most similar to the mouse angiopoietin-2 mRNA sequence with a 65% homology over a stretch of 395 bp. The highly homologous angiopoietin-2 (Ang2) and angiopoietin 1 (Ang1) are the members of a recently identified family of angiogenic factors essential in the process of recruiting support cells to stabilize the structure of blood vessels.5 7 The CDT6 sequence contained a 3' polyA tail and several putative polyadenylation signals (AAUAAA) in the 3' end. The deduced open reading frame of CDT6 lacked the start codon and therefore a 5' rapid amplification of cDNA ends (5'RACE) was performed on corneal RNA to identify the complete open reading frame. This resulted in an additional 58 bp, which contained a start codon in the expected reading frame. The complete cDNA sequence and deduced 346-amino-acid sequence of CDT6 are shown in Figure 2A. When a database similarity search was performed using the deduced amino acid sequence of CDT6 the best matches were the human and mouse Ang1, closely followed by the human and mouse Ang2. Sequence similarity was 69% over a stretch of 187 amino acids with human Ang1 (between residues 158 and 345) and 65% over a stretch of 188 amino acids (between residues 156 and 345) with human Ang2. The homology of CDT6 with the angiopoietins resides mainly in the COOH-terminal part of the protein, which contains a fibrinogenlike domain. In addition, sequence similarity with the coiled-coil domain of Ang2 was observed, between amino acids 46 and 111 (43% similarity). The amino acid similarity between CDT6 and human Angl-Ang2 in the fibrinogenlike domain is shown in Figure 2B. Five of the six cysteines of the angiopoietins in this domain were conserved in CDT6.

The three domains characteristic of the angiopoietins, an NH2-terminal hydrophobic leader sequence followed by a coiled-coil domain and a COOH-terminal fibrinogenlike domain are also present in CDT6. Whereas the fibrinogenlike
domains of the angiopoietins and CDT6 are of similar length, the coiled-coil domain of the angiopoietins is approximately twice the length of this domain in CDT6 (Fig. 3).

To explore the expression of CDT6 in the adult further, northern blot analysis of several adult human tissues was performed (Figs. 4A, 4B). Although CDT6 was readily detectable as a single band of approximately 2.3 kb in the human cornea, no expression could be detected in pancreas, liver, cerebrum, heart, seminal vesicles, brain cortex, or prostate (Fig. 4A). In addition, the human lens, the other avascular transparent cellular tissue of the human body, was assayed for CDT6 expression. Again, CDT6 expression could only be observed in the cornea but not in the lens cells or HUVEC (Fig. 4B). To determine in which of the three corneal layers CDT6 was expressed, RNA was isolated from corneal epithelium, corneal keratocytes, and corneal endothelium. CDT6 was highly expressed in the stromal layer but not in the epithelium or the endothelium (Fig. 5).

**DISCUSSION**

The integrity and transparency of the human cornea is vital for an undisturbed perception of visual information. Despite this important function of the cornea and its many unique features, only two cornea-specific expressed genes have been described in humans until now. These genes code for the epithelium-specific structural proteins, keratin K3 and keratin K12.

However, no cornea-specific gene products have been cloned from the stromal or endothelial layer that may contribute to features such as corneal avascularity and the low incidence of corneal neoplasia. As a first step toward the identification of such cornea-specific gene products, we constructed a cDNA library of human total cornea and screened this library for tissue-specific gene products. Besides the gene products we expected to find, such as the cornea-specific keratins and the highly expressed aldehyde dehydrogenase, a gene product was isolated that showed a remarkable homology with a recently described family of factors controlling angiogenesis, the angiopoietins. The homology between this cornea-specific gene product, which was named CDT6, and the angiopoietins was apparent on the level of primary amino acid sequence and on the level of protein structure. Similar to the angiopoietins, the CDT6 sequence coded for a COOH-terminal fibrinogen-like domain, a domain that is predicted to have a coiled-coil quaternary structure and an NH2-terminal hydrophobic sequence characteristic of secreted proteins. The two known
Total RNA was isolated from the separate layers of two human corneas. From the epithelial and stromal layer, 15% and 10% of the RNA was used, respectively. From the endothelial cell layer, all RNA was applied to the gel. RNA was blotted to nitrocellulose and hybridized with a labeled cDNA fragment of CDT6 and a 28S ribosomal RNA-specific probe. Note that the 28S ribosomal RNA signals in the stromal RNA are weak because of the small amount of total RNA in these lanes.

Angiopoietins, Ang1 and Ang2, are also secreted proteins and glycosylated. In the CDT6 sequence three putative N-glycosylation consensus sites are present suggesting that this protein is also glycosylated.

Angiopoietin 1 and Ang2 exert their role in angiogenesis by binding to the endothelial cell–selective transmembrane receptor tyrosine kinase Tie2. This receptor controls the capability of endothelial cells of recruiting support cells to encase the endothelial tubes, to stabilize the structure of blood vessels. Although Ang1 and Ang2 bind to the Tie2 receptor, only the binding of Ang1 leads to autophosphorylation of Tie2 and intracellular signal transduction, whereas Ang2 binds and blocks the Tie2 receptor. Angiopoietin 2 is therefore a naturally occurring antagonist of Ang1. The high-level tissue-specific expression of an angiopoietinlike factor such as CDT6 in the human cornea, a tissue normally devoid of blood vessels, suggests that CDT6, similar to Ang2, may play the role of negative regulator of angiogenesis. The expression in only the corneal stromal layer, the site of pathologic angiogenesis, supports this suggestion. However, we cannot fully exclude expression in other tissues, because the reversed northern and standard northern blot analysis would probably not detect low-level expression.

Similar to other angiogenic processes, the induction of corneal capillaries is thought to depend on a balance of positive and negative regulating factors. Under certain pathologic conditions this balance is shifted toward the positive regulating factors, and angiogenesis is induced. Several factors that induce corneal neovascularization have been described, including soluble adhesion molecules such as E-selectin, vascular cell adhesion molecule-1, and vascular endothelial growth factor. Although experimental corneal neovascularization is one of the most commonly used models of angiogenesis, the current knowledge of naturally occurring factors maintaining and restoring avascularity in the cornea is limited. Recently, it was shown that in rat vascular endothelial growth factor is required for the wound- and inflammation-related corneal neovascularization. Although the exact role of the newly isolated gene product CDT6 must be determined, the homology with the angiopoietins and the high tissue-specific expression in the corneal stroma suggest that it may contribute to the avascularity of the human cornea.

Acknowledgments

The authors thank Ger Pruijn and Sander Theunissen for their help with the computer analysis, Hans Westphal and Alec de Vos for helpful discussions, and the cornea bank of The Netherlands Ophthalmic Research Institute and Eurotransplant for providing the human corneas.

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