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Up-regulation of Osteonectin/SPARC in Age-Related Cataractous Human Lens Epithelia

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Purpose: To characterize gene expression patterns between epithelia isolated from cataractous and normal human lenses.

Methods: Reverse transcriptase differential display was used to identify differential expression between cataractous and normal epithelia. RT-PCR was used to compare pooled and individual RNA samples.

Results: One transcript, up-regulated in cataractous as compared to normal epithelia, was identified as osteonectin which is also known as SPARC (secreted acidic protein rich in cysteines). RT-PCR confirmed over-expression of this RNA. High levels of osteonectin mRNA were also detected in six individual epithelia dissected from cataractous lenses.

Conclusions: The present study provides evidence for up-regulation of osteonectin in human age-related cataract and suggests that osteonectin, a protein involved in cell-cycle control, extracellular matrix and Ca++ binding, plays an important role in human lens homeostasis and may be involved in processes leading to lens opacity.

This report provides evidence that expression of osteonectin/SPARC, whose deletion causes age-onset cataract in mice [1], is up-regulated in the cataractous human lens epithelium. The lens epithelium is essential for the growth, differentiation and homeostasis of the lens [2]. It contains the highest enzyme activities in the entire organ [3-6] and is capable of metabolic communication with the underlying fiber cells [7-9]. Damage to the lens epithelium and its enzyme systems is associated with cataract formation [10-14] and the development of lens opacity is associated with altered epithelial cell gene expression and/or protein synthesis. Some examples include: increased synthesis of Na,K-ATPase in response to increased membrane permeability [15]; altered protein synthesis and secretion in response to UV-B irradiation [16]; up-regulation of c-fos during UV-B induced apoptosis [17]; and decreased histone H3 and H4 expression and increased expression of protein kinase C, β-actin and γ-actin expression with UV light treatment [18].

We have previously reported the detection of gene expression changes between epithelia of age-related cataractous and normal human lenses by reverse-transcriptase differential display [19]. In this study, two specific genes, metallothionein II (up-regulated) and protein phosphatase 2a regulatory subunit (down-regulated) were found to be differentially expressed between lens epithelia isolated from cataractous versus normal donors.

In the present report, we have used differential display [20-21] and RT-PCR [22-24] to demonstrate up-regulation of osteonectin in lens epithelia dissected from age-related cataractous versus epithelia dissected from normal human lenses.

Osteonectin, also called SPARC (secreted protein acidic and rich in cysteines), is a 43 kDa glycoprotein-Ca^{++} binding protein which is believed to regulate cell growth through interactions with the extracellular matrix [25]. Endothelial cell injury in vitro is associated with increased secretion of osteonectin [26]. Osteonectin has been demonstrated to inhibit cell spreading on collagen and to induce cell rounding in cultured endothelial cells and fibroblasts [25]. It can modulate the activity of platelet-derived growth factor through a direct interaction [27]. These collective properties of osteonectin (Ca^{++} binding, cell matrix interactions and growth factor interactions) implicate the protein in processes potentially important for lens maintenance and cataract [28-32]. During the course of the work presented here, it was demonstrated that deletion of the osteonectin gene in mice results in age-onset cataract formation and lens disruption, indicating that mutation or deletion of osteonectin can result in cataract formation [1]. Up-regulation of osteonectin in cataractous human lenses suggests that it plays an important role in lens maintenance in this species and that it may be involved in the development of human cataract.

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METHODS

Isolation of RNA and Dissection of Lens Epithelia— Normal lenses and age-related cataractous lenses were dissected and the contaminating fiber cells were removed from the epithelia as previously described [33]. All epithelia were washed and examined to eliminate the possibility of blood contamination as previously conducted [34]. Cataractous epithelia were extracted and characterized by the same surgeon. For reverse transcriptase-PCR differential display, RNA samples were treated with RNase free DNaseI to remove possible DNA contamination [35]. Total RNA was prepared from pooled epithelia by phenol/guanidine isothiocyanate extraction as described [35]. Poly-adenylated RNA was isolated from individual epithelia by oligo dT chromatography using the Micro-fast track system (Invitrogen, Carlsbad, CA) as specified by the manufacturer.

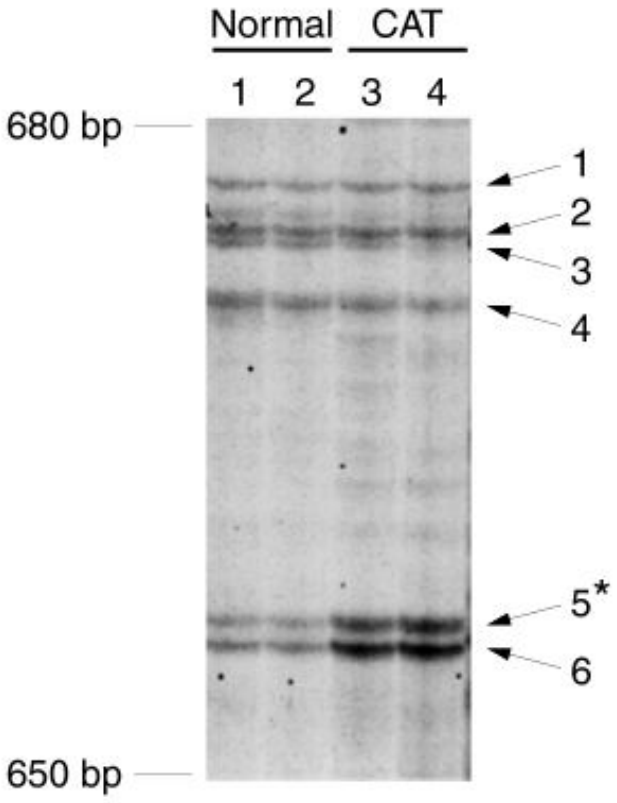
Reverse Transcriptase-Differential Display— Reverse transcriptase-differential display was performed by modification of the procedure originally described by Liang and Pardee [20-21].

First-strand cDNA Synthesis— RNA samples from 50 cataractous and 25 normal epithelia (200 ng) were reverse transcribed using 0.2 µM of an anchored primer (AP1) with sequence: 5'-**ACGACTCACTATAGGGC**TTTTTTTTTTT-TAA-3' (Genomyx, Foster City, CA) containing the T7 promoter sequence (bold), a T_{12} anchoring sequence and two 3' anchoring bases. First-strand synthesis was performed by incubation at: 25 °C x 10 min/42 °C x 60 min/70 °C x 15 min in the presence of 25 µM dNTPs, 10 mM DTT, 20 units of RNasin (Promega, Madison, WI) and 40 units of SuperScript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) in a volume of 20 µl reverse transcription buffer (50 mM Tris, pH 8.3, 6 mM $MgCl_2$, 100 mM KCl).

Amplification of double-stranded cDNA fragments— Double-stranded cDNAs were amplified in duplicate by the polymerase chain reaction (PCR) with the anchored first strand synthesis primer (0.2 µM) (5'-**ACGACTCACTATAGGG-C**TTTTTTTTTTTTAA-3') and an arbitrary annealing primer (AR1) (0.2 µM) of sequence 5'-**ACAATTTCACACAGG-A**CGACTCCAAG-3' containing the M13 (-48) reverse-primer sequence (bold). PCR was performed with 1 unit of AmpliTaq (Perkin Elmer) in the presence of 2.5 µCi [α-P^{33}]dATP (1000-3000 Ci/mmole) (New England Nuclear/DuPont, Boston, MA), 1.5 mM $MgCl_2$, 100 µM dNTPs in a reaction volume of 20 µl. PCR cycles were: 1 cycle of 95 °C x 2 min; 4 cycles of 92 °C x 15 s/46 °C x 30 s/72 °C x 2 min; 25 cycles of 92 °C x 15 s/60 °C x 30 s/72 °C x 2 min; one cycle of 72 °C x 7 min Following amplification, [α-P^{33}]-labeled cDNA fragments were separated by electrophoresis on a 4.5% polyacrylamide-8 M urea gel run on a Genomyx (Foster City, CA) sequencing apparatus and visualized by autoradiography on X-Omat film (Kodak).

Reamplification of Differentially Displayed Bands— A band of differing intensity between the cataract and the normal samples was excised from the gel. The resulting gel slice was directly subjected to PCR. cDNAs were bidirectionally amplified with 0.2 µM each full-length T7-promoter (5'-GTAATACGACTCACTATAGGGC-3') and M13-reverse (-48) sequencing primers (5'-AGCGGATAACAATTTC-ACACAGGA-3'). The PCR conditions and cycles used in this procedure were identical to those described for amplification of double-stranded cDNA fragments except that [α-P^{33}]dATP was omitted from the reaction mixture and annealing for the first four cycles was conducted at 50 °C.

Sequence analysis of the differentially displayed cDNA— The re-amplified differential display band was purified by Wizard PCR-preps (Promega, Madison, WI) as specified by the manufacturer and analyzed by electrophoresis on 1.0% agarose gels. The product was cloned into the TOPO TA Cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer and sequenced by fluorescent dye terminator cycle sequencing (Perkin Elmer-Applied Biosystems, Warrington WA1, Great Britain) as specified by the manufacturer using a primer (5'-GCCAGCTATTTAGGTGAC ACTATA-3') complementary to the TOPO TA vector SP6 sequence. Reactions were run and sequences analyzed on a model 377 Applied Biosystems sequencer using Applied



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CTCCAAGCATTTCATGAAAAAGCTGCTTCTTATTAATCATACAAACTCTCACC
                                                    -
CTCCAAGCATTTCATGAAAAAGCTGCTTCTTATTAATCATACAAACTCTCACC


ATGATGTGAAGAGTTTCACAAATCCTTCAAAATAAAAAGTAATGACTT
                                 
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Figure 1. Identification of osteonectin as differentially displayed between cataractous and normal human lenses. **(A)** Autoradiogram of the reverse transcriptase-PCR differential display gel using duplicate RNA samples isolated from cataractous (CAT, lanes 3 and 4) and normal (lanes 1 and 2) lens epithelia. Arrows indicate displayed bands (numbered) in the cataract and the normal samples. The differentially displayed band (number 5) that was further analyzed is marked with an asterisk. **(B)** Sequence alignment of differentially displayed band 5 with human osteonectin. The upper sequence is that determined for differential display band 5; the lower sequence is that reported for osteonectin. Numbering is from the start of translation. A one base-pair mismatch between the sequence of differential display band 5 and the reported sequence for osteonectin is shown in red.

Biosystems sequencing software (Applied Biosystems, San Francisco, CA). Sequences were analyzed using BLAST software (National Library of Medicine) and GenBank data.

Reverse Transcriptase-PCR— RT-PCR was performed by modification of established procedures [20]. Indicated amounts of RNAs were examined using the One Step RT-PCR system (Gibco-BRL, Gaithersburg, MD). The primer concentration used in these studies (200 nM) was chosen to insure that the amount of primers used in the reactions would not be limiting and was determined by performing control reactions at different primer concentrations (data not shown). Control reactions employed primers specific for αB-crystallin: (sense) (5'GCTGTCACCGCAGCCCCCAAGAAATAGAT-3') and (antisense) (5'-ATGGACATCGCCATCCACCACCCTGGAT-3'). Where indicated GAPDH (glyceraldehyde phosphate dehydrogenase) control reactions were also performed. The sequences of the GAPDH primers were: (sense) (5'-CCACCCATGGCAAATTCCATGGCA-3') and (antisense) (5'-CCACCTGGACTGGACGGCAGATCT-3'). PCR-cycling parameters (30-35 cycles) were chosen to ensure linear product formation over the amounts of RNA and other reagents indicated. αB-crystallin and GAPDH formation was determined to be linear for up to 40 PCR cycles under these conditions. Controls lacking reverse transcriptase (-RT) were performed under identical conditions with heat-inactivated RT. The sequences of the osteonectin primers were: (sense) (5'-CCTGAGGCTGTAACTGAGAGAAAG-3') and (antisense) (5'-GTGGGAGGGGAAACAAGAAGATAA-3'). Products were separated on 1.0% agarose gels and visualized by ethidium bromide staining. Reaction products were sequenced to ensure they were authentic.

RESULTS

Reverse Transcriptase-Differential Display and Identification of Osteonectin— Reverse transcriptase differential display was performed with duplicate RNA samples. Fifty pooled cataractous and 25 pooled normal epithelia were analyzed by differential display as described in Methods. The average age of the cataractous donors was 71 years. Forty-six percent of the donors were male. The average age and sex of the cataractous donors agreed well with that of the normal donors which averaged 68 years in age and were 44% male. The breakdown of the cataractous lenses was 36 mixed cataracts, 10 nuclear cataracts, 3 cortical cataracts and 1 posterior subcapsular cataract.

The differential display profile from approximately 650 bp to 680 bp is shown in Figure 1A. Indicated are six major bands (numbered) which were detected in both the cataractous and the normal RNA samples. Bands 1-4 were detected at approximately equal levels between the cataract and the normal samples while two bands, bands (5 and 6) were detected at much higher levels in the cataract as compared to the normal samples. Band 5, described in this study, was re-amplified as described in Methods and identified to contain human osteonectin. Its sequence aligned with the reported sequence [36] is shown in Figure 1B. It is identical to bps +1995 to +2095 (from the translation start site) of the reported 3' untranslated sequence for osteonectin mRNA [36] with the exception of a single indicated mismatch. This disparity from the reported sequence is most likely the result of misincorporation by TAQ polymerase since separately obtained osteonectin PCR products derived from other human RNA samples were 100% identical with the reported sequence (data not shown).

Confirmation of Osteonectin Over-expression Between Pooled Cataractous and Pooled Normal Epithelia— Over-expression of osteonectin was confirmed by RT-PCR with gene-specific primers. Different pooled RNAs were used for this procedure than were used in the original differential display procedure. The RNAs used for RT-PCR confirmation were prepared from eight normal epithelia from average age 54 year old donors and 19 cataractous epithelia from average age 71 year old donors. The breakdown of the cataractous lenses was 4 nuclear cataracts, 13 mixed cataracts, 1 cortical cataract and 1 posterior subcapsular cataract. RT-PCR was chosen to

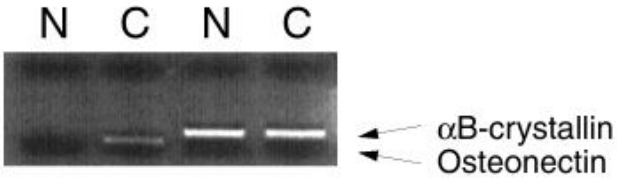

Figure 2. Confirmation of osteonectin over-expression between RNAs isolated from cataractous (C) and normal (N) samples. Ethidium bromide stained gel showing the levels of osteonectin in 50 ng of pooled normal RNA (lane 1) and 50 ng of pooled cataract RNA (lane 2) measured by RT-PCR after 30 PCR cycles. Shown as control are the levels of αB-crystallin in the normal (lane 3) and cataract (lane 4) samples amplified identically. Arrows indicate the positions of the 547 bp αB-crystallin control product and the 419 bp osteonectin product.

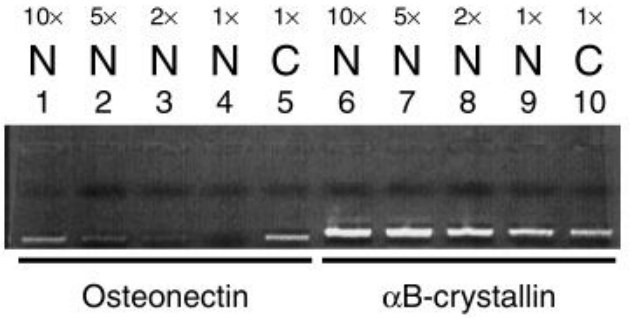

Figure 3. Re-confirmation of osteonectin over-expression between pooled cataract and increasing amounts of normal RNA. Ethidium bromide stained gel showing the levels of osteonectin and αB-crystallin after 35 PCR cycles between 50 ng (1X) of pooled cataract RNA, C, (lanes 5 and 10) and increasing amounts 50 ng (1X), lanes 4 and 9; 100 ng (2X), (lanes 3 and 8); 250 ng (5X), (lanes 2 and 7); and 500 ng (10X), (lanes 1 and 6) of normal (N) RNA. Underlined are the 419 bp osteonectin product (lanes 1-5) and the 547 bp αB-crystallin control product (lanes 6-10).

measure osteonectin expression over northern blotting since very small amounts of RNA were obtained.

The primers for osteonectin amplification were designed to produce a 419 bp product and were complementary to sense nucleotides $^{+}$1514 to $^{+}$1537 and antisense nucleotides $^{+}$1932 to $^{+}$1909 of the osteonectin sequence from the start of translation.

Figure 2 (lanes 1 and 2) compares the level of osteonectin transcript between 50 ng of cataract (lane 2) and 50 ng of normal (lane 1) RNA after 30 rounds of PCR amplification. The analysis was identically performed with primers specific for αB-crystallin as a control (Figure 2, lanes 3 and 4). Amplification of αB-crystallin under the conditions used in this study was determined to be linear for up to forty rounds of amplification. The osteonectin transcript was not detectable and the αB-crystallin transcript was only barely detectable at lower numbers (under 26 rounds) of PCR amplification cycles (data not shown). Consistent with the differential display results, the 419 bp osteonectin product was only detected in the pooled cataract sample (Figure 2, compare lanes 1 and 2), while approximately equal amounts of the 419 bp αB-crystallin control product were found between the pooled cataract and the pooled normal samples (Figure 2, compare lanes 3 and 4). The same results were obtained when another separately isolated pooled normal RNA preparation was compared with another separately isolated cataractous RNA preparation under identical conditions (data not shown). The αB-crystallin and the osteonectin PCR products were confirmed by sequencing to be authentic products. All RT-PCR products were dependent on reverse-transcriptase.

As a further confirmation of the levels of osteonectin transcript between cataractous and normal epithelia, the levels of αB-crystallin and osteonectin were compared again using the same RNA samples analyzed in the first RT-PCR comparison. In this experiment, (Figure 3), the levels of αB-crystallin and osteonectin were compared between a fixed amount (50 ng) of RNA from cataractous epithelia (Figure 3, lanes 5 and 10) and increasing amounts (up to 10 fold) of RNA from normal epithelia (Figure 3, lanes 1-4 and 6-9). Consistent with the first RT-PCR experiment, osteonectin was only detected in the cataractous RNA preparation after 35 PCR cycles when 50 ng of RNA from both samples were compared (Figure 3, compare lanes 4 and 5) while equal amounts of αB-crystallin were detected (Figure 3, compare lanes 9 and 10). However, when normal RNA is compared at 2 fold to 10 fold excess with respect to the cataractous RNA preparation, osteonectin can be detected at low levels in the normal RNA preparation (Figure 3, lanes 1-4). It is important to point out that the level of osteonectin detected using 10-fold more normal RNA than cataract RNA (Figure 3, lane 1) is less than half of the amount detected in the cataractous RNA preparation (Figure 3, lane 5) while the corresponding level of the αB-crystallin control transcript goes up approximately in proportion to the amount of RNA added (Figure 3, lanes 6-8).

Detection of Osteonectin mRNA in Individual Epithelia from Cataractous Lenses— In order to examine the individual variability of osteonectin expression, the levels of osteonectin mRNA were compared using 50 ng of RNA from 6 individual epithelia isolated from nuclear and mixed cataractous lenses (Figure 4). The exact ages and cataract types of the donors are listed above each lane of Figure 4.

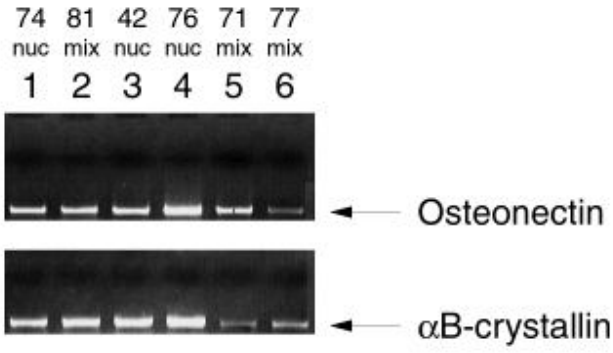

Figure 4. Osteonectin expression in six individual epithelia isolated from cataractous lenses. Ethidium bromide stained gels showing the relative levels of osteonectin detected by 35 RT-PCR cycles in 50 ng RNA in epithelia dissected from six cataractous lenses. The type of each cataractous epithelia is listed above each lane (**nuc**-nuclear; **mix**-mixed). Also indicated are the ages of the cataractous donors. Arrows indicate the 547 bp αB-crystallin control product and the 419 bp osteonectin product.

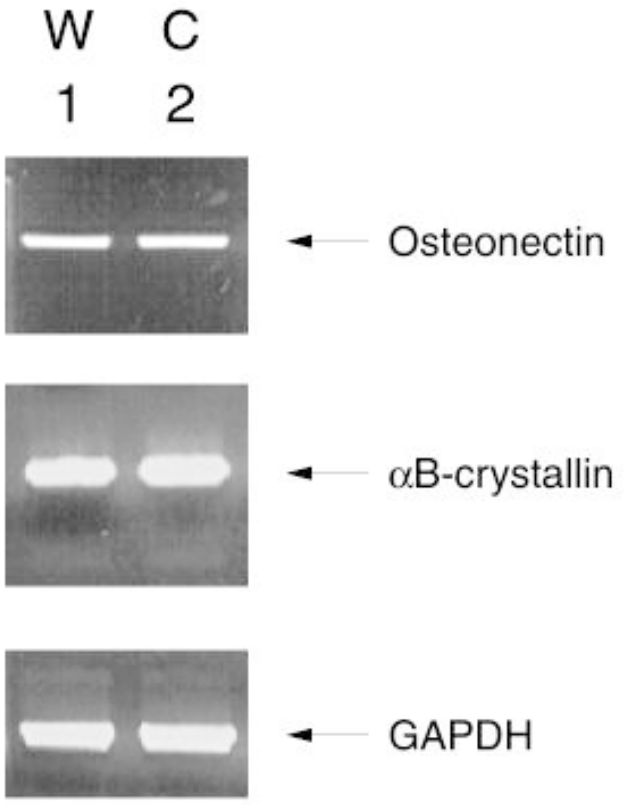

Figure 5. Spatial expression of osteonectin in normal lens epithelia. Ethidium bromide stained gels showing the relative levels of osteonectin (419 bp), αB-crystallin (547 bp) and GAPDH (600 bp) detected by 33 RT-PCR cycles using 100 ng RNA from pooled whole (W, lane 1) or central (C, lane 2) epithelial sections.

High levels of osteonectin were detected in all of the cataractous epithelia after 35 PCR cycles (Figure 4, lanes 1-5) except for one epithelia exhibiting lower expression than the rest (Figure 4, lane 6). All epithelia demonstrated high levels of αB-crystallin mRNA (Figure 4, lanes 1-4) except for two epithelia exhibiting lower amounts of αB-crystallin (Figure 4, lanes 5 and 6). These results suggest that high expression of osteonectin is a general phenomenon in individual cataractous lens epithelia. It is interesting to point out that one epithelium (Figure 4, lane 5) appears to express a higher level of osteonectin than αB-crystallin while another epithelium (Figure 4, lane 6) appears to express more αB-crystallin than osteonectin. These differences do not appear to be specific for cataract type since both of these epithelia were isolated from mixed cataractous lenses and one other mixed cataractous lens (Figure 4, lane 2) exhibited high levels of both transcripts.

Spatial Expression of Osteonectin in Normal Lens Epithelia— In the differential display and RT-PCR procedures whole normal epithelia (8-9 mm) were compared with central (5-6 mm) portions of cataractous epithelia. In order to be certain that up-regulation of osteonectin in cataractous as compared to normal epithelia is not the result of spatial gene expression differences between whole and central epithelial portions, osteonectin levels were compared by RT-PCR between equal amounts of RNA from 2 pooled 8-9 mm whole normal epithelia and 3 pooled 5-6 mm central epithelial samples. As controls, the levels of αB-crystallin and GAPDH were also examined in these samples.

This data is shown in Figure 5. Equal levels of all three transcripts were detected between the whole and central epithelial samples (compare lanes 1 and 2). These results indicate that over-expression of osteonectin is a result of cataract-specific differences between the cataractous and normal samples and is not a consequence of spatial differences in expression between whole and central epithelial portions.

DISCUSSION

Comparison of gene expression levels between epithelia isolated from cataractous and normal human lenses by reverse-transcriptase differential display identified an approximately 680 bp transcript (Fig. 1A, band 5) that was over-expressed in the cataractous as compared to the normal samples. Cloning and identification of this band identified it as osteonectin since the deduced sequence of the band was identical with the reported sequence with the exception of a single nucleotide mismatch.

Over-expression of osteonectin was confirmed by RT-PCR with gene-specific primers using additional cataractous and normal RNA preparations. RT-PCR was chosen over other methods to monitor the levels of osteonectin in the lens epithelia since very small amounts of RNA are routinely obtained from the dissected epithelia. Others have demonstrated the accuracy and sensitivity of RT-PCR for monitoring gene expression with small amounts of RNA [20]. αB-crystallin was used as a control in this study because it is an abundant transcript in lens epithelial cells and is produced in linear amounts over the amounts of RNA and PCR conditions used in the present work.

The RT-PCR results confirmed with separately isolated RNA samples the initial differential display results. Thus, analysis of 3 different pooled cataractous and 3 different pooled normal RNA preparations gave consistent results. Further RT-PCR analysis of six different individual epithelia from cataractous lenses indicated high levels of the osteonectin transcript in all six epithelia suggesting that over-expression of osteonectin is a general phenomenon in age-related human cataract. Further analysis with large numbers of epithelia will be required to evaluate the amount of individual variation for osteonectin expression and to determine whether there is any relationship between osteonectin expression and cataract type.

We are confident that the differences in osteonectin expression are epithelial-specific since all contaminating fiber cells were removed from the epithelia prior to RNA isolation. We are also confident that they represent cataract-specific differences since pooling of RNA from many epithelia prior to the differential display procedure should exclude artifacts resulting from individual variation and examination of osteonectin levels between whole and central epithelial sections showed an equal level of osteonectin expression. In addition, others have observed uniform immunostaining of osteonectin in normal bovine epithelia [37].

Comigrating with the major differential display band detected in the cataractous samples (Figure 1A, lanes 3 and 4), a lower intensity band was detected in the normal samples (Figure 1A, lanes 1 and 2). Consistent with the presence of osteonectin in normal epithelia, low levels of osteonectin were detected in normal epithelia by RT-PCR with a ten fold excess of normal RNA. Thus, in addition to its over-expression in cataract, osteonectin is also expressed at low levels in normal epithelia indicating an important function for osteonectin in normal lens maintenance. Up-regulation of osteonectin in the cataractous lens indicates that the gene is turned on in response to the presence of cataract and may be a protective response by the lens to ameliorate this condition. During the course of the present study it was demonstrated that deletion of osteonectin in knockout mice causes age-onset cataract and lens disruption [1]. Likewise, deletions or mutations in the osteonectin gene may also cause cataract in humans.

Although the exact functions of osteonectin in the lens are yet to be firmly established, it is believed to regulate cell growth through interactions with the extracellular matrix [25], to play an important role in cell-cycle control [25], and to bind Ca^{++} [25]. Since osteonectin also binds to collagen which is a major component of the lens capsule, it may have an important role in the assembly or stabilization of the lens capsule extracellular matrix. These properties of osteonectin implicate it in processes potentially important for lens maintenance and age-related cataract.

Age-related cataract involves multiple interrelated environmental, physiological and genetic components [5,10-13]. Identification of genes responding to the presence of cataract in the human lens epithelium points to specific

components and functions of the epithelium which are likely to be important for the maintenance of lens transparency. The results of the differential display analysis and the additional re-confirmation by RT-PCR collectively provide evidence for up-regulation of osteonectin in the cataractous human lens and they support the hypothesis that age-related cataract is associated with alterations in the expression of specific genes. Functional analysis of this and other differentially expressed genes in cataract will provide insight into the protective and regulatory mechanisms important for lens homeostasis and cataract.

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