EXTENDED REPORT

Expression of insulin-like growth factor binding protein-3 in pterygium tissue

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Background: Pterygium is a fibrovascular overgrowth from the conjunctiva onto the cornea. The pathogenesis of this common ocular surface disorder is not well understood and the only treatment is surgical removal.

Methods: DNA microarray analysis of primary pterygium tissue was carried out using uninvolved conjunctiva tissue as a comparison for gene expression levels. Real time polymerase chain reaction (PCR) was used to verify the mRNA level of expression for genes changed in pterygium. Western blot analysis and immunohistochemistry showed protein expression levels and the tissue distribution.

Results: Microarray analysis revealed that mRNA levels of a number of genes were changed in primary pterygium. In particular the gene for insulin-like growth factor binding protein-3, (IGFBP3), which modulates the effects of insulin-like growth factor on cells was significantly decreased. Both the message and protein expression of IGFBP3 in pterygium were decreased compared to normal, uninvolved conjunctiva.

Conclusion: Decreased levels of IGFBP3 protein have been strongly correlated with the presence of cancer. Identification of the low level of expression of IGFBP3 in pterygium suggests that the pathway controlling cell proliferation has lost an important control mechanism, which may explain the continued growth of pterygium.

Pterygium, a common ocular surface disorder, is a progressive growing fibrovascular tissue mass originating outside the cornea and which often invades the optical axis. In some severe cases, pterygium may lead to irregular corneal astigmatism and corneal stromal scarring with visual impairment. The biological mechanisms underlying pterygium are not well understood and the only therapy employed when it affects vision is surgical removal. Environmental conditions may be involved in establishing the conditions on the ocular surface that allow the development of primary pterygium and ultraviolet light exposure and other conditions that produce chronic irritation of the eye have been suggested.¹ In the search for biological explanations, investigators have examined the proliferative aspects of pterygium as well as some of the inflammatory events and potential changes in the regulation of matrix proteins.^{2–3}

In a recent study, cultures of pterygium fibroblasts were used to examine the expression of genes related to cell growth and apoptosis, and it was found that insulin like growth factor binding protein-2 (IGFBP2) was upregulated.⁴ The insulin-like growth factor binding proteins (IGFBPs) are members of the insulin-like growth factor (IGF) family, which have important roles in regulating proliferation, differentiation, and apoptosis. Of the six IGF binding proteins, IGFBP3 binds most of the plasma IGF as well as modulating interactions with the IGF receptor.⁵ High levels of circulating IGFBP3 are generally associated with a reduced risk of cancer while low levels of IGFBP3 increase the relative risk for developing colorectal, breast, prostate, renal, and lung cancer. Retinoic acid, interleukin 1 (IL-1), tumour necrosis factor α (TNF α), and p53 influence IGFBP3 expression level, as well as IGFBP3 induced apoptosis by binding to molecules like retinoic X receptor alpha (RXR α) binding partner, and orphan nuclear receptor (NUR77/NR4a1).^{6–9}

Here we report both mRNA and protein levels of IGFBP3 were downregulated in primary pterygium tissue compared to normal, uninvolved conjunctiva. Lower expression of

IGFBP3 in pterygium tissue suggests a possible mechanism for the higher growth rate in pterygium tissue.

MATERIALS AND METHODS

Patients and specimens

Pterygium tissues and remnants from the autologous upper bulbar conjunctiva grafts were collected immediately at the time of surgery and frozen in liquid nitrogen. The control conjunctival samples obtained from tissue remaining after repairing the excision were small, about 1 mg. Overall, the control conjunctival samples were from Chinese patients with an age distribution of 43–59 years, and included 11 males and five females. The pterygium samples were from seven Chinese and one non-Chinese with an age distribution of 42–57 years, and included four males and four females. The protocol was approved by the institutional review board of the Singapore Eye Research Institute and written informed consent was obtained from all participating patients.

DNA arrays

Gene expression analysis of the eight pterygium tissues and four pool of uninvolved conjunctiva were carried out using the Human Genome U133A GeneChip Array (Affymetrix Inc, USA). cRNA generated from 5 μ g of total RNA was prepared according to the sample preparation protocol (Affymetrix Inc, USA). Washing and staining was performed with a GeneChip Fluidics Station 450 and arrays were scanned using the GeneChip Scanner3000 (Affymetrix Inc, USA). The robust multiarray average (RMA) algorithm was applied to extract gene intensity.¹⁰ Cross array normalisation was performed

Abbreviations: FDR, false discovery rate; FITC, fluorescein isothiocyanate; IGF, insulin-like growth factor; IFBP, insulin-like growth factor binding protein; IL, interleukin; NUR77/NR4a1, orphan nuclear receptor; PCR, polymerase chain reaction; RMA, robust multi-array average; RXR α , retinoic X receptor alpha; SAM, significance analysis of microarrays; TNF α , tumour necrosis factor α

using the intensity based log ratio median method with the first array as the reference.¹¹ For selection of differentially expressed genes, the modified *t* statistic for significance analysis of microarrays (SAM) with the maximum standard deviation of the log ratio (over all available replicates for a gene) as the fudge constant was used.¹² Predicted false discovery rate (FDR) of 0.05 was used as a threshold for selection for differential expression. Selected Affymetrix probe set IDs were then annotated using NetAffx database (<http://www.Affymetrix.com/analysis/index.affx>). The data discussed in this publication have been deposited in NCBI's Gene expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/GEO/>) and are accessible through GEO series accession number GSE2513.

Reverse transcription and real time PCR

One microgram of total RNA, reversed transcribed using Oligo(dT)₁₅ primers and SuperScript II RNase H Reverse Transcriptase (Invitrogen, USA) was used as a template for the real time polymerase chain reaction (PCR) assay. Primers specific for human IGFBP2 (5'TCTACAATGAGCAGGAGG3' and 5'CCAGCCAGCCGGTGCCTGGC3'), IGFBP3 (5'AAAAA GCAGTGTGCGCCCTCC3' and 5'TCCACATTAACCTTGCAGCA G3'), hypoxanthine phospho-ribosyltransferase, HPRT (5'ATTCTTTGCTGACCTGCTGGAT 3' and 5'TCCCTGTT GACTGGTCATT3') were used in real time PCR. Real time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7700 Sequence

Detection System (Applied Biosystems). The thermal cycling conditions were as follows: 95°C for 10 minutes, 45 cycles at 95°C for 30 seconds, and 60°C for 1 minute. Data obtained from real time PCR were analysed using the comparative CT method as previously described by Livak.¹³ The paired *t* statistic with a *p* value <0.05 was used to determine whether the RT-PCR results for IGFBP2 and IGFBP3 in pterygium tissue were significantly different from normal conjunctiva.

Western blot analysis

Paired tissue samples of pterygium and uninvolved conjunctiva were obtained from three additional patients and immediately frozen. Five micrograms of total protein separated by 12% SDS-PAGE were transferred onto a 0.45 µm nitrocellulose membrane (Biorad). Membranes were blocked with 4% BSA/TBST before applying antibodies for anti-IGFBP3 (Abcam) (1:500) or anti-GAPDH (1:1000) (Santa Cruz) in 1% BSA/TBST incubation at 4°C for 16 hours, secondary antibody conjugated with HRP (1:1000) in 1% BSA/TBST were incubated for 30 minutes at room temperature. Chemiluminescent detection was performed using Supersignal West Pico (Pierce). GAPDH was used as an internal control.

Immunohistochemistry

A separate group of paired pterygium and conjunctival tissue samples from three patients were processed for immunohistochemistry. Sections were cut at 5 µm from blocks of fresh

Table 1 Mean fold change of genes of the IGF and IGFBP family in pterygium compared to conjunctiva

Probe set	Mean fold change	FDR	Symbol	Name
209540_at	1.54	0.2705	IGF1	Insulin-like growth factor 1 (somatomedin C)
209542_x_at	1.10	0.8128	IGF1	Insulin-like growth factor 1 (somatomedin C)
209541_at	1.08	0.8386	IGF1	Insulin-like growth factor 1 (somatomedin C)
211577_s_at	1.03	0.9089	IGF1	Insulin-like growth factor 1 (somatomedin C)
203628_at	-2.08	0.5335	IGF1R	Insulin-like growth factor 1 receptor
208441_at	-1.22	0.6846	IGF1R	Insulin-like growth factor 1 receptor
203627_at	1.06	0.8639	IGF1R	Insulin-like growth factor 1 receptor
202410_x_at	-1.18	0.6118	IGF2	Insulin-like growth factor 2 (somatomedin A)
210881_s_at	1.16	0.8536	IGF2	Insulin-like growth factor 2 (somatomedin A)
220561_at	-1.32	0.2680	IGF2AS	Insulin-like growth factor 2 antisense
201392_s_at	-1.03	0.9367	IGF2R	Insulin-like growth factor 2 receptor
201393_s_at	1.03	0.9320	IGF2R	Insulin-like growth factor 2 receptor
205302_at	-1.15	0.5585	IGFBP1	Insulin-like growth factor binding protein 1
202718_at	-1.24	0.6354	IGFBP2	Insulin-like growth factor binding protein 2
212143_s_at	-2.45	0.0344	IGFBP3	Insulin-like growth factor binding protein 3
210095_s_at	-1.83	0.0172	IGFBP3	Insulin-like growth factor binding protein 3
201508_at	1.27	0.4546	IGFBP4	Insulin-like growth factor binding protein 4
211959_at	1.21	0.6936	IGFBP5	Insulin-like growth factor binding protein 5
203424_s_at	-1.16	0.7712	IGFBP5	Insulin-like growth factor binding protein 5
211958_at	-1.09	0.8497	IGFBP5	Insulin-like growth factor binding protein 5
203425_s_at	-1.06	0.8318	IGFBP5	Insulin-like growth factor binding protein 5
203426_s_at	1.05	0.8679	IGFBP5	Insulin-like growth factor binding protein 5
203851_at	-1.06	0.8261	IGFBP6	Insulin-like growth factor binding protein 6
213910_at	1.32	0.2917	IGFBP7	Insulin-like growth factor binding protein 7
201163_s_at	1.20	0.6547	IGFBP7	Insulin-like growth factor binding protein 7
201162_at	1.08	0.8354	IGFBP7	Insulin-like growth factor binding protein 7
210118_s_at	1.39	0.2483	IL1A	interleukin 1
208200_at	-1.08	0.7697	IL1A	interleukin 1
202449_s_at	-1.13	0.5991	RXRA	Retinoid X receptor, alpha
202426_s_at	1.04	0.8956	RXRA	Retinoid X receptor, alpha
211258_s_at	1.06	0.7252	TGFA	Transforming growth factor, alpha
205016_at	-1.05	0.7765	TGFA	Transforming growth factor, alpha
205015_s_at	-1.02	0.9502	TGFA	Transforming growth factor, alpha
220406_at	1.20	0.6617	TGFB2	Transforming growth factor, beta 2
209908_s_at	-1.18	0.5414	TGFB2	Transforming growth factor, beta 2
209909_s_at	1.11	0.8485	TGFB2	Transforming growth factor, beta 2
220407_s_at	-1.00	0.9988	TGFB2	Transforming growth factor, beta 2
207113_s_at	1.09	0.8242	TNF	Tumor necrosis factor
211300_s_at	1.20	0.5727	TP53	Tumor protein p53 (Li-Fraumeni syndrome)
201746_at	1.11	0.6793	TP53	Tumor protein p53 (Li-Fraumeni syndrome)

Probe sets with significant changes are in bold face. The minus symbol indicates downregulation.

Figure 1 Graph shows comparative real time PCR of IGFBP3 and IGFBP2. HPRT normalised threshold cycle (delta Ct) is shown in bar graph. Raw data for plotting graph are shown on the right. The difference between the Ct of IGFBP3 in pterygium to conjunctiva is +1.77 cycle. IGFBP3 is 3.4-fold significantly downregulated (* $p=0.035$). IGFBP2 in pterygium is 1.83-fold downregulated compared to conjunctiva; however, the level of IGFBP2 expression was not statistically significant ($p=0.237$).

frozen pterygium tissue and matched uninvolved conjunctiva embedded in Optimum Cutting Temperature compound (Sakura, USA) and processed with an antibody against human IGFBP3 (Abcam, Cambridge, UK). Positive signals were detected by incubating with fluorescein isothiocyanate (FITC) conjugated anti-human IgG antibody (Santa Cruz, USA). Images were captured using 40 $\times$ Achrostat lens on an Axioplan2 microscope equipped with an AxioCam MRC (Carl Zeiss, Germany) camera.

RESULTS

The overall gene expression profiles of pterygium and conjunctiva were assessed using established procedures for Affymetrix DNA microarrays. IGFBP3 was identified as significantly downregulated in pterygium tissue in comparison with normal uninvolved conjunctiva. IGFBP3 transcripts (Affymetrix probe IDs: 210095_s_at and 212143_s_at) were 1.83-fold and 2.45-fold downregulated, respectively, when compared to control conjunctiva. The modified *t* test significances by SAM analysis for probes 210095_s_at and 212143_s_at were $p=0.0172$ and $p=0.0344$, respectively (table 1). Expression of IGFBP2 was found to be 1.24-fold downregulated in pterygium and the statistical analysis using SAM gave a p value of 0.6354, indicating that it was not significantly changed in pterygium (202718_at in table 1). The other IGF gene family members (IGFBP1 to IGFBP7), as well as the expression of IGF-I and IGF-II, were not significantly changed in pterygium samples. Although p53, TNF α , retinoid X receptor alpha (RXR α), and interleukin 2 (IL-2) are known to induce the expression of IGFBP3, these genes were also identified as not differentially regulated in overall global gene expression in pterygium tissue compared to uninvolved, normal conjunctiva.

Comparative real time PCR results showed that pterygium had a lower level of expression of IGFBP3, which was on average 3.4-fold less when compared to control conjunctiva (fig 1). Therefore, DNA microarray and real time PCR results corroborated each other and confirmed that the level of IGFBP3 mRNA in the pterygium tissue was significantly decreased compared to normal conjunctiva. Real time PCR results showed a slight downregulation of IGFBP2, with 1.83-fold less in pterygium compared to uninvolved conjunctiva, although the result was not statistically significant (fig 1).

Protein expression was examined in paired tissue samples of pterygium and uninvolved conjunctiva. Immunohistochemistry showed the localised expression of IGFBP3 in pterygium and conjunctival tissues. Positive

staining for IGFBP3 was primarily in the epithelial layer for both conjunctiva and pterygium (fig 2). Some positively stained cells were also present in the stromal layer of conjunctiva. The cytoplasm and perinuclear region of the epithelial layer was positively stained for IGFBP3. The staining intensity of IGFBP3 was less in pterygium epithelia compared to conjunctiva epithelium (fig 2A). In addition, western blot analysis confirmed a lower IGFBP3 protein expression in pterygium tissues when compared to uninvolved conjunctiva from the same eye. This result further corroborated the finding that protein levels of IGFBP3 were decreased in pterygium tissue (fig 2B).

DISCUSSION

The IGFBP family of genes has been identified as regulators of cell proliferation in many different cell types. Decreased expression of IGFBP3 in some tumour types has been reviewed.¹⁴ IGFBP3 was identified as one of the significantly downregulated genes in pterygium tissue compared to normal conjunctiva in the present DNA array experiment. Both the mRNA and protein level of IGFBP3 were confirmed to be downregulated in pterygium. The decreased expression of IGFBP3 in pterygium may result in reduced apoptosis and a higher rate of cell proliferation in pterygium tissue. Although not tumorigenic the slow growth may have a basis in several factors such as the decreased local availability of IGFBP3, which would also tend to explain why the other epithelial surfaces are not affected.

However, it is not clear if this is the cause or consequence of pterygium as the growth takes place usually over years before reaching a size that requires surgical removal. Tan and co-workers had previously reported high levels of cell proliferation in the subepithelial fibrovascular layer of pterygium, indicating that pterygium may be a disorder of excessive cellular proliferation in the fibrovascular tissue component.¹⁵ These findings support the idea that pterygium is a growth disorder rather than a degenerative condition of conjunctiva.¹⁶

Solomon and co-workers had previously reported that IGFBP2 was upregulated in pterygium fibroblast culture and that there were no differences in either mRNA level or protein levels of IGFBP3.⁴ Differences in the expression of IGFBP2 and IGFBP3 with the present results could be the result of two factors; firstly, the difference in the use of cultured cells in the work by Solomon and co-workers and the focus on only the fibroblasts in that study. Moreover, gene expression levels may be altered as a result of cell

Figure 2 (A) Immunohistochemistry of IGFBP3 in pterygium and paired conjunctiva. Staining of IGFBP3 (white areas) was primarily in the epithelial layer in both conjunctiva and pterygium. However, the conjunctiva reveals a great deal more staining than did the pterygium. Original magnification 400 $\times$. (B) Western blot results of three pterygium tissues compared with lower IGFBP3 expression in the paired conjunctiva tissue. The level of IGFBP3 detection in pterygium (P) and conjunctiva (C) tissue at 60 kDa is shown. GAPDH indicates the amount of total protein loaded in each lane. To visualise the band in P3 the protein loading in P3 was double of that in C3.

culture procedures. Secondly, the present data generated in both the microarray analysis and the real time PCR procedures include both pterygium epithelium and stroma. Thus, these major technical differences could significantly change the experimental outcome.

Genes functionally related to IGFBP3 were also examined in our microarray analysis. However, tumour suppressor protein p53, TNF α , and IL2, which can regulate the expression of IGFBP3 and IGFBP3 binding partner RXR α were not differentially expressed in pterygium. Thus, the downregulation of IGFBP3 in pterygium was probably not caused by the expression level of p53, TNF α , or IL2 in pterygium. Hence, the factors regulating the expression of IGFBP3 in pterygium remain to be elucidated. The expression of IGFBP3 was found to be downregulated in the primary pterygium. It could mean that the pathway controlling cell proliferation is affected in pterygium pathogenesis. In the future, the use of IGFBP3 protein could be explored as a possible therapeutic approach to inhibit the growth of pterygium.

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