Role of Periostin and Interleukin-4 in Recurrence of Pterygia

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PURPOSE. To identify the candidate genes for pterygia recurrence from a pterygia transcriptome and to analyze their transcriptional regulation and functional relationships.

METHODS. Transcriptional networks for pterygia recurrence were constructed using network analysis that was applied to 184 genes that showed a significant twofold change in the whole genome. Of the identified recurrence-related candidate genes in the major networks, periostin and IL-4 were analyzed for transcriptional relationships using pterygia-derived fibroblasts. Immunohistochemical analysis was used to study pterygia tissue. Effector candidate molecule for recurrence periostin was analyzed for cell adhesive function.

RESULTS. The pterygia transcriptome was divided into four major biological networks with high significance scores (P < 10−17). The classifier with the highest accuracy using the support vector machine algorithm was periostin, which was successfully linked to the network of cell cycle, connective tissue development and function, and cell morphology. Analyses using pterygia-derived fibroblasts showed that periostin was required for cell adhesion that was mediated by a presumed pterygia-related extracellular matrix protein, fibronectin. Periostin was found to be transcriptionally induced by IL-4. The IL-4-stimulated periostin induction was suppressed by MAP kinase/ERK kinase 1 inhibitor, indicating an involvement of the MAP kinase pathway. Pathologically, IL-4 was transcriptionally elevated in recurrent pterygia tissue and was localized to perivascular tissues and endothelial cells in the stroma of the subconjunctiva of pterygia.

CONCLUSIONS. Periostin is induced by IL-4 and is involved in the fibronectin-mediated pterygia fibroblast adhesion. These findings indicate that periostin probably promotes the recurrence of pterygia. (Invest Ophthalmol Vis Sci. 2010;51:139–143) DOI:10.1167/iovs.09-4022

A pterygium is an excessive fibrovascular proliferation of degenerated bulbar conjunctival tissue on the ocular surface. At present, surgery is the only method for treating pterygia, and recurrences are a major complication of the surgical treatment of pterygia. Although numerous approaches have been attempted to reduce recurrences, such as adjunctive mitomycin treatment, radiation, and amniotic membrane transplantation, recurrences still occur.1

We have determined the minimum gene set that would accurately differentiate primary from recurrent pterygia using a mathematical classification algorithm.2 We found that only three genes—periostin, tissue inhibitor of metalloproteinases-2 (TIMP-2), and 1-3-phosphoserine phosphatase homolog (PSPHL)—differentiate primary from recurrent pterygia.2

We have also reported on the numerous transcripts that are related to pterygia recurrence.2 However, the functional involvement in the genome or their significance in recurrence remains speculative. We have used knowledge-based analysis to understand the global interactions of the genes in the context of their function and regulation. To simplify the complex biological phenomena played by genes, network analysis has been reported to be a very efficient method.3 We have applied the Ingenuity Pathways Knowledge Base (Ingenuity Systems, Redwood City, CA, http://www.ingenuity.com), a biological network database created from known protein-protein interactions and the activation of signaling pathways, and found that the complex phenomena of pterygia recurrences can be simply summarized into four highly significant molecular networks. In this analyses, periostin and TIMP-2, which we reported as significant classifier genes in the support vector machine (SVM)-based classification algorithm,2 were statistically linked to the generated networks. Previously, TIMP-2 was shown to be involved in tissue remodeling processes of pterygia.1 Although periostin showed the highest classification accuracies of primary and recurrent pterygia,2 its functional characteristics are not yet well understood, and its involvement in the pathogenesis of pterygia has not been reported.

Thus, the purpose of this study was to determine the molecular function of periostin in pterygia pathology and the causative transcriptional event for periostin induction based on the generated networks. We shall show the function of periostin in the recurrence of pterygia and relate it to the role of IL-4 as its inductive signal.

MATERIALS AND METHODS

Patient Backgrounds and Specimen Collection

Pterygia heads were collected from 21 eyes of 21 Asian patients undergoing pterygium excision with conjunctival autografting at the Tottori University Hospital (Yonago, Japan) and the Ideta Eye Hospital (Kumamoto, Japan). Of these, 11 eyes of 11 patients showed primary pterygia and 10 eyes of 10 patients showed recurrent pterygia. Patients were 11 men and 10 women whose mean age was 68 ± 2 years (range, 52–85 years). Patients with primary cicatricial ocular surface disease, history of ocular allergic symptoms, and inflammatory ocular surface disease of unknown etiology were excluded. The study protocol conformed to the tenets of the Declaration of Helsinki, and the procedures used were approved by the Tottori University Ethics Committee. Signed informed consent was obtained from all patients.

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Transcriptional Analysis

PTerygia head tissues collected from primary and recurrence cases were transcriptionally analyzed using an oligo microarray (AceGene, Hitachi, Japan) corresponding to 30,336 human genes. Genes that were differentially induced or suppressed in the primary and recurrent pterygia cases were extracted from the whole genome using t tests. This set of genes was analyzed using a bioinformatics approach.

To gain insight into the transcriptional network of molecular events in the mechanism of recurrence, we applied commercially available analysis software (Ingenuity Pathway Analysis 3.0, based on the Ingenuity Pathways Knowledge Base; Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com). The resultant networks were evaluated by the significance score, which is expressed as the negative logarithm of that P-value. The obtained score in each instance indicated the likelihood that the assembly of a set of focus genes in a network could be explained by random chance alone.

Real-Time RT-PCR

Total RNA was isolated from the treated fibroblasts using a purification kit (RNaseasy Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol. One microgram of RNA samples was reverse transcribed using random hexamers and superscript III (Invitrogen, Carlsbad, CA). The cdNAs were amplified and quantified (LightCycler; Roche, Mannheim, Germany) with a one-step quantification of RNA kit (QuantiTect SYBR Green PCR kit; Qiagen). Sequences of the used real-time PCR primer pairs were as follows: IL-4: forward, 5'-CACCAGGTCTGCTAACAGC-3'; reverse, 5'-GCCCTGCAAGGTTCCTC-3'; peristin: forward, 5'-TTGAGTACCTGGAAGGAAAT-3'; reverse, 5'-AGATCCGCTGAAGTGGTTITG-3'; IL-13: forward, 5'-TTGAGGACTGCTAGAAACAG-3'; reverse, 5'-GGCTTGGCTCCACATGCT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5'-AGGCACATGCTGAGCAGC-3'; reverse, 5'-GCACCATGACACAAATCC-3'. To ensure equal loading and amplification, all products were normalized to GAPDH transcript as an internal control.

Establishment of Pterygia Tissue-Derived Fibroblast Line

Head tissues of pterygia were collected from four eyes of four patients without a history of pterygia surgery and were undergoing primary excision with conjunctival autografting at the Tottori University Hospital. Tissues were minced using a sterilized forceps and scissors and digested in 12.5 mg/mL collagenase (Nitta Gelatin, Osaka, Japan) at 37°C in a humidified atmosphere of 5% CO2 for 1 hour. The digested tissue was filtered and washed by centrifugation at 1500 rpm for 5 minutes and was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA) supplemented with 15% FBS (vol/vol) at 37°C in 5% CO2. The pterygia-derived conjunctival fibroblasts were cultured to at least the third passage before use.

Fibroblast Adhesion Assay

An adhesion assay was performed as described. Briefly, flat-bottom cell culture plates (Corning Costar, Cambridge, MA) were coated with 0 to 40 μg/mL purified human fibronectin (Gibco-BRL) in PBS at 4°C overnight. The plates were washed three times with PBS and blocked with 10 μg/mL heat-denatured bovine serum albumin (BSA; 85°C for 15 minutes; Sigma, St. Louis, MO) for 1 hour at 37°C. After blocking, the plates were washed with DMEM.

Fibroblasts, detached by enzyme treatment (Accutase; Innovative Cell Technologies, San Diego, CA), were incubated in DMEM for 30 minutes at 37°C in a tube rotator. The cells were then incubated in 0.1% BSA/PBS with antiperiostin (rabbit polyclonal antibody; 1:500; Biovendor, Brno, Czech Republic) or control IgG for 30 minutes at 4°C. After washing, the fibroblasts were seeded on fibronectin-coated plates (5 × 104 cells/well) in DMEM containing 1 μg/mL antiperiostin or control antibody and were incubated at 37°C for 1 hour. The medium was aspirated, and the plates were washed with PBS three times. The number of attached fibroblast was determined by dye (CyQUANT GR; Molecular Probes Inc., Eugene, OR) according to the manufacturer's protocol. Briefly, the solution (CyQUANT GR; Molecular Probes Inc.) was added to each well, and the fluorescence of the samples was measured at 520 nm with excitation at 480 nm fluorescence microplate reader (FL600; BioTek Instruments, Winooski, VT). Cell counts were calculated based on the calibration curve of fluorescence of diluted cell suspension.

Evaluation of Periostin Expression by Flow Cytometry

PTerygia fibroblasts were cultured with recombinant IL-4 (PeproTech, Rocky Hill, NJ) at the selected concentration for 48 hours. To inhibit MAP kinase activity, a highly selective inhibitor of MAP kinase/ERK kinase1 (MEK1), PD98059 (10 μM; New England Biolabs, Beverly, MA), was added to the media. Then the serum-starved fibroblasts were collected by enzyme treatment (Accutase; Innovative Cell Technologies) and incubated for 20 minutes at 37°C in a tube rotator. The fibroblasts were stained by incubation with antiperiostin antibody (1 μg/mL) or control IgG in 0.1% BSA/PBS for 30 minutes at 4°C. After washing, the cells were incubated with Alexa Fluor 488-antirabbit IgG (Molecular Probes, Inc.) for 20 minutes at 4°C. The expression of periostin on the surfaces of the fibroblasts was analyzed (FACSCalibur; Becton-Dickinson, San Jose, CA).

Statistical Analysis

Data are presented as the mean ± SEM. Statistical analyses were performed by unpaired Student’s t tests (two tailed) or ANOVA analysis.

Results

Construction of Network Model of Pterygia Recurrence

To understand the global interactions of the pterygia-related genes in terms of their function and regulation, we applied network analysis on the previously determined pterygia transcriptome. We hypothesized this would allow us to determine pterygia recurrence-related biological phenomena in a simplified form. To construct the network model, differentially induced or suppressed genes in the primary and recurrent pterygia cases were extracted from the whole genome. We focused on a gene set consisting of 184 genes that had a twofold change and that had been shown to contain valid transcriptional information to generate an efficient support vector machine classifier.

When this set of genes was analyzed (Ingenuity Pathway Knowledge Base of known signaling networks; Ingenuity Systems), four major biological networks with high significance scores (P < 10−17) were successfully generated (Table 1). Of these, periostin was placed in one of the major networks (network 4), which was annotated as cell cycle, connective tissue development and function, and cell morphology.

The most significant network (network 1) was annotated as cell-to-cell signaling and interaction, hematologic system development and function, and cell cycling. Interestingly, this network predicted vascular endothelial growth factor as the most linked gene, suggesting a role of network 1 in neovascularization processes. Another major network was network 2, which was annotated to function as immune response, cell-to-cell signaling and interaction, and hematologic system development and function and predicted IL-4 as the most linked gene. Another SVM classifier, TIMP-2, was listed in network 3, together with membrane metalloproteinase 7 (MMP7). Top function of the network 3 was annotated as cell death, immunologic diseases, and DNA replication/recombination/repair. Of the previously reported prognosis determinant candidate genes, PSPHL was not found in the generated networks.
**Increased Expression of IL-4 in Recurrent Pterygia**

Of all the genes either assigned or predicted in the networks, periostin was the SVM classifier with the highest accuracy.\(^2\) Periostin has recently been implicated as an adhesion molecule or a tissue remodeling mediator in Th2-type cytokine-mediated diseases,\(^3\) though its roles on the ocular surface remains unexplored. A representative Th2-type cytokine, IL-4, which was predicted in the calculated network 2, is known to induce adhesion molecules or tissue matrix modulators in colon carcinoma cells.\(^5\) Therefore, we hypothesized that IL-4 may contribute to the recurrence process by the induction of gene(s) in the observed networks.

**Induction of Periostin by IL-4**

To determine the roles played by IL-4 in the constructed networks, we tested whether IL-4 could induce the recurrence classifier, periostin, using pterygium-derived fibroblast cells. Re-combinant IL-4-stimulated fibroblasts were examined for periostin mRNA using real-time PCR. Our results showed that IL-4 induced periostin in a dose-dependent manner (Fig. 2A).

For the IL-4-activated genes, ERK1/2-mediated MAP kinase cascade is generally used.\(^7\) To examine an involvement of the ERK1/2 pathway in the transcriptional activation of periostin, IL-4-stimulated fibroblasts were treated with PD98059, a MEK1 inhibitor. PD98059 significantly inhibited the transcriptional activation of periostin by IL-4 (at 100 pg/mL and 1 ng/mL; \(P < 0.05\)), indicating an involvement of the MEK1/ERK1 pathway in periostin induction.

We next examined whether the transcriptional activation of periostin may actually translate into periostin expression. Flow cytometric analysis confirmed the expression of periostin by IL-4 (Fig. 2B) compared with control-treated fibroblasts (Fig. 2C). Consistent with the transcriptional outcome, PD98059 treatment inhibited periostin expression (Fig. 2D).

**Pterygia Fibroblast Adhesion Mediated by Periostin**

Finally, to understand the role of periostin in pterygia recurrence, we examined the functional role of periostin using pterygium-derived fibroblasts. Because pterygial tissues express large amounts of fibronectin, one of the cell matrix ligands of periostin, we tested whether periostin might promote fibronectin-mediated cell adhesion using a cell adhesion assay. Our results showed that fibronectin significantly promoted fibroblast adhesion in a dose-dependent manner (Fig. 3). When fibroblasts were treated with antiperiostin, fibronectin-medi-
ed-fibroblast adhesion was significantly inhibited. Thus, periostin was shown to mediate fibroblast adhesion in a fibronectin-rich environment, including pterygia tissue. This critical function of periostin in the pathogenesis of pterygia may promote recurrence.

**DISCUSSION**

In pterygial tissue, extracellular matrix protein and metalloproteinases (MMPs), including collagen types III and IV, laminin, fibronectin, and MMPs 8, 9, and 13, are abundantly expressed. During the recurrence process, the deposition of such ECM proteins continues and is accompanied by tissue-remodeling processes. These changes eventually lead to marked subepithelial fibrosis.

The results of this study demonstrated a new pathway, leading to pterygia recurrence, that involves IL-4 and periostin. Using knowledge-based bioinformatics, we identified an IL-4 network of immune responses and cell-to-cell signaling, presumably serving as a tissue fibrotic factor in pterygia pathogenesis. For example, in Th2-type diseases, including bronchial asthma, subepithelial fibrosis is a cardinal feature. IL-4 or IL-13 plays an important pivotal role in inducing ECM deposition and subepithelial fibrosis and in exacerbating airway hyperresponsiveness, eosinophil infiltration, and mast cell activation. However, we observed IL-13 transcripts were greatly reduced, though the reduction was not statistically significant. Because IL-13 is known to compete for IL-4 binding, this may potentiate IL-4 binding. An infiltration of mast cells has been recognized in pterygia tissue; however, a reasonable explanation for the infiltration has not been presented. Our findings have revealed a previously unrecognized, but presumably crucial, pathway for pterygia fibrosis.

A recent comprehensive analyses of pterygia transcriptome identified fibronectin as the top induced gene in the genome, followed by other ECM-related genes (e.g., collagen type III and versican). However, IL-4 induction has been unnoticed and hidden in our transcriptome and in others, probably because of its relatively low constitutive levels. Successful identification by network model prediction provided us with an important perspective in pterygia pathogenesis.

From an epidemiologic standpoint, exposure to ultraviolet light (UV) is one of most important factors for the development of pterygia. Indeed, UV exposure induces IL-4 expression in...
psoriatic tissue and T lymphocytes. Another possible explanation for the differential expression of IL-4 is a possible epigenetic modification of the IL-4 locus by DNA methylation and chromatin remodeling.

Our data suggest that the IL-4-induced periostin may play a critical role in pterygia recurrence. Periostin is an extracellular protein secreted by fibroblasts after injury. The FAS1 domain of periostin interacts with αvβ3 and αvβ5 integrins and stimulates a fibronectin-dependent motility of epithelial cells.

Recently, periostin has been gaining considerable interest in the process of cardiac remodeling or heart failure. Periostin associates with critical ECM regulators such as TGF-β, tenascin, and fibronectin and is a critical regulator of fibrosis by altering the deposition and attachment of collagen. Because fibronectin was listed as the top induced gene in the pterygia transcriptome, the IL-4-mediated periostin may constitute a significant component in the pathogenesis of pterygia. Moreover, IL-4 is known to stimulate fibroblasts to induce another recurrence classifier focus gene in network 3, TIMP2, which would further promote tissue remodeling.

To summarize, our results have identified new pathologic components in conjunctival fibrosis in pterygia recurrence. Expression of periostin after environmental perturbations, including IL-4 induction, can alter normal physiological interactions between fibroblasts and ECM protein, leading to fibrosis and scar formation. Thus, regulating IL-4/periostin induction may control the recurrence of pterygia.

References
