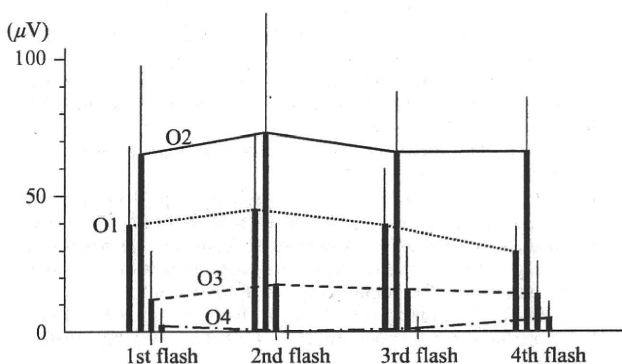


**Figure 5.** Mean peak times of O1, O2, O3, and O4 recorded in 28 normal subjects in response to repeated-flash stimuli (1st flash, etc.) after 30 min of dark adaptation. Repetition of the flash stimulus resulted in significant decrease in peak time of O2 ( $P = 0.000058$ ,  $0.0000076$ , and  $0.000043$ ), but increases in peak times of O3 and O4 ( $P = 0.0431$  and  $0.0145$  for O3;  $P = 0.0400$  and  $0.00381$  for O4). \* $P < 0.05$ ; \*\* $P < 0.01$  versus 1st-flash ERG.



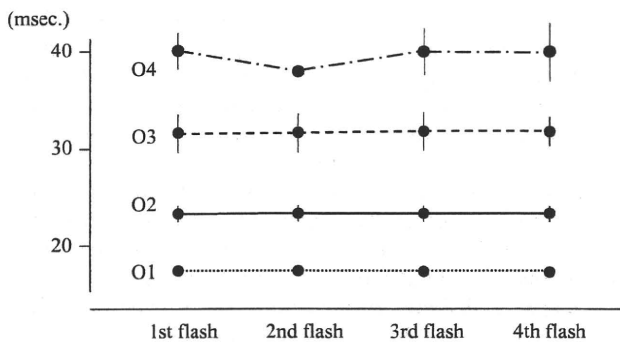
**Figure 6.** Mean amplitudes of O1, O2, O3, and O4 recorded in 28 normal subjects in response to repeated-flash stimuli (1st flash, etc.) after 10 min of light adaptation. Repetition of the flash stimulus did not result in any significant change in amplitude.

The peak time of O1 did not change significantly with repetition of the flash stimulus, but the peak time of O2 decreased significantly for the second, third, and fourth flashes compared with the first flash (Figs. 1, 3, and 5). The peak times of O3 and O4 were significantly longer in response to the second and third flashes, but not in response to the fourth flash, compared with the first flash (Fig. 5).

After light adaptation, the amplitudes and peak times of all four OPs did not vary in response to repeated flashes (Fig. 1), and none of the differences were significant compared with the first flash (Figs. 6 and 7). The amplitudes of O3 and O4 recorded after light adaptation were much smaller than those of O3 and O4 recorded after dark adaptation (Figs. 4 and 6).

## Discussion

Although OPs were discovered about 60 years ago,<sup>20,21</sup> their origins are still unclear. The results of animal experiments



**Figure 7.** Mean peak times of O1, O2, O3, and O4 recorded in 28 normal subjects in response to repeated-flash stimuli (1st flash, etc.) after 10 min of light adaptation. Repetition of the flash stimulus resulted in no significant change in peak times.

indicate that OPs may arise in bipolar cells,<sup>22,23</sup> in amacrine cells,<sup>24</sup> or in the inner plexiform layer of the retina.<sup>25-27</sup> Wachtmeister and Dowling<sup>26</sup> contributed to the knowledge of OPs by demonstrating that OPs reverse polarity in different layers of the retina and that the first OP (O1) reverses polarity at the border between the inner plexiform layer and the inner nuclear layer, more proximally than the other OPs. They proposed that OPs result from radial electrical flows in the retina. King-Smith and colleagues<sup>28</sup> recorded OPs separately from cone and rod systems and found that OPs originating from cone systems have shorter peak time than those originating from rod systems.

Many investigators have studied differences in OPs recorded under either scotopic or photopic conditions,<sup>16-19,29-33</sup> recorded in response to light stimuli of various colors,<sup>28,34-36</sup> or by using various locations of the active electrode.<sup>37</sup> The findings of some of these studies<sup>18,32,36</sup> suggest that O1 and O2 are generated mostly by cone systems and O3 and O4 mostly by rod systems. Tremblay and Lam<sup>37</sup> reported that the point of inversion of polarity is similar for O2 and O3 but different for O4. The findings of these studies suggest that both rod and cone systems may contribute to the generation of OPs, although each OP is generated by a unique combination of retinal components.

We recorded OPs both after 30 min of dark adaptation and after 10 min of light adaptation using repeated high-intensity flash stimuli with an interval of 5 s between stimuli. Under these conditions, the obtained electroretinograms contain both rod- and cone-driven responses. As a result, the reaction of OPs to the consecutive flash stimuli varied for O1, O2, O3 and O4.

O1 was recordable under both dark- and light-adapted conditions. On the other hand, though O3 and O4 were recordable with every stimulus under dark adaptation, they almost entirely disappeared after light adaptation (Figs. 1, 4, and 6). These results support the hypothesis that O1 is generated mostly by cone systems and O3 and O4 mostly by rod systems.<sup>18,32,36</sup>

The reaction of O2 to repeated-flash stimuli was unique among OPs. The striking characteristic of O2 was that its

amplitude was larger and peak time was shorter with the second and later flash stimuli. In other words, the O2 elicited by the first flash was different from the other O2s in both amplitude and peak time. These findings indicate that O2 is the most sensitive to the retinal condition (i.e., adaptation level of the retina) among the OPs.

Irifune<sup>38</sup> examined OPs in patients with diabetes and found abnormalities of OPs even in diabetic patients without diabetic retinopathy, concluding that these abnormalities may be enhanced by disturbances in dark adaptation in the early stages of diabetes. In the clinic, OPs recorded from an affected retina should be evaluated with care, because disturbances of either light or dark adaptation of the retina may influence the amplitude or peak time of OPs.

In conclusion, OPs were influenced by retinal adaptation. O1 was the least influenced by both the repeated flashes and light adaptation. O3 and O4 were easily attenuated with repetition of either flash stimuli or light adaptation. O2 was unique among OPs because the preceding flash or subtle light adaptation made the amplitude bigger and the peak time shorter.

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## 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.<sup>1</sup>

We have determined the minimum gene set that would accurately differentiate primary from recurrent pterygia using a mathematical classification algorithm.<sup>2</sup> 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.<sup>2</sup>

We have also reported on the numerous transcripts that are related to pterygia recurrence.<sup>2</sup> 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.<sup>3</sup> 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,<sup>2</sup> were statistically linked to the generated networks. Previously, TIMP-2 was shown to be involved in tissue remodeling processes of pterygia.<sup>1</sup> Although periostin showed the highest classification accuracies of primary and recurrent pterygia,<sup>2</sup> 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  $68 \pm 2$  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.<sup>2</sup> 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 terms of 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 (RNeasy 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'-CACCGAGTTGACCGTAACAG-3'; reverse, 5'-GCCCTGCAGAAAGGTTCC-3'; periostin: forward, 5'-TTGAG-ACGCTGGAAGGAAAT-3'; reverse, 5'-AGATCCGTGAAGGTGGTTTG-3'; IL-13: forward, 5'-TGGAGGACTTCTAGGAAAACGA-3'; reverse, 5'-CC-CTTTGCTCACCAGTCT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5'-AGCCACATCGCTCAGACAC-3'; reverse, 5'-GCCAATACGACCAAATCC-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% CO<sub>2</sub> 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% CO<sub>2</sub>. 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.<sup>4</sup> 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 × 10<sup>5</sup> 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.<sup>2</sup> We hypothesized this would allow us to determine pterygia recurrence-related biological phenomena in a simpler 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.<sup>2</sup>

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 determination candidate genes, PSPHL was not found in the generated networks.

TABLE 1. Transcriptional Network of Pterygia Recurrence

Network	Focus Genes	Predicted Genes	Score (-log [P])	Functions
1	<i>ACVR1B, ADRB2, AKAP5, AKAP11, ATP1B1, BTK, CA2, CD97, CHRM1, CYP3A5, DMTF1, GABRG3, GRK5, GRP, MMP1, NPAT, PPBP, THPO</i>	<i>CDKN3, CDKN1A, CKLFSF3, CXCL12, DLG4, E2F1, F2, GABRB3, GABRD, IL6, KIF2C, MAPK1, Prlpe, SMTN, SRC, ST5, <u>VEGF</u></i>	30	Cell-to-cell signaling and interaction, hematologic system development and function, cell cycle
2	<i>ACP5, CD1E, CD79B, CR2, IGHM, MS4A1, NCLN, PKNOX1, PLAC8, POU2F2, SATB1, TK2, USP33</i>	<i>ALDH1A2, ARG2, CCL24, CCL26, CD1B, CD1C, CEBPB, CLEC7A, CTLA2A, CYSLTR1, <u>IL4</u>, IL13, INSR, ISG20, LTA4H, MAOA, Retmla, SPRR2B, STAT4, SYNGR2, TFE3, Trim30</i>	19	Immune response, cell-to-cell signaling and interaction, hematologic system development and function
3	<i>AP1S1, APOC2, ARHGEF7, DNASE1L3, EHD1, EIF4F1, GSTM1, HIST1H1C, MMP7, SNAP29, TFAM, <u>TIMP2</u>, TLE4</i>	<i>AP1B1, AP1M1, ARL6IP, CIDEA, COL14A1, H2AFZ, HIPK1, HMG2, KRT15, MT1L, MYC, MYO9B, PDRG1, PLF2, PRKRIR, SLK, TAGLN2, TMSB4x, <u>TNE</u>, TP53, UBE2S</i>	19	Cell death, immunologic diseases, DNA replication, recombination, and repair
4	<i>BF, CEACAM7, DDX11, GDF2, GRIP1, LRMP, PIK3R1, <u>POSTN</u>, RAB27A, RNASEL, SWAP70, TRPC7</i>	<i>ABCG2, ANXA3, C12orf8, CSPG3, EGF, ESR1, FNTA, FSTL1, GJB2, HBP1, <u>HRAS</u>, HSPB8, Ims1, MYOD1, PFKFB1, PFKM, PPIB, RB1, RPS6KB2, SH2D3A, SYT3, TOM1L1, TRPC6</i>	17	Cell cycle, connective tissue development and function, cell morphology

Focus genes denote input genes assigned to the calculated networks.

Predicted genes are genes predicted for involvement in the calculated networks.

The most significant gene in the network was underlined.

Significant support vector machine classifiers periostin (POSTN) and TIMP-2 are shown in boldface italic.

### Increased Expression of IL-4 in Recurrent Pterygia

Of all the genes either assigned or predicted in the networks, periostin was the SVM classifier of recurrence with the highest accuracy.<sup>2</sup> Periostin has recently been implicated as an adhesion molecule or a tissue remodeling mediator in Th2-type cytokine-mediated diseases,<sup>5</sup> 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.<sup>6</sup> Therefore, we hypothesized that IL-4 may contribute to the recurrence process by the induction of gene(s) in the observed networks.

Initially, we used real-time PCR to determine whether the mRNA of IL-4 was transcriptionally induced in pterygia tissues. Analysis of all pterygia cases showed significant induction of IL-4 compared with normal controls (pterygia tissue,  $23.5 \pm 9.3$  relative copies; normal conjunctiva,  $0.7 \pm 0.5$  relative copies/GAPDH;  $P < 0.05$ ). Because tissue remodeling is a critical phenomenon in pterygia recurrence, we next examined whether IL-4 may indeed be induced after recurrence. In recurrence cases, the IL-4 mRNA levels were made worse than the primary cases (Fig. 1). In network 2, another Th2 type cytokine, IL-13, was detected. Therefore, we also examined the IL-13 mRNA levels. In contrast to IL-4, IL-13 was reduced in recurrent cases though the reduction was not statistically significant (primary pterygia,  $2.3 \pm 2.1$  relative copies; recurrent pterygia,  $0.07 \pm 0.06$  relative copies/GAPDH), further supporting an important role of IL-4 in network 2.

Immunohistochemical analysis was performed to confirm that IL-4 was translated in pterygia tissues. Consistent with the transcriptional analysis, IL-4 was prominently expressed in the recurrent tissue and localized mainly in the perivascular tissues and endothelial cells in the stroma of the subconjunctiva (Fig. 1B). Inflammatory cells, including macrophages or lymphocytes, also stained positive for IL-4 and were found focally in

the pterygia epithelia. In primary cases, IL-4 was barely detected.

### Induction of Periostin by IL-4

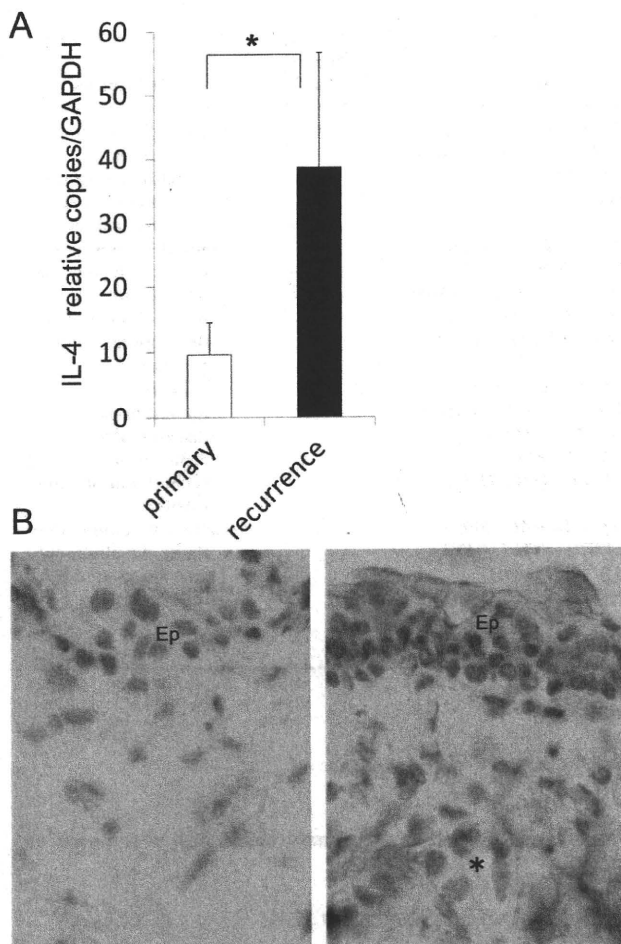
To determine the roles played by IL-4 in the constructed networks, we tested whether IL-4 could induce the recurrence classifier, periostin, using pterygia-derived fibroblast cells. Recombinant IL-4-stimulated fibroblasts were examined for periostin mRNA using real-time PCR. Our results showed that IL-4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

For the IL-4-activated genes, ERK1/2-mediated MAP kinase cascade is generally used.<sup>7</sup> 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 pterygia-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-mediated



**FIGURE 1.** Differential expression of IL-4 in primary and recurrent pterygia tissues. (A) Recurrent tissues shows significantly more periostin transcript ( $P < 0.05$ ;  $n = 11$  eyes for primary tissue;  $n = 10$  eyes for recurrent tissue). (B) Localization of IL-4 in primary and recurrent pterygia by immunohistochemistry. Upregulated IL-4 expression (brown) in epithelium and perivascular tissues of recurrent pterygia (right) compared with primary pterygia (left). Asterisks: inflammatory cells stained positive. Ep, conjunctival epithelium. Original magnification,  $\times 1000$ .

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

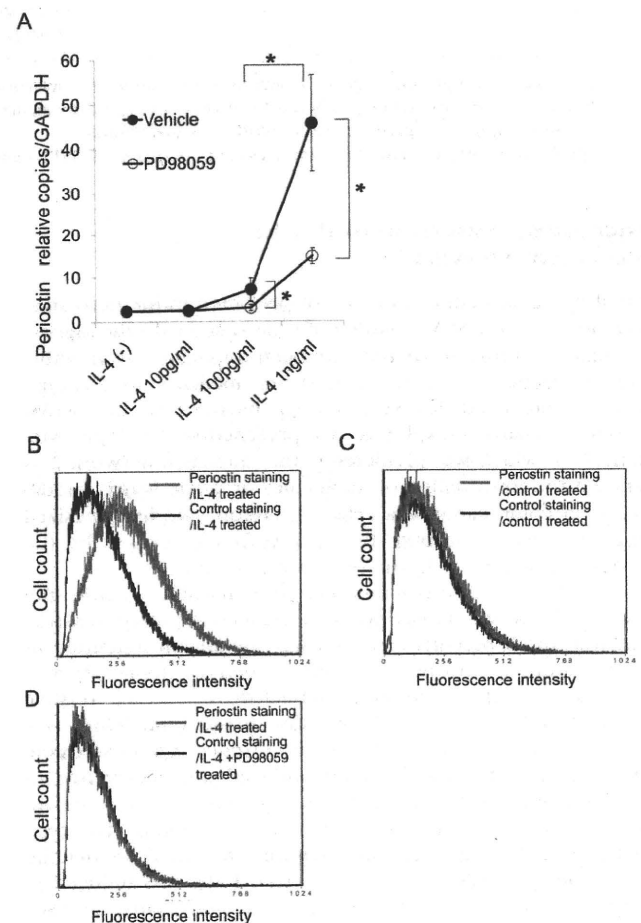
In pterygia tissue, extracellular matrix protein and metalloproteinases (MMPs), including collagen types III and IV, laminin, fibronectin, and MMPs 8, 9, and 13, are abundantly expressed.<sup>8</sup> During the recurrence process, the deposition of such ECM proteins continues and is accompanied by tissue-remodeling 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

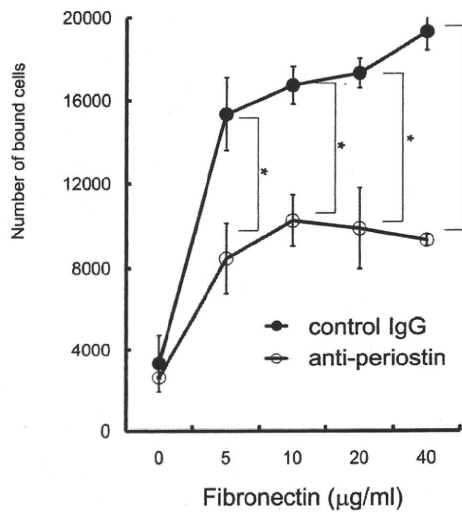
plays an important pivotal role in inducing ECM deposition and subepithelial fibrosis and in exacerbating airway hyperresponsiveness, eosinophil infiltration, and mast cell activation.<sup>5,9-12</sup> However, we observed IL-13 transcripts were greatly reduced, though the reduction was not statistically significant. Because IL-13 is known to compete for IL-4 binding,<sup>13</sup> 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.<sup>14-16</sup> 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).<sup>17</sup> 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



**FIGURE 2.** Periostin induction of pterygia fibroblast by IL-4. (A) IL-4 treatment induced periostin mRNA in a dose-dependent manner at a range of 0 to 1 ng/mL IL-4. Treatment with MEK 1 inhibitor PD98059 (10  $\mu$ M) significantly suppressed IL-4 induction.  $*P < 0.05$ ;  $n = 3$ . (B-D) Periostin protein expression by IL-4 was examined by flow cytometric analysis. Periostin was induced on pterygia fibroblasts by IL-4 (1 ng/mL) (B). No detectable expression of periostin was observed on nonstimulated fibroblasts (C). PD98059 (10  $\mu$ M) inhibited the expression of periostin (D). Data are representative of repeated experiments.



**FIGURE 3.** Requirement of periostin for fibronectin-mediated adhesion of pterygia fibroblast. Adhesion assay was used to examine the adhesive property of fibroblast to fibronectin-coated wells. Pterygia fibroblasts show fibronectin-dose dependent adhesion. Antiperiostin treatment significantly inhibited fibronectin-mediated adhesion. \**P* < 0.05; *n* = 3. Data are representative of repeated experiments.

psoriatic tissue and T lymphocytes.<sup>18</sup> 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.<sup>19</sup>

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  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins and stimulates a fibronectin-dependent motility of epithelial cells.<sup>20,21</sup>

Recently, periostin has been gaining considerable interest in the process of cardiac remodeling or heart failure.<sup>22-24</sup> Periostin associates with critical ECM regulators such as TGF- $\beta$ , tenascin, and fibronectin and is a critical regulator of fibrosis by altering the deposition and attachment of collagen.<sup>25</sup> Because fibronectin was listed as the top induced gene in the pterygia transcriptome,<sup>17</sup> 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*,<sup>7</sup> 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.

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# Induction of IL-6 in Transcriptional Networks in Corneal Epithelial Cells after Herpes Simplex Virus Type 1 Infection

Yuki Terasaka, Dai Miyazaki, Keiko Yakura, Tomoko Haruki, and Yoshitsugu Inoue

**PURPOSE.** To determine the transcriptional responses of human corneal epithelial cells (HCECs) after herpes simplex virus type (HSV)-1 infection and to identify the critical inflammatory element(s).

**METHOD.** Immortalized HCECs were infected with HSV-1, and the global transcriptional profile determined. Molecular signaling networks were constructed from the HSV-1-induced transcriptomes. The relationships of the identified networks were confirmed by real-time-PCR and ELISA. Contributions of the critical network nodes were further evaluated by protein array analyses as candidates for inflammatory element induction.

**RESULTS.** HSV-1 infection induced a global transcriptional response, with 412 genes significantly activated or suppressed compared with mock-infected HCECs ( $P < 0.05$ ,  $2 < \text{or } 0.5 >$  threshold). Infection by UV-inactivated HSV-1 did not induce significant transcriptional activity. Network analysis showed that the HSV-1-induced transcriptomes were associated with JUN N-terminal kinase, p38, extracellular signal-regulated kinase, and nuclear factor  $\kappa$ -B signaling pathways. These findings indicate that interleukin (IL)-6 and vascular endothelial growth factor (VEGF) probably serve as critical nodes of signaling events. ELISA and protein array analyses verified the induction of the inflammatory elements by HSV infection. Blocking the induction of IL-6 significantly reduced the expression of 21 cytokines, including CCL7, CCL8, CXCL6, transforming growth factor- $\beta$ 2, platelet-derived growth factor, interferon- $\gamma$ , IL-2, and VEGF, thus confirming the critical role of IL-6.

**CONCLUSIONS.** HCECs respond to HSV-1 infection by initiating mitogen-activated protein kinase-related transcriptional events, and IL-6 may serve to induce expression of an array of inflammatory mediators. (*Invest Ophthalmol Vis Sci.* 2010;51:2441-2449) DOI:10.1167/iovs.09-4624

Infection of corneal epithelial cells (CECs) by herpes simplex virus type (HSV)-1 can progress to blindness. The prompt use of anti-HSV-1 medications effectively arrests the initial viral infection and replication; however, the disease can still progress to a vision-threatening stage via a secondary wave of inflammatory responses. These responses are mediated by anti-HSV-1 factors or autoimmune responses induced by molecular

mimicry.<sup>1-3</sup> Herpetic stromal keratitis (HSK) is exacerbated by frequent reactivation of latent HSV-1 in the trigeminal ganglia, which can eventually lead to severe corneal opacity that requires corneal transplantation.<sup>4-6</sup>

The CECs are the primary target of HSV infections, and they serve as an innate barrier to deeper invasion before the development of acquired immunity. The CECs respond immediately to HSV exposure by releasing inflammatory mediators that recruit leukocytes, including neutrophils and macrophages. The primary responses are needed to establish T-lymphocyte-based acquired immunity. Thus, understanding how CECs respond to HSV infection is important for understanding how the eye reacts to a viral invasion.

CECs react to HSV-1 infection by expressing proinflammatory cytokines, including interferon (IFN)- $\beta$ , interleukin (IL)-6, tumor necrosis factor (TNF)- $\alpha$ , and IL-8.<sup>7-9</sup> The expression of IL-6, TNF- $\alpha$ , and IL-8 recruits neutrophils and mononuclear lymphocytes and activates their antiviral activity. This first wave, if uncontrolled, leads to corneal neovascularization, which further exacerbates the inflammatory responses and subsequently reduces vision.<sup>10-12</sup>

HSV-1 infections activate signal transduction, including the nuclear factor  $\kappa$ -B (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK) cascades.<sup>9,13-15</sup> These signaling events most likely result in the release of inflammatory mediators. However, the cellular inflammatory responses differ, depending on the type of host cells and species. HSV-1 infections alter the transcriptional responses of the host which leads to a global suppression of transcriptional events.<sup>3,16</sup>

Several hypothesis-based transcriptional analyses have been conducted that have provided considerable information. However, a global view of the responses of CECs as natural hosts to HSV-1 infection is lacking. Understanding how epithelial cells respond to HSV-1 in the perspective of the whole genome would help establish more efficacious antiviral therapy and management.

The purpose of this study was to gain insight into the complex intertwined biological events in CECs that follow an HSV-1 infection. A bioinformatics-based network analysis of global transcriptional responses was used along with a pathways database of molecular interactions.<sup>17</sup> Our data describe the critical roles of IL-6 in the HSV-1-induced MAPK cascade-related elements.

## MATERIALS AND METHODS

### Cells

A human corneal epithelial cell (HCEC) line was kindly provided by Kaoru Araki-Sasaki<sup>18</sup> (RIKEN BioResource Center, Tsukuba, Japan). The HCECs were propagated to confluence on 6- or 96-well plates in DMEM (Dulbecco's modified Eagle's medium; Invitrogen-Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and used at passages 4 to 6.

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## Viruses

Confluent monolayers of Vero cells were infected with HSV-1 (KOS strain; the generous gift of Kozaburo Hayashi, Immunology and Virology Section, Laboratory of Immunology, National Eye Institute, Bethesda, MD). The KOS strain was used, because it expresses VEGF, leading to corneal neovascularization, when inoculated onto the mouse eye.<sup>10</sup> After 1 hour of adsorption, the medium containing the virus was aspirated, and the monolayers were refed with fresh HSV-1-free medium. At maximum cytopathic effect, the medium was discarded, and the cells were frozen, thawed, and sonicated in a small amount of remaining medium. The supernatant, collected after centrifugation at 3000 rpm for 10 minutes, was overlaid onto sucrose density gradient (10%–60% wt/vol) and centrifuged with a swing rotor (SW28; Beckman, Fullerton, CA) for 1 hour at 11,500 rpm. The resultant visible band at a lower part of the gradient containing HSV-1 was washed, with centrifugation at 14,000 rpm for 90 minutes, and resuspended in a small volume of serum-free DMEM. The virus was then aliquoted and stored at  $-80^{\circ}\text{C}$  until use. The infectivity of the virus was determined by plaque titration assay, typically reaching up to  $1 \times 10^9$  PFU/mL.

For HSV-1 infection, the HCECs adsorbed the sucrose-density gradient-purified virus stock for 1 hour and then were refed with fresh medium.

## Microarray Procedures

HSV-infected HCECs were transcriptionally analyzed by using the whole human genome microarray (Agilent Technologies, Santa Clara, CA) corresponding to 41,000 human genes and transcripts. Mock-infected, UV-inactivated, HSV-infected HCECs were used as the control. Total RNA was isolated from the HSV-infected HCECs 12 hours post infection (PI) (RNeasy Mini Kit; Qiagen, Hilden, Germany), according to the manufacturer's protocol.

Cyanine-3-labeled cRNA was prepared from 0.25  $\mu\text{g}$  of RNA (One-Color Low RNA Input Linear Amplification PLUS kit; Agilent). Fragmented cRNA was hybridized to the whole human genome oligo microarray (G4112F; Agilent) by using a hybridization kit (Gene Expression Hybridization kit, G2545A; Agilent) and was scanned with a microarray scanner (G2565BA; Agilent). The acquired data were bioinformatically analyzed (GeneSpring GX 10; Agilent), and ANOVA was used to extract the genes that were differentially induced or suppressed after HSV infection.

## Pathways Analysis

The set of extracted genes was analyzed for transcriptional networks of molecular events (Ingenuity Pathways Analysis 7.0, IPA; Ingenuity Systems, Redwood, CA, computer program based on the Ingenuity Pathways Knowledge Base). The resulting networks were evaluated by the significance scores, which were expressed as the negative logarithm of the probability. The obtained score indicates the likelihood that the assembly of a set of focus genes in a network can be explained by chance alone.

## Real-Time RT-PCR

Total RNA was isolated from the HSV-infected HCECs and reverse transcribed (QuantiTect Reverse Transcription Kit; Qiagen). The cDNAs were amplified and quantified on a thermocycler (LightCycler; Roche Applied Science, Mannheim, Germany, with the QuantiTect SYBR Green PCR kit; Qiagen).

The sequences of the real-time PCR primer pairs were: VEGF: forward 5'-GCAGCTTGAGTTAAACGAACG-3', reverse 5'-GGTCCCGAAACCCTGAG-3'; IL-6: forward 5'-GATGAGTACAAAAGTCTCTGATCCA-3', reverse 5'-CTGCAGCCACTGGTTCTGT-3'; IFN- $\alpha$ 1: forward 5'-GGAGTTTGATGGCAACCAAGT-3', reverse 5'-CTCTCCTCTGCATCACACA-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'-AGCCACATCGCTCAGACAC-3', reverse 5'-GCCCAATACGACCAAAATCC-3'.

Equal loading and amplification were ensured by normalizing all products to the GAPDH transcript as an internal control.

## ELISA

The levels of secreted IL-6 and VEGF was determined by assaying supernatants collected from HSV-infected HCECs with commercial ELISA kits (Peprotech, Rocky Hill, NJ). Anti-human IL-6 antibody (clone: MQ2-13A5, Biolegend, San Diego, CA) was used to neutralize IL-6 activity.

## Cytokine Array Analysis

For inflammatory cytokine profiling after HSV infection, supernatants were collected from HCECs 12 hours PI and assayed with a protein array system (Human Cytokine Antibody Array; RayBiotech, Norcross, GA). This system determines the level of expression of 80 cytokines. The intensity of the chemiluminescence signals was digitized (LAS-1000plus; Fujifilm, Tokyo, Japan, and MultiGauge software ver.2.0; Fujifilm) and normalized by using the positive control signals in each membrane.

## Statistical Analyses

Data are expressed as the mean  $\pm$  SEM. Statistical analyses were performed by using *t*-tests or ANOVA, as appropriate.

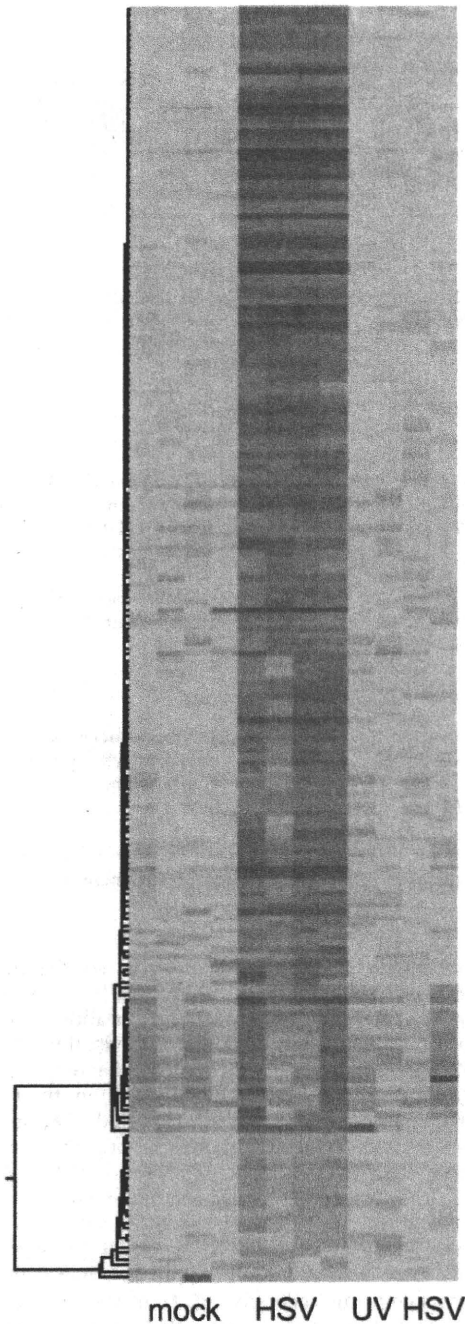
## RESULTS

### Microarray Analysis of HSV-Infected HCECs

To dissect the transcriptional responses of HCECs to HSV-1 infection, we first analyzed the IFN responses of HCECs by using real-time PCR. After HSV-1 infection, the level of IFN- $\alpha$ 1 transcript was significantly increased at 12 hours PI—that is, the IFN- $\alpha$ 1 expression relative to GAPDH was  $2.7 \pm 0.2$  (relative copies) at multiplicity of infection (MOI) 1 of HSV-1 and  $1.6 \pm 0.1$  (relative copies) for mock infection ( $P < 0.01$ ). The level was increased at 24 hours PI to  $31.3 \pm 7.7$  (relative copies) IFN- $\alpha$ 1/GAPDH at MOI 1 of HSV-1 and  $2.9 \pm 0.5$  (relative copies) for mock infection ( $P < 0.05$ ). At 6 hours PI, no appreciable increase was observed. These findings indicated that the response to the HSV infection occurred at 12 hours PI and increased thereafter.

Next, we conducted a global transcriptional profiling of HSV-infected HCECs by microarray analysis of HSV-infected, mock-infected, and UV-inactivated HSV-infected HCECs at the end of the inflammatory responses. We identified 13,594 genes that were differentially expressed in HSV-infected cells at 12 hours PI (ANOVA;  $P < 0.05$ ). To extract sets of virus-responsive genes, we then applied ANOVA with the threshold of twofold expression changes. This analysis resulted in the detection of 412 genes with significantly different expression in the three groups ( $P < 0.05$ ); 365 genes were upregulated and 47 were downregulated in the HSV-infected HCECs.

These genes were analyzed for hierarchical clustering. The resulting dendrograms showed that most of the genes were upregulated after HSV infection (Fig. 1). HSV-infected HCECs showed a distinctive expression profile, in contrast with barely discernible profiles in mock- or UV-inactivated HSV-treated HCECs. The upregulated genes at the highest ratio were *RAS*, dexamethasone-induced 1 (*RASD1*), family with sequence similarity 90, member A10 (*FAM90A10*), *LOC387763*, *FLJ00049*, v-maf musculoaponeurotic fibrosarcoma oncogene homologue A (*MAFA*), and growth arrest and DNA-damage-inducible, gamma (*GADD45G*), among the annotated network-eligible genes (Supplementary Table S1, <http://www.iovs.org/cgi/content/full/51/5/2441/DC1>). Of these, *RASD1*, *MAFA*, and *GADD45G* generally represent involvement of stress-induced pathways, including the MAPK cascade.



**FIGURE 1.** Clustering analysis of HSV-1 infection-induced transcriptome in an HCEC line. Four hundred twelve differentially expressed genes (ANOVA;  $P < 0.05$ ,  $2 < \text{or } 0.5 >$  threshold, 12 hours PI) were analyzed by using hierarchical clustering. Gene direction analysis showed that HSV-1 KOS strain infection, but not UV-inactivated HSV-1 infection, induced transcriptional alteration at 12 hours PI. The expression levels are color coded (red, activated; green, suppressed).  $n = 4/\text{group}$ .

The downregulated genes at the highest ratio were short-chain dehydrogenase/reductase family 42E, member 1 (HSPC105), zinc finger-BED-type containing 2 (ZBED2), and kelch-like 24 (KLHL24). In the HCECs exposed to UV-inactivated HSV, the transcriptional profile was almost identical with mock-infected HCECs, with the exception of 11 genes that were differentially expressed.

**Network Analysis of Altered Genes in HSV-Infected HCECs**

To obtain a global view of the HSV infection-induced phenomenon and determine therapeutic candidate inflammatory mediator gene(s) for herpetic keratitis, we analyzed the 412 extracted genes for signaling interactions by using a systematic biological approach. We successfully generated five major biological networks with high significance scores ( $P < 10^{-26}$ ) using a database of known signaling networks (Ingenuity Pathways Knowledge Base; Ingenuity Systems; Table 1).

Network 1 was the most significant network of focus genes: FOS, JUN, BCL2-associated X protein (BAX), and zinc finger protein 36 (ZFP36). Network 1 included genes annotated as cell cycle, cell death, and neurologic diseases. Network 2 contained those annotated as cancer, cellular growth and proliferation, and respiratory diseases, and were represented by the focus genes GADD45 and dual specificity phosphatase 1 (DUSP1), which are related to stressful GADD. These were classified as MAPK/extracellular signal-regulated kinase (ERK) cascade by network analysis. In this network, the upstream inflammatory mediators PDGF, CXCL1, and insulin-like growth factor (IGF)-2 were also upregulated.

Network 3 contained genes annotated as skeletal and muscular system development and function, cancer, and cell-to-cell signaling and interaction, and involved the upregulation of the arachidonic acid cascade mediator, phospholipase A2 (PLA2), and a transcriptional activator downstream of  $Ca^{2+}$  or cyclic AMP (cAMP) responsive element binding protein (CREBBP). Network 4 included genes annotated as connective tissue disorders, genetic disorders, and immunologic diseases, with upregulation of the inflammatory cytokines CCL5 and CXCL2, in addition to IL-6 induction.

Network 5 genes were annotated as cell cycle and skeletal and muscular system development and function and cancer, characterized by the induction of VEGF, endothelin 1 (EDN1), connective tissue growth factor (CTGF), heparin binding EGF-like growth factor (HBEGF), and cyclooxygenase (PTGS2).

Of the five signaling networks, we identified a proinflammatory focus gene, IL-6 in network 4, as the most significant canonical inflammatory mediator in terms of the number of interactions in the signaling networks. Because IL-6 generally serves as a critical inflammatory coordinator downstream of the pattern recognition receptors, which serve as a first line of defense against pathogens, we then analyzed the HSV-induced transcriptional networks in relation to IL-6.

To understand the transcriptional roles of IL-6 in the constructed networks, we merged networks 1 to 5 to give an overview (Fig. 2). Because IL-6 was centrally positioned in this view of the networks, we next explored whether it orchestrates the induction of the inflammatory mediators. In the merged network, we observed VEGF as a crucial node. VEGF has gained the interest of researchers for its involvement in corneal neovascularization at the later stage of HSK, and the neovascularization is typically followed by the establishment of epithelial lesions.<sup>10</sup> Therefore, we hypothesized that there is a direct relationship of IL-6 and VEGF in the epithelial transcriptome.

**Inductive Effect of IL-6 on VEGF in HSV-Infected HCECs**

We first determined whether IL-6 is expressed in HSV-infected HCECs by using real-time PCR. IL-6 was detected as early as 3 hours PI, and the level peaked at 12 hours (Fig. 3A). To confirm that IL-6 is translated, we used ELISA to assay the supernatants collected from the HSV-infected HCECs at 12 hours. Secreted IL-6 was elevated in HSV-infected HCECs in a dose-dependent manner, and the level increased until 24 hours PI (Fig. 3B). At

TABLE 1. Transcriptional Networks of HSV-Infected Corneal Epithelial Cells

Network	Focus Genes	Predicted Genes	Score = -log (P)	Functions
1	<i>ARF1, BAX, BCL2L11, Calpain, CAPN1, CCNG2, CD274, CDKN1C, CRABP1, EEF1D, FOS, GALNT11, GRASP, ID2, ING1, JUN, JUNB, MGEA5, MYCN, NFYA, OSBP2, PDCD1, PDCD4, SPRY4, VIM, ZFP36</i>	Caspase, Cyclin A, Cytochrome C, FGF, Hexokinase, Proteasome, Rb, Smad, Ubiquitin	46	Cell cycle, cell death, neurological disease
2	<i>ADM, CXCL1, DUSP1, DUSP4, DUSP6, IER2, GADD45, GADD45A, GADD45B, GADD45G, IGF2, JUN/JUNB/JUND, KLF2, MKP1/2/3/4, MXD1, THBD, Thyroid hormone receptor, PDGF-AB, PDGF-AA, PDGFA, PHLDA1, PNRC1, RASD1, VAV3</i>	ERK, GC-GCR dimer, JINK1I2, Laminin, N-cor, Notch, <i>Pa</i> , <i>Rar</i> , <i>Rxr</i> , <i>SWI-SNF</i> , VitaminD3-VDR-RXR	29	Cancer, cellular growth, and proliferation, respiratory disease
3	<i>ATAD4, BMF, Cbp/p300, CBX4, CREBBP, DLX2, EPHA4, GABARAPL1, MED26, MLL, MSX1, MYLK2, NOC2L, OSGINI, PDXK, PLA2, PLA2G6, SENP3, WISP2</i>	Actin, Calmodulin, <i>Ck2</i> , <i>ERK1I2</i> , <i>FSH</i> , Histone h3, Histone h4, Hsp70, <i>MAPK</i> , <i>Pka</i> , <i>Pld</i> , <i>PP2A</i> , <i>RGS2</i> , RNA polymerase II, <i>STAT5a/b</i> , Tubulin	29	Skeletal and muscular system development and function, cancer, cell-to-cell signaling and interaction
4	<i>CARD9, CCL5, CDKN2C, CXCL2, Cyclooxygenase, DUSP2, IER3, IFN-<math>\beta</math>, IL6, IRF9, MUC2, ND2, PIM2 (includes EG:11040), RGS16, RSAD2, SELPLG, SOCS1, TLR, TLR1, TNFAIP3</i>	<i>ALP</i> , Hsp27, <i>IFN-<math>\alpha</math></i> , <i>IFN-g</i> , <i>IgG</i> , <i>IL1</i> , <i>IL12</i> , <i>IRF</i> , <i>JAK</i> , <i>LDL</i> , MHC Class I, <i>NF-<math>\kappa</math>B</i> , <i>STAT</i> , <i>TGF-<math>\beta</math></i>	27	Connective tissue disorders, genetic disorder, immunological disease
5	<i>CTGF, CXCR4, DLL1, DUSP8, EDN1, EREG, ERN1 (includes EG:2081), MBTPS1, MYH3, Myosin, OASL, PTGS2, SNAI1, SP100, SYNJ1, THRA, VEGF</i>	<i>ADCY</i> , Calcineurin protein(s), G-protein- $\beta$ , <i>HBEGF</i> , <i>HCG</i> , <i>Ige</i> , <i>IKK</i> , <i>JNK</i> , <i>Me</i> , <i>MMP</i> , <i>Nos</i> , <i>p38</i> , <i>MAPK</i> , <i>p70 S6k</i> , <i>Pkc(s)</i> , <i>PLC</i> , <i>PLC-g</i> , Tyrosine kinase	26	Cell cycle, cancer, skeletal and muscular system development and function

Focus genes denote assigned input genes to the calculated networks. Predicted genes are genes predicted for involvement in the calculated networks.

12 hours PI, VEGF secretion was also significantly elevated in the HSV-infected HCECs (Fig. 4A). UV-inactivated HSV did not stimulate IL-6 and VEGF secretion (data not shown).

To examine the role played by IL-6, we evaluated HSV-infected HCECs for VEGF induction after blocking IL-6 with anti-IL-6 antibody (Fig. 4A) and found that a significantly lower level of VEGF was secreted from the infected HCECs. To determine whether the reduced VEGF level was due to a blockage of virus replication, we used real-time PCR<sup>19</sup> and a plaque assay to quantify the HSV-1 genome. Replication of the HSV-1 genome was not significantly affected by IL-6 blockade (cells control treated at MOI 0.1 yielded  $9.2 \pm 0.9 \times 10^3$  copies/ $\mu$ L and anti-IL-6-treated at MOI 0.1 yielded  $1.0 \pm 0.2 \times 10^4$  copies/ $\mu$ L,  $P > 0.05$ ). A plaque assay showed that the replication of the HSV-1 genome was not affected by the anti-IL-6 antibody (Fig. 4B). Thus, the decreased VEGF secretion induced by the IL-6 blockade appeared not to be the direct effect of altered HSV replication. In addition, when HCECs were exposed to recombinant IL-6 without HSV infection, they did not secrete VEGF (Fig. 4C). This finding indicates that activation of VEGF expression in HCECs requires both IL-6 and HSV-induced factors.

### Network Analysis of IL-6 and Related Inflammatory Cytokines in HSV-Infected HCECs

The outcome of these experiments (Figs. 3, 4) and the network analyses (Fig. 2) suggests that IL-6 plays a role in HSV-infected HCECs. In general, IL-6 is one of the major physiological mediators of acute phase reactions and is associated with or activates many inflammatory cytokines. Because IL-6 can orchestrate or modulate the inflammatory milieu of HCECs, we

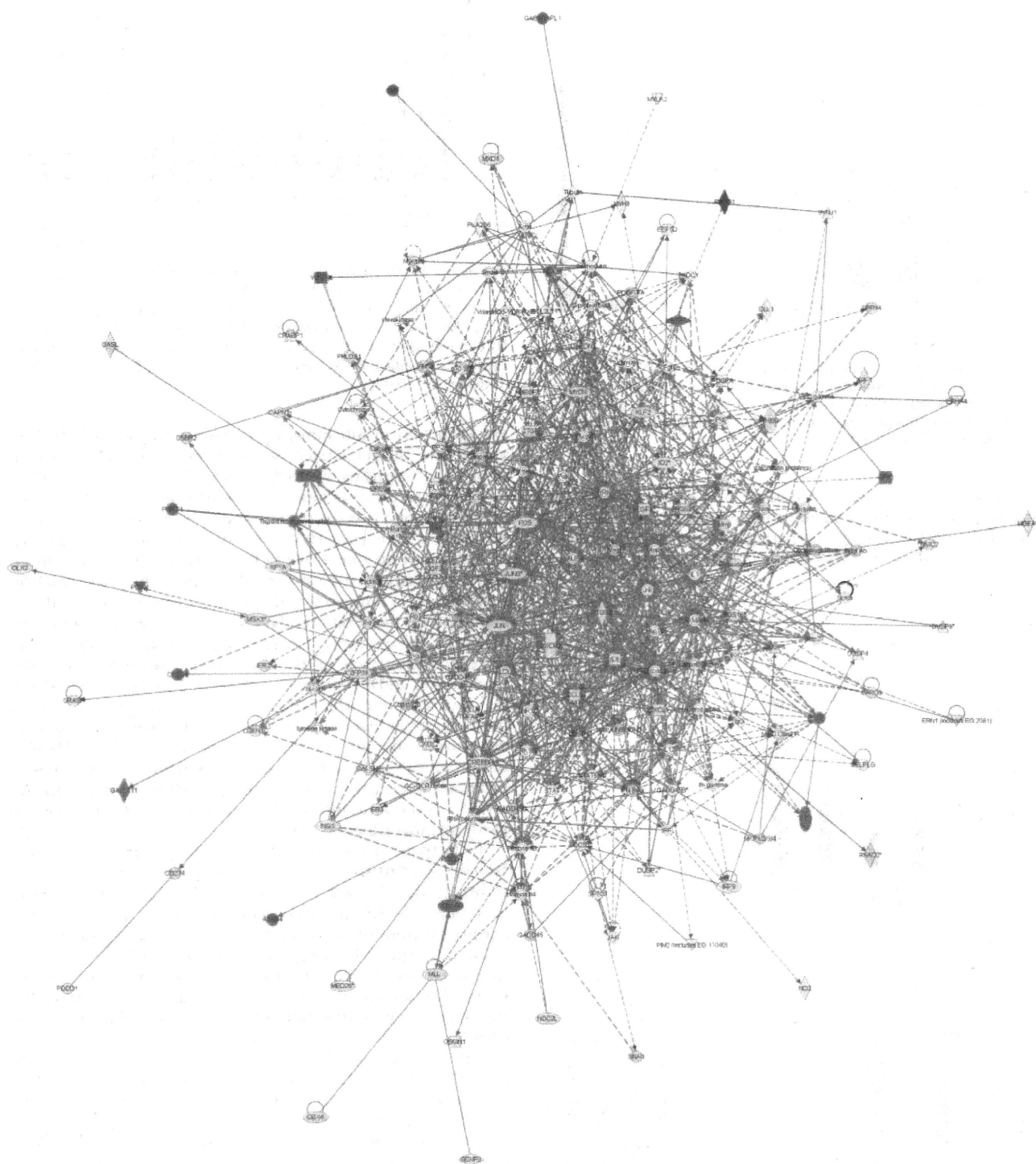
next determined the cytokine species that are dependent on IL-6. HCECs were infected with HSV at MOI 1, allowed 1 hour for adsorption, and then fed control IgG or anti-IL-6 antibody, added to the DMEM. After 12 hours of incubation, the supernatants of HSV-1-infected HCECs were collected and assayed with a cytokine array. After the HSV-1 infection, the release of many inflammatory cytokines, both reported ones and unrecognized ones, was induced. IL-6 was among the top five induced genes after *GRO*, *CCL7*, *IL-6*, *CCL8*, and *IL-8*, in descending order. This result again supports our proposal that *IL-6* plays a critical role in the HSV-induced transcriptome.

When 55 HSV-induced cytokines (normalized relative intensity >1.0) were analyzed for possible IL-6 dependency, we detected a significant reduction of 21 of them after anti-IL-6 treatment, including *CCL7*, *CCL8*, *CXCL6*, *TGF- $\beta$ 2*, and *PDGF* in descending order ( $P < 0.05$ ; Fig. 5). Consistent with the data in Figure 4, we confirmed an IL-6 dependence of VEGF.

To summarize the relationship of IL-6 and the IL-6-sensitive mediators, we further applied network analysis to them. When we used the representative network nodes with the significant edges identified in Figure 2, the interactions of IL-6 and the identified IL-6-sensitive cytokines (Fig. 5) were associated with to the MAPK cascade-related elements.

### DISCUSSION

We used a bioinformatics-based approach to analyze the response of HCECs to HSV-1 infection. Our results showed that HSV infection affected the expression of numerous genes, and most of the mRNAs were transcriptionally activated. This global transcriptional activation was also observed by Ka-

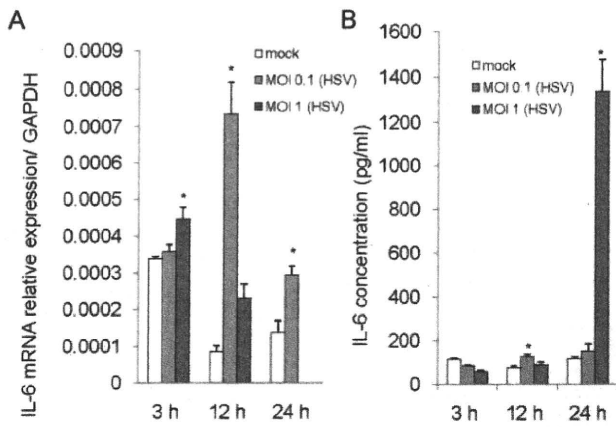


**FIGURE 2.** Pathway analysis of the biological processes underlying the HSV-1 infection-induced responses of HCECs. Networks 1 to 5 form the merged network. *Red*, activated; *green*, suppressed.

makura et al.,<sup>20</sup> who showed that most of the genes in the transcriptome of the HEp-2 epithelial cells were upregulated at 9 hours PI. Their results and our results are in striking contrast to the results of a microarray analyses of nonepithelial permissive cell lines (embryonic lung cells or HeLa),<sup>21-23</sup> which showed most of the cellular transcripts to be downregulated. These changes are considered to be mediated by transcrip-

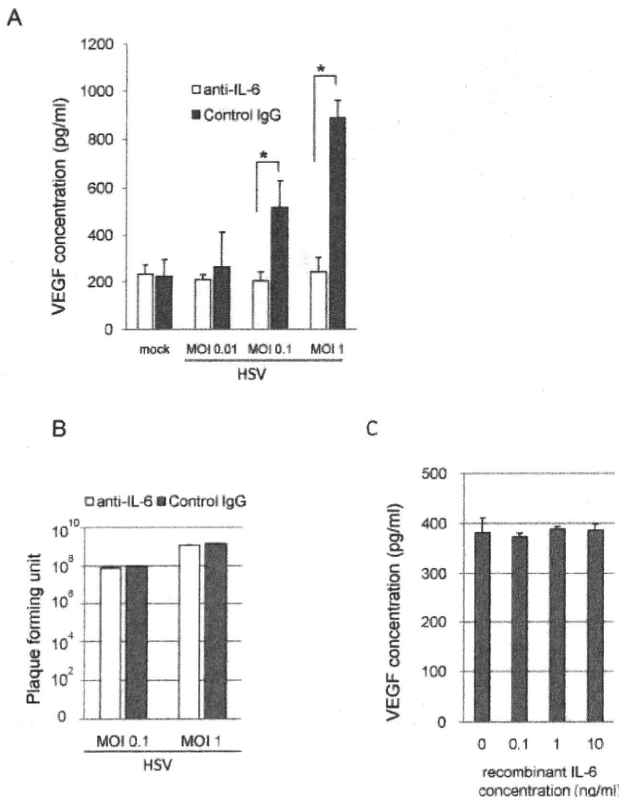
tional suppression of the host genes by viral proteins or immediate early genes.

In epithelial cells, including HCECs, our results and those of Kamakura et al.<sup>20</sup> showed a marked transcriptional upregulation, which may be related to or caused by epithelial-specific factors that act as a primary defense system and initiate the expression of an arsenal of proinflammatory mediators.

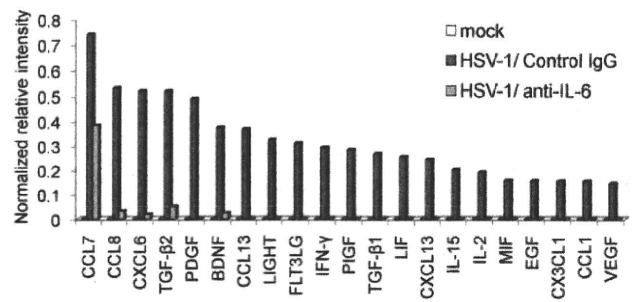


**FIGURE 3.** Kinetics of IL-6 induction in HCECs after HSV-1 infection. The mRNA of IL-6 is significantly induced at 3 hours PI and peaked at 12 hours PI, as determined by real-time RT-PCR (A). The IL-6 level in the infected supernatant was significantly elevated, as determined by ELISA (B). *n* = 6; \**P* < 0.05.

A global view of the HSV-induced host genes by network analysis clearly showed significant involvement of the JNK, p38, ERK, and NF- $\kappa$ B signaling pathways and related elements. Activation of NF- $\kappa$ B and JNK has been shown to be related to



**FIGURE 4.** Requirement of IL-6 and HSV-1 infection for VEGF induction in the HCECs. HSV-1 infection significantly induced VEGF at 12 hours PI which was suppressed by anti-IL-6 treatment (A). The supernatant of HSV-1-infected corneal epithelial cells was assayed for HSV-1 titration. There were no significant differences of virus titers in control IgG and anti-IL-6 treatment (B). Stimulation by recombinant IL-6 without HSV-1 infection failed to induce VEGF production by corneal epithelial cells (C). *n* = 4; \**P* < 0.01.



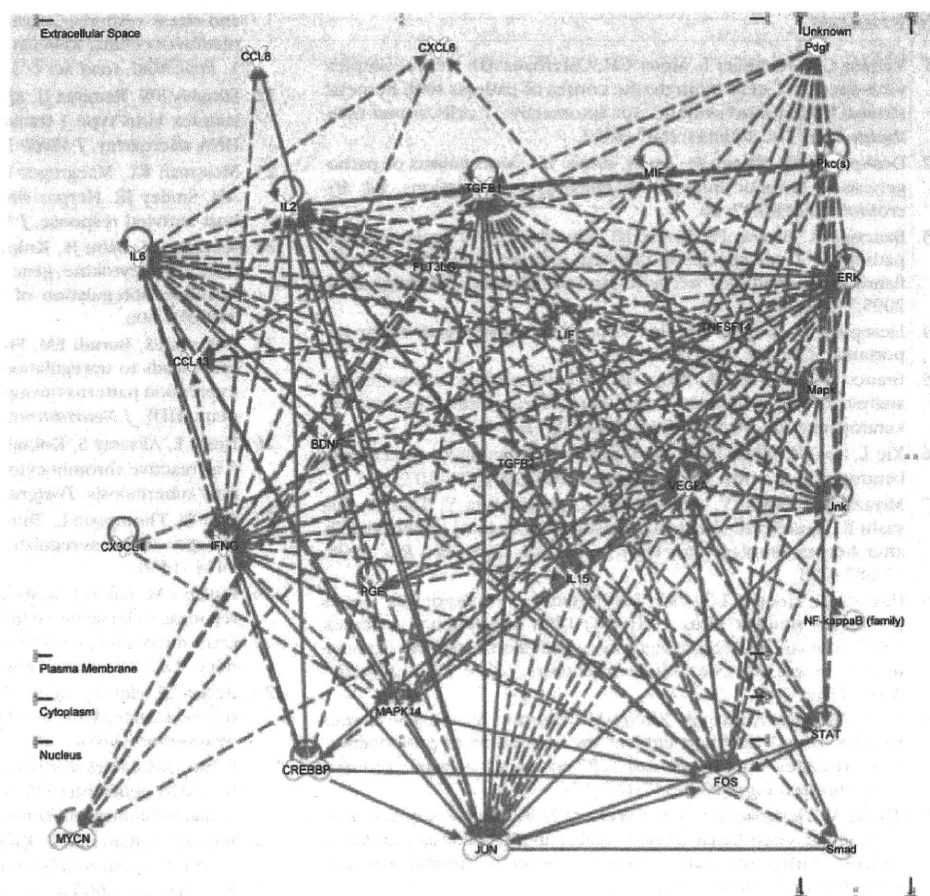
**FIGURE 5.** IL-6-sensitive induction profile of inflammatory cytokines by HSV-1-infected HCECs. HCECs adsorbed HSV-1 at MOI 1 for 1 hour and were reseeded with DMEM containing control IgG or anti-IL-6 antibody. After 12 hours of incubation, the supernatant of HSV-1-infected HCECs was assayed with a cytokine array. A panel of the inflammatory cytokines significantly suppressed by IL-6 blockade is shown. *n* = 4/group; *P* < 0.05.

the induction of inflammatory cytokines including IL-6, IL-8, and TNF- $\alpha$ .<sup>9</sup> In our attempt to understand the molecular relationship of signaling molecules and inflammatory cytokines, network analyses identified IL-6 as the most significant element. Although IL-6 has been noted to be an important inflammatory mediator after HSV infection<sup>24</sup> of epithelial cells, our analyses showed a new role of IL-6 at the whole-genome level. IL-6 is a pleiotropic cytokine and mediates acute phase reaction that influences antigen-specific immune responses.<sup>25,26</sup> IL-6 is an important B-cell differentiation factor as well as a convertor of T cells into cytotoxic T cells or Th17 lineage.<sup>27</sup> Considering the exacerbating role of IL-6 in the inflammatory response, the virulence of an HSV strain may be related to the inducibility of IL-6. For example, the MP strain has been reported to be a more potent inducer of IL-6 than the KOS strain<sup>8</sup> and is able to elicit more aberrant immune responses in the eye. Another important property of IL-6 is its neurotrophic function, which promotes neuronal survival.<sup>28</sup> The promotion of neuronal survival by IL-6 may induce ocular reactivation of latent HSV-1 in the trigeminal ganglion.<sup>29</sup>

In herpetic keratitis, IL-6 has been documented to be a factor that contributes to the massive neutrophil attraction to the corneal stroma.<sup>8,30,31</sup> Also, IL-6 has been reported to be related to corneal neovascularization via VEGF, another cardinal feature of herpetic keratitis.<sup>8,31,32</sup> The induction of VEGF in herpetic keratitis has been thought to be mediated in a paracrine manner by IL-6-producing bystander populations such as noninfected inflammatory cells.<sup>31</sup> However, the corneal epithelium has not been noted as an autocrine source of VEGF.

Our findings on HCECs' participation in an autoamplifying loop after infection provide a new and important perspective on epithelial function as a host defense mechanism. However, this effect has the potential to lead to HSK. Neovascularization is not usually observed in epithelial keratitis, but our findings suggest that the molecular background of HSK has already been set at an earlier stage of epithelial keratitis.

In the HSV-induced network (Fig. 2), IL-6 has been centrally placed in the transcriptional upregulation of CXCL1, FOS, JUNB, HBEGF, GADD45B, inhibitor of DNA binding 2 (ID2), and ZFP36.<sup>33-35</sup> Of these, FOS, JUNB, and GADD45 respond to environmental stresses by activating the MAPK pathways. FOS and JUN are especially critical transcription factors and are involved in numerous canonical pathways, including signaling by MAPK, acute phase responses, and signaling by the chemokines IL-2/IL-6/IL-17/PDGF, stress-activated protein kinase (SAPK)/JNK, TGF- $\beta$ , nuclear factor of activated T cells, and Toll-like receptor (TLR). FOS and JUN directly regulate the



**FIGURE 6.** Pathway analysis of IL-6-sensitive inflammatory cytokine induction and canonical pathways underlying HSV-1-infection.

critical inflammatory mediators IL-6, VEGF, IL-2, IL-8, and cyclooxygenase (Fig. 2).<sup>33,36-42</sup> When we analyzed the cytokine profiling sensitive to the blockade of IL-6, we found numerous previously unrecognized IL-6-sensitive genes, including *TGF-B2*, *PDGF*, *BDNF*, *CCL13*, *LIGHT*, *FLT3LG*, *IFN-G*, *PIGF*, *TGF-B1*, *IL-15*, *MIF*, and *CX3CL1*, together with previously reported genes, including *CXCL6*, *IL-2*, *CCL1*, *LIF*, *VEGF*, and *BDNF*.<sup>8,27,31,43-47</sup> These interactions were associated with IL-6, IL-2, IFN- $\gamma$ , TGF- $\beta$ 1, and VEGF (Fig. 6) and appeared to intricately regulate each other by interacting with ERK, MAPK, JNK, FOS, JUN, and MAPK14 (Fig. 6).

The induction of IL-6 is generally dependent on the activation of the NF- $\kappa$ B and p38 pathways.<sup>48</sup> HSV infection stimulates transcriptional activation of the NF- $\kappa$ B, CRE, and activator protein (AP)-1 recognition sites in the *IL-6* gene as early as 1 hour to 2 hours PI<sup>9,48,49</sup> and supports the suggestion that IL-6 induction at the very early stage of infection activates inflammatory mediators. However, the triggering mechanism of IL-6 has not been completely determined.

Keratocytes have been shown to express IL-6 after HSV infection in a TLR3- and TLR9-dependent manner without requiring transcriptionally competent HSV.<sup>8</sup> However, TLR9 stimulation requires very high levels of HSV DNA, far exceeding the concentration used in our study. In HSV-infected macrophages, IL-6 expression was shown to be mediated by RNA-activated protein kinase (PKR), which senses the accumulation of viral double-stranded RNA in the infected cells.<sup>48</sup> PKR-mediated IL-6 expression is independent of virion transacting protein 16 (VP16) or immediate early proteins, including infected cell protein (ICP)-0, -4, and -27, and requires transcriptionally competent HSV. This finding is consistent with our observation of defective transcriptional activation in UV-inac-

tivated HSV at the whole-genome level (Fig. 1). Induction of IL-6 expression in the presence of the HSV-induced transcriptome appears restricted to CECs or keratocytes as host cells.<sup>8,20,50-52</sup> A significant involvement of IL-6, which is characteristic of corneal cells or other host-derived factors, may specifically determine the transcriptional responses dependent on the functionality of the host cells.

Our microarray analysis detected a prominent upregulation of stress-related genes, including *RASD1*, *GADD45G*, and snail homolog 1 (*SNAI1*). In addition, cancer formation-related genes, including *MAFA*, zinc finger protein 296 (*ZNF296*), *IGF-2*, and gastrulation brain homeobox 2 (*GBX2*), among the top activated genes, were upregulated (Supplementary Table S1, <http://www.iovs.org/cgi/content/full/51/5/2441/DC1>).<sup>20,49,51,53</sup> The expression of the *RASD1* and *GADD45* proteins ZNF296 and IGF-2 are generally observed after HSV infection in microarray analyses depending on the cell type and the strain. In contrast, a strong expression of *MAFA* appears characteristic of CECs. *MAFA* belongs to the AP-1 transcription factor family, as do JUN and FOS, and is activated by phosphorylation by the p38 MAPK pathway.<sup>54</sup> It has strong cell-transforming/transactivation capabilities and has recently gained interest in oncogenesis.<sup>55</sup>

To summarize, HSV-infection of HCECs induces an inflammatome relating to transcriptional events involving ERK, MAPK, JUNK, and NF- $\kappa$ B and uses IL-6 as a critical element to regulate proinflammatory cytokine induction.

**Acknowledgments**

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# コンタクトレンズ関連角膜炎

## Contact Lens-Related Infectious Keratitis

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### はじめに

コンタクトレンズ (CL) は全国で約 2,000 万人が使用している。CL の進歩は非常に速く、約 40 年前からハード CL (HCL), 酸素透過性 HCL (RGPCCL), ソフト CL (SCL) (煮沸消毒), 頻回交換 SCL (FRSCL) (過酸化水素, マルチパーパススリューション: MPS), 1 日交換 SCL (1DaySCL), シリコンハイドロジェル SCL (過酸化水素, MPS) と次々に開発, 発売されてきた。また, 最近では乱視矯正 SCL, 老視用多焦点 SCL, カラー SCL, 角膜を大きく見せる SCL など非常に多様化している。現在のシェアは, 1DaySCL, FRSCL がともに約 600 万人で全体の半数以上を占めるといわれている。

CL 装用の最も重篤な合併症は角膜炎である。原因菌としては緑膿菌とアカントアメーバが最も多く、近年それらの急激な増加が問題となっている。緑膿菌の典型例を図 1 に示す。19 歳, 男性で SCL を装用したまま就寝, 2 日後にはこのような強い輪状膿瘍を呈した。緑膿菌の危険なところは急激な悪化である。アカントアメーバの典型例を図 2 に示す。20 歳の男性で 2 週間タイプの FRSCL ユーザーでケアの悪いケースであった。入院治療に 1 カ月以上を要した。アカントアメーバの問題は特效薬がなく治療までに非常に長時間を要することである。

これらの感染法の増加の背景には, SCL ケアの悪さ, CL ケース内の微生物汚染, MPS の弱い消毒効果などがあると考えられる。このような状況から, 日本コンタク

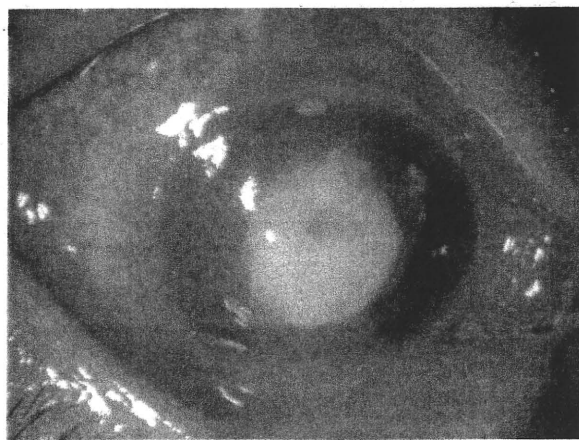


図 1 緑膿菌による角膜炎  
強い輪状膿瘍を認める。

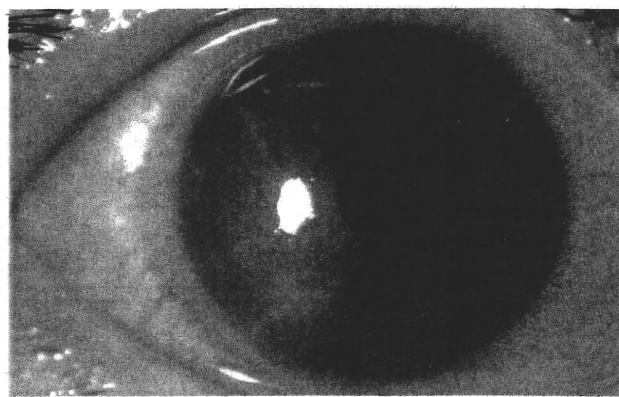


図 2 アカントアメーバ角膜炎  
放射状角膜神経炎を認める。

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トレンズ学会と日本眼感染症学会は共同でCL関連角膜感染症全国調査を行った。また、2009年、国民生活センターがMPSのアカントアメーバに対する消毒効果について報告を行った。これらの結果を踏まえてCL関連角膜感染症の動向について述べる。

### コンタクトレンズ (CL) 関連角膜感染症 全国調査

対象施設は全国224施設で、対象疾患はCL装用が原因と考えられる角膜感染症で入院治療を要した症例である。調査期間は平成19年4月から平成20年3月までの2年間で、担当医と患者へのアンケートを行った。担当医への調査項目は、年齢、性別、発症眼、自覚症状、初診時視力、前眼部所見、塗抹検鏡所見、分離培養結果、治療薬、外科的処置の有無、3カ月後の転帰、3カ月後の視力であった。患者に対するアンケート内容はCLの種類、装用時間、週当たりの装用日数、装用方法、消毒の種類、週当たりの洗浄回数、週当たりの消毒の頻度、こすり洗いの有無、レンズケースの交換頻度、定期検査の頻度、1日ディスプレイCLの使用期間、2週間FRSCLの使用期間、定期交換(1, 3カ月)SCLの使用期間、装用方法遵守の程度、処方された施設、購入

先であった。

#### 1. 対象症例数および性別・年齢

症例数は350例、男性が195例、女性が155例、年齢は9~90歳(平均28歳)であった。

#### 2. 発症眼

感染症の発症は右眼のみが160例(45.7%)、左眼のみが157例(44.9%)、両眼のものが33例(9.4%)であった。

#### 3. 細菌学的検査

塗抹検鏡検査が行われたのは278例(79.4%)であった。検体の採取部位別の結果を表1に示す。角膜病巣の検体からアカントアメーバが68例、グラム陰性桿菌が40例、グラム陽性球菌が20例で確認された。このほか、検鏡陽性数が多かった検体はCLケースで、角膜病巣と同様にアカントアメーバおよび、グラム陰性桿菌が多く検出された。

培養検査は333例(95.1%)で施行されていた。うちなんらかの微生物が検出されたのが228例、陽性率は68.5%であった。検体の採取部位別の結果を表2に示す。

表1 塗抹検鏡

菌種	グラム陽性球菌	グラム陽性桿菌	グラム陰性球菌	グラム陰性桿菌	糸状菌	アカントアメーバ
角膜病巣	20	16	5	40	2	68
結膜囊	2	2	0	3	0	0
眼脂	1	0	0	5	0	0
CL	5	1	2	8	0	6
CLケース	11	9	6	30	3	21
その他	1	0	1	0	0	2

(文献9より)

表2 分離培養にて検出された主要菌

菌種	黄色ブドウ球菌	表皮ブドウ球菌	コリネバクテリウム	緑膿菌	セラチア	その他のグラム陰性桿菌	アスペルギルス	アカントアメーバ
角膜病巣	3	5	6	70	3	4	0	56
結膜囊	1	3	4	3	1	1	0	0
眼脂	0	1	1	8	1	0	0	0
CL	4	2	2	20	5	13	0	4
CLケース	2	3	5	39	17	34	1	32
その他	0	1	0	5	1	3	0	1

(文献9より)

表 3 発症時使用していた CL

	症例数	%
1日ディスプレイザブル SCL	26	7.4
1週間連続装用ディスプレイザブル SCL	4	1.1
2週間 FRSCl	196	56.0
定期交換 (1カ月, 3カ月) SCL	56	16.0
従来型 SCL	9	2.6
カラー CL	17	4.9
ハード CL	17	4.9
オルソケラトロジーレンズ	2	0.6
無回答	23	6.6

(文献9より)

角膜病巣からは緑膿菌が70例, アカントアメーバが56例で同定されていた。その他黄色ブドウ球菌, 表皮ブドウ球菌, セラチアなどもみられたが, いずれも数例程度であった。CL ケースからの検出菌としては緑膿菌39例のほか, セラチア17例, その他のグラム陰性桿菌が34例とグラム陰性桿菌が多数検出されていた。アカントアメーバもCL ケースから32例で検出されていた。なお, 角膜病巣から検鏡・培養のいずれかでアカントアメーバが検出された症例は85例であった。

#### 4. 発症時使用していた CL の種類および装用方法

角膜感染症発症時使用していたCLの種類を表3に示す。FRSClが196例(56.0%)と過半数を占めていた。続いて症例数の多いものとして定期交換SCL 56例(16.0%), 1日ディスプレイザブルCL 26例(7.4%)があげられた。

#### 5. 使用していた消毒薬

CLの消毒薬の種類について, 回答のあった227例のうちMPSを使用していた例が212例(93.4%)であった。このほか過酸化水素が14例(6.2%), 煮沸消毒が1例(0.4%)みられたが, ヨード製剤を用いたという回答はなかった。

#### 6. ケアの方法

CLを外したのち再装用するまでのケアの方法に関し, CLの洗浄, 消毒, こすり洗い, CLケースの交換についてそれぞれ図3~6に結果を示す。CLの洗浄につい

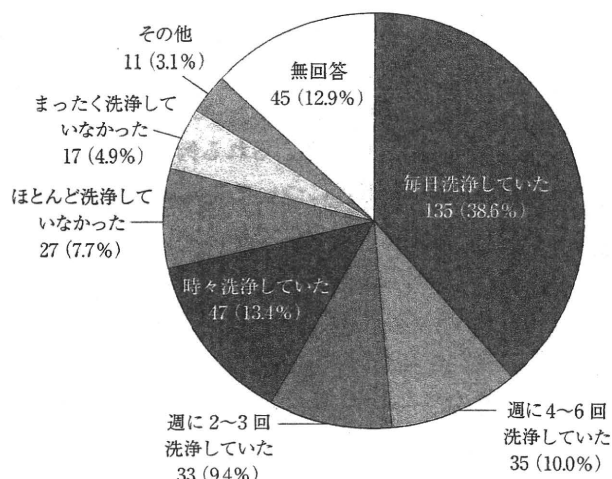


図3 CLの洗浄 (文献9より)

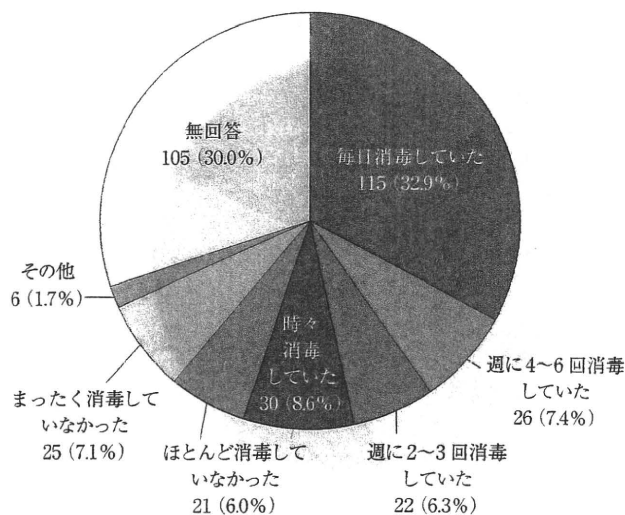


図4 CLの消毒 (文献9より)

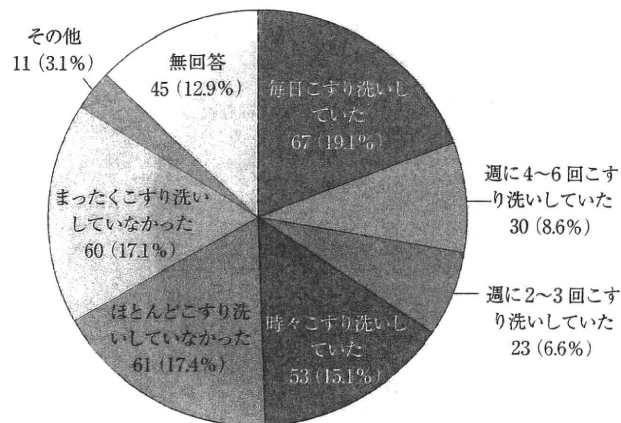


図5 CLのこすり洗い (文献9より)

ては、「毎日洗浄していた」は135例(38.6%)にとどまっていた。「時々洗浄していた」「ほとんど洗浄していなかった」「まったく洗浄していなかった」など、CLの洗浄が十分行われていないと考えられる例も一定数みられていた(図3)。CLの消毒についても洗浄とほぼ同様の結果であった(図4)。CLのこすり洗いについては「毎日こすり洗いしていた」は67例(19.1%)であり、「時々こすり洗いしていた」53例(15.1%)、「ほとんどこすり洗いしていなかった」61例(17.4%)、「まったくこすり洗いしていなかった」60例(17.1%)の3つを合わせると174例(49.7%)にもものぼった(図5)。レンズケースの交換については特に交換までの期間を決めていないものが多く、「不定期に交換していた」「ほとんど交換して

いなかった」「まったく交換していなかった」を合わせると174例(49.7%)と約半数を占めていた(図6)。

### 7. 定期検査

CLの定期検査の頻度について図7に結果を示す。「不定期に受けていた」、「ほとんど受けていなかった」、「まったく受けていなかった」など特定の受診間隔を定めていなかった例が162例(46.3%)を占めていた。

### 8. CLの使用期間

SCLは従来型を除き使用期間が定められている。この使用期間が遵守されているかについてレンズのタイプ別にアンケート結果をまとめた。1日ディスプレイザブルCL装用者26例のうち「1日」と規定どおりの使用期間を守っていたものは12例(46.2%)のみであった(表4)。同様にFRSCLについての結果を表5に示す。症例数の多いFRSCLにおいて「2週間以内」の使用期間であったものは74例であり、回答のあった177例のうち41.8%のみという結果であった。

### 9. CL装用方法の遵守

「CL装用方法を守っていたか?」という総括的な質

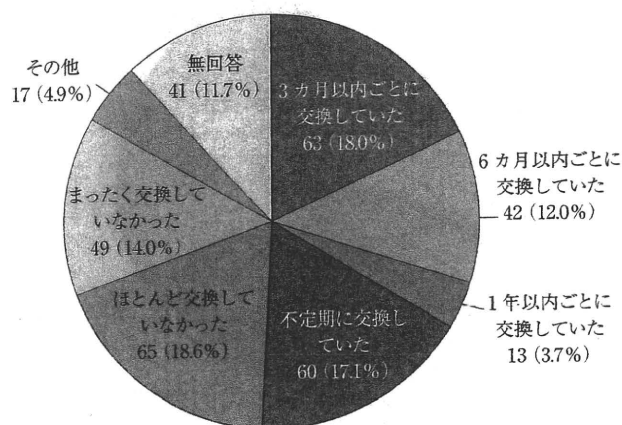


図6 CL ケースの交換 (文献9より)

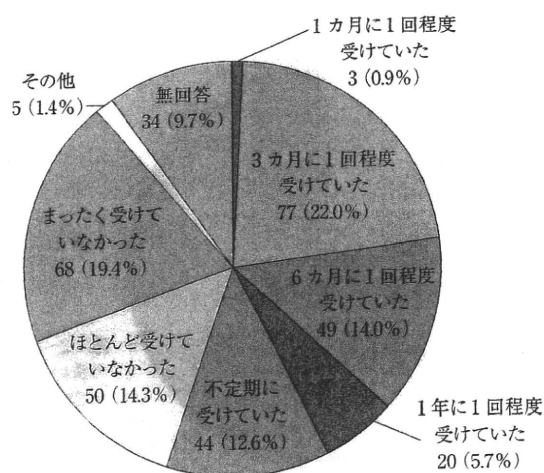


図7 CL の定期検査 (文献9より)

表4 1枚のCL使用期間(1日ディスプレイザブルSCL)

使用期間	症例数	%
1日	12	46.2
2~3日	6	23.1
4~7日	1	3.8
1週間を超え2週間以内	1	3.8
2週間を超え1カ月以内	3	11.5
1カ月を超える	2	7.7
無回答	1	3.8

(文献9より)

表5 1枚のCL使用期間(2W FRSCL)

使用期間	症例数	%
2週間以内	74	37.8
2週間を超え3週間以内	56	28.6
3週間を超え1カ月以内	26	13.3
1カ月を超える	21	10.7
無回答	19	9.7

(文献9より)