cDNA Microarray Analysis of Gene Expression Changes Induced by Dexamethasone in Cultured Human Trabecular Meshwork Cells

Takeshi Isibashi,1 Yasutaka Takaï,1 Kazubiko Mori,1 Shibeta Naruse,1 Hoyoku Nishino,2 Beatrice Y. J. T. Yue,3 and Shigeru Kinoshita1

PURPOSE. To profile gene expression changes induced by dexamethasone in cultured human trabecular meshwork (TM) cells and identify genes related to the occurrence of steroid-induced glaucoma.

METHODS. At confluence, dexamethasone (final concentration 10−7 M in 0.1% ethanol) or vehicle alone (control, 0.1% ethanol) was applied to cultured human TM cells from eyes of four normal subjects. After 7 days of application, a labeled cDNA probe was synthesized from extracted total RNA and hybridized to a human cDNA microarray containing 2400 genes. After hybridization, the tyramide signal was amplified, and the fluorescent signals on each microarray were scanned and analyzed.

RESULTS. In dexamethasone-treated TM cells, simultaneous analysis of 2400 human genes indicated a more than twofold increase in 30 genes. Five of them, myocilin (MYOC), decorin, insulin-like growth factor binding protein 2, ferritin L chain, and fibrillin-1C, were the most upregulated genes with higher-than-control expression levels in all four experiments. Their upregulation was further confirmed by semiquantitative RT-PCR. Downregulation, with fluorescent signals decreased to less than a half, was found in 34 genes. The dexamethasone-induced expression changes in most of these TM cell genes have not been reported previously.

CONCLUSIONS. cDNA microarray is a useful tool for gene expression analysis that confirms previous reports of upregulated mRNA expression of MYOC after treatment with dexamethasone in human TM cells. Changes in other genes subsequent to the treatment with dexamethasone may also reduce outflow facility, providing new insights into the pathogenesis of steroid-induced glaucoma. (Invest Ophthalmol Vis Sci. 2002;43: 5691–5697)

Corticosteroids such as dexamethasone are known to cause a form of open-angle glaucoma that involves increased resistance to aqueous humor outflow through the trabecular meshwork (TM).1,2 In the normal population, 34% to 42% of patients treated with topically or generally administered corticosteroids are termed "steroid responders," because markedly elevated intraocular pressure (IOP) develops after several weeks.3–5 By contrast, up to 90% of patients with primary open-angle glaucoma (POAG) are considered steroid responders.1,3,4 When steroid treatment is stopped, the IOP in most steroid responders usually returns to normal within several weeks, although in some cases it remains elevated1,3,4

Several possible mechanisms for steroid-induced elevation of IOP have been proposed, based on histologic studies of human TM from eyes with steroid-induced glaucoma and on experimental studies of organ-cultured human eyes and cultured TM cells. Proposed mechanisms include: accumulation or deposition of extracellular matrix material,3–10 decreased protease and stromelysin activities,11–12 reorganization of the TM cytoskeleton,13,14 increased nuclear size and DNA content,15 decreased phagocytotic capacity,16,17 and change in the synthesis of specific proteins.18 Moreover, it has been demonstrated by Polansky et al.19 and Nguyen et al.20 that the expression of myocilin protein (previously known as TIGR and GLC1A) is greatly enhanced by treatment with glucocorticoids in cultured TM cells. MYOC (myocilin), a gene isolated from a retinal cDNA library,21 has been linked to open-angle glaucomas.22 Because the linkage, there have been numerous reports regarding the myocilin protein and gene.22–26 Their functions however remain largely unknown.

cDNA microarray technology brings gene expression analysis to a genomic scale by permitting investigators to examine changes in the expression of literally thousands of genes simultaneously in a single experiment.27–29 and microarrays have already been used in ophthalmic research.30,31 We used a human cDNA microarray (Micromax; PerkinElmer Life Sciences Inc., Boston, MA) that is a glass slide cDNA microarray system designed for high throughput, high sensitivity, differential gene expression analysis.31 It consists of a 1 × 3-in. glass microarray presotted with 2400 known genes. The genes, characterized functionally on the basis of a Prosite motif search (Prosite is provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland, and is available at http://www.ncbi.nlm.nih.gov/Prosite), are readily identifiable through a gene database accessible on Perkin Elmer’s Web site (http://lifesciences.perkinelmer.com), linked to the National Institutes of Health GenBank (GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/Genbank).

To elucidate further the steroid response in outflow pathway cells, we used the gene microarray evaluate gene expression profile changes induced by dexamethasone in cultured human TM cells.

MATERIALS AND METHODS

Cell Culture

Normal human eyes from four different donors, 7, 28, 16, and 21 years of age, obtained less than 24 hours after death, were received from the

From the Departments of 1Ophthalmology and 2Biochemistry, Kyoto Prefectural University of Medicine, Kyoto, Japan; and the 3Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago College of Medicine, Chicago, Illinois.

Supported in part by Grant-in-Aid for scientific research from the Japanese Ministry of Education, Culture, Sports, Science and Technology and by Grants EY05628 and EY01792 from the National Eye Institute, Department of Ophthalmology and Visual Sciences, University of Illinois. Submitted for publication February 28, 2002; revised June 10, 2002; accepted June 28, 2002.

Commercial relationships policy: N.

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Corresponding author: Takeshi Ishibashi, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 6020841, Japan; tishibas@ophth.kpu-m.ac.jp.
Illinois Eye Bank, (Chicago, IL). The procedures for culturing TM cells from mammalian eyes, including human eyes, have been well established.32,33 Fourth-or fifth-passage human TM cells derived from donor eyes were cultured in 25-cm² tissue culture flasks with Eagle’s minimum essential medium including 10% fetal bovine serum, 5% calf serum, essential and nonessential amino acids, and antibiotics. At confluence, the cells were treated with 100 nM dexamethasone in 0.1% ethanol or 0.1% ethanol alone for 7 days.

**Microarray Hybridization and Data Analysis**

Total RNA was isolated from dexamethasone-treated and control TM cells with extraction reagent (TRIzol; Life Technologies, Inc., Rockville, MD), according to the manufacturer’s recommendations. A cDNA probe made from each 2 μg of total RNA was labeled by reverse transcription with either fluorescein or biotin. At probe creation, RNA of unlabeled plant genes was added for normalization. The cDNA probes were then mixed and cohybridized to the microarray for 16 hours. After stringent washes, the hybridized cDNA signal was amplified by the addition of streptavidin horseradish peroxidase (HRP) followed by cyanine 5-tyramide. Anti-fluorescein-HRP and cyanine 3-tyramide was then added sequentially. With a laser detection system (ChipReader; Virtek Vision Inc., Ontario, Canada), the fluorescent signals were scanned to determine differential gene expression between dexamethasone-treated TM cells and control cells. Local back-
TABLE 2. List of Human TM Cell Genes Upregulated More Than Twofold by Dexamethasone Treatment

<table>
<thead>
<tr>
<th>Name of Gene</th>
<th>Signal Ratio (DEX/Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample 1</td>
</tr>
<tr>
<td>MYOC*</td>
<td>3.21</td>
</tr>
<tr>
<td>Pigment epithelium-differentiation factor</td>
<td>13.46</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 2 (IGFBP2)*</td>
<td>1.53</td>
</tr>
<tr>
<td>Ferritin L chain*</td>
<td>3.01</td>
</tr>
<tr>
<td>Decorin*</td>
<td>2.03</td>
</tr>
<tr>
<td>Dihydropyridine dehydrogenase</td>
<td>2.74</td>
</tr>
<tr>
<td>Latent transforming growth factor-beta-binding protein-2</td>
<td>3.51</td>
</tr>
<tr>
<td>Transmembrane protein Jagged 1 (HJ1)</td>
<td>1.84</td>
</tr>
<tr>
<td>Fibrin-1 C*</td>
<td>2.06</td>
</tr>
<tr>
<td>KIAA0245</td>
<td>7.07</td>
</tr>
<tr>
<td>Ferritin H chain*</td>
<td>1.62</td>
</tr>
<tr>
<td>Alpha-2 strychnine binding subunit of inhibitory glycine receptor*</td>
<td>1.58</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 4 (IGFBP4)*</td>
<td>4.14</td>
</tr>
<tr>
<td>Monoamine oxidase*</td>
<td>1.35</td>
</tr>
<tr>
<td>Follistatin-related gene precursor</td>
<td>1.55</td>
</tr>
<tr>
<td>Splicing factor 5'p30c</td>
<td>1.39</td>
</tr>
<tr>
<td>KIAA0212</td>
<td>4.60</td>
</tr>
<tr>
<td>BCL2 adenovirus E1B 19kD-interacting protein 2</td>
<td>2.52</td>
</tr>
<tr>
<td>Elongation factor 1-alpha 1 (PTF-1)*</td>
<td>1.07</td>
</tr>
<tr>
<td>Fibroblast muscle-type tropomyosin*</td>
<td>1.78</td>
</tr>
<tr>
<td>Retinoic acid receptor*</td>
<td>1.68</td>
</tr>
<tr>
<td>KIAA0018</td>
<td>3.11</td>
</tr>
<tr>
<td>DNA-binding protein A variant*</td>
<td>2.53</td>
</tr>
<tr>
<td>Skeletal beta-tropomyosin</td>
<td>0.50</td>
</tr>
<tr>
<td>Thyroid autoantigen (truncated actin-binding protein)</td>
<td>1.40</td>
</tr>
<tr>
<td>Acidic calponin</td>
<td>1.68</td>
</tr>
<tr>
<td>Stearyl-CoA desaturase*</td>
<td>1.89</td>
</tr>
<tr>
<td>Pyrroline-5-carboxylate dehydrogenase (P5Cdh)</td>
<td>4.78</td>
</tr>
<tr>
<td>KIAA0347</td>
<td>0.11</td>
</tr>
<tr>
<td>KIAA0026</td>
<td>1.18</td>
</tr>
</tbody>
</table>

* Genes upregulated in four independent experiments.

Expression Profile Analysis of TM Cells Treated with Dexamethasone

Signal intensities of dexamethasone-treated and control TM cells in each of the four experiments (Fig. 1, samples 1–4) gave correlation constants of 0.91, 0.85, 0.79, and 0.88, respectively. Data points in the top left region of each scatterplot in Figure 1 represent genes with expression levels that were increased by treatment with dexamethasone. Those in the bottom right region are genes with decreased expression level. The signal ratio (dexamethasone/control) of each of the 2400 genes was calculated in each experiment. Genes with average ratios more than double that of the control and with dexamethasone signals stronger than in the control in at least three cases were defined as upregulated by dexamethasone. Genes with average ratios less than half that of the control were defined as downregulated. The 30 upregulated and 34 downregulated genes are listed in Tables 2 and 3, respectively. Our data disclosed that MYOC was the most upregulated gene. Its expression was increased 3.2-, 42.7-, 47.5-, and 2.4-fold (24.0 ± 12.2, mean ± SEM) in the four experiments. Expression levels of decorin and fibrillin-1 C, genes related to the extracellular matrices, and IGFBP2 and ferritin L chain were increased also, but to a lesser extent.

RT-PCR Confirmation of Gene Upregulation

To confirm the experimental results of the microarray, RT-PCR was performed on the five upregulated genes. Figure 2 shows...
the amplified fragments of RT-PCR products of MYOC, decorin, IGFBP2, ferritin L chain, fibulin-1C, and GAPDH in each of the four sample pairs. Relative mRNA expression levels were increased by treatment with dexamethasone, except for the ferritin L chain in sample 2 (Fig. 3). The RT-PCR results were similar to those from the microarray. The sample pair ratio values obtained with the microarray, however, were quite different from those obtained using RT-PCR. For example, respective MYOC mRNA expression ratios for the four sample pairs were 3.2, 42.7, 47.3, and 2.4 with microarray, and 4.1, 2.4, 1.5, and 1.7 with RT-PCR.

**DISCUSSION**

The present study, using a human cDNA microarray (Microflex, Perkin-Elmer) provides a survey of genes induced in cultured TM cells in response to treatment with dexamethasone. In the literature, there have been numerous reports concerning the mechanism of steroid-induced glaucoma, but the causes of the disease and the steroid response in the TM are not entirely clear. We discovered that after 7 days of treatment with dexamethasone, a number of genes were significantly upregulated and others were downregulated. Up-regulation of five of the genes was confirmed by semiquantitative RT-PCR. The latter results were similar to those of the microarray analysis in all genes except the ferritin L chain in sample 2, although there was no clear correlation of mRNA expression ratios between microarray and RT-PCR results. The disparity between the two methods may have occurred when...
genes were expressed at only low levels or when the probes made from total RNA hybridized the cDNA array with poor efficiency.

Consistent with those reported previously, our data showed that dexamethasone significantly induced the expression of MYOC (24.0 ± 12.2-fold),20,21 a gene that has been implicated in autosomal dominant juvenile glaucoma and POAG by genetic linkage analysis.22-25 These observations may lead to the hypothesis that increased expression of this protein is a key step in steroid-induced ocular hypertension, and there is structural evidence suggesting that extracellular myocilin could contribute to outflow resistance.20,54 However, elevated IOP does not develop in more than half of the patients receiving steroids. In POAG, myocilin gene mutations rather than the expression level may be more responsible, but again, the mutations are identified only in 3% to 5% of the POAG patient population.22-25 MYOC coding sequence variations are also extremely rare in patients with steroid-induced glaucoma.55 Judging from these reports, it seems difficult to explain the mechanism of steroid-induced glaucoma solely on the basis of myocilin protein accumulation in TM and/or MYOC mutations. The present study does not yield new findings regarding the relationship between MYOC and TM outflow resistance or steroid-induced glaucoma. The results nevertheless are in agreement with previous data19,20 on MYOC mRNA expression changes, validating the use of the microarray technology.

In fact, this study further revealed that decorin and fibulin-1 C, components of the extracellular matrix, were also induced by dexamethasone. Beside MYOC, decorin has been found to be present in human TM cells and is thought to serve important functions in the trabecular outflow pathway.59,60 The decorin increase may thus be related to the steroid-mediated reduction of the outflow facility. A similar induction of decorin production (maximally 4.8-fold) and decorin mRNA levels (to 2.3-fold) by dexamethasone was also observed in cultured human skin fibroblasts.57

Fibulin-1 was first described as an integrin-binding fibulin in human placenta by Argraves et al.58,59 These investigators showed that fibulin-1, a secreted glycoprotein, was incorporated into a fibrillar extracellular matrix when expressed in cultured cells or added exogenously to cell monolayers. Although there are no reports concerning the function of fibulin-1 in the TM, its gene is expressed in human TM,40 and conceivably it plays a role in the extracellular matrix assembly and, in turn, in regulation of the outflow pathway.

Microarray analysis showed that IGFBP2 and IGFBP4 gene expressions were also induced by dexamethasone, although IGFBP4 mRNA expression was not confirmed by RT-PCR. Insulin-like growth factors (IGFs) have been studied extensively in a variety of ocular and nonocular tissues. They are important in the regulation of normal, developmental, and pathologic processes, modulating both mitogenic and metabolic behavior in many tissues.41 IGFs are often bound to specific, high-affinity IGFBPs, seven of whose family members have been identified.42-44 It has been reported that IGFBPs modulate the biological actions of IGFs by either enhancing45 or inhibiting46 ligand–receptor interactions, and provide storage for IGFs in the extracellular matrix. In addition, IGFBPs themselves exhibit intrinsic bioactivity independent of IGF’s effects.47 Recently, it was reported that IGF-1 receptor and IGFBP5 are expressed at significant levels in human TM cells and that IGF may have an important role in TM maintenance.48 Although there are no reports as yet concerning expression of IGFBP2 or IGFBP4 in TM, upregulation of these genes by treatment with dexamethasone may affect the TM homeostasis and have an impact on the outflow.

Ferritin L chain and H chain were also induced by dexamethasone, and the mRNA expression induction of the L chain was confirmed by RT-PCR. Ferritin, the major intracellular iron storage protein in all organisms, is responsive to intracellular oxidative stress and reactive oxygen intermediates generated during inflammation.49 Cataract has been shown to occur in individuals with hereditary hyperferritinemia cataract syndrome, a condition in which ferritin L chain protein is systemically overexpressed.50 Although the function of ferritin in trabecular outflow is unknown, ferritin L and H chain mRNAs are expressed in human TM, the former especially at a high level.51 Upregulation of these ferritin chains in the TM may also play a part in the outflow facility reduction. As to the other upregulated genes identified (Table 2), it is at present difficult to speculate on their possible roles in TM cells and in the aqueous outflow system, because of incomplete knowledge regarding their functions.

Thirty-four genes were found to be downregulated by dexamethasone, and most of them probably do not have profound effect on aqueous outflow. One gene of note, however, is nitric oxide synthase (NOS). Its presence has been demonstrated in the TM both immunohistochemically52 and enzymatically.53 Sites of outflow resistance and regulation in the human eye are particularly rich in NOS.53 In addition, higher levels of hydraulic pressure have been shown to enhance basal production of nitric oxide (NO) in human trabecular cells,55 implicating NO as a physiological mediator in regulation of IOP. Abnormalities in NO or NO-containing cells have also been reported in POAG.54 These reports, together with the results of the microarray study, suggest that NOS gene downregulation by dexamethasone may be a factor involved in the reduction of outflow facility.

Chloride channel was identified as another downregulated gene. Intracellular Cl concentration is an important regulator of Na-K-Cl cotransporter activity in human TM cells, that may influence TM cell volume and consequently the aqueous outflow across the TM in vivo.55 The reduced chloride channel may elevate the intracellular Cl concentration, expand the TM cell volume, and increase outflow resistance.

In summary, this study identified a number of genes that are up- or downregulated by treatment with dexamethasone in cultured TM cells. Further work is needed to evaluate their functions in the TM, and the effects of dexamethasone-induced gene expression on aqueous outflow. The cDNA microarray (such as Micromax; Perkin-Elmer) is a powerful tool for analy-
sis of gene expression profile changes. It is useful and efficient in investigating not only steroid-induced glaucoma, but other ocular diseases of unknown causes as well.

Acknowledgments

The authors thank Andrew J. Quantock for help with the manuscript, and Chikako Mochida for technical support.

References


