Argon Laser Photocoagulation–Induced Modification of Gene Expression in the Retina

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PURPOSE. To generate a profile of genes expressed in the retina, RPE, and choroid after laser treatment and to identify genes that may contribute to the beneficial effects of laser photocoagulation in the treatment of angiogenic retinal diseases.

METHODS. Argon laser irradiation was delivered to the left eye of normal C57BL/6j mice (n = 30), with the right eye serving as the control in each animal. Three days after laser treatment, mice were culled, eyes enucleated, and the retinas dissected and pooled into respective groups. The total RNA of replicate samples was extracted, and expression profiles were obtained by microarray analysis. Data comparisons between control and treated samples were performed and statistically analyzed.

RESULTS. Data revealed that the expression of 265 known genes and expressed sequence tags (ESTS) changed after laser treatment. Of those, 25 were found to be upregulated. These genes represented a number of biochemical processes, including photoreceptor metabolism, synaptic function, structural proteins, and adhesion molecules. Thus angiotensin II type 2 receptor (Agtr2), a potential candidate in the inhibition of VEGF-induced angiogenesis, was upregulated, whereas potential modulators of endothelial cell function, permeability factors, and VEGF inducers, such as FGF-14, FGF-16, IL-1β, calcitonin receptor-like receptor (CRLR), and plasminogen activator inhibitor-2 (PAI2), were downregulated.

CONCLUSIONS. In this study, genes were identified that both explain and contribute to the beneficial effects of laser photocoagulation in the treatment of angiogenic retinal diseases. The molecular insights into the therapeutic effects of laser photocoagulation may provide a basis for future therapeutic strategies. (Invest Ophthmol Vis Sci. 2003;44:1426–1434)

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Angiogenic retinal diseases such as diabetic retinopathy, age-related macular degeneration, retinopathy of prematurity, retinal ischemia, and choroidal neovascularization are the most common cause of vision loss in the developed world. Although the underlying cause of these angiogenic retinal diseases is different, the factors initiating the angiogenic process are likely to be similar. One of the most important factors in the initiation of an angiogenic response is the upregulation of the expression of vascular endothelial growth factor (VEGF), a potent angiogenic and permeability factor, the upregulation of which has been shown in all patients who have neovascular eye diseases.1–4 Although there are several drug trials in progress currently, the only accepted treatment for these diseases is retinal laser photocoagulation, an ablative treatment that destroys the peripheral retina to preserve the central macula, which is responsible for 80% of human vision. A wide range of theories has been proposed to explain the beneficial effects of retinal laser photocoagulation in delaying retinal angiogenesis; however, the underlying molecular mechanisms remain to be elucidated.

It has been proposed that the therapeutic effects of laser photocoagulation are due to the destruction of photoreceptors, the highest oxygen consumers in the retina. Subsequently, these photoreceptors are replaced by glial cells, allowing increased oxygen diffusion from the choroid to the inner retina and thereby relieving inner retinal hypoxia.5,6 This improved oxygenation triggers a two-pronged cascade of events: (1) Constriction of the retinal arteries results in decreased hydrostatic pressure in capillaries and the constriction of capillaries and venules,7 and (2) the cellular production of VEGF is inhibited.8,9 Together, these effects are believed to result in the ultimate inhibition of neovascularization and a decrease in edema. However, altered gene expression and thus the altered regulation of cellular proteins in response to laser photocoagulation are likely to play an important role in achieving the desired therapeutic effects.

With the development of microarray technology, it is possible to monitor thousands of genes simultaneously, enabling the high throughput analysis of treatment methods, such as laser photocoagulation, on retinal gene expression. Such global investigations into altered gene expression can facilitate the identification of key regulatory factors and/or events that contribute to the therapeutic effects of laser photocoagulation in the inhibition of both neovascularization and the progression of retinal diseases. An examination of altered gene expression patterns in normal tissue also provides a baseline from which comparisons to the effects of laser photocoagulation in retinal disease models can then ensue.

In this study, the effects of laser photocoagulation on gene expression in the retina, RPE, and choroid were examined by using microarray technology. To validate the methodology, the expression profiles of selected genes were confirmed by quantitative PCR techniques. The molecular insights into the therapeutic effects of laser photocoagulation will not only increase...
our understanding of the mechanisms that underlie this treatment but will also identify genes for future gene therapy strategies.

Methods

Retinal Laser Photocoagulation of Mouse Retina

Female mice (C57BL/6j), aged 10 weeks, were used. The mice were treated and maintained in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of Western Australia Animal Ethics Committee. The mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg; Warner Lambert Co., Morris Plains, NJ) and xylazine (10 mg/kg; Bayer AG, Leverkusen, Germany), and the pupils dilated with 2.5% tropicamide (Alcon, Fort Worth, TX). Argon laser irradiation (514 nm; Coherent Radiation Systems, Palo Alto, CA) was delivered through a slit lamp ophthalmoscope (Carl Zeiss, Oberkochen, Germany) with a handheld coverslip serving as a contact lens. The laser spots (20 spots/retina) were placed at a setting of 50-µm diameter, 0.05-second duration, and 120-mW intensity and were scattered in the upper quadrant of the fundus. The left eye of each animal was laser treated, and the right eye remained untreated, serving as a control.

Perfusion of Laser-Treated Eyes with Fluorescence-Labeled Dextran

Two laser-treated mice were perfused with fluorescence-labeled dextran, as described previously. Briefly, the animals were anesthetized as just described. PBS (4 mL) was perfused through the left ventricle into the aorta to wash away the circulating blood, followed by 2 mL fluorescence-labeled dextran (Sigma, St. Louis, MO) for perfusion. The eyes were enucleated, fixed in 2% paraformaldehyde for 30 minutes, and flattened for fluorescence microscopy, as described previously.

Histology of Laser-Treated Eyes

Eyes (control, n = 2; laser-treated, n = 2) were harvested for histology 3 days after laser photocoagulation. The enucleated eyes were fixed in 4% paraformaldehyde for 2 hours and embedded in paraffin. Sections (~5 µm in thickness) were cut and stained with hematoxylin and eosin (H&E). The sections were then viewed under a light microscope to confirm the anticipated structural changes at the site of the laser burns.

Sample Preparation and Assessing RNA Quality

Enucleated control- and laser-treated eyes were harvested 3 days after laser treatment and maintained in RNA stabilization solution (RNALater; Ambion, Austin, TX) for a minimum of 2 hours. To maximize the solution’s penetration into the inner parts of the eye and to minimize RNA degradation, the eyes were slit before storage. The tissue was then dissected from the anterior segment and the lens. The resultant eyecups (comprising the retina and adjoining RPE layer and choroid) were pooled (n = 5 eyes per pool) to both maximize the amount of RNA obtained and to minimize bias due to biological variation. Total RNA was isolated with extraction reagent (TRiZol; Invitrogen, Life Technologies, San Diego, CA) and further purified using a kit (RNaseasy; Qiagen, Valencia, CA). The RNA concentration was determined spectrophotometrically.

Microarray Hybridization and Analysis

Biotinylated cRNA samples were prepared as described by the manufacturer (Affymetrix, Santa Clara, CA) and hybridized onto arrays (Test 3 Arrays; Affymetrix), which provided information on the quality of the cRNA product, background levels, and enabled maintenance of quality control of the hybridization technique and the scanning equipment. “Spiked” controls were added to the hybridization cocktail, to enable quality control of the hybridization process. Samples of sufficient quality were then hybridized onto the gene chip standard arrays (MG-U74Av2 GeneChip; Affymetrix). The sequence source for the MG-U74Av2 array was largely the C57BL/6J mouse strain, making it an appropriate array for this study. Replicate hybridizations of three independent pooled samples were performed by using separate chips for both control and laser-treated samples.

Statistical Analysis

The raw-image data were analyzed using the accompanying software (GeneChip Expression Analysis Software; Affymetrix) to produce perfect match (PM) and mismatch (MM) values to which we applied our own analysis. Normalization of each array was performed using a method that is intended to make the PM and MM quantiles of all arrays agree, referred to as quantile normalization. Normalized PM data were log transformed (base 2) and corrected for the effects of non-specific binding by a novel background correction (for more details of this analysis procedure, see Ref. 13).

The three replicate arrays of treatment and control were combined at the probe level, and the difference between the combined treatment and control arrays was calculated. For treatment arrays T1, T2, and T3 and control arrays C1, C2, and C3 the difference (d) between treatment and control for probe i of a given gene was formed:

\[ d_i = \frac{\text{T}_{i1} + \text{T}_{i2} + \text{T}_{i3}}{3} - \frac{\text{C}_{i1} + \text{C}_{i2} + \text{C}_{i3}}{3} \]

whereas the mean difference (\( \bar{d} \)) and SE over all probes (i = 1...n) for a given gene were calculated by

\[ \bar{d} = \frac{1}{n} \sum_{i=1}^{n} d_i \]

\[ \text{SE} = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} (d_i - \bar{d})^2} \]

A t statistic was formed for the difference between treatment and control for each gene: \( t = \frac{\bar{d}}{\text{SE}} \). Genes were ranked according to the size of their mean difference and \( t \) statistic. Ranking is performed on both statistics in an attempt to produce a list of genes with large mean differences and reasonable SEs. Requiring that the \( t \) statistic be large removes genes with large SEs from the list, and requiring that the mean difference also be large removes genes for which the \( t \) statistic is large, only because of very small SEs. Those genes for which both statistics are large relative to most genes are most likely to be differentially expressed.

Quantitative Real-Time PCR

Real time PCR (performed on three independent pooled samples) was used to confirm the gene expression data obtained in the microarray experiment. The RNA from eyecups of pooled groups of control and laser-treated eyes was reverse transcribed and used as a template in a real-time PCR reaction, using specific oligonucleotide primers and a fluorescent gel dye (SYBR green I; Applied Biosystems, Foster City, CA). The reaction was performed on a commercial system (RotorGene 2000; Corbett Research, Sydney, Australia). A standard curve was established for each PCR reaction. This was derived from the serial dilutions (10⁻³-10⁻⁷ pmol/µL) of a single-stranded, synthetic cDNA standard designed to represent the fragments of the genes listed later, and was used to calculate mRNA concentrations in the RNA samples. A “melt-curve” analysis of the PCR products was performed after the amplification, to demonstrate that only a single product was amplified.

Oligonucleotide primers were designed to span intronic sequences to exclude the possibility of amplifying genomic DNA during the limited extension time of the PCR protocol (15 seconds). Primer
**RESULTS**

**Changes of the Fundus, Retina-Choroidal Microvasculature, and Retinal Structure after Laser Photocoagulation**

The laser photocoagulation protocol (estimated to affect ≤25% of the fundus) adopted in this study produced laser burns of moderate whiteness, but without central heating bubble formation and subretinal or retinal hemorrhage (Fig. 1A). The retinal edema at the laser lesions subsided considerably at 3 days after laser treatment (white spot formation; Fig. 1B). Perfusion of the laser-treated eyes with fluorescence-labeled dextran demonstrated that laser photocoagulation did not affect the retinal vasculature (Fig. 1C, arrows). Histologic examination revealed that the structure of the inner retina was moderately affected by laser photocoagulation. There were significant changes in the outer retina evident by the disorganized outer nuclear layer and enlarged and/or migrating RPE cells. A few infiltrating macrophages (arrows) were present at the burn site (Fig. 1D) indicating the remnants of an inflammatory response. Bruch’s membrane remained intact, and no retinal or choroidal neovascularization was evident 3 days after laser photocoagulation.

**Microarray Analysis: Genes Modulated by Laser Photocoagulation**

Approximately 4 μg of total RNA was extracted per eyecup and confirmed to be intact by the generation of cRNA and subsequent hybridization to the microarrays. The 5′-to-3′ ratios of the mouse housekeeping genes GAPDH and β-actin as well as the spiked controls were all below 3, and background readings were below 130, demonstrating that high-quality RNA was obtained and that the cRNA synthesis worked efficiently (Table 1). Valid samples were then analyzed using the gene chips (MG-U74Av2; Affymetrix). Individually processed cRNA samples from pooled eyecups obtained from nontreated and 3-day post-laser-treatment eyes were hybridized onto separate gene chips. Genes considered differentially expressed as a result of laser treatment were those genes that had the highest mean differences  

\[
\Delta = \frac{\text{ratio}}{\text{SE}}, \quad \text{median (5′/3′ ratio), and t} \quad \text{statistics. Figure 2 shows a plot of t} \quad \text{versus SE for all genes (represented by small black dots) and for the genes with the highest t} \quad \text{and} \quad \Delta \quad \text{represented by the open circles. Values of t at or near zero represent genes that are not differentially expressed. Because t is} \quad \Delta \quad \text{divided by SE, genes with large SEs tend to have small t} \quad \text{ statistics. Genes for which a single animal has skewed the expression level measured by one of the three replicate chips have a larger SE and therefore a smaller t} \quad \text{statistic. This ensures that the gene appears much lower in the list of ranked t} \quad \text{statistics and greatly reduces the}
\]

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**Table 1. Assessment of RNA and cRNA Quality by UV Spectroscopy and Hybridization to Gene Microarrays**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Purity (260/280 ratio)</th>
<th>Background</th>
<th>5′/3′ Spiked Controls</th>
<th>5′/3′ Ratio β–Actin</th>
<th>5′/3′ Ratio GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>1.94</td>
<td>46.45</td>
<td>&lt;3</td>
<td>2.76</td>
<td>1.48</td>
</tr>
<tr>
<td>C2</td>
<td>1.98</td>
<td>46.72</td>
<td>&lt;3</td>
<td>2.02</td>
<td>1.29</td>
</tr>
<tr>
<td>C3</td>
<td>2.00</td>
<td>50.60</td>
<td>&lt;3</td>
<td>3.00</td>
<td>1.79</td>
</tr>
<tr>
<td>C4</td>
<td>2.12</td>
<td>55.57</td>
<td>&lt;3</td>
<td>2.31</td>
<td>1.78</td>
</tr>
<tr>
<td>A1</td>
<td>2.10</td>
<td>60.25</td>
<td>&lt;3</td>
<td>2.36</td>
<td>1.42</td>
</tr>
<tr>
<td>A2</td>
<td>1.96</td>
<td>62.57</td>
<td>&lt;3</td>
<td>2.19</td>
<td>1.20</td>
</tr>
<tr>
<td>A3</td>
<td>2.00</td>
<td>128.95</td>
<td>&lt;3</td>
<td>2.30</td>
<td>1.19</td>
</tr>
<tr>
<td>A4</td>
<td>2.00</td>
<td>54.83</td>
<td>&lt;3</td>
<td>2.81</td>
<td>1.42</td>
</tr>
</tbody>
</table>
possibility that it will be included in the final list of candidates for differential expression. Conversely, the largest t statistics occur for genes with the smallest SEs. Only those genes with large t and small SE, which are not highlighted, correspond to genes with low ds. The 265 genes highlighted in this figure were considered to be the most likely candidates for differential expression. It can be seen that the highlighted genes were distinct from the dense cloud of unchanged genes clustered about t = 0.

The 265 genes classified as differentially expressed 3 days after laser photocoagulation comprised 127 known genes and 138 ESTs. Most of these genes showed decreased expression after laser treatment (downregulation) with decreased expression that ranged between two- and fivefold. Figure 3 shows the distribution of genes regulated (both increased and decreased in gene expression) by argon laser photocoagulation. The largest groups comprised the ESTs (51%); regulatory molecules, enzymes, and transporters (8%); and extracellular matrix, tissue remodeling, adhesion molecules, and structural and cytoskeletal proteins (8%). The group identified as miscellaneous consists of nucleolar proteins, stress proteins, binding proteins, ubiquitin proteins, globins, globulins, and carrier proteins.

A subset of 25 genes (of which 13 were identified as ESTs) showed increased expression after laser treatment and included the angiotensin II type 2 receptor, rod transducin α, Hex (prh), AMOG, Supt5hp, α-crystallin, β-actin, dynamin, type 1 inosine monophosphate dehydrogenase, and several miscellaneous proteins of unknown function in the eye, such as inactive X-specific transcript, germline Ig variable region heavy chain, and a variable group of two-cell-stage gene families (Table 2). Figure 4 shows the distribution of these genes and again, the EST classification was the largest group of upregulated genes (52% of total number of upregulated genes).

**Confirmation by Real-Time PCR of Genes Modulated by Laser Photocoagulation**

Real-time PCR accurately quantifies the expression of genes and was used to validate a subset of genes highlighted by the microarray data. Our deliberate focus was on those genes that showed increased expression after laser treatment and genes involved in wound healing and repair.

The oligonucleotide primers and standards designed for the quantitative analysis were derived from the same sequence source as that for the probes. The real-time PCR, performed on two independent samples, confirmed the pattern of expression observed in the microarray data (Table 3) for 15 of the 16 genes analyzed. The multiplicity of change (ranging between no change to 70-fold differences shown by the real-time PCR), however, did not correspond to the change observed with the microarray (ranging between no change and 3.19-fold differences). The real-time PCR data from replicate samples was consistent. The exception to the above was the gene math3. The expression pattern of math3 identified in the microarray was not confirmed by real-time PCR. The microarray data showed that math3 was reduced in expression by 2.45-fold, whereas the real-time PCR data demonstrated a significant increase in math3 expression of almost 20-fold. For each gene analyzed by real-time PCR, the melt-curve confirmed that only a single product was amplified. The melting temperatures ($T_m$) of the products derived by amplification of the cDNAs were identical with the $T_m$ of the products derived from the stan-
Distribution of genes regulated by argon laser photocoagulation

Figure 3. The variety and proportions of gene classes found to be increased or decreased in expression, as determined by microarray analysis, in the retinal and RPE tissue of C57BL/6 mice after laser photocoagulation. The class defined as miscellaneous contains genes such as nucleolar proteins, stress proteins, binding proteins, ubiquitin proteins, globins, globulins, and carrier proteins.

dards, additionally confirming the identity of the observed products.

Figure 5 shows an example of the data obtained from a real-time PCR experiment for the angiotensin II type 2 receptor, where the standard amplicons of known concentration are represented in the smaller window within the graph. The two lines represent the amplification of Agtr2 in the control and treatment samples. These data show that the concentration of Agtr2 was seven times greater in the laser-treated samples than in control.

Table 2. List of Known Genes Upregulated by Laser Photocoagulation

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene Name</th>
<th>Change (×)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenic inhibitor</td>
<td>Agtr2</td>
<td>2.93</td>
<td>Evidence suggesting role in regeneration of neuronal axon injury; may be involved in neuronal cell differentiation and cell growth; involved in inhibition of VEGF expression and VEGF-induced angiogenesis</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>α-Transducin</td>
<td>2.33</td>
<td>Phototransduction</td>
</tr>
<tr>
<td>Neural growth/differentiation</td>
<td>Hex(�h)</td>
<td>2.64</td>
<td>Hexosaminase; deficiencies result in oculomotor defects and abnormal axonal morphology</td>
</tr>
<tr>
<td></td>
<td>μ-Crystallin</td>
<td>2.63</td>
<td>May serve as enzyme for amino acid metabolism in photoreceptors</td>
</tr>
<tr>
<td></td>
<td>IMPDH</td>
<td>2.17</td>
<td>Important for cyclic nucleoside metabolism in the photoreceptors; mutations associated with retinitis pigmentosa</td>
</tr>
<tr>
<td>ECM/tissue remodeling/adhesion molecules/structural/cytoskeletal proteins</td>
<td>AMOG</td>
<td>3.19</td>
<td>Adhesion molecule for glial cells</td>
</tr>
<tr>
<td></td>
<td>Sup5hp</td>
<td>2.64</td>
<td>Chromatin structural protein homologue</td>
</tr>
<tr>
<td></td>
<td>β-Actin</td>
<td>2.26</td>
<td>Structural protein; housekeeping gene</td>
</tr>
<tr>
<td></td>
<td>Dynaminn</td>
<td>2.14</td>
<td>Required for Notch-mediated cell interactions; binds to synaptophysin and may regulate endocytosis of synaptic vesicles</td>
</tr>
<tr>
<td>Regulatory molecules/enzymes</td>
<td>Inactive X-specific transcript</td>
<td>4.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Germline Ig variable region heavy chain</td>
<td>3.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Variable group of two-cell-stage gene families</td>
<td>2.54</td>
<td></td>
</tr>
</tbody>
</table>

List comprises genes upregulated after laser photocoagulation. ESTs are not included. Information provided includes the classes of genes (as presented in the accompanying pie chart in Fig. 4), the name of the genes with respective change multiples, as determined by microarray, and function.
DISCUSSION

The purpose of this study was to examine the short-term effects of argon laser photocoagulation on retina, RPE, and choroid tissue to determine whether laser photocoagulation alone directly stimulates factors involved in antiangiogenesis or inhibits those involved in angiogenesis. Although many theories have been proposed to explain the therapeutic effects of laser therapy in the treatment of angiogenic retinal diseases, a large-scale investigation of gene expression after laser photocoagulation has not yet been reported. In this study we sought to simulate clinical laser treatment (by treating a single quadrant of the fundus; see http://www.geocities.com/aaabadawy2001/#practical) with the aim of identifying key genes or regulatory pathways that may contribute to the beneficial effects of laser therapy.

In our study, three replicate microarray comparisons demonstrated consistent changes in the gene expression profiles. The direction of the regulation was also confirmed by quantitative PCR techniques in 15 of the 16 genes analyzed, although the absolute multiple of change differed between the two techniques. This is not surprising, because the normalization of spot intensity, the initial step in the microarray data analysis, often represses very large differences in the expression levels, whereas the quantitation by real-time PCR is linear over many orders of magnitude. Overall, the 94% confirmation of the gene array data demonstrated the reliability of our methods in which retinal tissue was pooled and the microarray experiment was performed in triplicate.

The histopathologic consequences of laser photocoagulation have been studied, and the immediate effects noted were edema, inflammation, and necrosis, resulting from the tissue damage, which appeared to settle considerably by 3 to 4 days after treatment. In our study, the genes involved in apoptosis and inflammation contributed less than 5% of the total number of genes modified by laser photocoagulation, indicating that these processes had settled by 3 days after laser treatment. The genes involved in tissue remodeling, extracellular matrix, and structural proteins were found to be modified by laser treatment. Altered expression of this class of genes was

![Figure 4. Distribution of genes and ESTs observed to be increased in expression, as determined by microarray analysis, after argon laser photocoagulation of the eyes of C57BL/6J mice.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932920/)

**Table 3. Validation of Microarray Data Using Quantitative PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR Data (1)</th>
<th>R</th>
<th>Microarray Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMOG</td>
<td>16.5 fold</td>
<td>0.997</td>
<td>3.19 fold</td>
</tr>
<tr>
<td>a-Crystallin</td>
<td>5.5 fold</td>
<td>0.999</td>
<td>2.63 fold</td>
</tr>
<tr>
<td>Dynamin</td>
<td>32.2 fold</td>
<td>0.999</td>
<td>2.14 fold</td>
</tr>
<tr>
<td>IMPDH</td>
<td>12.2 fold</td>
<td>0.992</td>
<td>2.17 fold</td>
</tr>
<tr>
<td>a-Transducin</td>
<td>18.0 fold</td>
<td>0.999</td>
<td>2.33 fold</td>
</tr>
<tr>
<td>Supt5hp</td>
<td>70.6 fold</td>
<td>0.994</td>
<td>2.64 fold</td>
</tr>
<tr>
<td>Agr2</td>
<td>7.0 fold</td>
<td>0.999</td>
<td>2.93 fold</td>
</tr>
<tr>
<td>b-Actin</td>
<td>2.5 fold</td>
<td>0.991</td>
<td>2.26 fold</td>
</tr>
<tr>
<td>C/EBP</td>
<td>11.5 fold</td>
<td>0.999</td>
<td>2.36 fold</td>
</tr>
<tr>
<td>Math3</td>
<td>19.2 fold</td>
<td>0.998</td>
<td>2.45 fold</td>
</tr>
<tr>
<td>GAPDH</td>
<td>No change</td>
<td>0.998</td>
<td>No change</td>
</tr>
<tr>
<td>HPRT</td>
<td>No change</td>
<td>0.991</td>
<td>No change</td>
</tr>
<tr>
<td>TGFa</td>
<td>No change</td>
<td>0.993</td>
<td>No change</td>
</tr>
<tr>
<td>IGF-I</td>
<td>No change</td>
<td>0.998</td>
<td>No change</td>
</tr>
<tr>
<td>VEGF</td>
<td>No change</td>
<td>0.999</td>
<td>No change</td>
</tr>
<tr>
<td>Opsin</td>
<td>No change</td>
<td>0.989</td>
<td>No change</td>
</tr>
</tbody>
</table>

List of genes further analyzed by quantitative PCR techniques. The data are presented providing the ratios of changes in intensity compared with control samples for the microarray data, and the ratios of changes in absolute concentration compared with control samples for the quantitative PCR data. R is derived from the standard curve generated in the quantitative PCR method from which the PCR data ratios are determined.

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Genes of particular interest to us were those that showed increased expression after laser photocoagulation and those particularly involved in the inhibition of angiogenesis. In this study, the most intriguing was angiotensin II type 2 receptor (Agtr2), which was significantly increased after laser photocoagulation. Angiotensin II is a vasoactive hormone that induces both endothelial cell proliferation23 and VEGF.24 These actions are mediated by the angiotensin II type 1 receptor.24–25 High levels of angiotensin II and VEGF have been reported in the vitreous fluid of patients with active proliferative diabetic retinopathy.26 In contrast, the angiotensin II type 2 receptor (Agtr2) has demonstrated antiproliferative effects,57–60 particularly in endothelial cells.29 Recent preliminary evidence has demonstrated a possible role for Agtr2 in the inhibition of VEGF-induced angiogenesis in the treatment of proliferative diabetic retinopathy. The significant increase in Agtr2 expression after laser photocoagulation may explain the success of laser therapy in the treatment of proliferative diabetic retinopathy.

At present the most used method of laser treatment in the clinic is the ablative treatment, which results in the destruction of the peripheral retina. The proliferating RPE cells, infiltrating macrophages, damaged photoreceptor cells, and affected choroidal cells, resulting from the laser photocoagulation, may all have contributed to the gene expression profiles observed in this study. Although the changing cell populations and tissue composition are part of the process of laser photocoagulation, the particular cell type(s) contributing to the different gene-expression patterns after treatment remain unknown. Localization studies identifying these cells are planned for future experiments. However, the stimulated cells (namely the proliferating RPE and infiltrating macrophages) may release factors that may act to inhibit angiogenesis. An example of a well-studied antiangiogenic factor expressed by proliferating RPE cells and macrophages is pigment epithelium–derived factor (PEDF).52

 expected, considering the degree of damage resulting from the laser burns and the initiation of wound-healing and repair (Fig. 1). The laser treatment was also shown to impact on genes involved in regulating cell function: genes involved in transcription, translation, and the cell cycle, as well as regulatory molecules (enzymes and transporters), showed decreased expression. Yet, some rescue of the neural retina and compensation of the loss of peripheral photoreceptor function was implied by the upregulation of inosine monophosphate dehydrogenase type 1 (IMPDH1), α-crystallin and Hex (pht), genes important for the metabolic activity of the photoreceptors;20–22, α-transducin for photoreceptor signal transduction; and genes involved in axonal growth, morphology, and synaptic function (such as dynamin).

The expression of genes involved in angiogenesis, inflammation, and apoptosis, and those encoding extracellular matrix proteins and growth factors have previously been shown to be upregulated in the retina of diabetic animals.21 Our data suggested that the beneficial effects of laser photocoagulation in the treatment of diabetic retinopathy may not only be the inhibition of angiogenesis but also the altered regulation of the genes involved in the inflammation and tissue damage associated with the disease. However, it is possible that the diseased state may respond differently to laser treatment and thus, future studies are directed at examining the effects of laser photocoagulation in models of angiogenic retinal diseases, such as diabetic retinopathy to determine the genetic response of the diseased tissue to laser treatment.

At present the most used method of laser treatment in the clinic is the ablative treatment, which results in the destruction of the peripheral retina. The proliferating RPE cells, infiltrating macrophages, damaged photoreceptor cells, and affected choroid cells, resulting from the laser photocoagulation, may all have contributed to the gene expression profiles observed in this study. Although the changing cell populations and tissue composition are part of the process of laser photocoagulation, the particular cell type(s) contributing to the different gene-expression patterns after treatment remain unknown. Localization studies identifying these cells are planned for future experiments. However, the stimulated cells (namely the proliferating RPE and infiltrating macrophages) may release factors that may act to inhibit angiogenesis. An example of a well-studied antiangiogenic factor expressed by proliferating RPE cells and macrophages is pigment epithelium–derived factor (PEDF).52

The real-time PCR analysis for angiotensin II type 2 receptor (Agtr2). The y-axis represents the fluorescence intensity and the x-axis the PCR cycle number. Using standards of known concentration, the concentration of RNA in the samples was determined from this graph. There was a sevenfold increase in Agtr2 expression as calculated from the average threshold cycle (Ct) values and using the standard curve. The standards were performed in duplicate and the samples were performed in triplicate. The variability between replicates was very low and demonstrates the accuracy and reproducibility of the data.
edema formation as vascular permeability is reduced, and a decrease in ischemic injury as a result of decreased nitric oxide production.

Other genes found to be downregulated by laser photocoagulation that may be beneficial in the treatment of ocular disorders were those genes encoding extracellular matrix proteins: α6-crystallin and stromelysin 1. These genes are induced by different types of stress, such as oxidative stress (in glaucoma) and diabetic retinopathy. 21, 40, 41 Stromelysin 1 has been associated with retinal pigment epithelial contraction and retinal detachment. 42

This study is a preliminary investigation in which laser photocoagulation modulated the expression of genes that may contribute to the therapeutic effects of this treatment in angiogenic retinal diseases. However, whether the expression pattern changes are reflective of the gene expression levels within the viable cells of the affected retina, RPE, and/or choroidal tissue or is the result of changes in tissue composition has yet to be determined. Further analysis is needed to identify the cell type(s) affected by laser treatment, to localize the gene expression and to determine whether these gene expression changes translate to functional changes. Future studies are also planned to examine the long-term effects of laser treatment and to compare these effects with those observed in models of angiogenic retinal diseases.

This study demonstrated that microarray analysis is a powerful tool for identifying cellular responses to different treatment strategies. Although, at present, global approaches are complemented with traditional gene expression studies, such as in situ hybridization, immunohistochemistry, and in vitro and in vivo functional studies, with the advances in sequencing of the human and mouse genomes, genome-wide expression monitoring with instant identification of individual genes and cluster analysis will improve our understanding of the underlying biological processes and may accelerate the identification of disease-causing and therapeutic genes.

References


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ERRATUM


The website referenced on page 786 was incorrect. The website should have been http://www.iovs.org/cgi/content/full/44/2/781/DC1.

The online version of this article was corrected on February 6, 2003, in departure from print.