In Vivo Retinal Gene Expression in Early Diabetes

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Purpose. Studies have demonstrated a causal role for specific molecules in the pathogenesis of diabetic retinopathy. Among the implicated mediators are growth factors such as vascular endothelial growth factor (VEGF) as well as adhesion molecules and proliferation- and apoptosis-related genes. However, a coordinated large-scale investigation of gene expression in the diabetic retina has not yet been reported. Here the retinal gene expression profile of diabetic and nondiabetic animals using cDNA microarrays were analyzed and compared.

Methods. Long–Evans rats were made diabetic with streptozotocin. Retinal gene expression was analyzed over 3 weeks using high-density nylon filter–based cDNA arrays. Genes were sorted into clusters according to their temporal expression profiles. They were also grouped according to their potential pathophysiological significance. The in vivo gene expression profiles of selected genes were verified via RNase protection assay.

Results. The rat GeneFilter contains a total of 5147 genes, of which 1691 are known genes and 3456 are expressed sequence tags (ESTs). On day 3, the expression of 27 known genes was increased by more than twofold. On days 7 and 21, the corresponding numbers were 60 and 12, respectively. A transient upregulation (>2-fold) in expression was seen in 627 of 5147 total genes. A subset of 926 genes exhibited a modest (<2-fold) decrease in expression. No genes showed a greater than twofold decrease in expression. Overall, the identity of the genes that were upregulated suggests that the response of the retina to the diabetic challenge contains an inflammatory component. Moreover, most regulatory activity occurs during the first week of diabetes.

Conclusions. The development of a rational therapy for diabetic retinopathy will be assisted by detailed knowledge regarding the molecular pathophysiology of the disease. Here, an expression profile of an underlying retinal inflammatory process in early diabetes was extracted. Beyond providing insight into the general nature of the response to a pathogenic challenge, gene expression profiling may also allow the efficient identification of potential drug targets and markers for monitoring the course of disease. (*Invest Ophthalmol Vis Sci.* 2001; 42:3047–3057)

Diabetic retinopathy remains a leading cause of vision loss and new-onset blindness. With the onset of diabetes, cellular and biochemical alterations within the retina are set into motion. Hyperglycemia triggers capillary basement membrane thickening, nonenzymatic glycation of extracellular matrix proteins and the activation of the aldose reductase/sorbitol pathway.[1] The biochemical changes include increased intracellular levels of diacylglycerol and the activation of protein kinase C.[2] Advanced glycation end products, adherent leukocytes, and reactive oxygen species have all been implicated in the pathophysiology of diabetic retinopathy.[3] It was recently demonstrated that diabetic blood–retinal barrier breakdown and endothelial cell injury and death are causally related to retinal leukocyte adhesion in the vasculature of the diabetic rat.[4] Leukocyte adhesion is mediated, at least in part, by intercellular adhesion molecule (ICAM) 1, CD18, and vascular endothelial growth factor (VEGF).[5]

Conventional research efforts have typically focused on a particular class of molecules at a time. A broader screen of potential regulatory pathways, using methods such as subtractive hybridization and differential display PCR, although desirable, can sometimes be cumbersome and inaccurate. The advent of high-density microarray technology,[6–9] with its capacity for simultaneously monitoring thousands of genes, provides a novel opportunity for a high-throughput analysis of diabetic retinal gene expression over time.

In the present study, the retinal gene expression profile of 5147 genes was studied in the retina during early diabetes and compared with the nondiabetic state. To validate the methodology, the expression profiles for selected genes were verified via RNase protection assay. Analysis of an in vivo disease model not only serves to identify disease-associated genes, but can also provide novel integrated information on the complex orchestration of gene expression throughout the genome in concert with underlying pathologic processes.

Materials and Methods

Experimental Diabetes

All animal experiments followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committees of the Children's Hospital. Pathogen-free Long–Evans rats weighing 200 to 250 g (Charles River, Wilmington, MA) received a single intraperitoneal injection of 60 mg/kg streptozotocin (Sigma, St. Louis, MO) in 0.05 mM citrate buffer (pH 4.5) after an overnight fast. Control animals received citrate buffer only. Animals with blood glucose levels > 250 mg/dl 24 hours later were deemed diabetic. All animals studied were deemed healthy, as determined by their food and water intake as well as general appearance and activity level, to assure that they were not ill after STZ injection. All animals had blood glucose levels between 250 and 350 mg/dl before they were killed. The rats were fed standard laboratory chow and were allowed free access to water in an air-conditioned room with a 12-hour light/12-hour dark cycle.

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RNA Extraction from Retinal Tissue

Enucleation was performed under deep anesthesia on days 3, 7, and 21 of diabetes. Seven animals ($n = 14$ retinas) were studied at each time point together with seven nondiabetic control animals. The retinas were carefully dissected away and immediately frozen in liquid nitrogen. The total RNA from each sample was extracted using the guanidinium isothiocyanate extraction method with RNAzol (Tel Test Inc., Friendswood, TX), as described previously. Briefly, the tissue was incubated with guanidinium isothiocyanate buffer on ice, extracted with phenol/chloroform/isoamyl-alcohol, and precipitated with sodium acetate and glycogen carrier in isopropanol. After resuspension of the RNA pellet, the samples were further purified by reprecipitation. The RNA from the seven animals in each group was pooled for the gene filter analyses.

RNA Labeling and Hybridization

For each labeling, 1 ng total RNA was reverse-transcribed in the presence of 50 μCi of [^{32}P]dCTP, 500 μg of oligo-dT, and 200 units of Superscript II RT (Life Technologies, Inc., Rockville, MD) and DNA polymerase I (Life Technologies, Inc.). The labeled, double-stranded cDNA was denatured and hybridized to the cDNA Gene Filter arrays. Briefly, the Gene Filters were prehybridized at 42°C in a roller oven (Hybaid; Midwest Scientific, St. Louis, MO) with 1.0 g/ml poly-dA (Research Genetics, Inc., Huntsville, AL) and 1.0 g/ml *Cot*I DNA (Life Technologies, Inc.) in 5 ml of Microhyb solution (Research Genetics, Inc.) for a least 2 hours. After an overnight hybridization with the radiolabeled probe, the filters were washed twice at 50°C in 2× SSC (1× SSC, 15 mM trisodium citrate, and 150 mM NaCl), 1% SDS for 20 minutes, and once at room temperature in 0.5× SSC, 1% SDS for 15 minutes. The filters were then exposed overnight to a phosphor screen and scanned at 50-μm resolution with a Phosphor Imager (MD Storm; Molecular Dynamics, Sunnyvale, CA). Scanned images were exported as .tiff (.gel) files for image analysis in Pathways 2.0 software (Research Genetics, Inc.). After each hybridization, the filters were stripped by boiling in 0.5% SDS solution and scanned for residual leftover hybridization.

Microarray cDNA Filters

Two rat microarrays were purchased from Research Genetics Inc. The clone selection on the filter was based on the following criteria: the clones contained the 3′ untranslated gene region, had an average size of 1 kDa, and originated from Oligo-dT primed libraries. Genes were selected and spotted ($n = 5147$) on a 5 × 7-cm nylon membrane by the manufacturer. The filter had 1691 known genes and 3456 ESTs (expressed sequence tags: partially sequenced and studied cDNAs). To avoid system variability that may be associated with the use of different filters, we performed sequential hybridizations on the same filter with each of the different cDNA probes. Alteration of hybridization results by repetitive stripping of the filter was analyzed by comparing the first and last hybridization of each filter using the same cDNA probe. For specific RNA probes duplicate gene expression profiles were compared with determine reproducibility between gene filters ($n = 2$).

The 5147 genes were normalized for overall background (average intensity of all genes) and compared on each filter using the Pathways 2.0 software. To exclude false-positive signals due to background noise, all spots with an intensity value less than twofold background were discarded. The numerical results (pixel intensties) were imported into an Excel spreadsheet. After raw data filtering, the total number of genes studied dropped from 5147 to 3375, whereas the number of known genes dropped from 1691 to 838. The maximal alterations in gene expression ranged from approximately one fourth to eightfold.

Data Analysis

To calculate the relative change in gene expression, the corresponding background-corrected spot intensity values were normalized to nondiabetic control. Genes whose change in expression level was less than one tenth relative to the nondiabetic control were excluded from further analysis. A value for "relative expression index" was then obtained by taking the base 2 logarithm of the ratio of the intensity values of day x over the corresponding value for the nondiabetic. These relative expression indices were used for cluster analysis using the ClustanGraphics3 software (Clustan Ltd., Edinburgh, United Kingdom). Direct hierarchical clustering based on the increase in sum of square (log2; Ward method) was used.[10]

RNase Protection Assay

^{32}P-labeled antisense riboprobes for transforming growth factor (TGF) β1 to β3 and macrophage inhibiting factor (MIF) 1 were prepared by T7 polymerase transcription from respective vectors according to the manufacturer's protocols (PharMingen; RiboQuant, San Diego, CA). All samples were simultaneously hybridized with an 18S riboprobe (Ambion, Austin, TX) to normalize for variations in isolation and loading of RNA. Protected fragments were separated on a gel of 5% acrylamide/8 M urea/1× Tris-borate-EDTA and quantified with a PhosphorImager.

Statistical Analysis

All results are expressed as means ± SD. The data were analyzed by ANOVA with post hoc comparisons tested using Fisher's protected least significant difference procedure. Differences were considered statistically significant when P values were <0.05.

Results

Quality of the Gene Expression Data

For nondiabetic and day 21 diabetic experiments, sample hybridizations were preformed in duplicate using separate RNA samples. This test revealed that the spot intensities for each gene were highly reproducible between the two separate experiments (data not shown). To determine whether the intensity of a hybridization signal corresponding to a particular cDNA was reproducible between GeneFilters, we compared duplicate gene expression profiles for specific RNA probes. The duplicate filters were compared by overlying each blot and checking with the Pathways software that the variation of spot intensities was within a ratio of two. With this approach, <0.15% variability in gene expression was observed between normalized duplicate hybridizations (% variability = number of cDNAs differentially expressed between duplicate hybridizations/total number of cDNAs per filter × 100). Similarly, using the same probe for two different hybridizations (first and eighth hybridization) with the same filter, no significant alteration in gene expression was observed.

General Gene Regulation

STZ injected, nondiabetic animals (nonconverters; blood glucose < 100 mg/dl) showed <0.2% variability in retinal gene expression compared with non–STZ-injected diabetic animals ($n = 7$; data not shown). All subsequent comparisons were to data generated from nondiabetic animals (day 0). On day 3 after the induction of diabetes, 27 known genes showed expression levels more than twofold. On day 7, the expression of 60 genes was increased more than twofold, whereas on day 21 the number was 14. Of the total 3375 genes that exhibited altered expression levels in diabetes, 177 were upregulated by more than twofold on day 3, 298 on day 7, and 82 on day 21 (see Table 1).

The temporal pattern of expression varied among genes. A group of 627 genes (12.2%), including 141 known genes, exhibited increased expression on day 3 that peaked on day 7, but decreased again by day 21. Seventy-three genes (including 16 known genes) showed a steady increase in expression from day 0 to day 21. A third group, comprising 189 unknown and

79 known genes, showed decreased expression on days 3 and 7 (<2-fold), but then rebounded on day 21.

Functional Expression Patterns

The expression pattern for known genes with characterized functions was initially examined. The analysis revealed a transient upregulation (>2-fold) of a variety of cell cycle genes and genes related to growth and differentiation (cytokines, growth factors, and related genes) as well as genes operative in inflammation. Genes were categorized into subsets of known gene function as follows:

1. general metabolic enzymes (free radicals, energy),
2. insulin/glucose metabolism related genes (transporters, secretion genes, resistance),
3. signal transduction (e.g., ras, PKC),
4. adhesion molecules,
5. apoptosis,
6. proliferation related genes (cell cycle),
7. growth and tissue remodeling,
8. extracellular matrix,
9. differentiation,
10. inflammation,
11. secreted growth factors, regulators, and
12. stress proteins.

A selection of relevant genes under each group heading is listed in Table 1.

Confirmation of the Gene Expression Results by RNase Protection Assay

To confirm the gene regulation observed on the filter, we performed an RNase protection assay for selected genes: TGF-β1 to -3 and MIF. Compared with the results generated by the GeneFilter array, there was no significant difference in the expression pattern ($P > 0.05$). Both methods showed an upregulation of RNA levels from day 0 to day 7, with stabilization at an intermediate level thereafter (Fig. 1).

Clustering of Genes According to Their Expression Pattern

The large-scale expression pattern of the genes as an ensemble, independent of their nominal function was studied. Hierarchical unsupervised clustering was used, where no predefined reference groups were created. This led to a cluster-tree model consisting of eight clusters of similarly behaving genes (Fig. 2). Clusters 2 and 3 exhibited only minimal changes (<1.5-fold change relative to the nondiabetic levels) but varied in the degree of alteration. Clusters 1 and 4 showed an initial decrease in expression at day 3, with expression levels normalizing again by day 21. Clusters 5, 6, 7, and 8 contained genes that were induced to varying degrees, with maximal expression at day 7 and a subsequent decrease by day 21.

Discussion

Diabetic retinopathy is the most common microvascular complication in patients with long-standing diabetes. It is a progressive disease that ultimately affects all retinal layers.[11] Although various key pathogenic factors have been identified, a large-scale investigation of the gene expression patterns in the diabetic retina has not yet been reported. We used gene filter technology to analyze the temporal dynamics and patterning of gene expression in the retinas of STZ-induced diabetic rats. The alterations in gene expression are likely diabetes related, because STZ nonconverters showed no difference in gene expression compared with nondiabetic animals. The gene expression profiles only correlate gene expression with early changes in the diabetic retina and do not address the causative role of specific genes in diabetic retinopathy. Some of the upregulated genes may be epiphenomena, perhaps ultimately serving as diagnostic or prognostic markers. Below we discuss selected genes whose expression pattern was altered and, which because of their previously reported functions, may play a role in the pathogenesis of diabetic retinopathy. The expression pattern of the genes discussed below is shown in Table 1.

A critical early event in the pathogenesis of diabetic retinopathy is leukocyte adhesion to the diabetic retinal vasculature.[4,5] Our analysis found that genes encoding neutrophil adhesion proteins, such as the cell adhesion-like molecule, integrin alpha, vassopressin V1, VCAM-1, the glycoprotein CD44, are upregulated early in the course of diabetes in the rat retina (day 3 and day 7), whereas genes that encode proteins that reduce adhesion, such as entactin I (or nidogen-1) are downregulated (<2-fold, days 3–21; Table 1). The suppression of entactin-1 synthesis was recently found to disturb the aggregation of ECM molecules involved in the formation of a functional basement membrane.[12] Calpactin II, a gene that codes for a molecule that interacts with integrins and regulates monocyte adhesion, is also upregulated in the retina early in diabetes (day 3). Interestingly, the gene that encodes phakoglobin, a protein that is reduced in areas where neutrophils adhere to the endothelium, was found to be downregulated in the diabetic rat retina. Platelet aggregation has been observed in human diabetes.[13] Genes that regulate platelet activation and subsequent adhesion, such as cyclophilin B[14] (day 7) and p72[15] (day 21), were found to be upregulated later in the course of diabetes in our study (Table 1). Therefore, genes that regulate neutrophil and platelet adhesion exhibit a temporal expression pattern in the diabetic rat retina consistent with their behavior in diabetes mellitus.

Another group of genes that was upregulated encodes for metabolic and detoxifying enzymes. Diabetic retinopathy is thought to result from chronic changes in the metabolic pathways of the retina. Hyperglycemia leads to increased intracellular glucose concentrations, accumulation of intermediary metabolites such as sorbitol, an increased lactate/pyruvate ratio, abnormal changes of the redox state (NADH/NAD^+ ratio), and ultimately irreversible cell damage.[16] Glucose autoxidation, protein glycation, and advanced glycation products all disturb the redox balance and have been proposed to result in oxidative stress that contributes to the development of diabetic complications.[17] The activities of enzymes such as aldose reductase, lactate dehydrogenase, and aldehyde dehydrogenase has been shown to correlate with the macroangiopathy in diabetes. Moreover, the inhibition of aldose reductase may prevent the development of complications such as cataract formation and certain aspects of retinopathy in diabetic animals.[18] We found that the transcription of genes involved in the above listed metabolic pathways was markedly upregulated in the retina in the STZ-rat model (Table 1). Interestingly, we observed that the gene that encodes the ovalbumin upstream promoter gamma nuclear receptor (rCOUP), a known repressor of the aldehyde dehydrogenase gene,[19] was downregulated (<2-fold; days 3–21), perhaps contributing to the increase in the transcription of the above enzyme. Genes that code for enzymes that reduce oxidative species, such as dihydropteridin reductase, were also downregulated early in the course of diabetes as well as the gene for superoxide dismutase (SOD), an important free radical scavenger. Excessive production of superoxide anion, due to reduced neutralization via the SOD enzymatic pathway, is thought to contribute to the pathophysiology of the endothelial dysfunction.[20] Moreover, adenosine receptor mRNA was upregulated in the retina early in diabetes. Adenosine is a product of the metabolism of ATP and is a

TABLE 1. Expression Pattern of Genes in Diabetes

Functional Group	Genes Analyzed	Gene Function	Day 3	Day 7	Day 21
Metabolic enzymes (free radicals, energy)	Alcohol dehydrogenase	Polyol pathway	↑↑ 2.73	↑↑↑ 5.26	↑ 1.76
	Superoxide dismutase (SOD) gene	Antioxidant gene radical scavenger	↑↑ 3.87	↑↑ 4.43	↑↑ 3.40
	Aldolase A	Polyol pathway	↑↑ 3.845	↑↑ 4.78	↑ 1.76
	Aldehyde reductase	Polyol pathway	↑↑ 2.01	↑ 1.75	↑ 1.21
	Microsomal aldehyde dehydrogenase	Polyol pathway, AGE formation	↑ 1.92	↑↑ 2.91	↑↑ 2.14
	Lactate dehydrogenase B	Polyol pathway	↑ 0.56	↑ 1.19	↑ 0.59
	CHOT	Creatinine transporter, energy	↑ 1.32	↑ 1.53	↑ 1.62
	Aldehyde dehydrogenase	Polyol pathway, AGE formation	↑ 1.05	↓ 0.77	↓ 0.97
	Adenosine receptor	Ischemia sensor	↓ 0.89	↑ 1.43	↑ 1.47
	Channel integral membrane protein 28 (aquaporin 1 edema)	Water channel protein	↓ 0.71	↑ 1.25	↑ 1.08
	Dihydropteridin reductase	Antioxidant (reduction of ROS)	↓ 0.98	↓ 0.86	↑ 1.02
	P-450 monooxygenase (CYP2J3)	Role in oxidative stress, redox potential	↓ 0.79	↓ 0.69	↓ 0.76
	Aquaporin 3	Water channel protein	↓ 0.97	↓ 0.69	↓ 0.78
	Diphor 1	Na-phosphate transport	↓ 0.81	↓ 0.73	↓ 0.86
	rCOUP	Represses aldehyde dehydrogenase promoter, regulates genes that regulate metabolic pathway	↓ 0.93	↓ 0.914	↓ 0.93
Insulin/glucose related (transporters, secretion, resistance)	Gamma subunit of sodium potassium ATPase	Glucose transport	↑ 1.55	↑ 1.56	↑ 1.70
	Clathrin-associated adaptor protein homolog (p47A)	Secretion	↑ 1.19	↑ 1.45	↑ 1.08
	Sortilin	Role in glucose transport/intolerance	↑ 1.47	↑ 1.71	↑ 1.96
	Insulin-responsive glucose transporter	Role in glucose transport/intolerance	↓ 0.88	↓ 0.87	↓ 0.95
	Glucose transporter	Glucose transport	↓ 0.79	↓ 0.69	↓ 0.77
	pp63 (phosphorylated *N*-glycoprotein)	Phosphorylated form inhibits insulin receptor	↓ 0.84	↓ 0.71	↓ 0.78
Signal transduction	AMP-activated protein kinase	Metabolic control during ischemia, prevents glucose uptake	↑↑↑ 5.63	↑↑↑ 5.45	↑↑ 3.06
	Phospholipase D	Platelet activation	↑↑ 2.51	↑↑↑ 5.24	↑↑ 3.06
	CaM-like protein kinase	Ca^{2+} signal transduction	↑↑ 3.21	↑↑↑ 6.49	↑↑ 2.95
	Smad2	TGF, interferons	↑↑ 2.20	↑↑ 4.55	↑↑ 2.23
	ragA/A-raf	VEGF is upregulated by cell contact via A-raf; proliferation pathways	↑↑ 2.65	↑↑ 2.88	↑ 1.52
	MAPK	In response to inflammatory stimuli	↑↑ 2.38	↑↑ 2.73	↑↑ 1.98
	SAP kinase-3	Upregulated in leukos in response to inflammatory stimuli; with JNK in apoptotic pathways	↑↑ 2.78	↑↑ 3.61	↑ 1.12
	Stat-3	TGF, interferons	↑ 1.46	↑↑ 3.80	↑↑ 2.72
	Rap 1B	ras pathway, platelet activation	↑ 1.91	↑↑ 2.33	↑↑ 2.89
	PPT-1	Low levels of protein phosphatase 1 inhibit death pathways	↑ 1.46	↑↑ 2.43	↓ 0.87
	Diacylglycerolkinase	Activator of PKC	↑ 1.68	↑ 1.71	↑ 1.81

(*continues*)

TABLE 1. (*continued*). Expression Pattern of Genes in Diabetes

Functional Group	Genes Analyzed	Gene Function	Day 3	Day 7	Day 21
Signal transduction (*continued*)	Serine-threonine kinase receptor type I	Phosphorylation of Smads; mediators of growth factor related signal	↑ 1.05	↑ 1.60	↑ 1.66
	Inositol 1,4,5,-triphosphate-3 kinase	PKC pathway	↑ 1.12	↑ 1.04	↑ 1.08
	14-3-3 zeta	Inhibits PKC	↑ 1.09	↓ 0.84	↓ 1.01
	PKC binding protein 15	Binds PKC	↓ 0.862	↑ 1.229	↑ 1.506
	rheb	ras pathway, loss = growth arrest	↓ 0.98	↓ 0.73	↓ 0.89
	Periodic acid soluble protein	Growth arrest	↓ 0.78	↓ 0.69	↓ 0.69
	Protein kinase C receptor	Role in growth factor activation	↓ 0.92	↓ 0.76	↓ 0.94
	Hck (hematopoetic cell tyrosine kinase)	In the FcgII pathway	↓ 0.89	↓ 0.76	↓ 0.90
	Smad4	TGF, interferons	↓ 0.95	↓ 0.71	↓ 0.80
	GADD 153	Growth arrest	↓ 0.46	↓ 0.79	↓ 0.53
	Periodic acid soluble protein	Growth arrest	↓ 0.78	↓ 0.69	↓ 0.69
	p72	Tyrosine kinase that is activated in macrophages after reactive oxygen damage	↓ 0.84	↓ 0.77	↓ 0.80
Stress proteins	hsp 27	Heat shock protein	↑↑ 3.5	↑↑↑ 5.97	↑↑ 3.95
	hsp 60	Heat shock protein	↑↑ 3.51	↑↑ 4.12	↑↑ 2.81
	Tau protein kinase I	Tau is phosphorylated in response to cellular stress stimuli	↑ 1.24	↑ 1.78	↑ 1.88
	a-synuclein (chaperone)	Folds proteins to inhibits loss of tertiary structure	↓ 0.97	↓ 0.80	↓ 0.74
	gp 46	Collagen binding protein	↓ 0.79	↓ 0.77	↓ 0.82
Differentiation genes	MIPP1 (multiple inositol polyphosphatase)	Chondral differentiation	↑↑ 2.23	↑↑ 2.54	↑↑ 2.96
	Minoxidil sulphotransferase	Keratinocyte differentiation	↓ 0.92	↓ 0.83	↓ 0.86
	CALLUS 1	Osteoclast growth	↓ 0.97	↓ 0.82	↓ 0.87
	rPLP	Prolactin-like protein	↓ 0.85	↓ 0.75	↓ 0.77
Apoptosis genes	TEGT	Bax inhibitor 1	↑↑ 2.29	↑↑↑ 5.67	↓ 0.82
	Neuronal death protein	Mediates apoptosis	↑ 1.48	↑↑ 3.68	↑↑ 2.41
	Bad	Bcl-2–associated death promotor protein	↑ 2.27	↑↑ 5.68	↑ 4.82
	p53	Apoptosis pathways	↑ 1.63	↑↑ 2.09	↑ 1.47
	GAPDH	Apoptosis pathways	↑ 1.91	↑↑ 5.68	↑ 4.82
	N-myc	Transcription factor	↑ 1.03	↑ 1.48	↑ 1.48
	DRAL	p53-induced apoptosis	↑ 1.62	↑ 1.75	↑ 1.80
	CD30	Integration of pathways, fas-TRAIL	↑ 1.04	↑ 1.13	↑ 1.22
Proliferation	NFG-inducible antiproliferative secreted protein (PC3)	Inhibition of proliferation	↑ 1.38	↑↑ 2.4	↑↑ 2.78
	Antiproliferative factor (BTG1)	Inhibition of proliferation	↓ 0.75	↓ 0.64	↓ 0.84
Proliferation, growth, tissue remodeling	Cyclin G	Cell cycle	↑↑↑ 5.07	↑↑↑ 6.108	↑ 1.10
	Cycline D1	Cell cycle	↑↑ 2.84	↑↑ 3.62	↑↑ 2.43
	CDc2 promotor region	Activation of cell cycle–related genes	↑↑ 2.08	↑↑ 2.29	↑↑ 2.43

(*continues*)

TABLE 1. (*continued*). Expression Pattern of Genes in Diabetes

Functional Group	Genes Analyzed	Gene Function	Day 3	Day 7	Day 21
Proliferation, growth tissue remodeling (*continued*)	Membrane-type matrix metalloproteinase	Remodelling	↓ 0.87	↓ 0.80	↓ 0.91
	a-prothymosin	Regulated by myc; involved in cell proliferation	↓ 0.92	↓ 0.69	↓ 0.75
	Perchloric acid soluble protein	Arrests in G1/S; inhibits protein synthesis	↓ 0.79	↓ 0.69	↓ 0.69
Extracellular matrix	Gelatinase A	Tissue degradation	↑↑ 2.18	↑↑ 4.82	↑ 1.53
	TIMP-1,2	Tissue degradation angiogenesis	↑↑ 2.22	↑↑ 4.02	↑ 1.53
	Hepsin	Downregulates trypsin-like activity	↑ 1.75	↑↑↑ 5.65	↓ 0.07
	Collagen alpha 1 type 1	Extracellular matrix	↑ 1.32	↑ 1.35	↑ 1.42
	Cathepsin	Inhibits matrix accumulation	↑ 0.84	↑ 0.78	↑ 0.87
	Laminin A	Builds connective tissue	↓ 0.98	↓ 0.86	↑ 1.025
	Fibronectin	Stabilizes and builds connective tissue	↓ 0.91	↓ 0.87	↓ 0.99
Inflammation	Cofilin	Induced by NO, necessary for phagocyte function	↑↑ 4.08	↑↑↑ 5.07	↑↑ 2.95
	p105 coactivator	Precursor for NF-κB	↑ 1.28	↑↑ 2.25	↑ 1.92
	MIF	Macrophage inhibiting factor	↑ 1.49	↑ 1.91	↑ 1.20
	Endothelin	Activates	↑ 1.40	↑ 1.93	↑ 1.97
Adhesion molecules	Integrin alpha 1	Forms surface receptors	↑↑ 2.31	↑↑ 2.48	↑↑ 2.89
	VCAM-1	Vascular cell adhesion molecule	↑↑ 3.20	↑↑ 3.26	↑↑ 3.84
	Cell adhesion-like molecule	Cell adhesion	↑↑ 3.21	↑↑ 3.26	↑↑ 3.84
	FAK (focal adhesion kinase)	Platelet activation-adhesion	↑↑ 2.41	↑↑ 2.73	↑ 1.44
	Cyclophilin B	Ca-dependent adhesion of platelets to collagen	↑ 1.14	↑ 1.15	↓ 0.83
	Calpactin II	Vascular disease	↑ 1.07	↓ 0.89	↓ 0.86
	p72-FcgRII	Platelet transduction and activation	↓ 0.83	↓ 0.77	↑ 0.81
	Entactin	Reduces adhesion, wound healing	↓ 0.97	↓ 0.80	↓ 0.91
	Phakoglobin	Reduced when PMNs stick to the endothelium	↓ 0.84	↓ 0.64	↓ 0.94
Growth factors and regulators	Thrombin	Thyrosine phosphorylation, MAPK, NF-κB regulator	↑↑ 2.16	↑↑ 2.91	↑↑ 2.98
	mta	Transcription factor, histone deacetylase	↑ 1.63	↑↑ 2.32	↑↑ 2.33
	IGF-1	Insulin-like growth factor 1	↑ 1.12	↑↑ 2.08	↑ 1.23
	IGFBP-5	Insulin-growth factor binding protein	↑ 1.28	↑ 1.38	↑ 1.50
	Interleukin 1β mRNA	Upregulates NO and VEGF synthesis	↑ 1.55	↑ 1.45	↑ 1.22
	FGF receptor activating protein (FRAG1)	Activates fibrobast growth factor	↓ 0.97	↓ 1.28	↑ 1.38

Genes were assigned to the following groups according to their function: metabolic enzymes (e.g., free radicals, energy), insulin/glucose-related genes (e.g., transporters, secretion genes, resistance), signal transduction (e.g., ras, PKC), adhesion molecules, apoptosis genes, proliferation, growth factors, tissue remodeling, extracellular matrix, differentiation genes, inflammation, and stress proteins. The standardized ratios between the measured point and nondiabetic controls are shown. The data are presented in the following 6 groups (ratio of change in intensity relative to nondiabetic, where expression levels were set as 1). The exact fold increase is also displayed. ↑↑↑: >5× (very large increase); ↑↑: 5> × >2 (large increase); ↑: 2> × >1 (increase); ↓: 1> × >0.5 (decrease); ↓↓: 0.5> × >0 (large decrease).

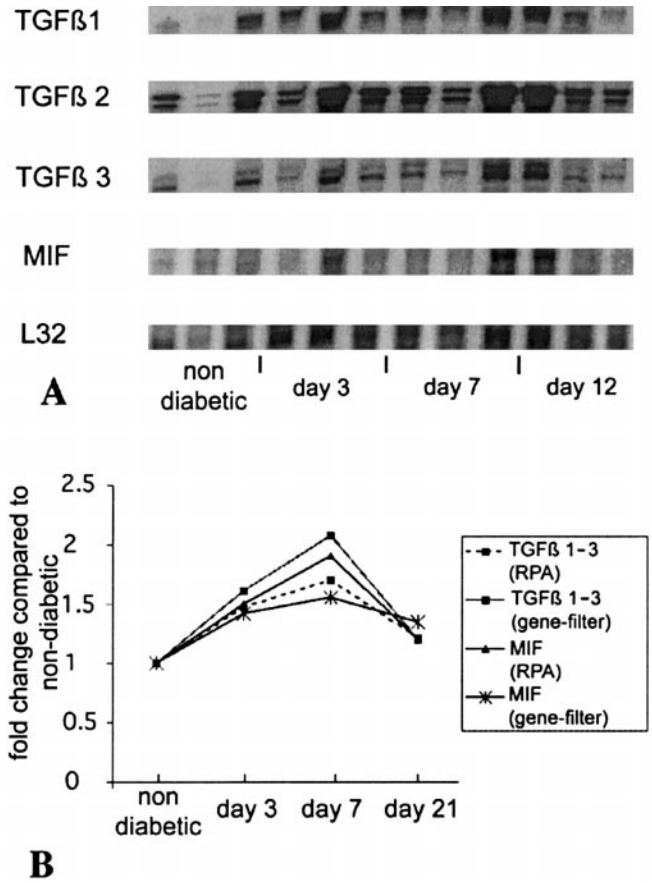

FIGURE 1. (**A**) RNase protection assay of the retinal MIF and TGFβ1 to 3 levels. Each lane represents pooled RNA from 7 eyes. (**B**) Comparison of gene expression for MIF and TGFβ (1 to 3) between RNA-protection assay and the gene filter. The values for the three TGFβ isoforms were added together up to facilitate comparison with the gene array where the three isoforms are represented by one data point. Both the gene filter array and with the RNA-protection assay show increased expression toward day 7 with stabilization and lower expression levels thereafter. There is no significant difference between the fold change in observed expression between the gene filter and the RNase protection assay ($P < 0.05$).

significant component of the retina's compensatory hyperemic response to substrate deprivation, as occurs in diabetes.[21] Adenosine receptor stimulation has a protective effect in ischemia, but its overstimulation may contribute to the cellular damage through reduction of blood flow, altered gene expression, the accumulation of superoxide radicals from adenosine catabolism, and the upregulation of nitric oxide synthase activity.[22] Notably, adenosine is also believed to increase the expression of vascular endothelial growth factor.[23–25]

Another striking finding was the increased expression of genes involved in signal transduction pathways that might play an important role in the pathogenesis of endothelial and pericyte dysfunction in diabetes. The protein kinase C (PKC) and the mitogen-activated kinase (MAPK) pathways have been implicated in diabetic retinopathy, either directly or indirectly via upstream growth factors, hyperglycemia, reactive oxygen species, and products of the sorbitol pathway.[26,27] PKC and MAPK have been proposed to trigger the cellular events necessary for the development of certain features of diabetic retinopathy, such as the breakdown of the blood retinal barrier and the death of endothelial cells and pericytes.[28,29] We found that the main activator of PKC, diacylglycerol kinase, was upregulated as early as day 3, and PKC-binding proteins that target PKC to its potential substrates, such as PKC binding protein beta15, were upregulated later in the course of diabetes (day 21), whereas inhibitors of PKC, such as 14 to 3–3 zeta, were downregulated (<2-fold, days 7 and 21). MAPK mRNA was also markedly upregulated, as were members of the ras pathway, such as ragA and A-raf, which play an important role in the activation of MAPK by growth factors. Interestingly, the ras family members also mediate the upregulation of VEGF via the MAPK pathway. In parallel, we observed a marked upregulation of genes that encode proteins involved in the transduction of growth factor signals, such as VEGF, TGF-beta, or various interleukins. Serine threonine receptor type I (STRI) mRNA increased early (day 3) in diabetic retinopathy. Through its intrinsic kinase activity, STRI phosphorylates intracellular proteins known as Smads that form heterodimers, translocate into the nucleus and mediate transcriptional responses for cytokines such as TGF-beta, interferons, and interleukins.[30] Smad 2 (<5-fold, days 3–21) and Stat 3 (<5-fold, days 3–7) were also found to be upregulated in our study, consistent with the activation of the TFG-β pathways. Mediators of the activation pathways for platelets, hematopoietic cell tyrosine kinase (hck),[31] Rap 1B[32] (<5-fold, days 7–21) and phospholipase D[33] or mediators in the activation of macrophages (tyrosine kinase p72[34]), or neutrophils (SAP kinase 3[35]) were also upregulated in our gene filter analysis. Similarly, stress proteins were upregulated. Hsp27 and Hsp60 transcription was markedly enhanced (<5-fold, days 3–21), perhaps as a consequence of the oxidative stress that the retina endures.[36,37] Tau protein kinase, which phosphorylates tau protein in response to cellular stress stimuli,[38] was also upregulated (<2-fold, days 3–21). In contrast, a-synuclein, a molecular chaperone that helps proteins maintain their tertiary structure, decreased less than twofold (days 3–21). Interestingly, despite its initial upregulation, hsp70 was significantly downregulated later in the course of diabetes in our study (<2-fold, day 21). Leukocyte-derived nitric oxide has been reported to have an important role in the endothelial dysfunction and the upregulation of adhesion molecules in endothelial cells that mediate the leukocyte extravasation in nonophthalmic tissues.[39] Because hsp70 is a heat shock protein that was reported to confer resistance to nitric oxide toxicity,[40] this downregulation may contribute to the leukocyte-mediated endothelial damage observed in the diabetic retina (Joussen AM, Poulaki V, Adamis AP, et al., unpublished results, 2001).

Another set of genes found to be upregulated in early diabetes were genes involved both in proliferation and apoptosis. One of the central manifestations of diabetic retinopathy is the proliferation of microvascular endothelial cells under the influence of growth factors that are upregulated throughout the course of diabetes.[41] In parallel, cellular stress results in endothelial and pericyte death.[42] We found that antiproliferative genes such as BTG-1,[43] growth arrest factors such as GADD-153 cell growth regulator,[44] growth arrest mediators such as rheb[45] (<2-fold, days 3–21), or the perchloric acid-soluble protein 1[46] were downregulated, whereas genes like N-myc[47] (<2-fold, days 3–21), CGR11[48] and alpha prothymosin[49] (<2-fold, days 3–21), which all stimulate proliferation, are upregulated or remain constant. Genes encoding cyclins, such as cyclin D1 (<5-fold, days 3–21) and G (>5-fold, days 3–7), were also upregulated, suggesting that the retinal cells are entering the cell cycle.[50,51] In addition to proliferation-related genes, we found that genes involved in apoptosis were also upregulated, such as the tumor suppressor gene p5 (<5-fold, day 7) and DRAL (<2-fold, days 3–7), a protein that induces apoptotic death through p53.[52] *N*-myc is also known to facilitate apoptotic death induced from a variety of stimuli.[53] Apoptosis promoting genes of the bcl-2 family, such as Bad,[54] were upregulated (<5-fold, day 7), whereas inhibitors of apop-

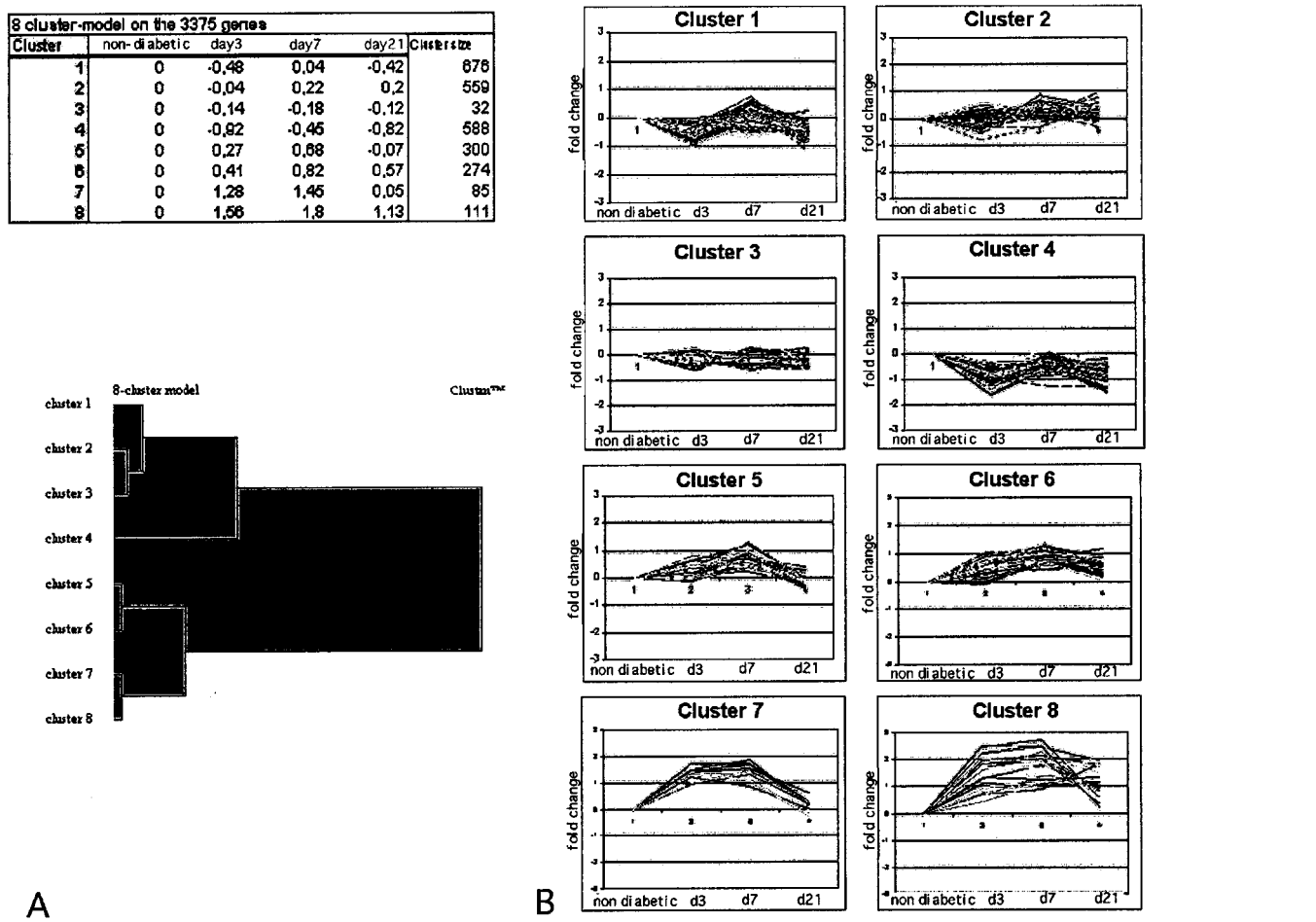

FIGURE 2. Eight-cluster model of 3375 genes. The hierarchical clustering of genes was based on their time course of expression. Four points are depicted: nondiabetic and 3, 7, and 21 days of diabetes. Log2 [expression level ratio relative to day 0 (nondiabetic)] values were used for Cluster analysis using the Ward method. The dendrogram was cut at the eight-cluster level. (**A**) Dendrogram of hierarchical clustering of the genes (intercluster relationships). The dendrogram was cut based on visual inspection. The numbers of the "branches" identify the clusters shown in the table in the upper left-hand corner. (**B**) Graphic demonstration of each cluster. Time courses of relative gene expression levels in the eight gene clusters.

tosis, such as the gene TEGT[55] were first upregulated (>5-fold, days 3–7) and then downregulated (<2-fold, day 21). CD30, another molecule that regulates several apoptotic pathways was also found to be upregulated. CD30 signals regulate a variety of apoptotic molecules such as Fas ligand, death receptor 3, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), TNFR-associated factor 1 (TRAF1), and cellular inhibitor of apoptosis 2 (cIAP2).[56] It is possible that growth factors, cytokines, and reactive oxygen intermediates produced by inflammatory cells upregulate the pro-apoptotic genes in the endothelial cells and pericytes and collaborate with ischemia and oxidative stress to induce apoptosis.

A central finding of our gene array study was the appearance of numerous mRNAs of genes with known roles in inflammation and wound healing after the induction of diabetes. We and others have previously demonstrated that early diabetic retinopathy shows features of an inflammatory disease.[4,5,57–59] In the present study, we found that the mRNA for macrophage inhibitory protein (MIF), a proinflammatory lymphokine involved in delayed hypersensitivity and phagocytosis, is significantly upregulated early in diabetic retinopathy.[60] It has been shown that insulin and glucose regulate MIF expression in cultured adipocytes in vitro, and MIF is believed to be involved in maintaining neutrophils in the vasculature and facilitating their adhesion and local release of cytokines.[61,62] Another inflammation-related gene that was upregulated was endothelin B (ETB) receptor (<2-fold, days 3–21). Its ligand, endothelin (ET), is a potent vasoconstrictor that acts as a permeability factor, perhaps in collaboration with VEGF, in diabetes. In nonophthalmic tissues, it works via a PKC-mediated mechanism and regulates extracellular matrix protein gene expression in target organs.[63] ET-receptor blockade can prevent short-term diabetes in the rat.[64,65] Thrombin was also upregulated (<5-fold, days 3–21). It plays an important role in platelet activation and adhesion, stimulates the expression of adhesion molecules in endothelial cells, and increases the production of cytokines that trigger the binding of leukocytes and platelets in the endothelium.[66] Therefore, thrombin mechanistically couples tissue damage to inflammation. In the same context, extracellular matrix genes such as laminin A, fibronectin, and collagen were downregulated. The tissue inhibitors for matrix metalloproteinases, TIMP-1, and TIMP-2 were upregulated as well as gelatinase A (<5-fold, days 3–7), possibly contributing to the extracellular matrix turnover that characterizes diabetes.[67]

Last but not least, the mRNAs for various growth factors and cytokines that are implicated in diabetic retinopathy were found to be upregulated in our study. We have previously shown that VEGF mRNA increases during the course of diabetes in the rat.[68] We now show that the mRNA for IGF-I, IGF-I

binding proteins (<2-fold, days 3–21), TGF-β (<2-fold, days 7–21), and IL-1β were upregulated early in the diabetic retina of the rat. TGF-β is believed to be produced by pericytes and to act as an autocrine factor, regulating their proliferation and their interaction with endothelial cells.[69,70] It also increases nitric oxide synthase activity in the endothelial cells, which in turn upregulates adhesion molecules and endothelin-1 expression.[71] The activity of the IGF axis depends on the dynamic balance between IGF and its binding proteins that modulate IGF bioactivity. Dysregulation of this axis can enhance vascular smooth muscle cell growth, migration, and extracellular matrix synthesis.[72] Recently, it was proposed that the sequestration of IGF-I by the IGF binding proteins contributes to the pathogenesis of microvascular disease in diabetes by lowering the amounts of this trophic factor.[73] Three IGF binding protein isoforms were elevated early in diabetes. We also found that the mRNA for IL-1β was upregulated in the diabetic rat retina. IL-1β is a chemoattractive cytokine and is believed to be an effector molecule in type I diabetes.[74] It has been shown to upregulate nitric oxide and VEGF synthesis[75] and to activate the transcription factor NF-κB in endothelial cells.[76] NF-κB is an important molecule in leukocyte activation and regulates apoptotic pathways in endothelial cells and the response to cellular stress. It is interesting to note that the p105 mRNA, a molecule that regulates the synthesis of the precursor for NF-κB, was also upregulated in our study (<5-fold, day 7).

Although most of the altered expression of known (named) genes after the induction of diabetes is consistent with the pathologic process, it should be noted that gene expression profiling of a tissue such as the pathologic retina does not allow one to deduce the cellular origin of the respective mRNAs, because of the cellular heterogeneity of the tissue specimen. Thus, the increase in many inflammation-related genes might simply reflect the influx of leukocytes into the diseased tissue.

Once sufficient databases of generic gene expression profiles become available, the signature expression profile of specific cell types can be extracted from whole-tissue profiles, thus enabling the monitoring of the change of cellular composition.[8] Expression profiling thus represents a global approach to characterize a pathologic process at the genomic scale but should be complemented with structure preserving methods such as in situ hybridization and immunohistochemistry. However, given the completion of the human genome sequencing project and the dramatic advances in functional genomic technologies that enable genome-wide expression monitoring with instant identification of the individual genes, the paradigm for studying the molecular basis for diseases is undergoing a major shift.[77] To fully embrace the advantages of a large-scale gene expression profiling, however, we have to extend our view from individual genes to global patterns (which would also include the expression of unnamed ESTs). At the simplest level of finding general patterns, a global interpretation of expression profiles can be achieved by grouping the genes based on the similarity of their expression behavior. Using hierarchical, unsupervised clustering, we found that an eight-cluster model fits the data well (Fig. 2). Members of the same clusters exhibit similarity with respect to both their expression levels during the observation period (qualitative temporal expression profile) and the overall change in their expression levels (quantitative expression level change). The premise of such cluster analysis has been that clusters contain genes that are likely to be coregulated or serve the same biological function. However, in a more integrative view that goes beyond merely assigning functions to genes, it has been suggested that genomic regulation is organized such that genes and proteins appear in functional modules. In conjunction with pathogenesis one could for instance define an "inflammation module," as a robust, coregulated set of genes that are used as an entity in a variety of tissue damage. With the development and spread of more encompassing microarrays, the systematic search for such regulatory modules, rather than individual genes, might lead to universal principles of pathogenesis and thus improve diagnosis and therapy.

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