The Transforming Growth Factor-β Pathway Is a Common Target of Drugs That Prevent Experimental Diabetic Retinopathy

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OBJECTIVE—Prevention of diabetic retinopathy would benefit from availability of drugs that preempt the effects of hyperglycemia on retinal vessels. We aimed to identify candidate drug targets by investigating the molecular effects of drugs that prevent retinal capillary demise in the diabetic rat.

RESEARCH DESIGN AND METHODS—We examined the gene expression profile of retinal vessels isolated from rats with 6 months of streptozotocin-induced diabetes and compared it with that of control rats. We then tested whether the aldose reductase inhibitor sorbinil and aspirin, which have different mechanisms of action, prevented common molecular abnormalities induced by diabetes. The Affymetrix GeneChip Rat Genome 230 2.0 array was complemented by real-time RT-PCR, immunoblotting, and immunohistochemistry.

RESULTS—The retinal vessels of diabetic rats showed differential expression of 20 genes of the transforming growth factor (TGF)-β pathway, in addition to genes involved in oxidative stress, inflammation, vascular remodeling, and apoptosis. The complete loop of TGF-β signaling, including Smad2 phosphorylation, was enhanced in the retinal vessels, but not in the neural retina. Sorbinil normalized the expression of 71% of the genes related to oxidative stress and 62% of those related to inflammation. Aspirin had minimal or no effect on these two categories. The two drugs were instead concordant in reducing the upregulation of genes of the TGF-β pathway (55% for sorbinil and 40% for aspirin) and apoptosis (74 and 42%, respectively).

CONCLUSIONS—Oxidative and inflammatory stress is the distinct signature that the polyol pathway leaves on retinal vessels. TGF-β and apoptosis are, however, the ultimate targets to prevent the capillary demise in diabetic retinopathy. ***Diabetes*** **58:1659–1667, 2009**

The clinical manifestations of diabetic retinopathy are consequences of a microangiopathy where retinal capillaries develop thickened basement membranes, lose pericytes and endothelial cells by accelerated apoptosis, form microaneurysms, become abnormally permeable, and eventually are transformed into acellular tubes of basement membrane no longer perfused (1–3). When a critical number of capillaries are affected, retinal edema and/or ischemia ensue. In principle, prevention should be attainable with correction of the hyperglycemia, but is not being attained today because the means of treating diabetes are still imperfect and do not easily maintain normoglycemia. Thus, retinopathy and its sight-threatening features continue to occur even among patients treated intensively (4).

There is an outstanding need for drugs that complement antidiabetic treatment by limiting the effects of residual hyperglycemia on retinal vessels. No such drugs are available for clinical use, even though several have been tested in human diabetic retinopathy. The clinical trials performed to date may have failed because the drugs were given too late, for too short a period, and/or at ineffective doses (5), but also because the drug targets may have been irrelevant to human diabetic retinopathy. These considerations encourage the development of approaches that would limit the risk of both false-negative and true-negative trials in the future.

One such approach could be to identify the molecular processes that are causally linked to retinal microangiopathy in animal models and then test whether the processes are active in human diabetic retinopathy and therefore represent efficient targets for new drug trials. We tested this approach using the streptozotocin diabetic rat, which models human diabetic retinopathy more accurately than the diabetic mouse (6). To capture comprehensively the effects of diabetes on retinal vessels, we examined the genome-wide gene expression profile of the vessels, which had not been investigated previously. The results of gene profiling can be translated to human diabetes because changes in gene expression can be studied accurately in postmortem human retinas (7,8), at variance with biochemical and posttranslational changes (8). We studied rats with 6-month duration of diabetes to capture events reflecting the ongoing injury induced by diabetes as well as the adaptive or maladaptive response to the injury that precedes capillary obliteration. The latter increases in the rat after 8–9 months of diabetes (3,9).

To identify the abnormalities with pathogenic importance, we compared the molecular effects of drugs well documented to prevent the histological features of microangiopathy in the diabetic rat. We reasoned that pathways that are attenuated by more than one drug and have relevant biological consequences can be attributed a candidate pathogenic role, to be verified and pursued in successive studies. In this work, we studied the aldose reductase inhibitor (ARI) sorbinil and aspirin because they protect the retinal vessels of diabetic rats (10,11) likely through different mechanisms. Sorbinil inhibits the first enzyme of the polyol pathway, which is activated when

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glucose levels are high, and can damage cells by multiple mechanisms (12). Aspirin is a pleiotropic drug that protects the retinal vessels against diabetes in multiple species (11,13) through mechanisms that do not appear to include its antiplatelet effects (11), but are otherwise unsettled. There was a reasonable expectation that the effects of the two drugs on molecular pathways would show differences as well as similarities and that the similarities, in turn, could identify essential pathways to be silenced or attenuated to attain prevention of the vascular damage induced by diabetes.

RESEARCH DESIGN AND METHODS

Procedures involving animals were approved by the animal care and use committee of the Schepens Eye Research Institute. Male Sprague-Dawley rats (5 weeks of age; Taconic Farms, Hudson, NY) were randomly assigned to one of the following groups: control, diabetic, diabetic treated with sorbinil (65 mg · kg^{-1} · day^{-1}; gift of Pfizer, Groton, CT), and diabetic treated with aspirin (30 mg · kg^{-1} · day^{-1}). Induction of diabetes with streptozotocin, administration of sorbinil and aspirin with the food, and treatment of diabetic rats with maintenance insulin were as previously described (10,11). Diabetic rats and age-matched nondiabetic control rats were studied after 3, 6, and 8 months of diabetes. A1C, plasma salicylates levels, and retinal levels of sorbitol and fructose were measured as previously described (6,10,11).

Isolation of retinal vessels and RNA extraction. Rat retinal vessels were isolated by hypotonic lysis of the fresh retina, a method documented to yield the retinal vascular network free of glia and neural contamination (10). The two retinas of each rat were dissected and incubated in ice-cold sterile distilled water for 1 h at 4°C. After incubation with DNase to dissolve DNA released from the lysed neural and glial cells, the vascular network was transferred to ice-cold PBS and cleaned of debris and remaining glial and neural elements by gentle pipetting. Total RNA free of contaminating genomic DNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) following the on-column DNase digestion protocol according to the manufacturer's instructions. The retinal vascular network was also prepared by trypsin digestion (3) of retinas fixed in formalin immediately after killing.

Oligonucleotide microarray hybridization and data analysis. Gene expression profiling of rat retinal vessels was performed using the GeneChip Rat Genome 230 2.0 array (Affymetrix, Santa Clara, CA). Each sample subjected to microarray hybridization consisted of the RNA extracted from the vessels of both retinas of one individual rat. Because the amount of RNA was insufficient for the standard hybridization protocol, each RNA sample was subjected to a round of amplification (Ovation biotin system; NuGEN Technologies, San Carlos, CA). GAGE R&R analysis documented that the amplification procedure did not introduce variance. All procedures for microarray analysis were carried out at the Harvard Medical School/Partners Healthcare Center for Genetics and Genomics Core Laboratory (http://www.hpcgg.org). Gene expression data were preprocessed with MAS 5.0, and probes that were consistently labeled as absent in all samples were excluded from subsequent analysis.

Analysis of the microarray data was performed using BADGE (Bayesian analysis of differential gene expression; http://genomethods.org/badge), a Bayesian approach to identify differentially expressed genes across two experimental conditions designed to yield high reproducibility at low sample size (14). The differential expression of each gene in two conditions is estimated by the fold change, and evidence of differential expression is measured by the probability that the fold change exceeds a fixed threshold, conditional on the data. To reduce the number of false positives due to multiple comparisons, the selection of significant changes of expression was based on a threshold on the posterior probability specified to achieve a preset false discovery rate, as determined by significance analysis of microarrays (15).

To identify the genes whose expression is affected by diabetes, only the 19,054 probe sets "present" in all samples (eight diabetic and nine controls) were included in the analysis. Probe sets with a probability >0.985 (equivalent to a false discovery rate <0.01) were selected as differentially expressed in diabetes. To determine which of the diabetes-induced gene expression changes were prevented by the two drugs, we selected the probes identified as differentially expressed in diabetes and compared their expression in the sorbinil-treated diabetic group ($n = 5$) versus the control group, and in the aspirin-treated diabetic group ($n = 9$) versus the control group. Genes for which the posterior probability of differential expression versus control was >0.95 were considered different from control, and the diabetes-induced changes were thus classified as not corrected by the drug. Conversely, when the posterior probability of differential expression versus control was <0.95, the expression was classified as similar to control and thus normalized by the drug. The threshold was higher than that used to identify differential expression caused by diabetes to increase the specificity of the conclusions about the drug effects.

The probe sets differentially expressed in diabetes were annotated using NetAffx (www.affymetrix.com/analysis), and the annotations were individually verified based on the latest UniGene cluster of the corresponding representative sequence. The differentially expressed genes identified through the annotation process were assigned to functional categories based on the information provided by Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene) and additional information from the published literature.

Real-time RT-PCR. cDNA was synthesized (8) from 1 μg of total RNA isolated from retinal microvessels and total retina of rats different from those studied in the microarray experiments. Real-time PCR was performed as previously described (16). Primers and probe sets for rat transforming growth factor (TGF)-β1 and ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motif type 1) were from Applied Biosystems (Foster City, CA). Relative expression was determined by the comparative $\Delta\Delta C_T$ method using β-actin as the endogenous control (16).

Western blotting. Fresh retinal microvessels and whole retinas were homogenized in RIPA buffer containing protease and phosphatase inhibitors (10). Immunoblotting was performed as previously described (10,16). The blots were sequentially probed for TGF-β receptor 1 (TGFβR1), cyclooxygenase (COX)-1, and nuclear receptor coactivator 3 (NCoA3). Ras GTPase activating protein (17) or β-actin were used to verify protein loading. For evaluation of Smad2 phosphorylation, blots were probed first for phosphorylated Smad2, stripped, and reprobed for total Smad2. The primary antibodies used are listed in supplemental Table 1, which is available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db08-1008/DC1.

TGFβR1 immunohistochemistry. Retinal trypsin digests were rehydrated in PBS and incubated in citrate buffer (pH 6.1; Dako, Carpinteria, CA) at 95°C for heat-mediated antigen retrieval. After permeabilization in 1% Triton X-100, quenching of endogenous peroxidase in 3% H_2O_2, and blocking in 10% normal goat serum, endogenous biotin (unmasked by the antigen retrieval procedure) was blocked by incubation in avidin and biotin–blocking solutions (Vector Laboratories, Burlingame, CA). The trypsin digests were then incubated overnight at 4°C with the TGFβR1 primary antibody (V-22; Santa Cruz Biotechnology) diluted to 1 μg/ml in 2% goat serum/0.1% Triton X-100 in PBS. The antigen-antibody complexes were detected by the avidin-biotin-peroxidase method (ABC Elite; Vector Laboratories) and visualized with diaminobenzidine. Each experiment included trypsin digests from each of the four groups of rats as well as a negative control obtained by substituting the primary antibody with an equivalent concentration of normal rabbit IgG. The trypsin digests were photographed at ×5 magnification (AxioVision imaging system; Carl Zeiss, Gottingen, Germany) to reconstruct the entire vascular network from each retina (11). The networks were used to identify for each rat four areas in the midretina with equivalent location and free of artifacts. The four areas were photographed at ×20 magnification using the same exposure and light setting to allow for comparison among samples. For evaluation of TGFβRI immunostaining, the images were grouped in panels of 12 images each, with each panel containing images from control, diabetic, and diabetic treated rats. The staining intensity of the capillaries within each image was scored on a continuous scale from 0 (no staining) to 4 (highest staining) by four masked observers. The scores attributed to each retina by the four observers were averaged to obtain one individual final score for each rat. The scoring was consistent among the four observers, as indicated by an overall coefficient of variation of 17%.

Membrane attack complex immunohistochemistry. Complement activation in rat retinal vessels was evaluated by membrane attack complex immunostaining on rat retinal sections as previously described (10).

Statistical analysis. The results of the effect of sorbinil and aspirin on diabetes-induced gene expression changes are summarized as the number of genes whose expression was normalized by the drug within each functional category. The significance of the deviation of this number from the null hypothesis of no gene being normalized was determined by means of a Fisher's exact test (2 × 2 contingency tables). Results of Western blotting and real-time RT-PCR are summarized as the means ± SD; the results in the diabetic and control group were compared by means of a two-tailed unpaired Student's t test. Results of TGFβRI immunostaining are summarized with the means ± SD; the results in the four groups of rats were compared with ANOVA followed by Fisher's protected least-significant differences test. StatView 5.0 software (SAS Institute, Cary, NC) was used for all analyses.

TABLE 1
Characteristics, retinal polyol pathway activity, and serum salicylate levels of study rats

	n	Body weight (g)	A1C (%)	Retinal sorbitol (nmol/mg protein)*	Retinal fructose (nmol/mg protein)*	Serum salicylates (mg/l)†
Control	50	629 ± 77	5.8 ± 1.5	1.17 ± 0.40	3.85 ± 1.45	Not tested
Diabetes	47	384 ± 50‡	13.3 ± 1.9‡	12.54 ± 8.64§	65.32 ± 13.16‡	Undetected
Diabetes with sorbinil	15	382 ± 48‡	12.3 ± 1.4‡	0.56 ± 0.25	6.40 ± 2.14	Not tested
Diabetes with aspirin	15	370 ± 43‡	12.9 ± 2.9‡	Not tested	Not tested	13.5 ± 7.8

Data are means ± SD. Statistical analysis was performed with ANOVA followed by Fisher's protected least-significant difference test. *Measurements were performed in four rats from each group; †measurements were performed in three diabetic rats treated with aspirin and one untreated diabetic rat; ‡$P < 0.0001$, §$P < 0.01$ compared with control rats.

RESULTS

The characteristics of the rats used in the microarray and related studies are presented in Table 1. The microarray study was based on 31 expression profiles of retinal microvessels isolated from diabetic rats ($n = 8$), diabetic rats treated with sorbinil ($n = 5$), diabetic rats treated with aspirin ($n = 9$), and control rats ($n = 9$). The duration of diabetes was 6 months. When compared with the nondiabetic rats, the retinal vessels of the diabetic rats showed significantly different expression of 127 known genes (131 probe sets) and 229 expressed sequence tags (ESTs) or genes with similarity to known genes but not yet fully characterized. The majority of the 127 known genes (105 upregulated and 22 downregulated by diabetes) could be grouped into several functional categories (Table 2).

The TGF-β pathway in diabetic retinal vessels. There was a prominent effect of diabetes on the expression of genes involved in signaling by members of the TGF-β family or affected by such signaling (Fig. 1 and supplemental Table 2). A total of 20 genes could be attributed to this category, which made the TGF-β pathway the single functional pathway most affected by diabetes in retinal vessels. Diabetes increased the expression of the type I receptor activin receptor–like kinase 5 (ALK-5), a widely distributed TGF-β type I receptor that in endothelial cells signals inhibition of migration and proliferation (18). ALK-1, a TGF-β type I receptor that is expressed more selectively in endothelium and induces migration and proliferation (18), was not upregulated by diabetes. There was increased expression of syndecan-2, which is upregulated by TGF-β and, in turn, upregulates the TGF-β type I and type II receptors (19); of CD44, also a target of TGF-β that facilitates the activation of TGF-β and signaling by the type I receptor (20); and of ubiquitin conjugating enzyme 9, the key enzyme of the sumoylation reaction, which is known to enhance TGF-β signaling (21). The diabetic retinal microvessels showed enhanced expression of connective tissue growth factor (CTGF), the main TGF-β effector in the induction of fibrosis (22); and of other genes prominently regulated by TGF-β, such as tissue inhibitor of metalloproteinase 1, the serine (or cysteine) proteinase inhibitor heat shock protein 47, prostaglandin-endoperoxide synthase 1 (COX-1), the histone acetyltransferase NCoA3, and thioredoxin-interacting protein (Txnip) (22–25). For some of these molecules, increased expression was confirmed at the protein level (supplemental Fig. 1).

Diabetic retinal vessels showed changes in gene expression pointing to increased signaling also by bone morphogenetic proteins (BMPs) (Fig. 1 and supplemental Table 2). There was increased expression of BMP receptor type 1a, also named ALK-3 and utilized by BMP-2 (26), and of periostin and frizzled homolog 1, both responsive to

TABLE 2
Functional clustering of the known genes differentially expressed in retinal vessels in diabetes

Functional category and known function*	Upregulated	Downregulated
TGF-β/BMPs pathway		
20 genes		
TGF-β activation	16	—
BMP activation	3	1
Oxidative stress		
7 genes		
Pro-oxidant	3	—
Antioxidant	2	1
Pro-/antioxidant	1	—
Inflammation and response to injury		
29 genes		
Inflammatory mediators	5	—
Acute-phase response proteins	9	—
IFN-γ pathway	8	—
Antigen presentation	3	1
Anti-inflammatory	3	—
Matrix and vascular remodeling		
35 genes		
Matrix	4	1
Cell adhesion	7	1
Actin organization	3	2
Vascular remodeling	17	—
Cell cycle		
7 genes		
Cell cycle progression	1	2
Cell cycle arrest	1	1
Proliferation inhibition	2	—
Apoptosis		
19 genes		
Proapoptotic p53 pathway	5	2
Proapoptotic	7	1
Antiapoptotic	2	2
Other		
39 genes		
Metabolism	8	1
Miscellaneous†	6	2
Function unknown‡	15	7

*The 127 known genes differentially expressed in diabetes were assigned to functional categories based on review of information from NCBI (National Center for Biotechnology Information) Entrez Gene integrated with data from published literature. Individual genes are listed in all pertinent categories based on their known functions. The complete list of the genes included in each functional category is presented in supplemental Tables 2–8. †Includes genes related to functions that cannot be assigned to any of the major categories; ‡as stated in bibliographical sources.

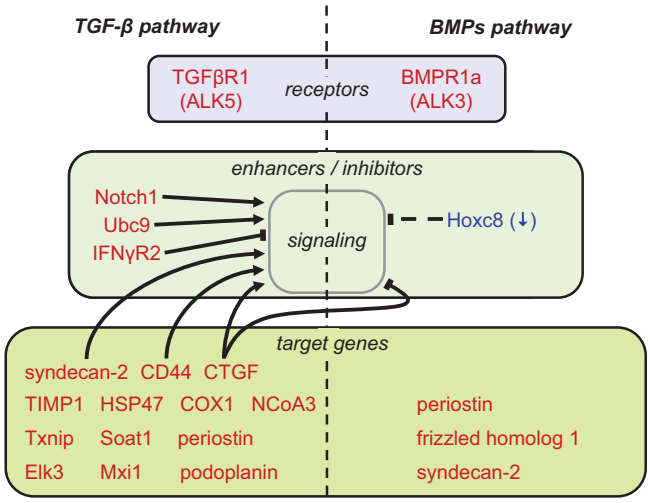

FIG. 1. Diabetes alters the expression of multiple genes of the TGF-β pathway (left side of the panel) and BMPs pathway (right side) in rat retinal vessels. Upregulated genes are shown in red, downregulated in blue. The expected effect of overexpression of genes on Smad signaling is shown by arrowed lines (stimulation) or blunt lines (inhibition). The expected effect of downregulation of Hoxc8 is release of inhibition, and thus activation of signaling (dotted blunt line). BMPR1a, BMP receptor 1a; HSP47, heat shock protein 47; IFNγR2, γ-interferon receptor 2; Mxi1, MAX interactor 1; Soat1, sterol O-acyltransferase 1; TIMP1, tissue inhibitor of metalloproteinase 1; Ubc9, ubiquitin conjugating enzyme 9.

vascular injury and known targets of BMP-2 (27,28). Facilitated BMP signaling was also suggested by the downregulation of the homeobox C8 (Hoxc8) transcription factor because Hoxc8 is a negative regulator of transcription of Smad1 and other BMP-specific receptor-regulated Smads (29). The array included probe sets for BMP-1 through -7; only the BMP-2 transcript was detected in all samples, and its levels were not modified by diabetes.

Of the three TGF-β isoforms present in mammals, the array showed the TGF-β2 and -β3 transcripts; the levels of neither were altered by diabetes. The TGF-β1 mRNA was undetectable in all samples tested. When measured by the more sensitive real-time RT-PCR, TGF-β1 mRNA levels were increased 1.5-fold in the retinal microvessels of diabetic rats ($P = 0.04$) (Fig. 2*A*). Increased levels of the TGF-β type I receptor ALK-5 was confirmed at the protein level (Fig. 2*B*). Because ALK-5 signals through Smad2 and Smad3, we sought evidence of ALK-5 activation by measuring Smad2 phosphorylation in the retinal vessels of diabetic and control rats. We found increased Smad2 phosphorylation after both 6- and 3-month duration of diabetes (Fig. 2*C*). Taken together, the data indicate that the complete loop for TGF-β1 signaling is enhanced in diabetic retinal vessels.

To learn whether the diabetes-induced changes in the TGF-β pathway extended to nonvascular cells of the retina, we measured the levels of the TGF-β1 transcript, TGF-β type I receptor, and Smad2 phosphorylation in the whole retina. We observed no changes induced by diabetes (Fig. 3*A–C*).

Genes related to oxidative stress, inflammation, and remodeling in diabetic retinal vessels. Diabetes increased the expression of molecules that generate oxygen-derived free radicals and decreased the expression of antioxidants (supplemental Table 3). Similar to the aorta of diabetic rats (30), the retinal vessels showed prominent upregulation of Txnip, a negative regulator of the antioxidant thioredoxin. Diabetes also increased the expression of genes involved in inflammation and response to injury (supplemental Table 4). The gene expression program was thus consistent with, and expanded, previous findings pointing to the occurrence of oxidative stress and inflammation in diabetic retinal vessels (9,31,32). Genes involved in tissue remodeling through formation of extracellular

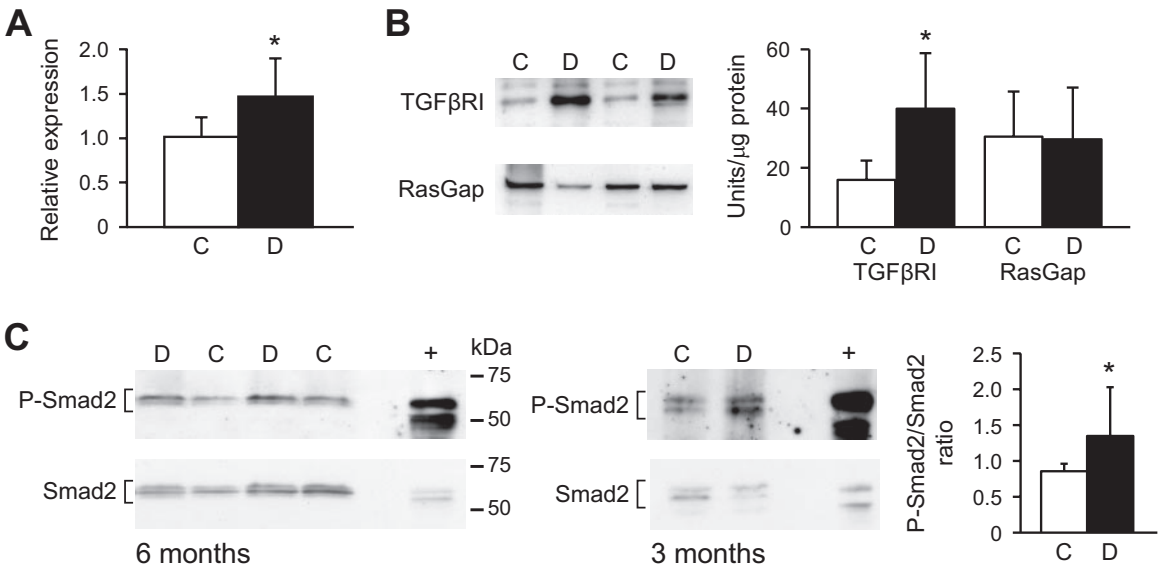

FIG. 2. Upregulation of the TGF-β1 signaling loop in retinal vessels of diabetic rats. Total RNA and protein lysates were prepared from retinal vessels isolated by hypotonic lysis from the retina of diabetic (D) and age-matched control (C) rats. *A*: TGF-β1 mRNA levels assayed by quantitative real-time PCR. Relative expression of TGF-β1 mRNA was calculated by the comparative C_T method using β-actin as endogenous control. Values are the means ± SD of $n = 6$ rats per group. *$P = 0.04$. *B*: Representative Western blot of TGFβR1 (ALK-5) and bar plot of the quantitative analysis. Blots were probed first for TGFβR1 followed by Ras GTPase activating protein (RasGAP) as control for loading. Values are the means ± SD of $n = 3–5$ rats per group. *$P < 0.04$. *C*: Representative Western blots of phosphorylated Smad2 (P-Smad2) and total Smad2 (Smad2) and bar plot of the P-Smad2–to–Smad2 ratio. The P-Smad2–to–Smad2 ratio was examined in the retinal vessels of rats with 6 months (*left panel*) and 3 months of diabetes (*right panel*). The 58–60-kDa bands detected in both retinal microvessels and the positive control (+; TGF-β–treated HepG2 cells) corresponds to Smad2; the 50-kDa band present in the positive control only corresponds to Smad3. The bar plot presents the pooled results obtained in rats with 6 and 3 months of diabetes. Values are the means ± SD of $n = 5–9$ rats per group. *$P = 0.05$.

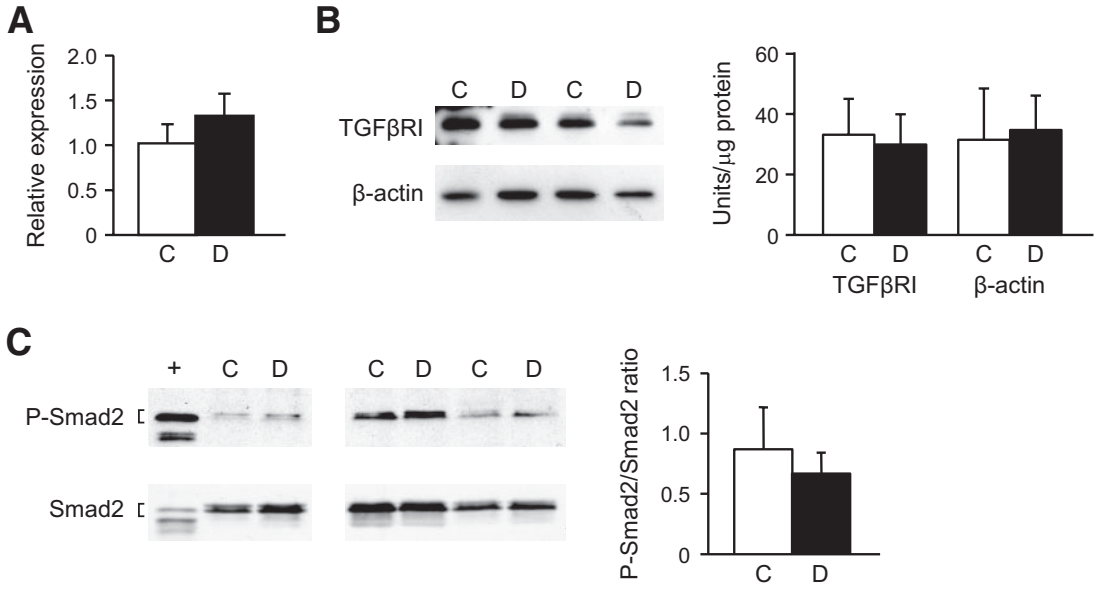

FIG. 3. TGF-β1 signaling is not increased in the neural retina of diabetic rats. Total RNA and protein lysates were prepared from the whole retina of diabetic (D) and age-matched control (C) rats. *A*: TGF-β1 mRNA levels assayed by quantitative real-time PCR. Values are the means ± SD of n = 4–5 rats per group. *B*: Representative Western blot of TGFβR1 (ALK-5) and bar plot of the quantitative analysis. Blots were probed first for TGFβR1 followed by β-actin as control for loading. Values are the means ± SD of five rats per group. *C*: Representative Western blots of phosphorylated Smad2 (P-Smad2) and total Smad2 (Smad2) and bar plot of the P-Smad2–to–Smad2 ratio. Values are the means ± SD of 8–14 rats per group.

matrix, regulation of cell adhesion, and organization of the actin cytoskeleton were upregulated in the diabetic vessels (supplemental Table 5). All changes related to actin were in the direction of increased polymerization and filament stability, which may contribute antiproliferative effects (33). Additional gene expression changes induced by diabetes pointed to inhibitory effects on cell cycle progression and cell proliferation (supplemental Table 6).

Proapoptotic pathways in diabetic retinal vessels. Multiple mechanisms of apoptosis appeared activated in diabetic vessels, many not suspected previously (supplemental Table 7). Changes in the expression of six genes of the p53 pathway converged in the proapoptotic direction. Another potential mechanism for direct death signals was the upregulation of Fas. TGF-β can induce apoptosis by multiple mechanisms (34), which include upregulation of Fas and activation of c-Jun NH_2-terminal kinase (34,35), the expression of which was also increased in the diabetic retinal vessels (supplemental Table 7). A potential mechanism for apoptosis by neglect was the downregulation of the G-protein–coupled receptor of lysophosphatidic acid Edg2 (endothelial differentiation gene 2), a survival factor for multiple cell types (36).

Concordant and discordant effects of the ARI sorbinil and aspirin on the diabetes-induced changes in gene expression in retinal vessels. The sorbinil and aspirin doses were previously documented to prevent the development of acellular capillaries in diabetic rats (10,11). In this study, the dose of sorbinil overnormalized sorbitol and reduced fructose accumulation by 96% in the retina of diabetic rats (Table 1), thus achieving almost complete inhibition of glucose flux through the polyol pathway. The aspirin dose, known to inhibit by 90% thromboxane B_2 formation during blood clotting in rats and thus exert an antiplatelet effect (11), resulted in serum salicylate levels (Table 1) 10- to 20-fold lower than those achieved by anti-inflammatory doses of aspirin (rev. in 11). Sorbinil normalized the diabetes-induced expression changes in 56% of the known genes and aspirin normalized those in 32%; the figures for the total number of probe sets (known genes and ESTs) were 48 and 47%, respectively.

Both sorbinil and aspirin attenuated significantly and to a similar extent the effects of diabetes on the TGF-β pathway (Fig. 4), preventing the expression changes of 55 and 40% of the genes, respectively. Of note, both drugs prevented the increased expression of TGF-β receptor 1 and CTGF. Likewise, sorbinil and aspirin reduced significantly and to a similar extent the effects of diabetes on proapoptotic pathways, with concordant correction of changes in the p53 pathway and concordant restoration of survival signals. In contrast, sorbinil reduced the changes in gene expression relevant to oxidative stress and inflammation strikingly more than aspirin (Fig. 4). Sorbinil led to normalization of expression of 71% of genes related to oxidative stress and 62% of those related to inflammation, whereas the figures for aspirin were 29 and 3%, respectively ($P < 0.0001$ for effect of sorbinil vs. aspirin on inflammation-related genes). The effect of sorbinil and aspirin on the expression of individual genes is reported in supplemental Tables 2–8.

The effects of sorbinil and aspirin on diabetes-induced activation of the TGF-β pathway were also tested on trypsin digests prepared from the retinas of rats with 8 months of diabetes. These preparations afforded the opportunity of studying the retinal vascular network fixed immediately after death and not subjected to hypotonic lysis while fresh. Figure 5 shows that the diffuse pattern of TGFβR1 immunoreactivity was increased in the retinal capillaries of diabetic rats compared with control rats ($P = 0.05$) and that both aspirin and sorbinil treatment prevented the increase ($P = 0.03$ and $P < 0.0001$ vs. diabetes, respectively), in agreement with the microarray results (supplemental Table 2). Of note, whereas aspirin returned TGFβR1 immunoreactivity to control values ($P = 0.62$ vs. control), sorbinil suppressed staining intensity below control values ($P = 0.015$ vs. control). Phosphory-

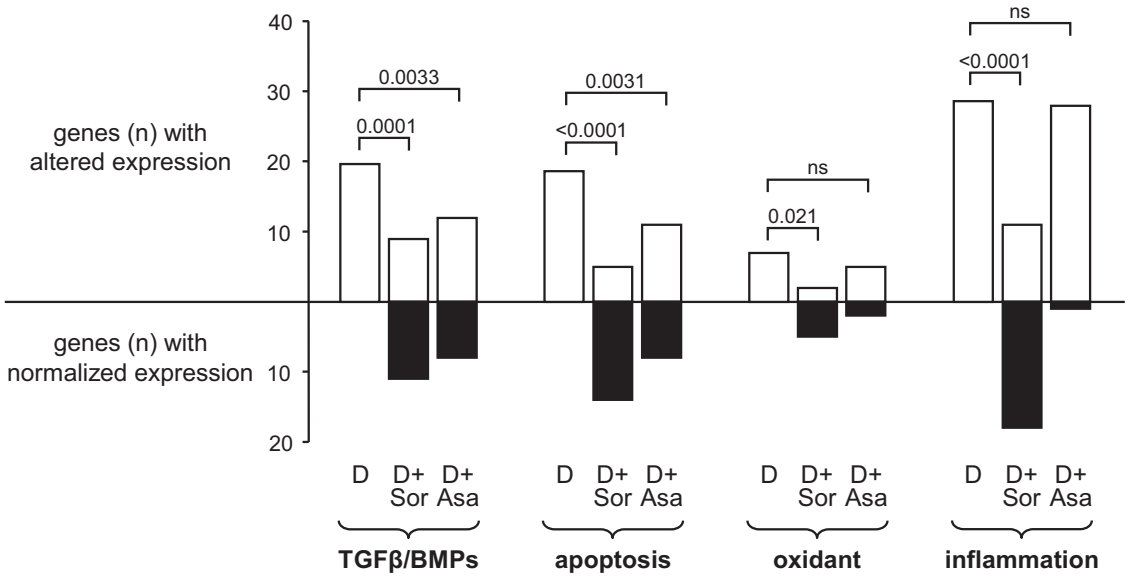

FIG. 4. Effects of sorbinil and aspirin treatments on the gene expression changes induced by diabetes in rat retinal vessels. Each functional category includes all genes pertinent to that category, as indicated in Table 2. Data were analyzed by Fisher's exact test. □, the number of genes differentially expressed in the retinal vessels of diabetic rats (untreated or treated) compared with control rats; ■ the number of genes whose expression is normalized by the drug (i.e., the expression in the retinal vessels of treated diabetic rats was not different from that in control rats). D, diabetic rats; D+Sor, diabetic rats treated with sorbinil; D+Asa, diabetic rats treated with aspirin; ns, nonsignificant.

lated Smad2 could not be detected in the fixed vascular preparations.

The discordant effect of sorbinil and aspirin on inflammation, as shown by the mRNA data, was consistent with the discordant effect on the increased levels of intercellular adhesion molecule 1 in the diabetic rat retina (11) and was confirmed by data on complement activation in retinal vessels. Supplemental Fig. 2 shows that aspirin, at variance with sorbinil (10), failed to prevent deposition of membrane attack complex in retinal vessels of diabetic rats.

The signature of diabetes on retinal vessels is tissue specific. We compared the effects of diabetes on gene expression in retinal vessels to those in Müller cells, the principal glia of the retina that shows signs of activation/reactivity in both human (37) and experimental (6) diabetes. We had previously examined the gene expression profile of Müller cells in the same model used in this study (rats with 6-month duration of streptozotocin diabetes) and on a comparable platform (GeneChip Rat Genome RG-U34A; Affymetrix) (16). Supplemental Table 9 shows that the functional gene categories affected by diabetes in retinal vessels differed from those affected in Müller cells. Of the genes related to TGF-β that we found affected by diabetes in retinal vessels, 80% were represented in the RG-U34A array, but only 6% of those were affected by diabetes in Müller cells. Of the genes related to apoptosis affected by diabetes in retinal vessels, 68% were represented in the RG-U34A array, and 0% of those were affected by diabetes in Müller cells. Only the inflammation-related genes showed overexpression in both the vascular and glial cell types (76% of the genes affected by diabetes in the vessels were represented in the RG-U34A, and 55% were upregulated by diabetes in Müller cells).

DISCUSSION

The results of this work indicate that retinal microangiopathy in diabetic rats is the product of several molecular pathways that are interconnected but not of equal pathogenic importance. Attenuation of increased activity of the TGF-β pathway and apoptosis, but not of oxidative stress and inflammation, appear to be required to prevent the microangiopathy. In view of its role in wound healing, extracellular matrix deposition, and fibrosis (22), TGF-β has over the years been an obvious candidate mechanism for the basement membrane thickening and matrix accumulation that are the hallmark of diabetes on blood vessels and vascular structures. However, the studies pointing to a role of excess TGF-β have been performed solely in relation to diabetic nephropathy (38).

Diabetic retinopathy has not been previously linked to excess TGF-β signaling, likely because the link has been more difficult to capture. We found increased TGF-β and TGF-β signaling selectively in microvessels but not in cells more abundantly represented in the retina, such as Müller glial cells (16) or neurons, that would make an increase manifest in whole retinal extracts (this work). The few suggestions to date of a contribution of TGF-β to diabetic retinal microangiopathy have come from the observations that the retinas of diabetic rats show the presence of oncofetal fibronectin (39), an isoform typically stimulated by TGF-β during tissue healing and remodeling, and the retinas of diabetic patients show expression of CTGF in pericytes, a shift to the vascular compartment from the microglial location of CTGF in the retinas of nondiabetic individuals (40). Our finding of increased Smad2 phosphorylation indicates that increased TGF-β signaling does in fact occur in retinal vessels of diabetic rats, and the concordant attenuation of such signaling by drugs that protect the vessels from the effects of diabetes through different mechanisms suggests that the increased signaling contributes to the vascular pathology. It may be argued that the diabetic retinal vessels showed overexpression of inflammation-related genes, whereas TGF-β is viewed, in general, as anti-inflammatory. In this respect, the increased TGF-β1 expression and activity in diabetes could even represent a compensatory response and, as such, be protective for the retinal vessels. However, the finding that

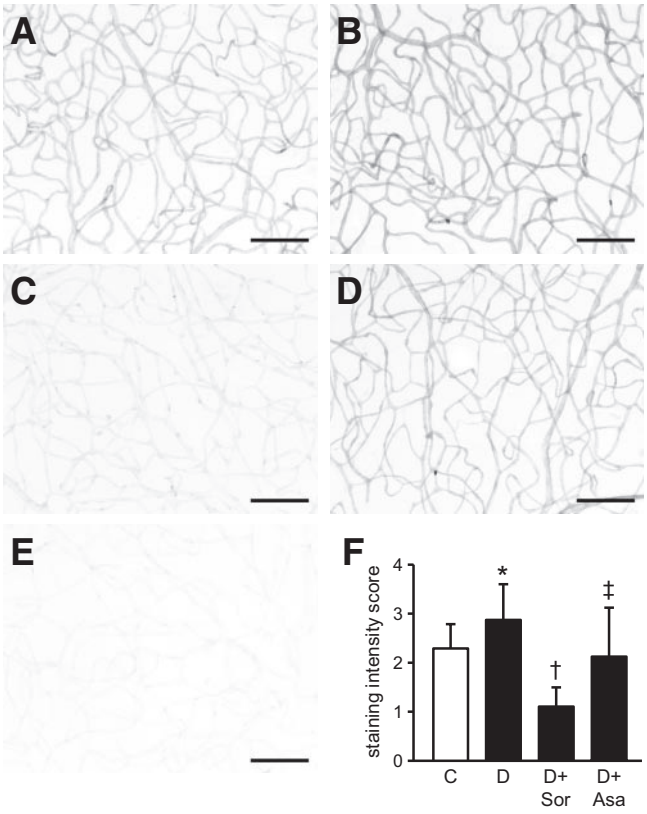

FIG. 5. Increased TGFβRI immunoreactivity in retinal vessels of diabetic rats is prevented by sorbinil and aspirin treatments. TGFβRI was detected by immunohistochemistry in retinal trypsin digests from diabetic rats (8 months of diabetes duration), diabetic rats treated with sorbinil or aspirin, and age-matched control rats. The effect of diabetes and of the two drugs on TGFβRI immunostaining was quantitated by four masked observers. *A–C*: Representative photographs of midretina fields showing TGFβRI immunostaining of retinal capillaries. *A*: Control, score 2.0 ± 0.7 (mean ± SD of scores by the different masked observers). *B*: Diabetes, score 3.8 ± 0.04. *C*: Diabetes treated with sorbinil, score 1 ± 0.0. *D*: Diabetes treated with aspirin, score 2.3 ± 0.7. *E*: Negative control. Scale bar = 100 microns. *F*: Bar plot of the quantitative analysis of staining intensity. Values are the means ± SD of the final scores computed for each individual rat. C, control rats, $n = 11$; D, diabetic rats, $n = 10$; D+Sor, diabetic rats treated with sorbinil, $n = 6$; D+Asa, diabetic rats treated with aspirin, $n = 6$. *$P = 0.05$ vs. control rats; †$P < 0.02$ versus control rats, diabetic rats, and diabetic rats treated with aspirin, ‡$P < 0.04$ versus diabetic rats.

both sorbinil and aspirin attenuated the TGF-β pathway, but only sorbinil reduced inflammation, indicates that the TGF-β pathway was upregulated independently of the genes related to inflammation. This agrees with findings in Müller cells, which show in diabetic rats changes in the expression of genes related to inflammation but not to TGF-β (16). Fibrosis is not necessarily driven by inflammation, which might explain the general lack of efficacy of anti-inflammatory mediators in the treatment of fibrotic disease (41). In diabetes in particular, TGF-β upregulation could be induced and sustained by high glucose levels, which stimulate TGF-β promoter activity in vascular cells (42).

Comparison of the effects of sorbinil and aspirin led to the identification of the signature of the polyol pathway on retinal vessels. The ARI prevented or attenuated changes in all functional categories of genes, indicating that the polyol pathway mediates most molecular abnormalities induced by hyperglycemia in rat retinal vessels, including TGF-β overexpression. The role of the polyol pathway in mediating glucose-induced increases in TGF-β has been documented in cultured cells (43). Only the ARI, and not aspirin, prevented the pro-oxidant and proinflammatory changes in gene expression, consistent with the discordant effects of the two drugs on other indicators of diabetes-induced oxidative stress and inflammation (this work and 10,11). This finding, combined with the known pro-oxidant effects of the polyol pathway (44) and the fact that reactive oxygen species—induced by hyperglycemia or other stimuli—beget inflammation (45), points to the combination of oxidative stress and inflammation as the distinct signature of the polyol pathway on retinal vessels.

The effects of aspirin indicated that attenuation of pro-oxidant and proinflammatory gene expression is not necessary to prevent retinal vascular cell apoptosis and vessel histopathology (11) in diabetes. We cannot exclude that aspirin may have attenuated aspects of inflammation not reflected in the gene expression data, but we note that the concentration of aspirin achieved in our rats (two orders of magnitude lower than those exerting typical anti-inflammatory effects) (rev. in 11) also failed to attenuate complement deposition in diabetic retinal vessels. However, it remains conceivable that attenuation of oxidative stress and/or inflammation may be sufficient to prevent retinopathy if these processes in diabetes are upstream of, and contribute to, apoptosis. What the effect of aspirin highlights is that in diseases resulting from the contribution of multiple pathogenic events, the attenuation of some of the events is sufficient to prevent the ultimate phenotype, despite persistence of the other known contributors. An example of such interplay was recently documented in a model of Alzheimer's disease (46). The mechanism(s) whereby aspirin attenuated the effects of diabetes on the TGF-β and pro-apoptotic pathways in retinal vessels remain speculative. The dose effective in our diabetic rats was 20-fold lower than the dose reported to decrease the excess TGF-β and CTGF expression induced by diabetes in the rat kidney and by high glucose in cultured mesangial cells (47). Comparison with the selective antiplatelet agent clopidogrel had suggested that our dose of aspirin protects retinal vessels in diabetes by mechanisms other than antiplatelet activity (11). Aspirin could have worked by inhibiting the activity of COX-1 in vascular cells, which expressed higher levels of the enzyme in diabetes. However, it is not known whether increased COX-1 is harmful to vascular cells, and the fact that aspirin prevented the overexpression of COX-1 (not a previously known effect of aspirin) suggests that the effects on COX-1 were downstream of some other beneficial effect. At concentrations consistent with those achieved in our experiments, aspirin induces NO release from endothelial cells (48).

Our findings have several translational implications. Knowledge that the combination of oxidative stress and inflammation is the distinct signature of the polyol pathway on retinal vessels makes it possible to seek such a signature in human diabetes to help determine whether there is a rationale for adjunct therapy with ARIs. The action of aspirin, which prevents the vascular histopathology resulting from a complex process by attenuating only selected pathways, establishes a valuable paradigm for development of diversified drug approaches to the complications of diabetes. The observation that increased TGF-β signaling begins early in diabetic retinal vessels proposes that capillary remodeling also begins early. Early onset of pathological remodeling can be one of the reasons for the "memory" that retinal vessels carry of exposure to

the diabetic milieu (5,49), which translates into poor reversibility of even the initial lesions of diabetic retinopathy and justifies emphasis on prevention (5). Targeting the TGF-β pathway may become a rational strategy in the prevention of the two main microvascular complications of diabetes, retinopathy and nephropathy. In view of the homeostatic importance of TGF-β, the targeting modalities would need not to interfere with physiological activity. Partial attenuation of overactivity appears to be sufficient to limit the consequences on tissues. Phenotypes attributable to increased TGF-β signaling can be rescued by inactivation of a single *TGF-β1* allele (50), and in our study prevention of retinal diabetic microangiopathy did not require complete normalization of gene expression changes in the TGF-β pathway. Angiotensin II blockers may prove effective anti–TGF-β drugs insofar as they were recently reported to reduce the progression of vascular pathologies caused by excessive TGF-β signaling (51). This effect of angiotensin II blockers, coupled with our findings of an overactive TGF-β pathway in diabetic retinopathy, may need to be taken into account in interpreting the encouraging results obtained with candesartan in the primary prevention of retinopathy in normotensive type 1 diabetic patients (52). Orally active inhibitors of TGF-β signaling are in development (53) and would add options for highly targeted interventions. The next steps in these studies are to define the effects of selective normalization of TGF-β signaling in retinal vessels and ascertain whether the TGF-β pathway is overactive in human diabetic retinopathy.

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