Effect of Dicarbonyl Modification of Fibronectin on Retinal Capillary Pericytes

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PURPOSE. To determine effects of α-dicarbonyl modification of an extracellular matrix protein on retinal capillary pericyte attachment and viability.

METHODS. Primary cultures of bovine retinal pericytes (BRPs) were seeded on either normal fibronectin (FN) or FN modified by methylglyoxal (MGO) and glyoxal (GO). Apoptosis was measured by flow cytometry along with caspase-3 activity. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) and Akt/PKB were evaluated by Western blot analysis. Cellular glutathione and reactive oxygen species were measured. αB-crystallin was measured by Western blot analysis and, to determine its role in apoptosis, experiments were conducted using BRPs that were transiently transfected with αB-crystallin.

RESULTS. Cultures seeded on MGO- or GO-modified FN showed a significant reduction in the number of viable cells, an increase in the number of apoptotic cells, and increased caspase-3 activity, which correlated with the extent of FN modification. Pericytes seeded on either type of modified FN showed phosphorylation of p38 MAPK and dephosphorylation of Akt/PKB. Cultures seeded on dicarbonyl-modified FN had reduced glutathione and increased levels of reactive oxygen species compared with those on a normal matrix. Cells on the altered matrices had reduced αB-crystallin levels as well. Transient transfection of rat αB-crystallin into BRPs significantly reduced the apoptosis triggered by α-dicarbonyl-modified FN.

CONCLUSIONS. These observations indicate that modification of FN by α-dicarbonyl compounds triggers apoptosis through a combination of increased oxidative stress and reduction of αB-crystallin. This mechanism may contribute to loss of pericytes in diabetic retinopathy and contribute to the resultant vascular lesions. (Invest Ophthalmol Vis Sci. 2004;45:1983–1995) DOI:10.1167/iovs.03-0995

Selectiive degeneration of pericytes in the retinal capillary vessels is a distinguishing feature of early retinal vascular damage in diabetes. Loss of protective pericytes can result in decreased capillary tonicity, formation of microaneurysms and vessel dilation.1–7 Normally, retinal capillary pericytes extend long cytoplasmic processes over the surface of endothelial cells to form interdigitating contacts between the two cell types. This interaction facilitates the maturation, remodeling, and maintenance of the vascular system through secretion of growth factors and continuing modulation of the extracellular matrix.4–8 Pericytes are also thought to influence regulation of vascular permeability.9

Several mechanisms, including oxidative stress and glycation, may account for the loss of pericytes from retinal vessels in diabetes. These mechanisms appear to be interrelated. During protein glycation, reactive oxygen species (ROS) are produced as byproducts, and advanced glycation end product (AGE) synthesis is accelerated by oxidative stress.10–13 Numerous studies have shown an increase in oxidative stress in diabetic retina.13–16 Treatment with antioxidants has been shown to inhibit initial lesions, supporting a role for oxidative stress in diabetic retinopathy.14,15 Although mechanisms of oxidative damage are not clear, studies implicate oxidative stress-induced signaling pathways in diabetic complications.18,19 Glycation, the reaction of aldehydes and ketones with amino groups on proteins, produces alterations collectively known as AGEs.20–22 Extracellular AGEs are toxic to retinal capillary cells, especially to pericytes. AGEs accumulate within retinal capillary basement membranes (BMs) or within capillary cells in diabetes.23,24 and receptors for AGEs (RAGE) were detected in diabetic retinas.25–27 AGE-induced pericyte death presumably involves AGE-RAGE interaction.28 Additional studies have shown that RAGE binds AGEs administered to diabetic rats27 and that receptor-mediated internalization of AGEs increases oxidative stress in vascular cells.29 In addition to these receptor-mediated events, experiments with pharmacological agents provide indirect evidence for involvement of AGEs in the pathogenesis of diabetic retinopathy.30,31

Several studies have documented the overexpression of retinal BM proteins in diabetes, which may explain the observed thickening of capillary BMs in that disease.3,25,26 Demonstration of enhanced fibronectin (FN) synthesis in diabetic retinal capillaries33,34 was of particular interest in our study. Capillary cells are anchored to matrix adhesion molecules in BM through receptors belonging to a superfamily of integrins. Integrins bind to RGD peptide domains on BM proteins.35 Arginine in this peptide sequence is likely a target for glycation in diabetes. Kalfa et al.36 showed that BM protein modification by glucose reduces pericyte cell proliferation. Others found impaired attachment of pericytes to BM proteins that are secreted by high-glucose-treated endothelial cells.37 These observations indicate that modification of BM proteins can affect retinal capillary cells and suggest that abnormalities of normal cellular attachment could cause functional anomalies, such as increased permeability. Impaired cell attachment may also lead to anoikis, a process in which programmed cell death is induced by the loss of cell-matrix interaction.
Recent studies have shown that the α-dicarbonyl compounds, methylglyoxal (MGO) and glyoxal (GO) produce AGE-like compounds on proteins 38,39 suggesting that these compounds are major modifiers of proteins in vivo. 40 MGO is produced by several mechanisms, the major one being nonenzymatic conversion of the glycolytic intermediates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. GO is produced by various oxidative pathways, including oxidation of polyunsaturated fatty acids. 41 These α-dicarbonyls react strongly with arginine and, to a lesser extent, with lysine and cysteine in proteins. Plasma concentrations of these dicarbonyls are increased in diabetes, 42 and several products of the reaction between the α-dicarbonyls and protein have been isolated and detected in vivo. 38,39,43 The elevation of MGO and GO, and modification of BM proteins in diabetes coupled with the high susceptibility of pericytes to AGEs prompted our study with cultured pericytes. Because we believe that modification of BM proteins by α-dicarbonyls is an important factor in retinal vascular damage in diabetes, we initiated these in vitro studies to determine how dicarbonyl-modified FN affects adherent pericytes.

Materials and Methods

Reagents were obtained from the following sources: caspase-3 substrate (Ac-DEVD-AFC) from Calbiochem (La Jolla, CA); FN active fragment (RGDS) from Peptides International (Osaka, Japan); and FN (from bovine plasma), MGO, and aminoguanidine (AG) from Sigma-Aldrich (St. Louis, MO). MGO was purified further by distillation twice under low pressure and temperature. Peroxidase-conjugated rabbit anti-mouse immunoglobulin was obtained from Dako (Carpinteria, CA); methylene blue dye binding. Data are from three experiments.

Cell Cultures

Bovine retinal pericytes (BRPs) were isolated as described. 44 Retinas were dissected from bovine eyes and washed in phosphate-buffered saline (PBS) that contained 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich). The retinas were homogenized in Eagle’s minimum essential medium (EMEM; Mediatech, Herndon, VA) using a Teflon pestle and mortar, and the homogenate was passed over a 90-μm mesh. The capillaries were collected by centrifugation and digested at 37°C with type I collagenase (2 mg/mL; Worthington Biochemicals, Lakewood, NJ) for 1 hour. The dissociated pericytes were cultured in Dulbecco’s Modification of Eagle’s medium (DMEM; Mediatech) containing 10% fetal calf serum (Invitrogen-Gibco, Carlsbad, CA), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The medium was changed every other day. In all experiments, cells between passages 3 and 5 were used. The isolated pericytes had distinct morphologic characteristics as reported in the literature. They displayed α-smooth muscle actin staining and were not recognized by antibodies against von Willebrand Factor VIII antigen.

Dicarbonyl Modification of FN

Culture plate wells or dishes were coated with FN (2 μg/cm²) and incubated at room temperature for 1 hour. The bound FN was then modified by incubation with sterile solutions of 10, 50, and 100 μM MGO or 10 or 50 μM GO in PBS at 37°C for 1 week. The plates were washed three times with sterile PBS to remove any unbound dicarbonyl before use in experiments. Controls were plates coated with FN and incubated without addition of the dicarbonyl compounds.

Pericytes Grown on Modified FN

Pericytes (BRP) in DMEM containing 1% FCS were seeded onto treated or control culture plate wells or dishes and allowed to attach to the
FN-coated surface (60%–80% confluent). The cells were then incubated for various times (16–48 hours) for experiments.

FN-Bound Cells

Adherent cells were measured by a modification of the method of Bar-Shavit as described by Orlando and Cheresh. BRPs in DMEM with 1% FCS at a density of 4 × 10⁴ cells per well were seeded on unmodified or dicarbonyl-modified FN. After 2 days, apoptotic cells were quantitated by flow cytometry, to measure binding to annexin V. (A) Cells seeded on MGO-modified FN; (B) cells seeded on GO-modified FN; (C) comparison of apoptosis in cells seeded on α-dicarbonyl-modified FN in the presence or absence of amino-guanidine (AG). Inclusion of 50 μM AG in one set of treated samples confirmed that MGO- and GO-derived products triggered apoptosis.

FIGURE 3. Apoptosis of pericytes on α-dicarbonyl-modified FN. BRPs (1.2 × 10⁵ cells/well) were seeded on unmodified or dicarbonyl-modified FN. After 2 days, apoptotic cells were quantitated by flow cytometry, to measure binding to annexin V. (A) Cells seeded on MGO-modified FN; (B) cells seeded on GO-modified FN; (C) comparison of apoptosis in cells seeded on α-dicarbonyl-modified FN in the presence or absence of amino-guanidine (AG). Inclusion of 50 μM AG in one set of treated samples confirmed that MGO- and GO-derived products triggered apoptosis.

RGDS Peptide Modification and Incubation with BRPs

RGDS peptide (23 nanomoles) in 0.1 M sodium phosphate buffer (pH 7.4) was incubated with 230 nanomoles MGO or 230 nanomoles of GO for 3 days at 37°C. Both the modified and native peptides were added to cultures of BRPs (2 × 10⁵ cells) and incubated for 4 hours at 37°C. The cells were then seeded onto FN-coated wells, and after 16 hours, the number of attached cells was measured with methylene blue, as described earlier.

Apoptosis Assay

BRPs (1.2 × 10⁵) were seeded onto dicarbonyl-treated or control wells and incubated for 2 days as described earlier. All attached cells, as well as detached cells in the culture media, were collected together from each treatment. The cells were then centrifuged, washed with 1.0 mL cold Hanks’ balanced salt solution (HBSS) containing 2% BSA, resus-
pended in 100 μL of binding buffer containing 1 μL of FITC-Annexin V and 10 μL of propidium iodide, and incubated for 15 minutes at room temperature. Binding buffer (400 μL) was added, and the cell populations were analyzed by flow cytometry (FACScan; BD Biosciences Immunocytometry Systems, Braintree, MA). The number of apoptotic cells was expressed as a percentage of the total cells in each sample.

Caspase-3 Assay
BRPs (5 × 10⁶) were seeded on 100-mm dishes coated with MGO- or GO-modified FN or with unmodified FN for 1 day, as described earlier. The cells were then lysed on ice in 200 μL of buffer containing 0.71% NP-40, 71 mM Tris (pH 7.5), 0.71 mM EDTA, and 212 mM NaCl. Samples corresponding to 40 μg protein were applied to a black plastic 96-well microplate and incubated for 1 hour at 37°C in 21 mM HEPES buffer (pH 7.4) containing 105 mM NaCl, 5.25 mM dithiothreitol (DTT), and 50 μM Ac-DEVD-AFC in a total volume of 200 μL. The fluorescence product was measured at 505 nm (excitation, 400 nm) by a microplate fluorescence reader (Gemini XS; Molecular Devices). Human recombinant caspase-3 was used as the positive control.

Cellular Glutathione Level
BRPs were seeded on MGO- or GO-modified or unmodified FN for 2 days in DMEM containing 1% FCS. The cells were detached by trypsinization (0.05% trypsin and 0.53 mM EDTA), and approximately 8 × 10⁵ cells were suspended in 1.0 mL HBSS and incubated with 100 μM monochlorobimane for 15 minutes at 37°C. After incubation, the cell suspension was centrifuged at 200g for 5 minutes at room temperature, and the pellet was resuspended in 800 μL HBSS. A microplate fluorescence reader measured the fluorescence at 460 nm (excitation, 360 nm) of each cell suspension. Basal autofluorescence, measured in cells not incubated with the probe, was subtracted from all samples.

Intracellular ROS
BRPs in DMEM containing 1% FCS were seeded on MGO- or GO-modified or unmodified FN. After 2 days, the adherent cells (1 × 10⁶ cells) were loaded with 10 μM CM-H2DCFDA in 1.0 mL HBSS by incubation with shaking for 45 minutes at 37°C. The samples were centrifuged at 200g for 5 minutes at room temperature, the superna-
Fluorescence in the cell suspension was measured at 530 nm (excitation-480 nm) by a microplate fluorescence reader.

**p38 MAPK Phosphorylation**

To determine whether modified FN induces phosphorylation of p38 MAPK, we seeded BRPs onto six-well plates treated with dicarbonyl-modified FN or unmodified FN for 16 to 24 hours. Immunoblot analysis with a p38 MAP kinase (Thr180/Tyr182) antibody kit (PhosphoPlus; Cell Signaling) was used to assay p38 MAPK. The adherent cells were washed twice with ice-cold PBS and lysed in 100 μL of SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue). Cell lysates were prepared as described earlier and subjected to SDS-PAGE (12% reducing gels). The proteins were transferred electrophoretically to nitrocellulose membranes and treated with an anti-phospho-p38 MAP kinase (Thr180/Tyr182) polyclonal antibody (1:1000 dilution) in 5% BSA, PBS, and 0.05% Tween-20 (buffer A). All samples were incubated overnight at 4°C and then reacted with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution in buffer A) for 1 hour at room temperature. The membrane was washed with buffer A and developed with chemiluminescence (ECL; Pierce, Rockford, IL). Scanning densitometry quantified signal intensities in the immunoblots (NIH Image software, available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

**Akt Phosphorylation**

In other experiments, BRPs were seeded onto six-well plates with modified or unmodified FN to determine whether MGO- or GO-modified FN affects phosphorylation of Akt/PKB. After 24-hour incubation, Akt phosphorylation was assessed with an Akt (Ser473) antibody kit (PhosphoPlus; Cell Signaling). The adherent cells from each well were lysed, and the proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes as for MAPK. The membranes were incubated overnight first with anti-phospho-Akt (Ser 473) and Akt antibody (1:1000 dilution in buffer A) at 4°C, then with HRP-conjugated rabbit anti-mouse antibody (1:2000 dilution in buffer A) for 1 hour at room temperature. Finally, the membranes were washed with buffer A, developed with ECL (Pierce), and assessed by scanning densitometry, as described for p38 MAPK.

**Detection of αB-crystallin**

BRPs were seeded on unmodified or modified FN for 24 hours, and the adherent cells were washed twice with ice-cold PBS and lysed on ice.
in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1/100 g/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysate was dispersed by sonication for 20 seconds in a sonifier (Branson, Danbury, CT) set at 20% amplitude. The suspension was centrifuged at 15,000 g for 10 minutes at 4°C. Transfection was verified by separating the lysate proteins on 15% SDS-PAGE under reducing conditions. The proteins were transferred electrophoretically to a nitrocellulose membrane (250 mA, 1 hour), and the membrane was incubated first at 4°C overnight with an anti-aB-crystallin monoclonal antibody (1:1000 dilution in buffer A) and then with HRP-conjugated rabbit anti-mouse IgG for 1 hour. The membrane was washed with buffer A and developed with enhanced chemiluminescence (Pierce).

Confocal Microscopy

BRPs (2 × 10⁵) were seeded on 100 μM MGO- or 50 μM GO-modified FN or on unmodified FN in glass-bottomed microwell dishes (MatTek Corp., Ashland, MA). The cells were fixed for 10 minutes in 3.7% paraformaldehyde-PBS and permeabilized with 0.1% Triton X-100 for 10 minutes. To block nonspecific antibody binding, cells were incubated in 3% BSA and PBS for 1 hour at room temperature. Cells were then incubated for 2 hours with 1:1000 diluted monoclonal antibody against αB-crystallin (Stressgen). An FITC-conjugated goat anti-mouse IgG was used as the secondary antibody. To visualize F-actin, 2.5 U of Texas red phalloidin was added along with the secondary antibody. Slides were examined under a laser scanning microscope (LSM 5 Pascal; Carl Zeiss Meditec, Oberkochen, Germany) using a 63× oil-immersion lens (Plan-APOCHROMAT; Carl Zeiss Meditec). XY-images were collected and exported from the laser scanning image browser into image-management software (Photoshop; Adobe, San Diego, CA).

Cloning of Rat αB-crystallin

The full-length cDNA for rat αB-crystallin was isolated by RT-PCR from the total RNA of rat dorsal root ganglion. The primers used in the PCR amplification were: forward primer, 5'-CTAGCTAGCCACCATGGACATCGCATCCACCACG-3', and reverse primer, 5'-CCGCTCGAGCTGTTCTGACGCTGC-3'.

These primers introduced unique restriction enzyme sites (NheI and XhoI) to be used for subcloning the PCR product into the mammalian expression vector pcDNA3.1(-) (Invitrogen). The cDNA was verified by DNA sequence analysis.
Transient Transfection of \( \alpha \)-B-crystallin into BRPs

BRPs (3 \( \times \) 10^5) were transiently transfected with 5 \( \mu \)g pcDNA3-FLAG plasmid (BD Biosciences-Clonetech, Palo Alto, CA) that contained rat \( \alpha \)-B-crystallin. We used a cell line optimization kit (Nucleofector; Amaxa Biosystems) according to the manufacturer’s instructions. The cells were suspended in solution T (100 \( \mu \)L) and transferred to a cuvette. Five micrograms of cDNA was added, and transfection was performed using a T20 Program with transfection equipment (Amaxa Biosystems). The transfection was confirmed by Western blot analysis using a monoclonal antibody for \( \alpha \)-B-crystallin as described earlier.

Apoptosis in \( \alpha \)-B-crystallin–Transfected Cell

Transfected and nontransfected BRPs (1.0 \( \times \) 10^5) were seeded on 100 \( \mu \)M MGO- or 50 \( \mu \)M GO-modified FN or on unmodified FN for 16 hours, then washed with DMEM (1% FCS) to remove floating cells. After 2 days, apoptosis was measured by annexin V binding, as described earlier.

Protein Measurement

Proteins were measured with a protein assay kit (Bio-Rad, Hercules, CA) using BSA as a standard.

Statistical Analyses

Fisher’s PLSD test (Statview 5.0; SAS Institute, Inc., Cary, NC) evaluated differences among different treatment groups. We considered a level of \( P < 0.05 \) to be significant. Unless indicated, all experiments were performed at least three times with similar results. The values in graphs correspond to the mean of at least three samples. Bars indicate SD.

RESULTS

FN applied to culture dishes was modified by exposure to MGO or GO. Figure 1 shows that the chemical modifications could be detected immunohistochemically by a monoclonal antibody against argpyrimidine (developed with rhodamine conjugated rabbit anti-mouse IgG) and an antibody against glyoxal-AGEs (developed with FITC-conjugated goat anti-rabbit IgG). When MGO and GO react with proteins, they produce many structurally different AGEs. The antibodies used in this experiment recognize only specific products. The patchy staining in Figure 1 may be due to the inability of our antibodies to recognize all modifications and/or insufficient penetration of antibodies to bind modifications in FN-coated culture plates. It is highly likely that FN is uniformly modified by both MGO and GO under the conditions used in our experiments.

In all our cell culture experiments, direct comparisons of the effect of MGO-modified FN to that of GO-modified FN could not be made, because the experiments were performed at different time points, and the primary cells that we used responded slightly differently, although with the same pattern and trend, to the same treatment at different times.
Methylened blue staining enabled us to determine the number of viable pericytes seeded on matrices of dicarbonyl-modified FN. After 2 days of exposure to FN modified by 100 μM MGO, the number of viable cells decreased approximately 30%. FN modified by GO at a 50-μM concentration reduced the number of viable cells by 42%. The decrease in the number of cells on modified FN suggests detachment and/or apoptosis of the cultured pericytes.

Because cell death could result from modification of the RGD peptide in FN, which is the cell-anchoring domain on FN, we incubated RGDS peptide with MGO or GO for 3 days and then added it to pericyte cultures for 4 hours. The incubated cells were then seeded onto FN-coated wells. There was a significant reduction in viable cells (60%) when the unmodified peptide was used, indicating competition of the RGD domain for FN binding and cell anchoring (Fig. 2). Modification of RGDS by MGO marginally reduced this competition (25%), and the GO-modified peptide had even less effect (~13%). These data suggest that α-dicarbonyl modification of RGD peptide is only partially responsible for the decrease in viability of cells seeded on dicarbonyl-modified FN. There was a possibility that unreacted MGO would cause cell death. Addition of 230 nanomoles of MGO had no direct effect on cell viability.

Cell death due to apoptosis could also account for the loss of viable cells from α-dicarbonyl-modified FN matrix. We measured the number of apoptotic cells with annexin V staining by flow cytometry. Figure 3 shows that apoptosis in BRPs increased after 2 days of exposure to MGO-modified FN. The extent of apoptosis increased directly with the MGO concentration from 10 to 50 μM. With 100 μM MGO, the effect was slightly less than with 50 μM, possibly because of cell detachment. Results with GO-modified FN were similar, and a significant increase in apoptosis occurred when cells were seeded on FN that had been modified by 50 μM GO. The effect of FN modified by 10 μM GO was modest, but a trend toward an increase in apoptosis was evident. To determine whether the MGO and GO effects were due to products from the reaction of the dicarbonyls with FN, we added AG (a compound that blocks AGE formation from α-dicarbonyl compounds) during modification of FN with α-dicarbonyls. AG completely blocked the increase in apoptosis, both from MGO and GO (Fig. 3C). These data indicate that the modifications of FN by α-dicarbonyls enhance apoptosis in BRPs.

We verified the participation of caspase-3 activation in this process. Caspase-3 activation occurred in BRPs seeded on either MGO- or GO-modified FN, and the extent of activation related to the amount of α-dicarbonyls used for FN modification (Fig. 4). We noted a slight drop with 10 μM MGO, which corresponded to the observed number of apoptotic cells (Fig. 3). As noted earlier, this may be due to detachment of cells from the modified FN matrix.

Because an increase in intracellular oxidative stress could promote apoptosis, we measured intracellular reduced GSH. Figure 5A shows that cells seeded on 50 μM MGO-modified FN had a 40% reduction of GSH compared with those on unmodified FN. Cells on the GO-modified FN also showed significant reduction of GSH, more than 53% with 50 μM GO-modified FN. Although the effect on ROS generation was not as dramatic, we found increased ROS in cells incubated on either MGO- or GO-modified FN (Fig. 6). As noted for apoptosis and caspase-3 activation, reduction of ROS content in cells on FN modified by 100 μM MGO was slightly less than that on FN modified by the lower (50 μM) dicarbonyl concentration.

Diverse stimuli activate p38 MAPK by upstream kinases that phosphorylate tyrosine and threonine residues. Phosphorylation of these two sites, which can be detected with an anti-phospho-p38 MAPK antibody, is generally accepted as an indication of heightened p38 MAPK activity. Figure 7 shows representative immunoblots from pericytes seeded on MGO- or GO-modified FN. Phosphorylation appeared more extensive in GO-FN cells, where phospho-p38 MAPK increased 37% and 65% in cells seeded on 10 and 50 μM GO-modified FN. Phosphorylation increased relative to cells on unmodified FN after 16 and 24 hours of contact with the carbonyl-modified
matrices. Although the phosphorylation was not as dramatic in MGO-FN cells, there was a measurable increase (26%) in the 100 μM/H9262 samples after 16 hours. This effect was somewhat reduced with 100 μM/H9262 M MGO-FN at 24 hours (5%), but with 50 μM/H9262 M MGO-FN it increased and reached 13% in the same period.

Apoptosis is regulated by several signal-transduction pathways, including the Akt/protein kinase B pathway. To determine whether contact of BRPs with α-dicarbonyl-modified FN affects cell survival signaling by Akt during apoptosis, we measured phosphorylation of Akt. Figure 8 compares results from cells seeded on MGO- and GO-modified FN with those on unmodified FN. The levels of Ser473-phosphorylated Akt remained unchanged in controls after 24 hours. In contrast, phosphorylated Akt decreased significantly in cells seeded on both 50 μM MGO-modified FN and 50 μM GO-FN.

α-crystallin is thought to be an anti-apoptotic protein51 and a molecular chaperone.52 Many cell types contain α-crystallin, but it is most abundantly expressed in the ocular lens. We found that after 24 hours of incubation, pericytes seeded on modified FNs (100 μM MGO or 50 μM GO) had significantly reduced levels of α-crystallin (Fig. 9). This was confirmed by confocal microscopy. As can be seen in Figure 10, the immunoreactivity of α-crystallin appeared to decrease in cells seeded on MGO- or GO-modified FN when compared with those seeded on unmodified FN, and it overlapped with the immunoreactivity for actin.

We next wanted to determine whether overexpression of αB-crystallin protects BRPs from apoptosis. Accordingly, we transfected BRPs transiently with cDNA encoding rat αB-crystallin. Nearly 20% to 30% of cells were transfected, and the transfection was confirmed by Western blot analysis. As can be seen in Figure 11, transfected cells had a ninefold increase in αB-crystallin relative to nontransfected ones. The transfection also appeared to be protective, since transfected cells seeded on dicarbonyl-modified FN showed little or no increase in apoptosis. Flow cytometry 2 days after application to FN and modified FN plates showed that overexpression of rat αB-crystallin almost completely inhibited MGO-FN- or GO-FN-
induced apoptosis in BRPs (Fig. 11). Although we do not yet know the exact pathways affected in BRPs apoptosis, our data indicate several possible mechanisms, as indicated schematically in Figure 12.

**Discussion**

Loss of pericytes from retinal capillaries is the earliest event in diabetic retinopathy and possibly accounts for the appearance of pericyte ghosts within the vessels. This initial lesion is succeeded by a loss of endothelial cells, leaving acellular capillaries. Pericytes are normally surrounded by extracellular matrix proteins from the capillary BM. The mechanisms of pericyte death in diabetes remain unclear, but because the BM is an essential component of healthy capillaries, we suspected that modification of BM proteins might initiate the vascular damage in diabetic retinopathy.

Dicarbonyl compounds, such as MGO and GO, become elevated in the plasma of diabetic individuals, and these compounds are highly reactive with proteins. We find that both MGO and GO can modify FN in such a way as to trigger apoptosis in pericytes. A novel aspect of our observation is that these compounds trigger apoptosis through d-dicarbonyl modification of the substratum and not by damaging pericytes directly. Several previous studies showed that sugar-modified proteins or dicarbonyl compounds had direct toxic effects on cultured pericytes, but these studies used concentrations of sugars or AGE-proteins far higher than that could be present in vivo. We used concentrations of d-dicarbonyls in the range of 10 to 100 μM to modify FN, and these agents did not contact the cells directly. Concentrations within the micromolar range of sugars or AGE-proteins far higher than that could be present in vivo. We used concentrations of d-dicarbonyls in the range of 10 to 100 μM to modify FN, and these agents did not contact the cells directly. Concentrations within the micromolar range

**Figure 11.** Apoptosis in pericytes overexpressing aB-crystallin. BRPs were transiently transfected with full-length rat aB-crystallin and lysed, and overexpression of aB-crystallin was confirmed in 2.5-μg protein samples by Western blot analysis (top). SDS-PAGE was performed on a 15% gel. The percentage increase in aB-crystallin in transfected cells is shown (top). Transfected and non-transfected cells were seeded on unmodified or dicarbonyl-modified FN. Cells were allowed to attach for 16 hours, unattached cells were removed by changing the medium, and apoptotic cells (annexin-V-bound cells) were quantified at 48 hours by flow cytometry. Data are the mean result of three experiments.

**Figure 12.** Conceptual mechanisms for pericyte apoptosis by d-dicarbonyl modifications. Dicarbonyl-derived products on FN could induce an oxidative stress resulting from the loss of GSH and an increase in ROS. These changes, in turn, could promote phosphorylation of p38 MAPK and dephosphorylation of Akt, as well as reduction of aB-crystallin, culminating in activation of caspases and subsequent apoptosis.
approximate reported physiological levels. MGO levels as high as 300 μM have been recorded in mammalian cells. Because α-dicarboxyls are extremely reactive, the actual levels may be far higher than reported. Like MGO, plasma GO is also increased in diabetes. The continued presence of these reactive compounds in diabetes increases the possibility for modification of long-lived proteins, such as those within BM.

After finding that α-dicarboxyl-modified FN induced apoptosis in BRPs, we focused our studies on the mechanisms involved. We recorded a decrease in viable cells when pericytes were applied to a matrix of α-dicarboxyl-modified FN. Pericytes anchor to FN through integrin molecules, where the integrin-binding site is the RGD peptide. Because arginine is highly susceptible to reaction with α-dicarboxyls, we anticipated that arginine modification was responsible for failure of pericyte attachment to FN and eventual cell death. In fact, a recent report from another laboratory has indicated that MGO modification of RGD peptide leads to a decrease in cell adhesion and spreading. Unexpectedly, our competition experiments with RGDS peptide suggested that modification of RGD by MGO and GO could account for only a minor reduction in viable cells. Thus, we suspected that apoptosis might be triggered directly by α-dicarboxyl modification of the culture substrate.

Apoptosis in our cultured pericytes cannot be due to failure to adhere to FN. When we used flow cytometry to measure apoptosis, we allowed the cells to attach to α-dicarboxyl-altered FN for 16 hours, then washed away any detached cells and measured apoptosis in the remaining cells after 2 days. Addition of aminoguanidine during the incubation of FN with α-dicarboxyls completely blocked subsequent apoptosis. In addition, both MGO- and GO-modified FN triggered an increase in caspase-3 activity in the cultured pericytes. Taken together, these data suggest that apoptosis in the pericytes resulted from a specific interaction of the cells with α-dicarboxyl-derived modifications of FN.

Activation of p38 MAPK is implicated in stress-induced apoptosis. We find activation of p38 MAPK in BRPs incubated on α-dicarboxyl-modified FN, indicating that the modified FN evoked a stress-induced response. The decline in cellular GSH along with an increase in ROS levels suggests that oxidative stress contributes to this stress-induced p38 MAPK activation. However, we cannot rule out participation of other MAPKs.

Integrin-mediated protein kinase B (PKB/Akt) phosphorylation is recognized as an important mechanism of cell survival, and dephosphorylation of Akt by protein phosphatase 2A (PP2A) is implicated in apoptosis. The mechanism by which PKB/Akt prevents apoptosis is not fully understood. It is generally believed that PKB/Akt acts by phosphorylation of downstream targets, such as those within BM. We previously have reported that α-B-crystallin is upregulated by p38 MAPK activation in contrast to findings in this study that α-B-crystallin is downregulated when p38 MAPK is activated. Differences in cell type and stress may be reasons for this discrepancy. However, our findings are in accord with Andley et al. who noted that α-B-crystallin protects cells from apoptotic stimuli, we find that overexpression of α-B-crystallin blocks apoptosis in cells seeded on α-dicarboxyl-modified FN. α-B-crystallin may act at more than one site to inhibit apoptosis. It was shown to prevent activation of caspase-9 and maturation of caspase-3, suggesting that it affects both mitochondrial and death receptor pathways. Our data with pericytes confirm that α-B-crystallin is an antiapoptotic protein. The close association of α-B-crystallin with actin suggests a role for α-B-crystallin in actin polymerization and supports the observation of Wang and Spector that α-crystallin can stabilize actin polymers and prevent stress-induced aggregation of actin.

In summary, we find that adherence to dicarboxyl-modified FN leads to apoptosis in BRPs. The initiation of apoptosis appears related to increased oxidative stress and weakening of the antiapoptotic repertoire within the cells. Our data indicate a biochemical basis for pericyte apoptosis that may explain why an increase in α-dicarboxyl compounds in diabetes could initiate damage to retinal vessels. It is possible that α-dicarboxyl-mediated apoptosis described in this study is one among several biochemical pathways that contribute to pericyte death in diabetes. Further understanding of these early events in diabetic retinopathy ultimately may help in the prevention and management of this difficult disease.

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