Expression of Apoptosis Markers in the Retinas of Human Subjects with Diabetes

Ahmed M. Abu El-Asrar, Lieve Dralands, Luc Missotten, Ibrabim A. Al-Jadaan, and Karel Geboes

PURPOSE. To investigate the expression of the apoptotic mediators in the retinas from human subjects with diabetes mellitus.

METHODS. Ten donor eyes from five subjects with diabetes mellitus, and eight eyes from four nondiabetic subjects without known ocular disease serving as control subjects were examined. Immunohistochemical techniques were used with antibodies directed against glial fibrillary acidic protein (GFAP), caspase-3, Fas, Fax ligand (Fasl), Bax, Bcl-2, survivin, p53, extracellular signal-regulated kinases (ERK1/2), and p38.

RESULTS. In retinas from all subjects without diabetes, weak Bcl-2 immunoreactivity was confined to GFAP-positive glial cells in the nerve fiber layer. Weak immunoreactivity for ERK1/2 was noted in a few nuclei in the inner nuclear layer and in a few Müller cell processes. Cytoplasmic immunostaining for survivin was noted in the retinal pigment epithelial cells. There was no immunoreactivity for the other antibodies tested. All diabetic retinas showed cytoplasmic immunoreactivity for caspase-3, Fas, and Bax in ganglion cells. Fasl immunoreactivity was detected in GFAP-positive cells. Upregulation of Bcl-2 immunoreactivity was noted in GFAP-positive cells in nerve fiber and ganglion cell layers, and Bcl-2 induction was noted in Müller cell processes. Strong immunoreactivity for ERK1/2 was observed in many nuclei in the inner nuclear layer in GFAP-positive cells in the nerve fiber and ganglion cell layers and numerous Müller cell processes. Survivin immunoreactivity was not altered in the diabetic retinas. There was no immunoreactivity for p53 and p38.

CONCLUSIONS. Ganglion cells in diabetic retinas express several proapoptosis molecules, suggesting that these cells are the most vulnerable population. Glial cells in diabetic retinas are activated and express several antiapoptosis molecules in addition to the cytotoxic effector molecule Fasl, suggesting a possible role of glial cells in induction of apoptosis in ganglion cells. (Invest Ophthalmol Vis Sci. 2004;45:2760-2766) DOI: 10.1167/iovs.03-13592

Increased apoptosis of neural retinal cells in experimental diabetes in rats and diabetes mellitus in humans was recently documented. At particular risk are retinal ganglion cells, which demonstrated a 10% decrease in cell number in diabetic rats compared with control eyes. Apoptotic cells did not colocalize with endothelial cells. These data confirmed that nonvascular cells, most likely ganglion cells, become apoptotic in the diabetic rat retinas. Apoptotic death requires synthesis of new proteins by degenerating cells and can be prevented by protein synthesis inhibitors.

The molecular basis for the apoptogenic effects of diabetes in the retina is not yet identified. The molecular events regulating apoptosis are complex and involve genes that are both proapoptotic and antiapoptotic. A number of mediators are involved in apoptosis, including caspases, Fas/Fas ligand (Fasl), Bax/Bcl-2, survivin, and p53.

Central to the implementation of apoptosis is a class of cysteine aspartate-specific proteases of the interleukin-1β-converting enzyme family known as caspases. They exist as proenzymes that are proteolytically processed to their active forms in response to an apoptosis-inducing stimulus. Activated caspases cleave each other’s precursors into mature, active enzymes in a proteolytic cascade. Activated caspases kill cells by degrading structural elements and DNA repair enzymes and by indirect activation of chromosomal endonucleases. Fas (CD95) is a type I transmembrane glycoprotein belonging to the tumor necrosis factor-α receptor superfamily. On ligation with agonistic antibody or the natural Fasl, Fas trimerizes and recruits several proteins that share a death domain that leads to the formation of a specific death-inducing signaling complex at the intracellular region of the Fas receptor. The recruitment of caspase-8 to the death-inducing signaling complex results in proteolytic activation of the enzyme, which, in turn activates a series of other caspase members. Fasl is a type II transmembrane glycoprotein that induces apoptosis in target cells in both the membrane-bound form and the soluble form. Cell survival and apoptotic death have been shown to be regulated by genes of the bcl-2 family. Thus, several studies demonstrated a protective, antiapoptotic effect of Bcl-2 protein in neural cells both in vitro and in vivo. Several proteins have been identified that share homology with Bcl-2. Some of these, such as Bax, render cells more susceptible to apoptotic stimuli. Survivin is a protein that inhibits apoptosis and regulates cell division. It inhibits apoptosis by either directly or indirectly interfering with the function of caspases. The nuclear phosphoprotein p53 is a key determinant in the process of apoptosis in many cell types, acting to promote apoptosis. p53 is a DNA-binding transcription factor originally recognized as a tumor suppressor, and mutations in this gene are found in approximately half of all human tumors. Transfer of information for cell death or survival programs is organized by the cascades of kinases, by which several adaptive and protective or pathogenic proteins are functionally activated by phosphorylation. Among the signal transduction pathways involved in cell fate, mitogen-activated protein kinases (MAPKs) occupy a central place. Members of the MAPK family include extracellular signal-regulated kinases (ERK, p44 MAPK/ERK1, and p42 MAPK/ERK2), c-Jun NH2-terminal protein kinases (JNK), and p38 MAPK. JNK and p38 MAPKs are strongly activated by stress signals such as inflammatory cytokines.

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We obtained 10 human donor eyes postmortem from five subjects with diabetes mellitus. Donor eyes were used in the study in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. No subject had a history of retinal disease or of ocular disease, as determined by gross examination. The iris, lens, and vitreous were gently removed. The retina and uveal tissue were dissected from the surrounding tissue, including the cornea, iris, lens, and vitreous. The sections were fixed in 4% paraformaldehyde and embedded in paraffin.

**METHODS**

Monoclonal and polyclonal antibodies were used in the study (Table 1). Details of specificity and use are provided in Table 1. The specificity of the antibodies used is indicated in Table 2. The sections were incubated with the monoclonal and polyclonal antibodies listed in Table 1. The specificity of the antibodies used is indicated in Table 2. The sections were incubated with the monoclonal and polyclonal antibodies listed in Table 1. The specificity of the antibodies used is indicated in Table 2.

**Table 1.** Monoclonal and Polyclonal Antibodies Used in the Study

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Incubation Time</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-cleaved caspase-3</td>
<td>1:25</td>
<td>120 minutes</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-Fas (B-10)</td>
<td>1:50</td>
<td>Overnight</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>Anti-Fasl (N-20)</td>
<td>1:50</td>
<td>30 minutes</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>Anti-Bax (B-9)</td>
<td>1:50</td>
<td>30 minutes</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>Anti-Bcl-2 (clone 124)</td>
<td>1:10</td>
<td>30 minutes</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-survivin (D-8)</td>
<td>1:20</td>
<td>30 minutes</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>Anti-p53 (clone DO-7)</td>
<td>1:10</td>
<td>30 minutes</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-phospho-ERK1/ERK2 MAPK (E10)</td>
<td>1:30</td>
<td>Overnight</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-p38 MAPK</td>
<td>1:50</td>
<td>Overnight</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-GFAP (pc)</td>
<td>1:300</td>
<td>30 minutes</td>
<td>Dako</td>
</tr>
</tbody>
</table>

| pc, polyclonal antibodies; mc, monoclonal antibodies. |

Because retinal neural cell death in diabetes is thought to be due to an apoptotic mechanism, it is important to know which apoptotic mediators are specifically expressed during apoptosis of retinal cells in diabetes. Therefore, we used immunohistochemical techniques to study the expression of the apoptotic markers caspase-3, Fas, Fasl, Bcl-1, survivin, p53, ERK2/2, and p38 in the retinas from diabetic patients and in the retinas from subjects without diabetes.

**Table 2.** Specificity of the Antibodies Used in the Study

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-cleaved caspase-3</td>
<td>Detects the large fragment of activated caspase-3 (17–20 kDa)</td>
</tr>
<tr>
<td>Anti-Fas</td>
<td>Detects a peptide mapping at the carboxyl terminus of Fas of human origin</td>
</tr>
<tr>
<td>Anti-Fasl</td>
<td>Detects a peptide mapping at the amino terminus of Fasl of rat origin</td>
</tr>
<tr>
<td>Anti-Bax</td>
<td>Maps at amino acids 1–171 representing all but the carboxyl terminal 21 amino acids of Bax of mouse origin</td>
</tr>
<tr>
<td>Anti-Bcl-2</td>
<td>Reacts with the Bcl-2 oncoprotein</td>
</tr>
<tr>
<td>Anti-survivin</td>
<td>Raised against a recombinant protein corresponding to amino acids 1–142 of survivin of human origin</td>
</tr>
<tr>
<td>Anti-p53</td>
<td>Reacts with a 55-kDa protein. The epitope recognized is located between the N terminal amino acids 1 and 45 and possibly between amino acids 37 and 45 of the human p53 protein</td>
</tr>
<tr>
<td>Anti-phospho-ERK1/ERK2 MAPK</td>
<td>Detects endogenous levels of p44 and p42 MAP kinase (ERK1 and ERK2) dually phosphorylated at threonine 202 and tyrosine 204</td>
</tr>
<tr>
<td>Anti-p38 MAPK</td>
<td>Detects total p38 MAP kinase (phosphorylation-state-independent) levels</td>
</tr>
<tr>
<td>Anti-GFAP</td>
<td>Reacts strongly with human GFAP.</td>
</tr>
</tbody>
</table>
One case (case 5) had no retinopathy. Documented to be present in two cases (cases 1 and 2). The results is given in Table 4. Similar findings were noted in retinas from subjects without diabetes. Retinas from subjects without diabetes showed weak immunoreactivity for GFAP very close to internal limiting membrane in nerve fiber layer, and ganglion cell layer (data not shown). Weak Bcl-2 immunoreactivity was noted in a few scattered nuclei in the inner nuclear layer was continuous, with immunostaining in cell processes. Based on the morphologic assessment of cell types, increased immunostaining for ERK1/2 in diabetic retinas was associated with glial cells. The distribution and intensity of survivin immunoreactivity was not altered in the diabetic retinas compared with retinas from subjects without diabetes. There was no immunoreactivity for p53 and p38.

### DISCUSSION

There are three important findings of the present study: (1) Ganglion cells in diabetic retinas expressed the apoptosis-promoting factors caspase-3, Fas, and Bax; (2) glial activation in diabetic retinas was associated with upregulation of ERK1/2 signaling pathway that has a protective effect on apoptotic signaling from death receptors. In addition, glial cells in diabetic retinas showed upregulation of the antiapoptotic marker Bcl-2 and expressed the cytokotic effector molecule FasL; and (3) retinal pigment epithelial cells in diabetic and nondiabetic retinas expressed the antiapoptotic protein survivin. Distinct members of the caspase family are involved in both the initiation and execution phases of apoptosis, with the initiator caspases coupling cellular signaling pathways to caspase activation and the downstream effector caspases being responsible for the cleavage of cellular substrates. Among them, caspase-3 is the executioner caspase known to play a central role in the proteolytic cascade during apoptosis. The detection of activated caspase-3 is a very reliable way to identify cells destined to die by apoptosis, even before many of the morphologic characteristics (e.g., DNA fragmentation) are present. In the present study, the executioner caspase-3 immunoreactivity was observed in ganglion cells in diabetic retinas. Our observations are consistent with previous reports.
showing caspase-3 activation in the retinas of diabetic animals and diabetic patients. Several studies demonstrated that caspase-3 is involved in the apoptotic death of retinal ganglion cells induced by ischemia, excitotoxicity, axotomy, and chronic ocular hypertension. Inhibition of caspase-3 activity reduced apoptotic cell death induced in retinal cells by excitotoxicity and ischemia.

In diabetic retinas, Fas immunoreactivity was observed in ganglion cells, and FasL immunoreactivity was localized to glial cells. These findings indicate that both Fas death system components are available for interaction, possible Fas-mediated ganglion cell death, and a possible role of glial cells in the induction of apoptosis in ganglion cells. Our results are in agreement with a previous report showing that levels of Fas and FasL were elevated in the retinas of diabetic rats. In vitro studies showed that cultured astrocytes express FasL, and that FasL expressed in astrocytes was functional, as astrocytes induced apoptosis in neuronal cells through FasL. The crucial role of the Fas/FasL death receptor system in apoptotic neuronal cell death after ischemia has recently been shown. Rat

**Table 4. Summary of Staining Results**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Retinas from Four Subjects without Diabetes</th>
<th>Retinas from Five Subjects with Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-cleaved caspase-3</td>
<td>- (8/8)</td>
<td>+ (10/10)</td>
</tr>
<tr>
<td>Anti-Fas</td>
<td>- (8/8)</td>
<td>+ (10/10)</td>
</tr>
<tr>
<td>Anti-FasL</td>
<td>- (8/8)</td>
<td>+++ (10/10)</td>
</tr>
<tr>
<td>Anti-Bax</td>
<td>- (8/8)</td>
<td>+ (10/10)</td>
</tr>
<tr>
<td>Anti-Bcl-2</td>
<td>+ (8/8)</td>
<td>+ + (10/10)</td>
</tr>
<tr>
<td>Anti-survivin</td>
<td>+ + (8/8)</td>
<td>+ (10/10)</td>
</tr>
<tr>
<td>Anti-p53</td>
<td>- (8/8)</td>
<td>- (10/10)</td>
</tr>
<tr>
<td>Anti-phospho-ERK1/ERK2 MAPK</td>
<td>+ (8/8)</td>
<td>+++ (10/10)</td>
</tr>
<tr>
<td>Anti-p38 MAPK</td>
<td>- (8/8)</td>
<td>- (10/10)</td>
</tr>
<tr>
<td>Anti-GFAP</td>
<td>+ (8/8)</td>
<td>+++ (10/10)</td>
</tr>
</tbody>
</table>

- , no staining; +, weak staining; ++, intense staining; ++++, very intense staining.

* Both eyes were examined in each subject.

**Figure 1.** Photomicrograph of a retina from a diabetic subject. This is a negative control slide that was treated identically with the omission of the primary antibody showing no staining. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar, 10 μm.

**Figure 2.** Photomicrographs of retinas from nondiabetic subjects. (A) Immunohistochemical staining for Bcl-2 showing weak immunoreactivity confined to the innermost retina in the nerve fiber layer (NFL; arrow). (B) Immunohistochemical staining for ERK1/2 showing weak immunoreactivity in a few nuclei in the inner nuclear layer (INL; arrow) and in a few Müller cell processes (arrowhead). (C) Immunohistochemical staining for survivin showing cytoplasmic immunoreactivity for survivin in retinal pigment epithelial cells (RPE; arrow). Abbreviations are defined in Figure 1. Scale bar, 10 μm.
FasL and FasL expression were increased in human diabetic retinas and that Bax immunoreactivity in ganglion cells was consistent with a previous report showing that Bax levels are increased oxidative stress, accumulation of advanced glycation end products, and increased levels of glutamate, which have been shown to increase the expression of proapoptosis molecules and lead to apoptosis in neuronal cells. In vivo and in vitro studies demonstrated that oxidative stress induced by high glucose leads to activation of caspase-3 and apoptosis in dorsal root ganglion neurons. The apoptosis induced by high glucose could be effectively inhibited by pretreatment with caspase-3 inhibitors. In vitro studies showed that advanced glycation end products increase the expression of proapoptosis markers caspase-3 and Bax and increase apoptosis in cultured rat retinas. Glutamate treatment of cultured rat retinas induces caspase-3 expression and apoptotic cell death in ganglion cells, and an intervention to caspase-3 provides effective protection to retinal neurons against glutamate excitotoxicity. These observations establish that a metabolic abnormality characteristic of diabetes is sufficient to upregulate the expression of the apoptosis-promoting factors and stimulate a death pathway in retinal ganglion cells.

In the present study, diabetic retinas showed ERK1/2 upregulation in glial cells. Similarly, upregulation of ERK1/2 expression was also detected in glial cells in retinas of glaucomatous human eyes and in retinas of experimental endotoxin-induced uveitis. Our observations further document glial activation in retinas of human subjects with diabetes. The glial cells and the ERK pathway may be chronically activated in diabetic retinas because of the continuous presence of extracellular stimulatory factors, such as ischemia, oxidative stress, glutamate excitotoxicity, which are implicated in diabetic retinopathy. Glucos, glutamate excitotoxicity, and oxidative stress have been associated with the activation of ERK1/2 in brain glial cells. Recently reported studies have established the concept that activation of ERK1/2 confers survival advantages to cells in the face of activation of apoptotic pathways. Our observation of activated ERK signaling in glial cells in diabetic retinas suggests that the activity of this kinase pathway may account, in part, for the relative protection of glial cells against damage, whereas retinal ganglion cells undergo apoptosis. In vitro studies demonstrated that ischemia and elevated hydrostatic pressure induce apoptosis in retinal ganglion cells, whereas cocultured glial cells survive the same stress conditions. Several studies demonstrated that the ERK1/2 signaling pathway is involved in the pathogenesis of diabetic retinopathy. ERK activity increased in the retina of diabetic animals compared with normal control animals. Pouali et al. demonstrated that hyperglycemia-induced retinal vascular endothelial growth factor expression requires ERK1/2 and that inhibition of the ERK1/2 pathway reduces retinal hypoxia-inducible factor-1α levels, and suppresses retinal vascular endothelial
growth factor upregulation, and blood-retinal barrier breakdown in diabetic animals. Moreover, advanced glycation end products were found to stimulate vascular endothelial growth factor expression and activate the transcription factor hypoxia-inducible factor-1α in an ERK-dependent pathway.59

In the nondiabetic retinas, weak Bcl-2 immunoreactivity was confined to GFAP-positive cells in the innermost retinal layers. Previous reports demonstrated that Bcl-2 immunoreactivity was detected in Müller cells in human,60 rabbit,61 and rat62 retinas. Diabetic retinas showed Bcl-2 upregulation in glial cells in nerve fiber and ganglion cell layers and Bcl-2 induction in Müller cells processes. This result is in contrast to a previous study, in which Mizutani et al.60 demonstrated that in the adult human retina, Bcl-2 levels are not modified by diabetes. The differences in technique, and the antibodies may account for this discrepancy. Upregulation of Bcl-2 immunoreactivity in retinal Müller cells has also been observed in a mice model of neurotoxin-induced retinal neuronal degeneration,61 after optic nerve transection in rats,62 and in rats with inherited retinal dystrophy.63 The increased expression of the antiapoptotic marker Bcl-2 in glial cells in diabetic retinas may contribute to preventing glial cells from undergoing apoptosis.

In our study, we showed that survivin, a protein that inhibits apoptosis,75 was expressed by retinal pigment epithelial cells in diabetic and nondiabetic retinas. The distribution and intensity of survivin immunoreactivity was not modified by diabetes. Similarly, αB-crystallin, a member of the small heat shock proteins that exerts an antiapoptotic effect was reported to be constitutively expressed by retinal pigment epithelial cells.64 The expression of these antiapoptotic proteins in retinal pigment epithelial cells may reflect the fact that retinal pigment epithelial cells have minimal mitotic capacity, are in a postmitotic state, and have to survive for the lifetime of the organism.65

In conclusion, these observations suggest that diabetes induces an apoptogenic environment in the retina, that retinal ganglion cells are the most vulnerable population, and that the Fas death system and glial cells may be involved in the induction of cell death by apoptosis in ganglion cells. In addition, our results suggest that glial cells and retinal pigment epithelial cells are prevented from undergoing apoptosis. The use of antiapoptotic agents could play a role in the treatment of diabetic injury in the retina.

Acknowledgments

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References

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