

# Caspase Activation in Retinas of Diabetic and Galactosemic Mice and Diabetic Patients

Susanne Mohr,<sup>1,2</sup> Xia Xi,<sup>1</sup> Jie Tang,<sup>1</sup> and Timothy S. Kern<sup>1,2</sup>

**Apoptosis of retinal capillary cells begins early in diabetes and likely contributes to the capillary obliteration that is an important feature of diabetic retinopathy. Caspases are proteolytic enzymes that are closely involved in the induction and execution phases of apoptosis, but their role in the development of diabetic retinopathy has not been studied previously. Our study focused on the measurement of activities of multiple caspases in retinas of mice at different durations of diabetes. Several caspases (including caspases-1, -2, -6, -8, and -9) were activated as early as 2 months of diabetes. The caspases activity pattern changed with increasing duration of disease, suggesting a slowly developing caspases cascade. Activities of executioner caspases (e.g., cas-6 and -3) became elevated after longer duration of diabetes, and the induction of cas-3 activity was associated with the duration of diabetes at which capillary cells begin to show evidence of undergoing apoptosis. Retinas from patients with type 2 diabetes likewise showed a significant increase in activities of cas-1, -3, -4, and -6. For comparison, retinal caspases were also measured in experimental galactosemia, another model that develops a diabetic-like retinopathy. The pattern of caspases activation differed between diabetes and galactosemia, but cas-1 activity became elevated soon after elevation of blood hexose concentration in both. Caspases offer new therapeutic targets to test the role of apoptosis in the development of diabetic retinopathy. *Diabetes* 51:1172–1179, 2002**

**T**he background stages of diabetic retinopathy are believed to lead to the advanced, sight-threatening stages of retinopathy as a result of a progressive decrease in perfusion of the retinal vasculature and resulting ischemia of the retina. Histologic analysis of retinal areas that were nonperfused *in vivo* has indicated that nonperfused vessels are acellular (1). How the capillary cells die is unclear, but both retinal capillary pericytes and endothelial cells have been found by us to

die by a process consistent with apoptosis in humans and animals with diabetes and in experimental galactosemia (another model that develops a diabetic-like retinopathy) (2). Accelerated death of the retinal capillary cells precedes development of other lesions characteristic of retinopathy, suggesting that it might play a critical role in the development of diabetic retinopathy (2), but this remains to be positively demonstrated.

Capillary cells are not the only retinal cells that undergo apoptotic death in diabetes. A greater-than-normal frequency of nonvascular cells, seemingly Müller (glial) cells and ganglion cells, have also been reported to become terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive in retinas of humans and animals with diabetes (3). The rate of apoptosis of the retinal vascular and nonvascular cells in diabetes is low (but statistically greater than normal), consistent with the very slow development of diabetic retinopathy.

Caspases, a family of cysteine proteases, are known to be critically involved in two activities: activation of proinflammatory cytokines and the initiation and execution of apoptosis (4,5). To date, 14 members of this family have been identified and divided into three subfamilies depending on their sequence homology: 1) the caspases-1, or former interleukin-1 $\beta$  converting enzyme, family; 2) the cas-2, or ICH-1, family; and 3) the cas-3, or CPP32, family (6,7).

Caspases such as cas-2, cas-6, cas-7, cas-8, cas-9, cas-10, and, especially, cas-3 have been most frequently identified with apoptosis (8,9). Cas-1 and its member of the subfamily have been implicated both in apoptosis and in inflammatory processes. Caspases implicated in apoptosis can be divided further into initiators and executioners (10). The exact order of the executioners and the place of other caspases in the apoptotic pathway are still controversial, but cas-3, -6, and -7 are generally regarded to be executioners. During apoptosis, caspase precursors (zymogens) become activated by internal cleavage at a conserved aspartate residue to form a heterodimer or tetramer of active p20 and p10 subunits (11). Activated caspases can process their own and other caspases, therefore leading to the idea of a cascade mechanism for signal transduction. The extent to which this happens *in vivo* is still a fertile area of research. Several proteins are known to be cleaved by caspases during apoptosis: lamin A, lamin B, poly (ADP-ribosyl) polymerase, and topoisomerase I (12–14).

Because apoptosis of retinal capillary cells likely contributes to capillary “dropout” and thus retinal ischemia in diabetic retinopathy, we were interested in the caspases that might be involved in the initiation and/or execution of this apoptotic process. These studies were conducted in

From the <sup>1</sup>Department of Medicine, Case Western Reserve University, Center for Diabetes Research, Cleveland, Ohio; and the <sup>2</sup>Department of Ophthalmology, Case Western Reserve University, Center for Diabetes Research, Cleveland, Ohio.

Address correspondence and reprint requests to Susanne Mohr, Case Western Reserve University, Department of Medicine, Division of Clinical and Molecular Endocrinology, Center for Diabetes Research, BRB 435, 10900 Euclid Ave., Cleveland, OH 44106. E-mail: sxm38@po.cwru.edu.

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BREC, bovine retinal endothelial cell; DMEM, Dulbecco's modified Eagle's medium; EBM, endothelial cell basal medium; FITC, fluorescein isothiocyanate; ECGF, endothelial cell growth factor; FBS, fetal bovine serum; rMC, retinal Müller cells; STZ, streptozotocin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

TABLE 1  
Characteristics of the experimental group of diabetic, galactosemic, and control mice at 2, 4, and 8 months' duration

Experimental group	Duration (month)	Number of animals	Ghb (%)	Glucose levels (mg/dl)
Normal	2	10	3.2 ± 0.4	130 ± 19
Diabetic	2	9	8.4 ± 2.4	378 ± 70
Galactosemic	2	9	6.2 ± 0.4	134 ± 12
Normal	4	10	3.3 ± 0.5	121 ± 34
Diabetic	4	9	8.1 ± 0.4	445 ± 84
Galactosemic	4	10	6.0 ± 0.74	149 ± 23
Normal	8	10	3.2 ± 0.6	137 ± 34
Diabetic	8	9	10.2 ± 1.7	475 ± 50
Galactosemic	8	8	6.3 ± 0.2	142 ± 12

Data are mean ± SD.  $P < 0.05$ .

diabetic mice, galactose-fed mice, and autopsy samples collected from patients with diabetes. Furthermore, our study looked for changing patterns of caspase activities with increasing duration of hyperglycemia. Understanding the caspase network and identifying initiator caspases are expected to be important to evaluate the role of caspases in the development of diabetic retinopathy

## RESEARCH DESIGN AND METHODS

**Materials.** Caspase substrates for cas-1 (YVAD-AFC), cas-2 (VDVAD-AFC), cas-3 (DEVD-AFC), cas-4 (LEVD-AFC), cas-5 (WEHD-AFC), cas-6 (VEID-AFC), cas-8 (IETD-AFC), and cas-9 (LEHD-AFC) were purchased from Calbiochem. Primary antibody against mouse cas-1 was from Zymed, and antibody against subunits of cas-3 was from New England Biochemicals. All secondary antibodies were obtained from Santa Cruz. In Situ Cell Death Detection Kit was from Promega. Annexin V was purchased from R&D Systems. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), endothelial cell growth factor (ECGF), and penicillin-streptomycin were from Fisher. Endothelial cell basal medium (EBM), plasma-derived horse serum, heparin, and all other chemicals were obtained from Sigma.

### Methods

**Animals.** Male mice (C57BL/6) that weighed 20 g were randomly assigned to be made diabetic, galactosemic, or control. Diabetes was induced by streptozotocin (STZ) injections (60 mg/kg body wt i.p. on 5 consecutive days), and insulin was given as needed to achieve slow weight gain without preventing hyperglycemia and glycosuria (0.1–0.2 units of NPH insulin subcutaneously, 2–3 times a week). Experimental galactosemia was produced by feeding a diet of Teklad 7004 (Harlan, WI) enriched with 30% D-galactose. Animals were caged in pairs, had free access to food and water, and were maintained under a 14-h on/10-h off light cycle. Body weight was measured weekly. Treatment of animals conformed to the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research. Shortly before the animals were killed, the severity of blood hexose elevation was estimated by measuring the level of nonenzymatic Ghb using affinity chromatography (Glyc-Affin; Pierce, Rockford, IL). Animals in whom Ghb was less than twice the normal value were excluded from additional experiments.

Animals were killed after 2, 4, and 8 months of diabetes or galactosemia. These durations precede the appearance of lesions of retinopathy in mice (T.S.K., unpublished observation). Age-matched normal animals served as controls. The retinas of both eyes were isolated and immediately resuspended in 200  $\mu$ l of lysate buffer (100 mmol/l HEPES buffer [pH 7.5] containing 0.1% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, 10% sucrose, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, and 1  $\mu$ g/ml leupeptin) and placed on ice for 30 min. After 10 s of sonication on ice, lysates were centrifuged at 9,000g for 5 min at 4°C. The supernatants were frozen for additional experiments. Protein measurement was done using the BioRad assay.

**Human tissue.** Human eyes were obtained through the National Disease Research Interchange (Philadelphia, PA). The donors remained anonymous, but age, presence or absence of diabetes, and cause of death were noted. Severity of diabetes and severity of retinopathy were not available, but none of the patients had laser photocoagulation. The characteristics of the patients with diabetic and nondiabetic control subjects are presented in Table 3. The eyes of the donors with and without diabetes were enucleated, packed on wet

ice, and obtained by our laboratory 15 ± 3 h after death. For the experiments described below, the retina was isolated and two pieces from the temporal/superior part of the retina were resuspended in 200  $\mu$ l of lysate buffer, placed on ice for 30 min, sonicated for 10 s, and centrifuged at 9,000g for 5 min at 4°C, followed by protein measurement of the supernatant, as described above.

**Tissue culture.** Retinal Müller cells (rMC-1 cells/4 × 10<sup>6</sup> cells) were grown in 100-mm Petri dishes in 10 ml of DMEM (5 mmol/l glucose) supplemented with 10% FBS and 1% penicillin/streptomycin (growth medium) at 37°C/5% CO<sub>2</sub> overnight. The next day, medium was replaced by 10 ml of DMEM containing 2% FBS, 1% penicillin/streptomycin, and either 5 or 25 mmol/l glucose (treatment medium). Medium was changed every other day. Only cells from passage 20 and lower were used for the experiments.

Bovine retinal endothelial cells were isolated as described (15). Isolated endothelial cells were grown in 100-mm Petri dishes coated with 0.1% gelatin in 10 ml of EBM medium supplemented with 10% plasma-derived horse serum, 5% ECGF, 1% penicillin/streptomycin, and 5% heparin (growth medium) at 37°C/5% CO<sub>2</sub> until 75% confluence. Then, medium was replaced by 10 ml of EBM medium containing 2% FBS, 5% ECGF, 1% penicillin/streptomycin, 5% heparin, and either 5 or 25 mmol/l glucose (treatment medium). Medium was changed every other day. Only cells from passage 8 and lower were used for the experiments.

At times indicated, cells were lysed in 200  $\mu$ l of lysate buffer, sonicated for 10 s, and centrifuged at 9,000g for 5 min at 4°C, followed by protein measurement of the supernatant, as described above.

**Annexin V staining.** rMC-1 cells (1 × 10<sup>5</sup>) were grown on coverslips and incubated in treatment medium containing 5 or 25 mmol/l glucose as described above. Annexin V staining was done according to the manufacturer's instructions. Briefly, after incubation of rMC-1 cells for 48 and 96 h, the medium was removed and cells were washed twice with annexin V binding buffer (2 ml). For staining, cells were incubated in 500  $\mu$ l of annexin V staining solution (1:50 dilution of annexin V-phycoerythrin in annexin V binding buffer) for 15 min in the dark at 37°C/5% CO<sub>2</sub>. The annexin V staining solution was removed, and coverslips were immediately mounted on slides using an antifading fluorescent mounting medium (Vector Shield) and analyzed by fluorescence microscopy (Zeiss Axiophot; Karl Zeiss, Thornwood, NY) at excitation of 540 nm and emission of 600 nm.

**Measurement of caspase activity.** Caspase activity was measured as described previously (16). Briefly, equal amounts of proteins (usually 15  $\mu$ g) were incubated in lysate buffer containing the fluorogenic caspase substrate (2.5  $\mu$ mol/l) in a total volume of 100  $\mu$ l at 32°C for 1 h. Cleavage of the substrate emits a fluorescence signal that was quantified by a Tecan Spectra FluorPlus fluorescence plate reader (excitation: 400 nm, emission: 505 nm). Activities of cas-4 and cas-5 were averaged together, because evidence suggests that they cleave similar substrates (17). The average value is represented as cas-4/5.

**Western blots against caspase.** Equal amounts of proteins (50  $\mu$ g) were separated in a prepared 4–20% SDS gradient gel (BioRad) by electrophoresis and blotted onto nitrocellulose membrane (100 V, 90 min). The membrane was washed three times (5 min) with PBS containing 0.05% Tween 20, blocked with 5% dry milk in PBS/0.05% Tween 20 (2 h, room temperature), and incubated with a primary antibody (usually 1:1,000 dilution in PBS/0.05% Tween 20) overnight at 4°C. After primary incubation, the membranes were washed five times (5 min) and incubated with secondary antibody (1:3,000 dilution) for 1 h at room temperature. After washing (5 × 5 min) of the membranes in PBS/0.05% Tween 20, membranes were developed using enhanced chemiluminescence (Amersham).

**In situ cell death of retinal capillary cells.** Cell death in retinal trypsin digests was detected with the TUNEL method using fluorescein isothiocyanate (FITC)-based In Situ Cell Death Detection Kit. The retinal vasculature was isolated by the trypsin digest technique, as reported previously by us, after 5 days of formalin fixation (2). The digestion buffer contained 0.2 mol/l sodium fluoride to inhibit DNA fragmentation as a result of DNases contaminating the crude trypsin preparation. The digests were rehydrated in PBS and stained according to the manufacturer's instructions. In each experiment, the negative control received only the label solution without the terminal transferase, and the positive control was exposed to DNase (1  $\mu$ g/ml in 40 mmol/l Tris-HCl, 6 mmol/l MgCl<sub>2</sub> buffer [pH 7.5]) for 10 min at room temperature before the TUNEL reaction. TUNEL-positive cells were detected by the presence of FITC-positive stain using a Zeiss Axiophot fluorescence microscope.

### Statistical analysis

Experimental groups were compared statistically using the Mann-Whitney *U* test (two-tailed). Means with  $P < 0.05$  were considered statistically significant.

TABLE 2  
Galactosemia-induced changes in retinal caspase activities

Caspase	2 Months	4 Months	8 Months
Cas-1	14 ± 3.8	6.2 ± 3.7	-7.3 ± 5.4
Cas-2	NS	NS	NS
Cas-3	NS	NS	NS
Cas-4/5	6.4 ± 4.2	NS	NS
Cas-6	NS	4.4 ± 3.3	NS
Cas-8	NS	NS	NS
Cas-9	NS	NS	5.0 ± 2.2

Data are means ± SD of caspase activities that were significantly different from normal ( $P < 0.05$ , Mann-Whitney  $U$  test, two-tailed). NS, not statistically significant. Caspase activities were measured of retinas from galactosemic and normal mice at different durations as described in RESEARCH DESIGN AND METHODS. Results represent the percentage increase in caspase activities compared with normal age-matched animals.

**RESULTS**

**Animals.** Blood hexose levels in diabetic and galactosefed mice were elevated, as evidenced by significantly elevated GHb percentage and abnormal body weight compared with normal age-matched animals. Diabetic animals also had significantly elevated blood glucose levels. Table 1 summarizes the characteristics of the experimental groups at different durations (2, 4, and 8 months).

**Caspase activation in the retina of diabetic mice.** It was shown previously that apoptosis of retinal cells precedes the development of morphologic lesions of diabetic retinopathy. Activation of caspase is necessary to initiate or execute apoptosis. To identify caspase or a possible caspase cascade involved in the apoptotic process in the retina of diabetic patients, we measured caspase activities of eight caspases (cas-1, -2, -3, -4, -5, -6, -8, and -9) in the retinas of diabetic and normal animals using commercially available substrates. The activities detected using substrates for the human cas-4 and -5 likely represent the activity of mouse cas-11 (for which no substrate is available), because human cas-4 and -5 are close homologues of mouse cas-11 (17). Figure 1 shows the time course of caspase activation in the retina of diabetic mice compared with normal age-matched animals.

At 2 months' duration of diabetes, activities of cas-1, -2, -4/5, -6, -8, and -9 were significantly elevated in comparison with normal age-matched animals (Fig. 1, top panel). Activities of initiator caspases such as cas-2, -8, and -9 increased by 7.5–15% above normal at this duration of diabetes. The activity of the executioner cas-6 increased by 15%, whereas cas-3, another executioner caspase and the most common marker for apoptosis, was not yet activated. The activities of cas-1 and -4/5, members of the subfamily that is most closely associated with proinflammatory cytokine production, were strongly elevated (6 and 16%, respectively) at 2 months of diabetes.

At 4 months of diabetes, the pattern of caspase activities changed. Cas-1 activity slightly increased up to 8%, whereas all other caspase activities, especially those of other initiators, declined (but were still significantly elevated above normal). The activity of cas-2 could not be detected anymore. The activity of cas-3 (3.5%) was significantly detectable for the first time (Fig. 1, middle panel).

At 8 months' duration of diabetes, members of the cas-1 subfamily, such as cas-1 and -4/5, were still active. Activ-

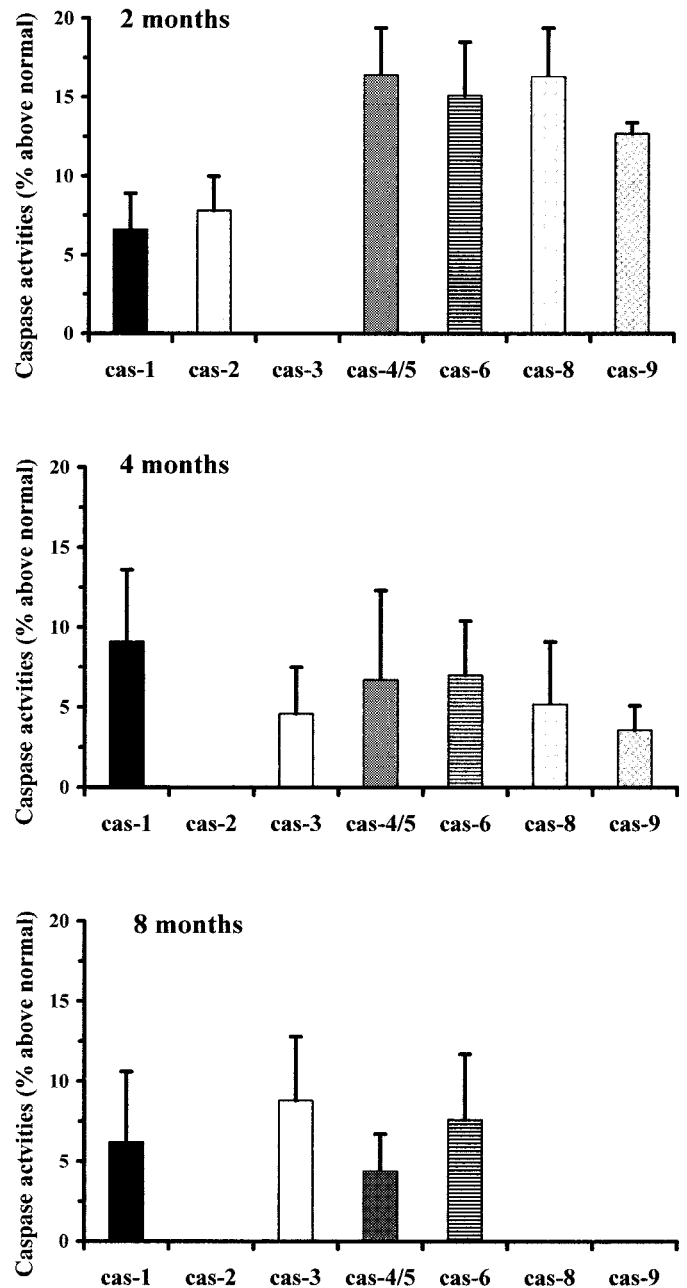


FIG. 1. Caspase activities in retinas of diabetic mice at different durations of diabetes. Retinas of diabetic ( $n = 9$ ) and normal ( $n = 10$ ) C57BL/6 mice were isolated at 2, 4, or 8 months. Cas activities of retinal lysates were measured as described in RESEARCH DESIGN AND METHODS. Activities are presented as the percentage (mean ± SD) above normal. Only means ± SD of caspases activities that were significantly different from normal with  $P < 0.05$  (Mann-Whitney  $U$  test, two-tailed) are presented.

ities of all initiator caspases were undetectable at this duration. The activity of the executioner cas-3 increased up to 8%, whereas that of the executioner cas-6 remained unchanged (Fig. 1, bottom panel).

**Relation of cas-1 activity to the severity of diabetes.** Figure 2 shows a linear correlation of cas-1 activity in the retinas of diabetic animals ( $n = 9$ ) with the severity of diabetes at 2 months' duration. The correlation was statistically significant ( $P < 0.025$ ) with a correlation factor of  $r = 0.742$ .

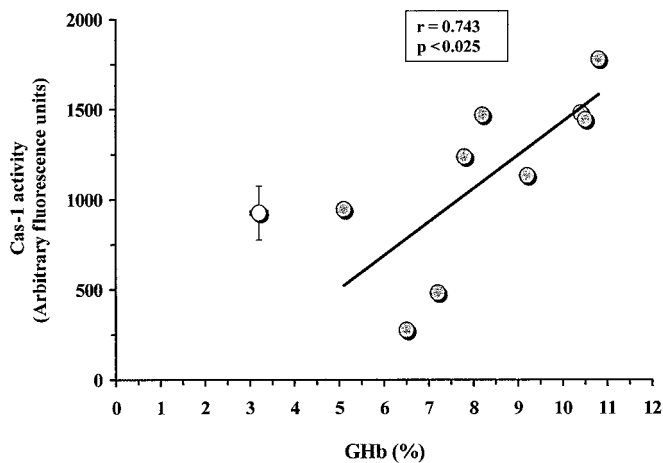


FIG. 2. Correlation of cas-1 activity with the severity of diabetes. Cas-1 activity (presented as arbitrary fluorescence units) was plotted against percentage of GHb of individual diabetic mice (gray dots,  $n = 9$ ) at 2 months' duration of diabetes. Linear regression, correlation factor ( $r$ ), and significance ( $p$ ) present correlation of cas-1 activity with GHb. Cas-1 activity and GHb% of normal age-matched mice (hollow dot,  $n = 10$ ) are presented as the mean  $\pm$  SD.

**Galactosemia-induced caspase activation in the retina.** Because we identified caspase activation in the retina of diabetic animals, evaluation of caspase activity in another model for diabetic retinopathy, experimental galactosemia, would provide insight on which caspases were closely associated with the development of lesions like those characteristic of diabetic retinopathy.

At 2 months of galactose feeding, only cas-1 and -4/5 were strongly activated (14 and 6.4% above normal, respectively). Thereafter, cas-6 was activated at 4 months and cas-9 at 8 months of galactosemia. Thus, the pattern of caspase activation observed in retinas of galactosemic animals differed from those observed in diabetic animals. Cas-1, -4/5, and -6 activities disappeared in the retinas of galactosemic animals with longer duration. At 8 months of galactosemia, cas-1 activity even dropped below normal (-7.3%). Cas-3 activity was not detected in the retinas of galactose-fed mice at these different durations (Table 2).

**Confirmation of cas-1 and -3 activation by Western blot.** Evaluation of which caspases are activated on the basis of measurement of enzyme activities depends heavily on the specificity of the substrates for individual caspases. To confirm activation of several caspases, we assessed the cleavage of the caspase proenzyme into its subunits by Western blot.

Figure 3 shows the proenzyme and the active p20 subunit of cas-1 in the retinal lysates of normal, diabetic, and galactosemic mice at 2 and 8 months, respectively (Fig. 3A and B). In the retinas of normal animals, the p20 cas-1 subunit could not be detected at any time point.

Cas-3 activation was confirmed using a cas-3 antibody specifically detecting cleavage products of cas-3, such as p20 (inactive) and p17 (active) subunits. The smaller p10 subunit of cas-3 cannot be detected by this antibody. Activation of cas-3 into its active subunits occurs via an intermediate inactive p20 subunit that needs to be cleaved further to the active p17 subunit.

As Fig. 4 shows, the active p17 subunit of cas-3 could be detected in the lysates of retinas of animals that were diabetic for 8 months. In contrast, neither normal nor

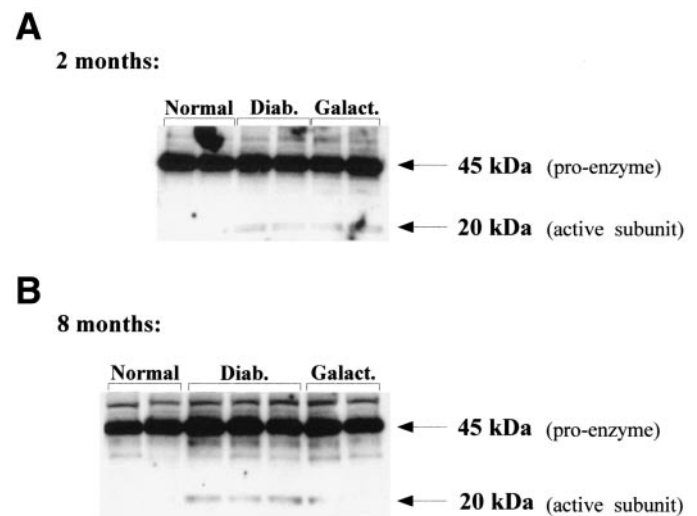


FIG. 3. Diabetes- and galactosemia-induced cas-1 activation. Lysates from retinas of diabetic, galactose-fed, and normal animals at 2 (A) and 8 (B) months' duration of diabetes were separated in a 4–20% gradient gel under reducing conditions and probed for cas-1 as described in RESEARCH DESIGN AND METHODS. The figure shows the inactive proenzyme (45 kDa) as well as the active subunit (20 kDa) and represents the result of two individual animals of each experimental group.

galactosemic animals showed any cas-3 cleavage products at 8 months' duration. The lack of an active cas-3 subunit in the retinas of galactose-fed animals is consistent with the absence of cas-3 activity in the retina of these animals. Cas-6 activation also was confirmed by Western blot (data not shown).

**Association of cas-3 activation with the detection of apoptotic cells within the retinal vasculature of diabetic mice.** The frequency of TUNEL-positive cells in retinal capillaries of diabetic mice was slightly greater than normal at 24 weeks:  $2.3 \pm 0.9$  TUNEL-positive capillary cells in diabetic animals ( $n = 4$ ) compared with  $1.0 \pm 0.8$  TUNEL-positive capillary cells in normal animals ( $n = 4$ ;  $P < 0.05$ ). The frequency of TUNEL-positive cells in the retinal vasculature tended to be greater at 48 weeks of diabetes ( $7.9 \pm 1.6$  in diabetic animals and  $1.3 \pm 0.6$  in normal; both  $n = 2$ ).

**Caspase activation in retinas of diabetic patients.** Caspase activities were measured in lysates of human retinas obtained postmortem from patients with ( $n = 5$ ) and without ( $n = 4$ ) diabetes. Table 3 summarizes the characteristics of the patients.

Consistent with the findings in diabetic mice, the activities of some caspases in retinas of patients with diabetes,



FIG. 4. Activation of cas-3 at 8 months' duration of diabetes. Lysates from retinas of diabetic, galactose-fed, and normal animals at 8 months' duration of diabetes were separated in a 4–20% gradient gel under reducing conditions and probed against the cleaved subunits of cas-3 as described in RESEARCH DESIGN AND METHODS. The antibody detects (shown by the positive control) both the inactive p20 subunit and the active p17 subunit of cas-3. No specific stain was detected in either the normal controls or the galactosemic animals. The figure represents the result of two individual animals of each experimental group.

TABLE 3  
Characteristics of patients with and without diabetes

	Age/sex	Cause of death	Type of diabetes
Nondiabetic	64/M	MI	—
Nondiabetic	37/M	MVA	—
Nondiabetic	67/F	CA	—
Nondiabetic	73/M	MI	—
Diabetic	52/M	MI	DM 2
Diabetic	70/F	ICH	DM 2
Diabetic	59/F	CA	DM 2
Diabetic	79/M	CA	DM 2
Diabetic	70/F	MI	DM 2

MI, myocardial infarct; MVA, motor vehicle accident; CA, cancer; ICH, intracerebral hemorrhage; DM 2, non-insulin-dependent diabetes mellitus (type 2).

such as cas-1 (5.2%), cas-3 (5%), cas-4 (4.5%), and cas-6 (13%), were significantly greater than normal ( $P < 0.05$ , two-tailed). Activities of other caspases, however, remained equivalent to that observed in normal patients (Fig. 5).

**Distinct caspase activation in retinal Müller and endothelial cells.** For the experiments described above, caspase activities were measured from lysates of whole retinas. To identify possible caspase cascades in different cell types, we studied the effect of hyperglycemia on caspase activation in vitro using two different cell types. The rat rMC-1 and primary bovine retinal endothelial cells (BRECs) were incubated in high glucose (25 mmol/l) and low glucose (5 mmol/l) for up to 7 days. Because of the faster growth of rMC-1 cells, cells were incubated for only 5 days to avoid apoptosis induced by overgrowth or starvation. Figure 6A shows that high glucose induced the activation of cas-1 family members in rMC-1 cells, as well as cas-8 (6.4%/day 3) and subsequent cas-9 (6.1%/day 5). Hyperglycemia led to a strong activation of the executioner cas-6 (17.3%/day 5) and a modest activation of cas-3. However, the activity of cas-3 induced by hyperglycemia varied and became completely suppressed in higher pas-

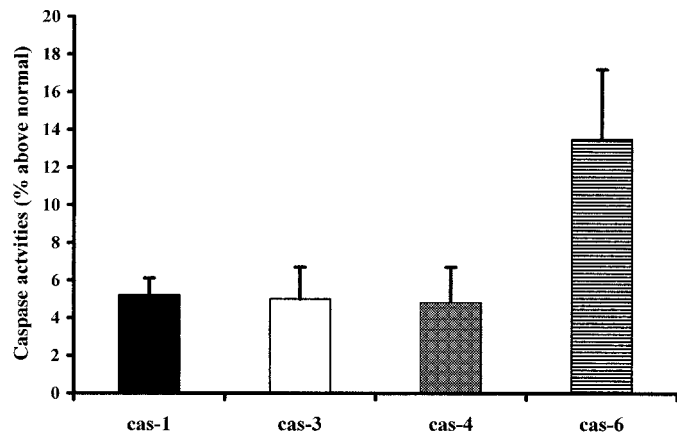


FIG. 5. Caspase activities in retinas of patients with diabetes. Retinas of patients with and without diabetes were isolated, and caspase activities were measured as described in RESEARCH DESIGN AND METHODS. Activities are presented as the percentage (mean  $\pm$  SD) above normal. Only means  $\pm$  SD of cas activities that were significantly different from normal with  $P < 0.05$  (Mann-Whitney  $U$  test, two-tailed) are presented.

sages, possibly as a result of changes in the SV 40 transfected cell line.

In contrast, hyperglycemia did not activate any member of the cas-1 subfamily in BRECs. Cas-3 activity was strongly induced (22%) at 7 days of high glucose treatment (Fig. 6B). Activation of cas-8 (11.3%/day 5) was stronger in BRECs than in rMC-1 cells, but activity of cas-9 (3.1%/day 5) was not significantly induced in endothelial cells.

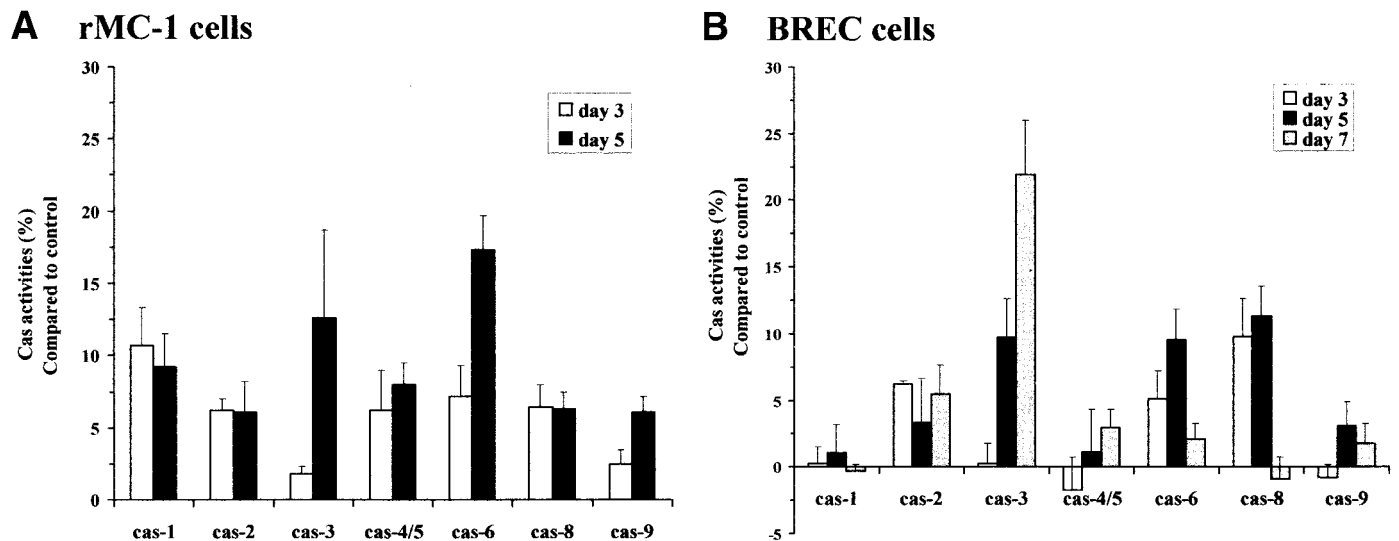
**Caspase inhibition prevents high glucose-induced apoptosis in rMC.** Inhibition of caspase activities using the broad-spectrum caspase inhibitor zVAD-fmk (50  $\mu$ mol/l) inhibited hyperglycemia-induced apoptosis in rMC-1 cells, as shown by annexin V staining (Fig. 7). DMSO (solvent) had no effect on glucose-induced apoptosis.

DISCUSSION

Apoptosis of retinal capillary cells and subsequent nonperfusion of these capillaries have been postulated to play an important role in the development of diabetic retinopathy (2). Caspase activation is crucial to initiate and execute apoptotic processes within cells (6,8). Our study reveals that, indeed, caspases become activated in retinas of diabetic, galactose-fed mice and in retinas of patients with diabetes, and inhibition of caspases prevented cell death caused by elevated glucose concentrations. To determine caspase activation in our study, we actually measured caspases activities, rather than relying on techniques that are able to detect only structural evidence of caspase activation (e.g., Western blot). Measuring caspase activities has the advantage of determining whether activation of a specific caspase actually led to an active, functional enzyme. Detection of a cleaved caspase active subunit (activation) does not always guarantee increased enzymatic activity, because caspases are proteolytic enzymes that contain a highly reactive cysteine in their active site and are known to be very sensitive toward oxidative as well as nitrosative stress (18). However, Western blots were done to test the specificity of the substrates used.

Initiator caspases, such as cas-8, -9, and -2, are known to be able to activate proteolytically executioner caspases, such as cas-3, -6, or -7 (19,20). Consistent with this, a time course at different stages of diabetes has shown a distinct pattern of caspase activation and deactivation in the retina. The increase in caspase activities was modest but statistically significant and consistent with the slow rate of apoptosis and the slow development of diabetic retinopathy. The activation of several initiator caspases occurred early in the course of diabetes. Apoptosis initiator caspases induce distinct signaling pathways. Diabetes activates cas-8 and -9, suggesting the activation of both an extrinsic and an intrinsic pathway. Cas-8 is known to become activated via death receptors leading to two possible subsequent pathways that induce apoptosis (21–23). In the first, type I (extrinsic), cas-8 is strongly activated via a tight death-induced signaling complex formation at the receptor. The strong activation of cas-8 allows the direct activation of cas-3. In the second, type II (extrinsic/intrinsic), cas-8 is less strongly activated, and the signal is amplified via the mitochondria, in which cas-9 is activated by Apaf-1 and cytochrome c that has been

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**FIG. 6.** Hyperglycemia-induced caspase activation in rMC and endothelial cells. rMC-1 cells (A) and BRECs (B) were incubated in low (5 mmol/l) and high (25 mmol/l) glucose for the times indicated. Caspase activities of cell lysates were measured as described in RESEARCH DESIGN AND METHODS. Activities are presented as the percentage (mean  $\pm$  SD) compared with control (5 mmol/l glucose). The figure represents the result of  $n = 11$  (rMC-1) and  $n = 4$  (BREC) independent experiments.

released from the mitochondria subsequently activating downstream executioners (24–26). In addition, DNA damage, oxidative stress, and drugs can activate both cas-9 isoforms (mitochondrial and nuclear) directly (intrinsic) (27,28). Very little is known about cas-2, but it has been found to be an inducer of apoptosis in neuronal cells and retinal ganglion cell (29,30). Because of its long prodomain, it has been considered an upstream caspase binding to anchor molecules of death receptors, but other reports have also demonstrated a very downstream activation of cas-2 (31).

The activation of cas-3, a downstream executioner, occurred later than the activation of other caspases. Cas-3 is the executioner caspase known to activate the apoptotic endonuclease DFF40, which is responsible for the DNA laddering, a characteristic of apoptosis (32,33). Compared with the late activation of cas-3, our results have demonstrated an earlier activation of cas-6, another executioner. Reports have shown rapid and slow activation of cas-6, suggesting that the place of cas-6 in a cascade might depend on the cell type (34,35). Cas-6 activation has been closely associated with neuronal apoptosis and the development of neuronal diseases such as Alzheimer's disease (34,36). Likewise, perhaps the early activation of cas-6 is related to the early detection of apoptotic retinal ganglion cells in diabetic rats (3).

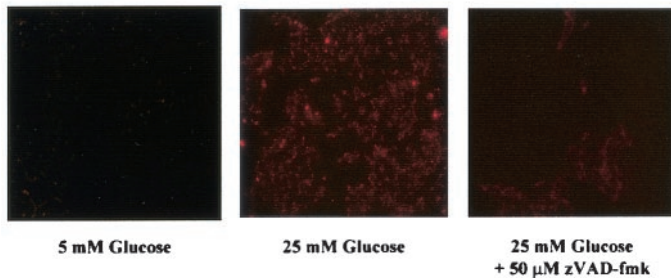
The pattern of caspase activation in the retina differed between diabetic and galactose-fed animals. Cas-9 activation seemed to be delayed in the retinas of galactose-fed mice compared with diabetic mice, and activation of cas-2 and -8 were not detectable in galactosemia at the durations used for the study. Perhaps this difference between diabetes and galactosemia contributes to the observed different response of the two retinopathies to aminoguanidine therapy (37): aminoguanidine was found to inhibit both capillary cell apoptosis and capillary degeneration in diabetes but did not have an effect in galactose-fed animals. It is interesting that common to both diabetes and galactosemia was the early activation of cas-1 and its family

members, raising the possibility that activation of this caspase subfamily might play an important role in the formation of histological lesions observed in the two models for diabetic retinopathy. The finding of cas-1 and -4 activation in retinas of patients with diabetes strengthens this idea and is consistent with a growing awareness that diabetic retinopathy has several characteristics of a chronic inflammatory disease (e.g., increased nitric oxide production, vaso-occlusion, and vascular permeability) (38–40).

How cas-1 and its family members cas-4, -5, and -11 (cas-11 is the mouse homologue to human cas-4 and -5) become activated in diabetes and their role in apoptosis are still uncertain. Some reports have shown that activation of cas-1 is involved in the induction of fibroblast apoptosis, and cas-1 activation also seemed to be related to apoptosis in several neurodegenerative diseases and subsequent ischemic events (41,42). Cas-1 has also been shown to activate cas-3 as well as cas-6 (43). However, the major function of cas-1 has been closely related to proinflammatory cytokine production, such as interleukin-1 $\beta$  and -18 (44,45). Some reports have shown that cas-11 is necessary to activate cas-1 in mice, which is consistent with our finding of an early induction of a cas-11-like activity (17,46).

Our *in vitro* studies have demonstrated that high glucose itself is able to induce the activation of the proinflammatory cas-1 family in rMC cells. Cas-8 and -9 were significantly activated in these cells, indicating an involvement of the mitochondria and type II (extrinsic/intrinsic) signaling pathway. In contrast, elevated levels of glucose did not activate members of the cas-1 family in retinal endothelial cells but led to a strong cas-8 response, suggesting a possible type I (extrinsic) signaling pathway (especially because the cas-9 activation was not significant). The *in vitro* studies of retinal endothelial and Müller cells strongly indicate that high glucose treatment induces different caspase cascades in these cell types, suggesting that the findings in retinal homogenates represent different

Annexin V Staining of rMC-1 cells



**FIG. 7. Caspase inhibition prevents hyperglycemia-induced apoptosis in rMC.** rMC-1 cells were incubated in low (5 mmol/l) and high (25 mmol/l) glucose in the presence or absence of zVAD-fmk (50 μmol/l) for 3 days. Cells were stained with annexin V as described in RESEARCH DESIGN AND METHODS. The figure represents the result of three similar experiments.

caspase cascades occurring in different cell types. Full understanding of caspase cascades and cell type specificity will require more selective substrates and antibodies. As expected, inhibition of caspases prevented high glucose-induced cell death, strengthening that elevated glucose clearly causes caspase-dependent apoptosis but not necrosis.

In total, our data suggest that diabetes or elevated glucose activates several slowly progressing cascades of caspases, in which initiator caspases activate a sequence of other caspases that results in multiplication of the death signal within a cell and ultimately leads to activation of executioner caspases and cell death. The degree of caspase activation by diabetes may seem small but is statistically significant. So far, we are not able to compare the increase in caspase activities with other chronic diseases (where apoptosis is known to be important, e.g., arthritis and Alzheimer's disease), because these studies do not assess enzymatic activity, only immunohistochemical demonstration of increased caspase cleavage. Our in vitro demonstration that inhibition of caspase activity can prevent cell death caused by elevated glucose offers a new potential target, at which the role of apoptosis in the pathogenesis of diabetic retinopathy might be evaluated. Identifying the role of both the proinflammatory and proapoptotic caspase network might offer new therapeutic strategies for the inhibition of this retinal disease. However, the long-term use of caspase inhibitors has not been evaluated in any disease and will require more extensive studies.

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**REFERENCES**

- Bresnick G, Engerman R, Davis MD, de Venecia G, Myers FL: Patterns of ischemia in diabetic retinopathy. *Trans Am Acad Ophthalmol Otolaryngol* 81:694-709, 1976
- Mizutani M, Kern TS, Lorenzi M: Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest* 97:2883-2890, 1996
- Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW: Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. *J Clin Invest* 102:783-791, 1998
- Alnemri E, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J: Human ICE/CED-3 protease nomenclature (Letter). *Cell* 87:171, 1996
- Alnemri ES: Mammalian cell death proteases: a family of highly conserved aspartate specific cysteine proteases. *J Cell Biochem* 64:33-42, 1997
- Nicholson DW, Thornberry NA: Caspases: killer proteases. *Trends Biochem Sci* 22:299-306, 1997
- Zhivotovsky B, Burgess DH, Vanags DM, Orrenius S: Involvement of cellular proteolytic machinery in apoptosis. *Biochem Biophys Res Commun* 230:481-488, 1997
- Salvesen GS, Dixit VM: Caspases: intracellular signaling by proteolysis. *Cell* 91:443-446, 1997
- Stennicke HR, Salvesen GS: Biochemical characteristics of caspases-3, -6, -7, and -8. *J Biol Chem* 272:25719-25723, 1997
- Cohen GM: Caspases: the executioners of apoptosis. *Biochem J* 326:1-16, 1997
- Orth K, O'Rourke K, Salvesen GS, Dixit VM: Molecular ordering of apoptotic mammalian CED-3/ICE-like proteases. *J Biol Chem* 271:20977-20980, 1996
- Orth K, Chinnaiyan AM, Garg M, Froelich CJ, Dixit VM: The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J Biol Chem* 271:16443-16446, 1996
- Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM: Yama/ CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 81:801-809, 1995
- Porter AG, Ng P, Janicke RU: Death substrates come alive. *Bioessays* 19:501-507, 1997
- Yaccino JA, Chang YS, Hollis TM, Gardner TW, Tarbell JM: Physiological transport properties of cultured retinal microvascular endothelial cell monolayers. *Curr Eye Res* 16:761-768, 1997
- Mohr S, McCormick TS, Lapetina EG: Macrophages resistant to endogenously generated nitric oxide mediated apoptosis are hypersensitive to exogenously added nitric oxide donors: dichotomous apoptotic response independent of caspase-3 and reversal by the MEK inhibitor PD098059. *Proc Natl Acad Sci U S A* 95:5045-5050, 1998
- Kang SJ, Wang S, Hara H, Peterson EP, Namura S, Amin-Hanjani S, Huang Z, Srinivasan A, Tomaselli KJ, Thornberry NA, Moskowitz MA, Yuan J: Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *J Cell Biol* 149:613-622, 2000
- Mohr S, Zech B, Lapetina EG, Brune B: Inhibition of caspase-3 by S-nitrosation and oxidation caused by nitric oxide. *Biochem Biophys Res Commun* 238:387-391, 1997
- Colussi PA, Harvey NL, Kumar S: Prodomain-dependent nuclear localization of the caspase-2 (Nedd2) precursor. A novel function for a caspase prodomain. *J Biol Chem* 273:24535-24542, 1998
- Colussi PA, Harvey NL, Shearwin-Whyatt LM, Kumar S: Conversion of procaspase-3 to an autoactivating caspase by fusion to the caspase-2 prodomain. *J Biol Chem* 273:26566-26570, 1998
- Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapani JA, Tomaselli KJ, Litwack G, Alnemri ES: In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc Natl Acad Sci U S A* 93:7464-7469, 1996
- Boldin MP, Goncharov TM, Goltsev YV, Wallach D: Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. *Cell* 85:803-815, 1996
- Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scalfidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM: FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 85:817-827, 1996
- Krammer PH: CD95's deadly mission in the immune system. *Nature* 407:789-795, 2000
- Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X: Cytochrome c and dATP-dependent formation of Apaf-1

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- caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479–489, 1997
26. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X: Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90:405–413, 1997
  27. Ritter PM, Marti A, Blanc C, Baltzer A, Krajewski S, Reed JC, Jaggi R: Nuclear localization of procaspase-9 and processing by a caspase-3-like activity in mammary epithelial cells. *Eur J Cell Biol* 79:358–364, 2000
  28. Ochs K, Kaina B: Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent. *Cancer Res* 60:5815–5824, 2000
  29. Troy CM, Rabacchi SA, Friedman WJ, Frappier TF, Brown K, Shelanski ML: Caspase-2 mediates neuronal cell death induced by beta-amyloid. *J Neurosci* 20:1386–1392, 2000
  30. Kurokawa T, Katai N, Shibuki H, Kuroiwa S, Kurimoto Y, Nakayama C, Yoshimura N: BDNF diminishes caspase-2 but not c-Jun immunoreactivity of neurons in retinal ganglion cell layer after transient ischemia. *Invest Ophthalmol Vis Sci* 40:3006–3011, 1999
  31. Li H, Bergeron L, Cryns V, Pasternack MS, Zhu H, Shi L, Greenberg A, Yuan J: Activation of caspase-2 in apoptosis. *J Biol Chem* 272:21010–21017, 1997
  32. Liu X, Zou H, Widlak P, Garrard W, Wang X: Activation of the apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease). Oligomerization and direct interaction with histone H1. *J Biol Chem* 274:13836–13840, 1999
  33. Wolf BB, Schuler M, Echeverri F, Green DR: Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. *J Biol Chem* 274:30651–30656, 1999
  34. LeBlanc A, Liu H, Goodyer C, Bergeron C, Hammond J: Caspase-6 role in apoptosis of human neurons, amyloidogenesis, and Alzheimer's disease. *J Biol Chem* 274:23426–23436, 1999
  35. Hirata H, Takahashi A, Kobayashi S, Yonehara S, Sawai H, Okazaki T, Yamamoto K, Sasada M: Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. *J Exp Med* 187:587–600, 1998
  36. Pellegrini L, Passer BJ, Tabaton M, Ganjei JK, D'Adamio L: Alternative, non-secretase processing of Alzheimer's beta-amyloid precursor protein during apoptosis by caspase-6 and -8. *J Biol Chem* 274:21011–21016, 1999
  37. Kern TS, Tang J, Mizutani M, Kowluru RA, Nagaraj RH, Romeo G, Podesta F, Lorenzi M: Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of diabetes and galactosemia. *Invest Ophthalmol Vis Sci* 41:3972–3978, 2000
  38. Kowluru RA, Engerman RL, Kern TS: Abnormalities of retinal metabolism in diabetes or experimental galactosemia VIII. Prevention by aminoguanidine. *Curr Eye Res* 21:814–819, 2000
  39. Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clermont AC, Aiello LP, Ogura Y, Adami AP: Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. *Proc Natl Acad Sci U S A* 96:10836–10841, 1999
  40. Antonetti DA, Barber AJ, Khin S, Lieth E, Tarbell JM, Gardner TW: Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Penn State Retina Research Group. *Diabetes* 47:1953–1959, 1998
  41. Pasinelli P, Borchelt DR, Houseweart MK, Cleveland DW, Brown RH Jr: Caspase-1 is activated in neural cells and tissue with amyotrophic lateral sclerosis-associated mutations in copper-zinc superoxide dismutase. *Proc Natl Acad Sci U S A* 95:15763–15768, 1998
  42. Friedlander RM: Role of caspase 1 in neurologic disease. *Arch Neurol* 57:1273–1276, 2000
  43. Ali A, Mundle SD, Ragasa D, Reza S, Shetty V, Mativi BY, Cartlidge JD, Azharuddin M, Qawi H, Dar S, Raza A: Sequential activation of caspase-1 and caspase-3-like proteases during apoptosis in myelodysplastic syndromes. *J Hematother Stem Cell Res* 8:343–356, 1999
  44. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, et al.: Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376:37–43, 1995
  45. Ghayur T, Banerjee S, Hugunin M, Butler D, Herzog L, Carter A, Quintal L, Sekut L, Talanian R, Paskind M, Wong W, Kamen R, Tracey D, Allen H: Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production. *Nature* 386:619–623, 1997
  46. Wang S, Miura M, Jung YK, Zhu H, Li E, Yuan J: Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell* 92:501–509, 1998