Advanced glycation end products can induce glial reaction and neuronal degeneration in retinal explants

A Leclere-Collet, L H Tessier, P Massin, V Forster, G Brasseur, J A Sahel, S Picaud

Background/aims: Neuronal degeneration has been reported to occur in diabetic retinopathy before the onset of detectable microvascular abnormalities. To investigate whether advanced glycation end products (AGE) could be directly responsible for retinal neurodegeneration, retinal explants were incubated with glycated bovine serum albumin (BSA).

Methods: Retinal explants obtained from non-diabetic adult rats were incubated 4 days with or without 200 μg/ml glycated BSA. Neuronal apoptosis was quantified by terminal dUTP nick end labelling (TUNEL) and immunostaining with anti-cleaved caspase-3 antibody. Expression of glial fibrillary acidic protein (GFAP) was localised by immunofluorescence.

Results: TUNEL and cleaved caspase-3 positive cells increased significantly by 2.2-fold and 2.5-fold in retinal explants incubated in glycated BSA (p<0.05), respectively. The ganglion cell layer was the most sensitive retinal layer to the glycated BSA. Neuronal degeneration was confirmed by increased GFAP labelling in Müller glial cells from retinal explants treated with glycated BSA.

Conclusion: These results suggest that AGE could induce retinal neurodegeneration in the absence of blood perfusion. Cells in the ganglion cell layer appeared to be the most sensitive as in diabetic retinopathy and its animal models. AGE toxicity could therefore contribute to the early pathological mechanisms of diabetic retinopathy.

Neuronal cell depletion was observed decades ago in diabetic patients. Although diabetic retinopathy (DR) is classically considered a retinal microangiopathy, loss of colour vision, of contrast sensitivity, electroretinogram abnormalities, and neuronal apoptosis were detected in diabetic patients before capillary closure and microaneurysms, suggesting that DR could have a neuropathic component that may occur early in the disease. These observations have been confirmed by animal studies showing that neuronal apoptosis and glial reaction could occur after only 1 month of experimental diabetes in streptozotocin induced diabetic rats. The mechanisms by which diabetes induces neuroglial reactions remain unknown. These neuroglial reactions are unlikely to be the result of ischaemic necrosis since they occur very early in diabetic rats before the onset of any known vascular occlusion. However, neuronal cell death due to increased vascular permeability cannot be ruled out, since some increase in permeability can occur after 2–4 weeks of experimental diabetes in rats. The advanced glycated end products (AGE) can provide the early molecular pathogenic mechanisms responsible for neuronal apoptosis and neuroglial reactions. Their involvement in the development of DR is supported by the prevention of DR by blockers of AGE formation in animal models. The potential retinal toxicity of AGE was consistent with apoptosis of cultured cortical neurons and with their accumulation in the retina of diabetic patients. The present study was performed to assess whether AGE can directly trigger neuronal apoptosis in the retina, independently of blood vessel circulation. AGE were applied to retinal explants, as they both preserve the retinal cellular architecture and limit the influence of the blood vasculature.

MATERIALS AND METHODS

Male non-diabetic Long Evans rats (Charles River Laboratories, France) aged 7 weeks were used for this study. Animal studies conformed to the principles of laboratory animals (NIH publication no 85–23, revised 1985), and the French law on animal protection.

Retinal explants were obtained according to the protocol described by Pinzon-Duarte et al. The choroid was gently peeled away from the retinal pigment epithelium (RPE), leaving the RPE attached to the neurosensory retina. The retina was transferred onto a polycarbonate membrane (Transwell, Corning, Netherlands) and cultured in Dulbecco’s Modified Eagle Medium containing 10% fetal calf serum and 10 μg/ml gentamicin. For each animal, one retina was used as a control retinal explant and the other retina was incubated in 200 μg/ml glycated BSA (Sigma Chemical Co, St Louis, MO, USA, 95% purity) for 4 days, a period providing the lowest variability when inducing apoptosis in such ex vivo retina. This glycated BSA concentration was selected because similar concentrations (250 μg/ml; 100 μg/ml) were used previously to demonstrate AGE neurotoxicity and AGE role in increased retinal expression of vascular endothelium growth factor (VEGF). Furthermore, circulating AGE ranged in diabetic patients between 1–120 μg/ml, which was considered equivalent to 4–480 μg/ml glycated BSA by the authors of the study. No albumin was added to the control condition because the culture medium already contains albumin (2700 μg/ml) from the fetal calf serum such that the addition of glycated BSA represented less than 8% increase of its concentration.

The retinal tissue was fixed and processed for immunohistochemistry and terminal dUTP nick end labelling-fluorescein-isothiocyanate (TUNEL) labelling, as previously described. Retinal explant sections were labelled with anti-cleaved active caspase-3 antibody (Cell Signalling Technology, Herts, UK, 1:100), anti-vimentin antibody (Chemicon International, Temecula, CA, USA, 1:200).

Abbreviations: AGE, advanced glycation end products; BSA, bovine serum albumin; DAPI, diaminobenzidine; DR, diabetic retinopathy; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; GLAST, L-glutamate/aspartate transporter antibody; INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RAGE, AGE receptor; RPE, retinal pigment epithelium; TUNEL, terminal dUTP nick end labelling; VEGF, vascular endothelium growth factor.
anti-glial fibrillary acidic protein (GFAP) antibody (Dako, Trappes, France, 1:50), anti-L-glutamate/L-aspartate transporter antibody (GLAST) (Chemicon, 1:200), and anti-glutamine synthetase antibody (Chemicon, 1:400). Nuclear labelling was achieved by incubating the sections in diamidino-phenyl-indole (DAPI) solution (Sigma, solution stock: 500 ng/ml concentration, 1:400).

Cells were counted under the 40× objective on the microscope (Olympus, Melville, NY, USA) in five visual fields (250 μm long) from the retinal explant section. The number of cells positive for the TUNEL staining or the cleaved caspase-3 immunolabelling was normalised to the DAPI labelled retinal cell nuclei. Data were statistically analysed with the Mann-Whitney rank sum test.

RESULTS

AGE induced retinal cell apoptosis

The treatment with glycated BSA for 4 days induced a 2.1-fold increase in the number of TUNEL positive cells (glycated BSA: 14.44% (SD0.62%), n = 7; control: 6.63% (0.46%), n = 7, p <0.001), which was statistically significant in all nuclear retinal layers (fig 1A, B, E). Small groups of adjacent TUNEL positive cells were furthermore observed in the inner part of the outer nuclear layer (ONL) in four retinal explants treated with glycated BSA (n = 7) (fig 1B), whereas these characteristics were not observed in control retinal explants (fig 1A).

To confirm the retinal toxicity elicited by glycated BSA, retinal explant sections were labelled with an earlier marker of apoptosis, the anti-cleaved active caspase-3 antibody (fig 1C, D). The treatment with glycated BSA induced a 2.4-fold increase in active caspase-3 positive cells (glycated BSA: 8.59% (0.39%), n = 7; control: 3.48% (0.44%), n = 7, p <0.001), statistically significant in all nuclear retinal layers (fig 1F). These data suggest that a glycated protein can induce neuronal apoptosis in the retina as early as 4 days of incubation.

AGE induced glial reaction

Neuronal apoptosis is classically associated to a glial reaction which is demonstrated by a local increase in GFAP expression in the retina at the site of the lesion.13 In control retinal explants, GFAP was restricted to the innermost retinal layer, anti-glial fibrillary acidic protein (GFAP) antibody (Dako, Trappes, France, 1:50), anti-L-glutamate/L-aspartate transporter antibody (GLAST) (Chemicon, 1:200), and anti-glutamine synthetase antibody (Chemicon, 1:400). Nuclear labelling was achieved by incubating the sections in diamidino-phenyl-indole (DAPI) solution (Sigma, solution stock: 500 ng/ml concentration, 1:400).

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where astrocytes are located, and to the outer plexiform layer (OPL). Limited GFAP positive Müller cell processes were occasionally observed (fig 2A, C). In contrast, in retinal explants treated with glycated BSA, all Müller cell processes were extensively GFAP positive from their end tip at the inner limiting membrane to their opposite end at the outer limiting membrane. The GFAP staining was not locally restricted but extended throughout the entire retinal explant sections (fig 2B, D). No change in GLAST and glutamine synthetase was detected following the AGE treatment (data not shown). The increase in GFAP expression showed that a treatment with a glycated protein can induce a glial reaction.

**DISCUSSION**

Electrophysiological measurements in diabetic patients and animal models generally have located the earlier deficits to the inner retina but some series have indicated visual dysfunction in the outer retina and RPE. Histologically, although neuronal apoptosis has been prominent in the 4 day glycated BSA incubation with a maximum of the effect of glycated BSA on these cells. The presence of a large mass of TUNEL positive materials may further indicate that the Mueller cell change could result from a direct exposure after 4 days of incubation in glycated BSA. This observations were consistent with those obtained following incubation of retinal cultures with the AGE precursor glyoxal. However, apoptotic cells accounted for only 14% in the 4 day glycated BSA incubation with a maximum of 20% in the GCL, whereas Reber et al reported up to 50% retinal cell death after only 9 hour glyoxal incubation with a similar higher sensitivity of the GCL. The difference may be the result of the different mechanisms involved, as glyoxal cannot only generate AGE but also reactive species, whereas in our experiment, cell toxicity can only result from the incubated glycated BSA. Our results indicated further that retinal cell death can occur by activation of caspase-3, although they do not exclude the contribution of other death pathways like caspase-independent mechanisms.

In diabetic patients and animal models, Müller glial cells are also affected as indicated by their increased GFAP expression. Under our experimental conditions, Müller glial cells also exhibited a major upregulation of GFAP expression after 4 days of incubation in glycated BSA. This Müller cell change is likely to result from the neuronal apoptosis induced by glycated BSA. However, we cannot exclude that the Müller cell change could result from a direct effect of glycated BSA on these cells. The presence of a large mass of TUNEL positive materials may further indicate that glial cells have impaired phagocytic abilities, as previously reported for resident peritoneal macrophages in streptozotocin induced diabetic animals or in vitro following AGE incubation.

Neuroglial alterations are early events in the development of DR. Our study using retinal explants suggest that neuroglial lesions could result from AGE diffusion into the retinal tissue independently of major vascular perturbations like occlusions. Our study further underlines that AGE do not induce neuroglial reactions 16-33 in diabetic retinopathy. The molecular pathways of AGE induced neuroglial reactions are not known but could be related to AGE receptors like RAGE receptors, that are located in the inner retina in humans and rats19 or the galectin-3 receptor that is located in Müller cells in rats. Further studies will investigate the molecular mechanisms of AGE induced neural apoptosis in the retina.

**REFERENCES**

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