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<td>掲載誌・巻号・ページ (Citation)</td>
<td>Experimental eye research, 90(1):17-25</td>
</tr>
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<td>刊行日 (Issue date)</td>
<td>2010-01</td>
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<tr>
<td>資源タイプ (Resource Type)</td>
<td>Journal Article / 学術雑誌論文</td>
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<td>版区分 (Resource Version)</td>
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<td>権利 (Rights)</td>
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<td>DOI</td>
<td>10.1016/j.exer.2009.09.003</td>
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PDF issue: 2018-11-23
Title page

Full title: Altered expression of aquaporins 1 and 4 is temporally synchronized with neurodegenerative events in retinas of spontaneously diabetic Torii rats.

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Abstract

Evidence is mounting that not only microangiopathy, but also neurodegenerative events occur in the retinas of humans and rodents with early diabetes. Diverse pathologies are known to alter the amount and/or location of glial expression of the water-selective channels aquaporins (AQPs) 1 and 4. However, the temporal relationships among glial activation, the altered expression of the AQP proteins and neuronal death in the retinas of diabetic animals remains to be investigated. Male spontaneously diabetic Torii (SDT) rats reportedly develop diabetes by 40 weeks of age at the latest and manifest proliferative diabetic retinopathy at 50 weeks or later. This study compared temporal changes in neuroretinal apoptosis, glial fibrillary acidic protein (GFAP) expression and the expression of AQPs 1 and 4 between SDT rat retinas and age-matched Sprague-Dawley (SD) rat retinas. Cell death was detected by terminal deoxynucleotidyl transferase-mediated deoxy-uridine triphosphate nick end-labeling on retinal flatmounts and activated caspase 3 immunofluorescence of retinal cryosections. The expression of GFAP and AQPs 1 and 4 were assessed by immunohistochemistry of cryosections and retinal flatmounts. Diabetes started to develop around 15 weeks in SDT rats. Apoptotic cells in the ganglion cell layer and the inner nuclear layer were significantly more numerous in 40-week-old SDT rat retinas than in either age-matched SD rat retinas or 10-week-old SDT rats. GFAP immunoreactivity was confined to the nerve fiber layer both in SD and SDT rats at 10 weeks, whereas it spanned the whole retina in SDT rats, but not in SD rats, at 40 weeks. AQP1 was expressed in the outer retina, whereas AQP4 was expressed in the
perivascular and end feet of the Müller cells and astrocytes in the inner retina in the control SD rats and the SDT rats at 10 weeks. The perivascular AQPs shifted from AQP4 to AQP1 in 40-week-old SDT rats that exhibited marked hyperglycemia. Thus, the development of diabetes increases neuroretinal apoptosis, and this is temporally synchronized with an altered expression pattern of GFAP and water selective channels AQP1 and 4 in SDT rats.

**Keywords**: spontaneously diabetic Torii rat, diabetic retinopathy, neuronal apoptosis, retinal neurodegeneration, aquaporin, water channel, glial fibrillary acidic protein.
Introduction

Diabetic retinopathy (DR) is one of the most serious complications of diabetes mellitus and is the leading causes of postnatal blindness in developed countries (Congdon et al., 2004). Evidence is accumulating that DR comprises not only microangiopathy, which is characterized by blood-retinal barrier breakdown, capillary obliteration and subsequent neovascularization, but also glial dysfunction and neuronal death in the retina. This includes altered expression of glial fibrillary acidic protein (GFAP) in Müller cells and astrocytes (Barber et al., 2000), impaired glutamate metabolism (Kowluru et al., 2001; Li and Puro, 2002), and accelerated neuronal apoptosis (Barber et al, 1998; Nakanishi et al., 2006). However, diabetes-induced phenotypic changes in neuroglial elements still remain to be investigated.

Aquaporins (AQPs) are a family of integral membrane proteins that allow water to cross the plasma membrane (Agre and Kozono, 2003) and they are critically involved in the maintenance of ionic and osmotic balance in the central nervous system (CNS) in response to osmotic gradients and differences in hydrostatic pressure (Tait et al., 2008). Among the 13 isoforms of the AQP protein family identified so far, at least four AQPs are found to be expressed in the neural retina, AQP0, AQP1, AQP4 and AQP9. AQP0 is expressed in subpopulations of bipolar cells, amacrine cells and retinal ganglion cells (RGCs) (Inadiev et al. 2007a). AQP1 is normally expressed in the outer retina in photoreceptors and in distinct amacrine cells (Kim et al., 1998; Nagelhus et al., 1998; Iandiev et al., 2005), while AQP4 is expressed predominantly in the perivascular and vitreal end feet of Müller cells and in astrocytes in the inner retina (Nagelhus et al., 1998; Verkman et al., 2008; Goodyear et al., 2009). AQP9 is expressed in
catecholaminergic amacrine cells (Iandiev et al., 2006a) and RGCs (Dibas et al., 2007). Among these, AQPs 1 and 4 received much attention, since it has been demonstrated that in the CNS, glial AQPs not only facilitate water flux into and out of the brain parenchyma (Manley et al., 2000) but also modulate neuronal excitability and enhance astrocyte migration (Kong et al., 2008; Saadoun et al., 2005a, 2005b; Tait et al., 2008). In addition, the amount and/or location of glial expression of AQPs 1 and 4 in the retinas of a variety of animal disease models were found to be altered, which include ischemia/reperfusion (Iandiev et al., 2006b) and streptozotocin (STZ)-induced diabetes (Iandiev et al., 2007b).

With regard to diabetes, a microarray study demonstrated that AQP1 gene expression is upregulated in the retinas of diabetic rats (Gerhardinger et al., 2005). Iandiev et al. (2007b) showed that AQP1 immunoreactivity was enhanced in glial cells located in the innermost retinal layers and those surrounding the superficial vessels in STZ-induced diabetic rats. Perivascular AQP4 expression in the superficial vessel plexus was reportedly reduced (Iandiev et al., 2007b) but unaltered in the inner nuclear layer (INL) (Pannicke et al., 2006; Iandiev et al., 2007b). Upregulation of AQP1 has also been described in brain astrocytes after acute brain injury and in peri-tumoral astrocytes (Kaur et al., 2006; Tait et al., 2008). Thus, glial cell-mediated water transport, which is critical for maintaining neuronal activity, may be either compromised by diabetes-induced metabolic stress or altered as a compensatory mechanism, especially in the inner retina where RGCs reside. However, the temporal relationships among glial activation, the altered expression of AQP proteins and neuronal death in the retinas of diabetic animals, especially of those that spontaneously develop diabetes, have not been evaluated.
The purpose of this study was to examine time-dependent changes in cellular apoptosis in the inner layer, as well as GFAP expression and the localization of AQPs 1 and 4 in the retinas of both spontaneously diabetic Torii (SDT) rats and those of age-matched Sprague-Dawley (SD) rats. The SDT rat is a novel substrain of the SD rat that spontaneously develops diabetes (Shinohara, et al, 2000). Male SDT rats exhibit hypoinsulinemia after 10 weeks and marked hyperglycemia and glucosuria by 20 weeks of age, and the cumulative incidence of diabetes is almost 100% by 40 weeks of age (Shinohara, et al, 2000; Masuyama, et al, 2004). In addition, some SDT rats reportedly develop severe ocular complications at 50 weeks of age or later that resemble several features of proliferative DR in humans, including retinal neovascularization, tractional retinal detachment with fibrous proliferation and massive hemorrhages in the anterior chamber (Yamada, et al, 2005; Kakehashi, et al, 2006) as well as retinal leukostasis (Matsuoka et al., 2007).

This study demonstrates that the accelerated apoptosis in the inner retina is temporally synchronized with an altered expression pattern of GFAP and AQPs 1 and 4 at relatively early time points in SDT rats.

Materials and methods

Animals and confirmation of diabetes mellitus

Male SDT rats (n=33) and normal SD rats (n=24) between eight and ten weeks of age were purchased from CLEA Japan, Inc. (Tokyo, Japan). They were housed in the Kobe University animal facility under standard conditions of room temperature (24±2°C) a 12:12 h light-dark cycle with ad libitum access to food and tap water. The following experiments were approved by the Animal Care Committee of Kobe University
Graduate School of Medicine and followed the Association of Research in Vision and Ophthalmology Resolution on Care and Use of Laboratory Animals.

Blood glucose was assessed by a Lifescan meter® (LifeScan Inc., Drive Milpitas, CA), urinary glucose by Diasticks® (Bayer Medical Co., Ltd., Tokyo, Japan) and glycosylated hemoglobin (Hb) A1c by DCA2000 (Bayer Medical Co., Ltd., Tokyo, Japan). Body weight, blood glucose and urinary glucose were measured weekly and at sacrifice (10, 20, or 40 weeks of age). HbA1c was measured at 10, 20, and/or 40 weeks of age. The rats were confirmed as diabetic if their blood glucose level was above 13.89 mM or urinary glucose was positive.

**Retinal preparation and terminal deoxynucleotidyl transferase-mediated deoxy-uridine triphosphate nick end-labeling (TUNEL) staining**

Retinas were dissected, flat-mounted and subjected to TUNEL staining using the ApopTag Peroxidase In Situ Apoptosis Detection kit (Chemicon Inc. Temecula, CA) as previously described (Barber et al., 1998; Nakanishi et al., 2006). Six eyes each of 10-, 20-, and 40-week-old SDT rats as well as six eyes of 40-week-old SD rats were used for TUNEL staining. We omitted 10- and 20-week-old SD rats from the TUNEL assay because previous studies by us (Nakanishi et al., 2006) and others (Barber et al., 1998) already demonstrated only a few TUNEL-positive cells in the retinas of normal SD rats at these ages, and we wished to reduce the number of rats for sacrifice.

To detect TUNEL-positive cells, each retina was visually scanned with a high power (40×) objective under microscope in a masked fashion. The entire retinal area was measured by tracing the outline of retinal images using an image analysis system (Micro Analyzer®, Japan Poladigital, Tokyo, Japan). The total number of TUNEL-positive cells
was counted for each flat-mounted retina and was expressed as the number per unit area of 0.5 cm² (Barber et al., 1998; Nakanishi et al., 2006).

**Immunofluorescence in retinal cryosections and whole mount retinas**

Immunofluorescence studies for retinal cryosections and whole mount retinas were performed as previously described (Kanamori et al., 2005; Nakanishi et al., 2006).

In brief, eyes were enucleated for cryosections, embedded in 20% sucrose optimal cutting temperature compound (Sakura Finetek USA, Torrance, CA), snap-frozen and stored at -80 °C until use. Retinal cryosections 5 µm in thickness were collected on silane-coated microscope slides. After fixing in 2% paraformaldehyde and blocking with 10% goat serum in PBS, the sections were incubated overnight at 4 °C with primary antibody. Following washing, the sections were incubated with tetramethyl rhodamine isothiocyanate-conjugated AffiniPure F(ab')2 fragment goat anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) or fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:200, Jackson ImmunoResearch) together with nuclear counterstaining with bisbenzamide (Hoechst 33258: Sigma Aldrich Japan, Tokyo, Japan) at room temperature for 1 h. After extensive washes, the sections were coverslipped with Permafluor aqueous mounting medium (Lab Vision Corporation, Fremont, CA).

For whole-mounted retinal immunolabeling, dissected retinas were fixed for 10 min in 2% paraformaldehyde, blocked in 10% goat serum in PBS with 0.1% Triton for 2 h and transferred to primary antibodies in block solution for three days at 4 °C. After extensive washing in PBS with 0.3% Triton, the retinas were transferred to the
appropriate secondary antibody for 24 h at 4°C. The tissues were mounted on microscope slides with the mounting medium and air dried.

Immunostaining with exclusion of the primary antibody was also conducted as a negative control.

The following primary antibodies were used: rabbit anti-cleaved caspase 3 (1:800, Cell Signaling Technology, Beverly, MA), mouse anti-GFAP (1:200 for cryosections and 1:50 for whole mounts, Millipore, Billerica, MA), rabbit anti-AQP1 (1:200; Millipore) and rabbit anti AQP4 (1:200, Santa Cruz Biotechnology, CA).

For caspase cryosection immunostaining, five eyes each from 10-, 20-, and 40-week-old SDT rats as well as five eyes from 40-week-old SD rats were used. We omitted 10- and 20-week-old SD rats for the same reason as for the TUNEL assay described above. For GFAP and AQPs 1 and 4 double immunolabeling, three eyes each of 10-, 20-, and 40-week-old SD and SDT rats were used.

**Image analysis**

Retinal images were viewed and captured using a high power (40×) objective of a fluorescence microscope with a built-in digital camera (Biozero BZ-8000; Keyence, Osaka Japan) or a confocal laser scanning microscope (LSM 510 Meta, Carl-Zeiss, Oberkochen, Germany).

To determine the cleaved caspase-3 immunopositive cell ratio, ten serial sections 50 µm apart centered at the plane through the optic nerve were selected. The ratio of cleaved caspase 3-positive cells to total cells stained with Hoechst dye was counted for each section, and the average was defined as the ratio of cleaved caspase 3-positive cells for the specific retina. The mean of the five retinas was compared between each group.
Statistical Analyses

Data are expressed as means ± standard deviation (SD). Statistical analyses were performed by Stat View version 5.0 software (SAS Institute, Cary, NC). Body weight, blood glucose and HbA1c levels were compared between SD and SDT rats of the same age with the Mann-Whitney nonparametric test. Time courses of changes in the number of TUNEL-positive cells and the ratio of cleaved caspase 3-positive cells were analyzed using non-repeated measures analysis of variance (ANOVA) with the Bonferroni test. All statistical values were judged significant if the $p$ value was less than 0.05.

Results

Rat metabolic conditions

The mean body weight of the SD rats was 397.0 ± 32.6 g at 10 weeks, 509.7 ± 10.8 g at 20 weeks and 647.5 ± 16.4 g at 40 weeks, whereas that of the SDT rats was 414.7 ± 17.7 g, 475.0 ± 50.5 g, and 413.2 ± 34.7 g at the corresponding ages. There was a statistically significant difference between the mean body weight of the SD rats and that of the SDT rats at 40 weeks of age (P<0.0001).

The mean blood glucose of the SD rats was 7.58 ± 2.54 mM at 10 weeks, 7.72 ± 1.62 mM at 20 weeks and 6.97 ± 1.24 mM at 40 weeks, whereas that of the SDT rats was 7.21 ± 1.88 mM at 10 weeks and 15.53 ± 3.99 mM at 20 weeks. All 40-week-old SDT rats exhibited marked hyperglycemia that was beyond the scale of the measurement (>27.78 mM). There was a significant difference in mean blood glucose between the SD and SDT rats at 20 weeks (P=0.0004).
The mean HbA1c of the SD rats was 2.6±0.3% at 10 weeks, 2.8±0.2% at 20 weeks and 2.6±0.3% at 40 weeks, whereas that of the SDT rats at the corresponding ages was 2.8±0.2%, 8.8±5.2% and 10.8±2.8%. The difference was significant at 20 and 40 weeks of age (P=0.014 and P<0.0001, respectively).

All but one of the 11 SDT rats that were sacrificed at 10 weeks exhibited normoglycemia and normal urine contents at every examination. Six of eleven SDT rats that were sacrificed at 20 weeks showed hyperglycemia at death. The time at onset of diabetes was 12 weeks in one, 13 weeks in one, 14 weeks in one and 19 weeks in three. These six rats were subjected to the TUNEL assay. All 11 SDT rats that were sacrificed at 40 weeks became diabetic between 17 and 23 weeks.

Accelerated cellular apoptosis in the inner layers in the 40-week-old SDT rat retinas

Earlier studies provided evidence that apoptosis of retinal neurons and glial cells is enhanced in chemically induced diabetic animals (Barber et al., 1998; Nakanishi et al., 2006). In order to test whether cellular apoptosis in the inner retinal layers increased along with the onset of diabetes in SDT rats, TUNEL and cleaved caspase 3 immunolabeling analyses were conducted.

The number of TUNEL-positive cells, which were located away from vasculature, per 0.5 cm² retina was 7.7 ± 1.9, 9.3 ± 3.2 and 34.7 ± 8.4 in SDT rats at 10, 20, and 40 weeks of age, respectively, whereas it was 6.0 ± 2.7 in SD rats at 40 weeks of age. The number of TUNEL-positive cells in the 40-week-old SDT rat retinas was significantly greater than that in the 10- and 20-week-old SDT rat retinas and the age-matched SD rat retinas (Fig. 1; p<0.0001, non-repeated measures ANOVA with Bonferroni test).
Caspase 3 activation was also enhanced in cells residing in the inner retina of the SDT rats at 40 weeks of age (Fig. 2). The ratio of cleaved caspase 3 immunoreactive cells relative to the total number of cells stained with Hoechst dye in the ganglion cell layer (GCL) and in the INL was 8.7 ± 2.3%, 11.2 ± 4.5%, 23.7 ± 6.3% and 7.5 ± 2.0% in the 10-, 20-, and 40-week-old SDT rats and the 40-week-old SD rats, respectively. The retinas of SDT rats at 40 weeks showed approximately 2.5-fold more caspase 3 activation than those of SDT rats at 10 weeks (P<0.0001) and 20 weeks (P=0.0002), as well as compared with those of SD rats at 40 weeks (P<0.0001).

These findings indicate that neuronal and glial cell apoptosis were enhanced in the inner retinas of SDT rats at 40 weeks.

**Altered pattern of immunoreactivity of AQPs 1 and 4 in retinas of SDT rats at 40 weeks.**

Double immunolabeling for AQP1 and GFAP in retinal cryosections revealed that in both SD and SDT rats at 10 weeks of age, AQP1 immunoreactivity was observed preferentially in the outer retinal parenchyma between the outer plexiform layer (OPL) and the photoreceptor layer (PRS) in addition to the punctate AQP1 immunoreactivity within vessels that represents red blood cell immunoreactivity for APQ1 as previously reported (Fig. 3) (Kim et al., 1998; Nagelhus et al., 1998; Iandiev et al., 2005). Scattered immunoreactivity for AQP1 was also faintly detected around the superficial vessels and at the inner limiting membrane (ILM) (Fig. 3). Sharp GFAP immunoreactivity was confined to the innermost retinal layer where astrocytes reside (Fig. 3), which is also compatible with prior observations (Barber et al., 2000). These immunolabeling patterns for AQP1 and GFAP were not altered in the retinas of SD rats at 40 weeks of age (Fig.
3). In contrast, the retinas of SDT rats at 40 weeks of age obtained additional immunoreactivity for AQP1 strongly around the superficial vessels and at the ILM and faintly in the inner plexiform layer (IPL; Fig. 3). In addition, GFAP immunoreactivity spanned the entire retina from the NFL through the outer nuclear layer (ONL) to the PRS as in a variety of retinal injuries previously reported (Fig. 3) (Barber et al., 2000; Kanamori et al., 2005). The merged figure shows the co-localized expression of AQP1 and GFAP around the superficial vessels and at the IPL of the SDT rats at 40 weeks of age (Fig. 3).

Whole mount retinal immunolabeling confirmed this co-localization of AQP1 and GFAP expression in the 40-week-old SDT rat retinas (Fig. 4). At 10 weeks of age, both SD and SDT rat retinas showed only scattered, punctate pattern of AQP1 immunoreactivity of red blood cells in the nerve fiber layer (NFL) (Fig. 4). In these retinas, a tentacle-like stellate pattern of GFAP immunoreactivity wrapped around the superficial vessels, which represents the astrocyte immunoreactivity for GFAP (Barber et al., 2000; Kanamori et al., 2005). These immunoreactivity patterns were not altered in the retinas of SD rats at 40 weeks of age (Fig. 4). In contrast, the retinas of SDT rats at 40 weeks of age displayed additional AQP1 immunoreactivity around the superficial vessels as well as faintly at the astrocytic processes, which was co-localized with GFAP immunoreactivity (Fig. 4). The GFAP-immunoreactivity itself was partially disrupted and weaker compared to the SDT rat retinas at 10 weeks or the SD rat retinas at 40 weeks (Fig. 4). This disorganized pattern of GFAP immunoreactivity at the superficial retinal layer is comparable with changes in retinas under a variety of stresses as in previous studies (Barber et al., 2000; Kanamori et al., 2005). In a deeper plane of focus within the INL, a punctate pattern of GFAP immunoreactivity was observed that
represents the Müller cell’s gain of GFAP immunoreactivity (Fig. 4) (Barber et al., 2000; Kanamori et al., 2005). In addition, AQP1 immunoreactivity was also enhanced in glial cells adjacent to the vessels in this layer (Fig. 4).

Double immunolabeling for AQP4 and GFAP in retinal cryosections revealed that AQP4 immunoreactivity was essentially located in the OPL and the ILM as well as around the vessels residing in the inner retina both in SD and SDT rats irrespective of age as in previous reports using normal rodent retinas (Fig. 5) (Nagelhus et al., 1998; Verkman et al., 2008; Goodyear et al., 2009). Observable changes in retinal AQP4 expression were a moderate reduction in immunoreactivity around the superficial vessels and the ILM in the SDT rats at 40 weeks of age (Fig. 5). The altered expression of GFAP in the 40-week-old SDT rat retinas was consistent with the findings in the sections for AQP1 and GFAP double immunostaining as mentioned above.

No consistent alterations of AQP1 and 4 and GFAP expression were detected in the SDT rats at 20 weeks of age (data not shown).

**Discussion**

The present study confirmed previous observations that male SDT rats started to exhibit hyperglycemia at 20 weeks of age, and all became diabetic by 40 weeks (Shinohara et al., 2000; Masuyama et al., 2004). Along with the development of diabetes, cellular apoptosis of the inner retinal parenchyma was enhanced in SDT rats. In parallel with this change, the expression pattern of the glial stress marker GFAP and the selective water channels AQPs 1 and 4 was altered. In control SD rats at all ages tested and SDT rats at 10 and 20 weeks of age, AQP1 was expressed in the outer retina, particularly in the PRS, whereas AQP4 was expressed predominantly around the
superficial vessels and at the ILM. In a clear contrast, the SDT rats at 40 weeks of age exhibited additional AQP1 immunoreactivity at the ILM and around the vessels in the inner retina, whereas the perivascular AQP4 immunoreactivity was reduced.

Growing evidence shows that neural apoptosis in the retinal GCL and INL is elevated in diabetics (Barber et al., 1998; Gastinger et al., 2006; Nakanishi et al., 2006). RGC apoptosis reportedly took place as early as 15 days after the onset of diabetes in mice that received STZ injections (Martin et al., 2004), at 1 month in STZ-induced diabetic rats (Barber et al., 1998; Nakanishi et al., 2006) and in several spontaneous diabetic mice (Barber et al., 2005; Cheung et al., 2005; Ning et al.; 2005). The expression of pro-apoptotic proteins such as BAX and caspase was also upregulated in retinas of diabetic animals (Feit-Leichman et al., 2005; Kowluru, 2005). A previous report using human donor eyes disclosed that RGCs in diabetic retinas expressed caspase 3, Fas and BAX, whereas Müller cells amplified the pro-survival proteins Bcl-2 and p44/p42 mitogen-activated protein kinase in addition to the pro-apoptotic FasL (Abu-El-Asrar et al., 2004). The present findings of more TUNEL-positive and activated caspase 3 immunoreactive cells in the inner retinas of SDT rats at 40 weeks of age (with the former being less frequent than the latter) are consistent with these earlier works (Gastinger et al., 2006; Nakanishi et al., 2006). The possible reasons for more caspase 3 immunoreactive cells than TUNEL-positive cells include that extensive fixation with alcohol and xylene may destroy protein antigenicity and that cells with activated caspase 3 may not always undergo DNA fragmentation, which is the final apoptotic process (Gastinger et al, 2006).

By contrast, two papers demonstrated an absence of apoptotic neuronal death in the retinas of alloxan- or STZ-induced diabetic mice (Asnaghi et al., 2003; Gaucher et al.,
2007). This discrepancy may be due to the duration of the diabetes, since Martin et al (2004) found neuroretinal apoptosis at 2, 6 and 12 weeks after diabetes induction, while Asnaghi et al (2003) failed to find it at 10 and 24 weeks. Alternatively, the use of insulin may have affected the results, because the diabetic mice without retinal neural apoptosis received regular insulin injections (Asnaghi et al., 2003; Gaucher et al., 2007) and both the mice and the rats with RGC apoptosis did not (Martin et al., 2004; Cheung et al., 2005). Insulin may reduce retinal neuronal apoptosis not only indirectly via glycemic control but also via its direct neuroprotective ability (Nakamura et al., 2001), since Asnaghi et al (2003) demonstrated insulin’s protection of RGCs in STZ diabetic rats even with incomplete glycemic control.

We could not identify the cell type that underwent apoptosis. Although apoptotic cells were localized away from vasculature and confined to the GCL and INL, these included both neuronal and glial cells. As for neuronal cells, RGCs and amacrine cells, the latter of which reside both in the INL (conventional amacrine cells) and the GCL (displaced amacrine cells), are known to be apoptotic in diabetic rodent retinas (Gastinger et al., 2006). However, it is often difficult to identify the apoptotic retinal neuronal cells by immunohistochemical co-localization with a marker protein because most proteins, including cellular makers, are cleaved and degraded by the time caspase 3 is activated, as pointed out by Gastinger et al (2006). They demonstrated that, at most, 8% and 6% of active caspase 3-positive cells were co-immunostained with NeuN (a neuronal marker) and tyrosine hydroxylase (an amacrine cell marker), respectively, in STZ-induced diabetic rat retinas (Gastinger et al., 2006). Retrograde labeling can also be used to analyze RGC survival in various types of acute injury that cause substantial retinal neurodegeneration (Kanamori et al., 2009). However, the neuronal loss in diabetic
retinas is too slow and limited to be reliably counted with this method.

The AQPs are integral membrane proteins whose main function is to transport water across cell membranes in response to osmotic gradients (Agre and Kozono, 2003; Verkman et al., 2008; Tait et al., 2008; Goodyear et al., 2009). Analysis of knockout mice lacking individual AQPs suggests their involvement in retinal signal transduction and retinal swelling following injury (Da and Verkman, 2004; Li et al., 2002; Yuan et al., 2009). The present findings of polarized expression of AQPs 1 and 4 in the control rat retinas are consistent with previous investigations (Kim et al., 1998; Nagelhus et al., 1998; Iandiev et al., 2005; Verkman et al., 2008; Goodyear et al., 2009). Moreover, along with the development of diabetes and increased inner retinal apoptosis, re-distribution of the expression of AQPs 1 and 4 occurred in the SDT rat retinas, consistent with a previous observation by Iandiev et al. (2007b) using STZ-induced diabetic rat models; specifically, the expressional shift from AQP4 to AQP1 in glial cells adjacent to the superficial vessels. The perivascular AQP4 to AQP1 shift and the perivascular gain of AQP1 expression in retinas were also observed in rodent models of ischemia/reperfusion injury (Iandiev et al., 2006b) and high salt loading (Qin et al., 2009). However, the glial gain of immunoreactivity for AQP1 was limited around the superficial vessels in these models, while the perivascular expression of AQP1 at the INL was also enhanced in the SDT rats at 40 weeks of age (Fig. 4). SDT rats may present more radical glial reactivity after the development of diabetes, which may be involved in the anginogenesis that reportedly occurs in SDT rats at the later time course. In addition, the upregulation of AQP1 expression at the INL and IPL in the 40-week-old SDT rat retinas may corroborate with a previous microarray experiment by Gerhardinger et al. (2005), in which AQP1 gene expression in isolated Müller cells
taken from STZ-induced diabetic rats was elevated 2.4-fold compared to control rats. Taken together, glial cell-mediated water transport in the retina may be altered with the development of diabetes in SDT rats as it is in STZ-induced diabetic rats (Iandiev et al., 2007) and in rats with high salt loading (Qin et al., 2009).

The expressional shift from AQP4 to AQP1 and the reduced expression in the AQP4 protein at the superficial vessels may occur in a pathology- or stress-type dependent fashion, since a variety of pathological conditions have been demonstrated to cause distinct patterns of AQP4 expression. For example, perivascular AQP4 expression was increased in the chick model of form-deprivation myopia (Goodyear et al., 2008). AQP4 gene expression was upregulated with combustion smoke exposure (Zou et al., 2009). However, elevated intraocular pressure and intravitreal injection of endothelin-1 reduced both mRNA and protein levels of retinal AQP4, possibly because of the enhanced ubiquitin-dependent proteasome degradation of AQP4 (Dibas et al., 2008). In a rat model of branch retinal vein occlusion, expression of the AQP4 gene was reduced but no overall mislocation of the protein was detected (Rehak et al., 2009).

The reason why AQP4 expression and localization varies among pathologies is unknown mainly because the function of any AQP proteins in the neural retina is not yet fully understood. Previous observations of co-localized expression of AQP4 and a distinct potassium channel named Kir4.1 in Müller cells initially led to the idea that AQP4 may play a key role in K⁺ buffering during light-neural signal transduction (Iandiev et al., 2006b, 2007b). Iandiev et al. (2006b, 2007b) speculated that an uncoupling of AQP4-mediated water transport from K⁺ currents may lead to an alteration of the water flux across the membranes of Müller cells and deep retinal vessels and result in water accumulation in the cytoplasm. However, recent patch-clamp
studies using AQP4 knockout mice provided evidence against a functional interaction between AQP4 and Kir4.1 (Ruiz-Ederra et al., 2007; Zhang et al., 2008). Ruiz-Ederra et al. (2007) detected no changes in Kir4.1 expression in AQP4 knockout mice, nor did they find any significant differences in K⁺ current magnitude or current-voltage relationships between wild-type and AQP4 knockout mice in isolated Müller cells. It may be of interest to note that microangiopathy resembling human proliferative DR is known to occur only after 55 weeks of age in SDT rats (Yamada et al., 2005; Kakehashi et al., 2006). No fluorescein leakage was detected until this time point, which suggests that the expressional alteration in AQP1 and 4 does not directly link to retinal edema development in this spontaneously diabetic model. This may be consistent with a recent observation by Saadoun et al. (2009) of no alteration of blood brain barrier integrity or brain morphology in AQP4 knockout mice.

On the other hand, recent studies provided evidence that AQPs are responsible for cellular migration and angiogenesis (Saadoun et al., 2005a, 2005b; Tait et al., 2008). Saadoun et al. (2005b) showed that targeted AQP1 gene disruption in mice reduces angiogenesis in vivo. Cell migration involves the transient formation of membrane protrusions termed lamellipodia and cell membrane ruffles at the leading edge of the cell (Saadoun et al., 2005a, 2005b; Tait et al., 2008). These require rapid local changes in ion flux and cell volume, probably accompanied by rapid transmembrane water flux. AQPs may play a critical role in water entry into lamellipodia. Since some SDT rats develop retinal and optic disc neovascularization at 55 weeks or later as mentioned above, the preceding upregulation of AQP1 at the ILM might be involved in the subsequent neovascularization.

The association of elevated neuroretinal cellular apoptosis and the expressional shift
of the AQP proteins is a matter of particular interest (Chen et al., 2008). In the CNS, AQP4 knockout markedly reduced brain swelling in mouse models of cytotoxic brain edema such as acute water intoxication and ischemic stroke (Manley et al., 2000; Tait et al., 2008). Reduced glial cell water permeability due to AQP4 deficiency may be responsible for brain protection in cytotoxic edema to some degree (Tait et al., 2008). In this regard, the downregulation of perivascular AQP4 in the NFL of SDT rats at 40 weeks of age may reflect a compensatory mechanism for metabolic stress induced by diabetes. In addition, AQPs may be involved in glial cell dysfunction and death through an interaction with glutamate transporters, which were shown to be co-expressed with AQP4 (Nielsen et al., 1997). In the CNS, regulation of extracellular glutamate levels crucially depends on the sodium-dependent glutamate transporters located in astrocytes, which couple the reuptake of glutamate to Na\(^+\) and water molecules (Zeng et al., 2007). Zeng et al. (2007) reported remarkable reductions in glutamate transporter 1 expression and glutamate uptake, as well as less astrocytic injury triggered by glutamate in AQP4 knockout mice. Since glutamate is a major neurotransmitter in the retina, is tightly regulated by Müller cells, and is known to lead to excitotoxicity to RGCs at excessive concentrations (Kowluru et al., 2001; Li and Puro, 2002), the altered AQP protein localization may influence the glutamate transport properties of Müller cells and glutamate clearance in the retina. Further studies are needed to address this issue.

In conclusion, the development of diabetes led to neurodegeneration, and this was temporally synchronized with glial activation and altered expression of the water selective channels AQPs 1 and 4 in SDT rat retinas.
Acknowledgment

This study was supported in part by Grant-in-Aid No. 1630499 (A.N., M.N.), No. 19390444 (A.N., M.N.), No. 17591835 (M.N.), No. 20592043 (M.N.) and a grant for 21st Century COE program ”Center of Excellence for Signal Transduction Disease: Diabetes Mellitus as Model” (A.N.), from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government, and by Uehara Memorial Foundation (M.N.). M.N. is a recipient of the 12th ROHTO Award for Ophthalmic Research.
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growth factor, aquaporin 4, nitric oxide synthases and vascular permeability in the retina
Figure legends

Figure 1
Terminal dUTP nick end-labeling (TUNEL) staining on retinal flatmounts. a, representative photograph of retinal flatmount and TUNEL staining (insert). Arrowheads indicate TUNEL-positive cells. Scale bar indicates 10 μm. b, quantification of the number of TUNEL positive cells per unit retinal area. SDT stands for spontaneously diabetic Torii rats, whereas SD for Sprague-Dawley rats. Bars indicate means ± SD. *, p<0.0001 (non repeated measures ANOVA with Bonferroni test).

Figure 2
Cleaved caspase3 immunofluorescence in retinal cryosections. a through d, representative pictures. Red, cleaved caspase3 immunoreactive cells; blue, nuclei stained with Hoechst dye. Scale bar indicates 20 μm. a, spontaneously diabetic Torii (SDT) rat retina at 10 weeks. b, SDT rat retina at 20 weeks. c, SDT rat retina at 40 weeks. d, Sprague-Dawley rat retina at 40 weeks. e, graph showing the ratio of cleaved caspase3 immunoreactive cells to total Hoechst-stained nuclei in GCL and INL in ten retinal sections per eye (n=5). Bars indicate means ± SD. *, p=0.0002; **, p<0.0001 (non-repeated ANOVA with Bonferroni test). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. IR, immunoreactivity

Figure 3
Aquaporin (AQP) 1 immunofluorescence in retinal cryosections. SD stands for
Sprague-Dawley rats, whereas SDT for spontaneously diabetic Torii rats. GFAP, glial fibrillary acidic protein. ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRS, photoreceptor layer. Scale bar indicates 50 μm.

Figure 4
Aquaporin (AQP) 1 immunofluorescence on retinal flatmounts. SD stands for Sprague-Dawley rats, whereas SDT for spontaneously diabetic Torii rats. GFAP, glial fibrillary acidic protein. NFL, the superficial microscopic focal plane within the nerve fiber layer. INL, the deep microscopic focal plane within the inner nuclear layer. Scale bar indicates 50 μm. Arrowheads indicates AQP1 immunoreactivity of red blood cells, whereas arrows perivascular AQP1 immunoreactivity.

Figure 5
Aquaporin (AQP) 4 immunofluorescence in retinal cryosections. SD stands for Sprague-Dawley rats, whereas SDT for spontaneously diabetic Torii rats. GFAP, glial fibrillary acidic protein. ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRS, photoreceptor layer. Scale bar indicates 50 μm. Note an arrow indicating reduced AQP4 immunoreactivity around a superficial vessel.
Fig. 2

The images show layers of tissue labeled with different cellular markers:
- GCL (Ganglion Cell Layer)
- IPL (Inner plexiform layer)
- INL (Inner nuclear layer)
- OPL (Outer plexiform layer)
- ONL (Outer nuclear layer)

**Images (a-d):**
- **Image (a):** Green and blue staining in the GCL, IPL, INL, OPL + ONL layers.
- **Image (b):** Similar staining patterns as in (a).
- **Image (c):** Different staining patterns with red and blue in the GCL, IPL, INL, OPL + ONL layers.
- **Image (d):** Minimal staining compared to (a) and (b).

**Graph (e):**
- The graph displays the Cleaved caspase-3 IR ratio (%) for different time points:
  - 10w SDT rats
  - 20w SDT rats
  - 40w SDT rats
  - 40w SDT rats

- The bars show a significant increase in the IR ratio from 10w to 40w SDT rats.
- The 40w SDT rats show a decrease compared to 40w SDT rats.
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**Legend**

- **AQP1**: Red
- **GFAP**: Green
- **INL**: Yellow

**Fig. 4**

*Scale bar: 50 μm*