Published in final edited form as:

Brain Res. 2009 March 3; 1258: 25–33. doi:10.1016/j.brainres.2008.12.026.

Impaired neurogenesis in adult type-2 diabetic rats

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Abstract

Type-2 diabetes is an adult onset condition that affects millions of people worldwide. The ensuing hyperglycemia renders multiple organs to various complications and increases the risk of learning and memory impairment. The Goto-Kakizaki (GK) rat developed from normoglycemic Wistar-Kyoto (WKY) rat is a model for type-2 diabetes, with insulin resistance developing around 12 weeks of age. We presently analyzed the neural progenitor proliferation and survival of the newly generated cells in the dentate gyrus (DG) and the subventricular zone (SVZ) of 6 and 18 week-old GK and WKY rats. At 6 weeks of age, both GK and WKY cohorts showed similar blood glucose levels (112 ± 14 mg/dL) and similar rates of neural progenitor proliferation. At 18 weeks of age, the GK rats showed significantly increased blood glucose levels (by $92 \pm 12\%$; $p < 0.05$) and higher number of proliferating neural progenitor cells compared to WKY rats (by $183 \pm 16\%$ in SVZ and by $36 \pm 5\%$ in DG; $p < 0.05$ in both cases). In both the neurogenic areas, $52 \pm 9\%$ of the newly formed cells survived to 3 weeks in the 18 weeks old WKY rats, but in the GK rats only $16 \pm 7\%$ of the new cells survived to 3 weeks. When cultured from the DG of the 18 week old rats in the presence of FGF2 and IGF1, the GK cohort yielded significantly lower number of neurospheres than the WKY cohort (by $69 \pm 7\%$; $p < 0.05$). These results indicate that hyperglycemic environment induces proliferation of adult neural progenitors, but detrimental to their survival. Impaired neurogenesis might be a promoter of the decreased brain function in type-2 diabetes.

Keywords

Hyperglycemia; Animal model; Neural Progenitors; Proliferation; Survival; Growth factors; Neurospheres

1. INTRODUCTION

Diabetes is a major health concern with approximately 200 million people suffering in the world. This disease occurs in two major forms; while the early-onset type I diabetes is characterized by insulinopenia, commonly caused by the destruction of the insulin producing beta islet cells of the pancreas, the adult-onset type II diabetes results from peripheral insulin resistance. A commonality between the 2 conditions is the hyperglycemia which is detrimental to cognition and other neurological functions (Toth et al., 2007; Kodl and Seaquist, 2008).

One of the major complications of diabetes is decreased learning and memory functions. In mammalian brain, neurogenesis occurs throughout the life in 2 major areas of brain viz., the

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dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Gage, 2000; Alvarez Buyla et al. 2002). The new neurons formed by the proliferation of the neural progenitor are thought to replace the dead neurons in the hippocampus and olfactory bulb (Taupin, 2008). In addition, a plethora of growth factors, environmental conditions and diseases have been shown to affect these cells both positively and negatively (Wiltout et al., 2007).

Recent studies showed that neurogenesis, synaptic plasticity and learning potential will be significantly compromised in the rodent model of type 1 diabetes (Zhang et al, 2008; Stranahan et al, 2008). We currently evaluated the effect of type-2 diabetes on adult brain neural progenitor proliferation and survival using the Goto-Kakizaki (GK) rat which is a spontaneous, non-obese, carrier of the disease created by selective breeding of random Wistar-Kyoto (WKY) rats that showed hyperglycemia (Goto et al, 1975, Ahmad et al, 2008).

2. RESULTS

2.1. Hyperglycemia in GK Rat

At 6 weeks of age, the fasting blood sugar levels were observed to be similar (112.1 ± 14.4 mg/dL) in the GK and WKY rats ($n = 30/\text{group}$) (Fig. 1). At 10 weeks of age, the blood sugar level was not statistically different between the two cohorts or when compared with their respective 6 weeks old levels (Fig. 1). In the WKY rats, the blood glucose level remained unchanged at further ages (Fig. 1). Where as, the GK rats became overtly hyperglycemic by 16 weeks showing significantly higher blood sugar levels than the WKY rats (by $91 \pm 15\%$; $p < 0.05$; $n = 18/\text{group}$) (Fig. 1). The GK rats remained in the hyperglycemic state at 20 weeks of age (Fig. 1). At 6 weeks of age, the body weights were observed to be 154 ± 12 g (GK; $n = 30$) and 182 ± 14 g (WKY; $n = 30$). At 18 weeks age, the body weights were observed to be 372 ± 41 g (GK; $n = 18$) and 464 ± 58 g (WKY; $n = 18$). Both cohorts of rats were fed on normal rat chow.

2.2. GK rats showed normal progenitor proliferation in the pre-hyperglycemic age

To study the proliferation of neural progenitors, cohorts of 6 weeks and 18 weeks old GK and WKY rats were injected with 5-bromo-2-deoxyuridine (BrdU; 50 mg/Kg; *i.p.* twice a day) for 5 days and killed on day 6 for immunostaining. When compared between the 6 weeks old WKY and GK rats, the number of proliferating neural progenitors was not significantly different in either SVZ or DG ($n = 6/\text{group}$) (Fig. 2 and Fig. 4). Immunostaining for the immature proliferating neural marker doublecortin (DCX) also showed a similar result (Fig. 2).

2.3. Hyperglycemia increased proliferation, but decreased survival of neural progenitors

At 18 weeks of age (after they became hyperglycemic), the GK rats showed a significantly higher progenitor proliferation compared to the age-matched WKY rats ($n = 6/\text{group}$) in both SVZ (by $197 \pm 21\%$; $p < 0.05$) and DG (by $44 \pm 8\%$; $p < 0.05$) (Fig. 3 and Fig. 5). We also studied the survival of the newly formed cells by allowing a different cohort of GK and WKY rats ($n = 6/\text{group}$) to survive for 3 weeks after the 5 day BrdU injections. Despite the high level of proliferation, the number of neural progenitors that survived for 3 weeks were significantly lower in the GK compared to WKY rats. The rate of survival was $41 \pm 11\%$ in the SVZ and $44 \pm 9\%$ in the DG of the WKY rats (Fig. 5). Where as in the GK rats, the survival of the newly proliferated progenitors to 3 weeks was only $12\% \pm 4\%$ in the SVZ and $15 \pm 6\%$ in the DG ($n = 6/\text{group}$) (Fig. 5).

2.4. Impaired responsiveness of GK rat neural progenitors to growth factors

To understand whether the increased proliferation and decreased survival of the neural progenitors in the hyperglycemic rat were due to intrinsic cellular factors or the surrounding environment, we developed neurosphere cultures from the 18 week old GK and WKY rats. We could passage (the cultures were passaged once a week) the neurosphere cultures developed from the SVZ for more than 15 cycles in case of WKY rats; but the GK rat SVZ neurospheres stopped growing after the 3rd passage (data not shown). The neurospheres cultured from the DG of both WKY and GK rats survived for at least 15 passages and the number and size of spheres formed were not significantly different between the 2 cohorts in the absence of exogenously added FGF2 and IGF1 (Fig. 6). When the WKY cultures were grown the presence of growth factors FGF2 and IGF1 (20 ng/ml each), the spheres increased significantly compared to control (by $117 \pm 14\%$ in the presence of FGF2 and by $302 \pm 41\%$ in the presence of FGF2+IGF1; $p < 0.05$ in both cases; $n = 8/\text{group}$) (Fig. 6). However, the GK spheres showed no such increase in the presence of FGF2 alone or FGF2+IGF1 (Fig. 6).

3. DISCUSSION

In brief, results of the present study shows that adult type-2 diabetic rats show increased proliferation but decreased survival of the neural progenitors. Furthermore, neural progenitors cultured from adult diabetic rats show an impaired responsiveness to growth factors.

Diabetes is known to promote cognitive impairment and vascular dementia in humans (Biessels et al., 2008). Hemoglobin A1c level which is a marker of the long-term hyperglycemia was shown to correlate with cognitive decline in humans (MacLulich et al., 2004). Although the precise mechanisms that mediate this cerebral dysfunction are not known, chronic inflammation and oxidative stress seen in diabetes (MacLulich and Seckl, 2008) are known to promote neuronal death and inhibit neurogenesis (Das and Basu, 2008; Ekdahl et al., 2008; Wiltout et al., 2007). Neurogenesis in mammals which continues throughout the life helps to replace neurons in the DG of hippocampus that play a role in memory functions. We currently show that neurogenesis is severely impaired in the adult type-2 diabetic GK rats. Interestingly in these animals the proliferation of neural progenitors in both the neurogenic regions increased significantly, but their survival diminished severely. The decreased survival of the newly formed cells might be acting as a feed-forward mechanism to increase the proliferation in a vain attempt to maintain new neuron formation at a constant level.

The precise mechanisms that impair the survival of the newly formed cells are not known at present, but our studies showed that lack of responsiveness to growth factors might be a cause. While the progenitors cultured from the normoglycemic rats formed more and bigger neurospheres when exposed to FGF2 and IGF1, progenitors from the hyperglycemic rats failed to respond to added growth factors. Growth factors are known to play a significant role in proliferation, survival and phenotypic maturation of the neural progenitors under *in vivo* and *in vitro* conditions (Palmer et al., 1995; Martens et al., 2002; Nakatomi et al., 2002; Aberg et al., 2003; Dempsey et al., 2003; Naylor et al., 2005; Yan et al., 2006; Kalluri et al., 2007; Wiltout et al., 2007). Of importance, our laboratory and others showed that infusion of FGF2 and IGF1 into rodent brain significantly increases neural progenitor proliferation under normal and ischemic conditions (Palmer et al., 1999; Tureyen et al., 2005; Kalluri et al., 2005). While FGF2 is thought to be essential for cell division, IGF1 was shown to promote survival of the newly formed cells (Kalluri et al., 2005, 2007). Both these growth factors are known to be essential for maintaining the pluripotency of neurosphere cultures (Richards et al., 1992). In adult neural progenitors, IGF1 induces the phosphorylation of phosphatidylinositol-3-kinase/AKT and glycogen synthase kinase which are known to promote cell survival (Wiltout et al., 2007). As type-2 diabetic GK rats have a disrupted insulin signaling pathway, the neural progenitors from these animals might not have responded to IGF1 which acts through insulin

receptors. However, it is surprising that the hyperglycemic rat progenitors also failed to respond to FGF2 which acts via distinct FGF receptors leading to phosphorylation of the down-stream MAP-kinase ERK. Hence, decreased neurogenesis in type-2 diabetic brain might be due to an inherent defect in the neural progenitors/stem cells to respond to different growth factors. Our previous studies showed that FGF2 enhances progenitor proliferation more robustly in the presence of IGF1 (Kalluri et al., 2007). Increased proliferation in rats infused with FGF2 was shown to be associated with enhanced post-ischemic memory formation and retention (Nakatomi et al., 2002). Hence, disrupted response to FGF2 might contribute to the memory deficits seen in diabetics.

Impaired neurogenesis is not restricted to type-2 diabetes. Recent studies showed that genetically type-1 diabetic mice and STZ treated rats also show decreased neurogenesis (Zhang et al., 2008; Beauquis et al., 2008). Type 1 diabetes induced by streptozotocin injection in rodents was also shown to decrease neural progenitor proliferation (Beauquis et al., 2008; Stranahan et al., 2008). Interestingly, high-fat diet which accelerates diabetes also impairs hippocampal neurogenesis in rodents (Lindqvist et al., 2006). Several studies showed that diabetic humans show declined episodic learning and memory which could be attributed to hippocampal dysfunction (Messier et al., 2005). One of the contributing factors for the impaired hippocampal function might be disruption of neurogenesis mediated by increased glucocorticoids levels known to be present in diabetics. Long-term exposure to high levels of corticosterone is known to disrupt learning in animals (Oitzl et al., 1998) and maintaining physiologic levels of corticosterone was shown to enhance neurogenesis leading to restoration of LTP and reversal of learning deficits in type-2 diabetic mice (Stranahan et al., 2008).

Inflammation and oxidative stress which are commonly seen in diabetic subjects are known inhibitors of neurogenesis (Pluchino et al., 2008; Ekdahl et al., 2008; Kim et al., 2008). Treatment with the anti-oxidant and anti-inflammatory compounds like curcumin and indomethacin was shown to restore the neurogenesis inhibited by inflammation and oxidative stress (Kim et al., 2008; Monje et al., 2003). A recent study from our laboratory showed that the anti-diabetic thiazolidinediones (TZD) rosiglitazone and pioglitazone (PPAR γ agonists) significantly decreased stroke-induced brain damage and neurological dysfunction in type-2 diabetic mice by preventing inflammation (Tureyen et al., 2007). Interestingly, PPARs are known to activate the molecular pathways STAT3 and Wnt signaling which control proliferation, differentiation and phenotypic specification of neural progenitor cells (Cimini and Ceru, 2008). Due to their capability to control blood sugar and lipid balance, rosiglitazone and pioglitazone are FDA-approved for type-2 diabetes treatment. As these TZDs control inflammation and oxidative stress, they might provide a better microenvironment for the neural progenitors to proliferate and form mature neurons in the diabetic brain.

In conclusion, our studies for the first time demonstrated a disruption of neurogenesis in adult type-2 diabetic animals which might be a factor responsible for the compromised cognitive and memory functions seen in diabetics. Compounds that could increase neurogenesis might be potentially beneficial in diabetes treatment.

4. EXPERIMENTAL PROCEDURES

4.1. Rats

The WKY and GK rats were obtained at 4 weeks of age from Taconic Animal Farms (Germantown, NY, USA). Animals were housed and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*, US Department of Health and Human Services Publication number 86–23 (revised in 1986) and all procedures were approved by the animal care committee of the University of Wisconsin-Madison. Blood sugar levels were estimated

twice every week using a blood glucose monitoring system (FreeStyle, Abbott Park, IL, USA) using blood obtained from the hind paw. Rats were fasted overnight for blood sugar testing.

4.2. Progenitor proliferation and survival

Neural progenitor cells were estimated with immunohistochemistry in the DG and SVZ of rats as described earlier (Kalluri et al., 2007; Yan et al., 2007). BrdU (Sigma Chemical Co., MO, USA) dissolved in sterile saline was injected *i.p.* (50 mg/Kg) twice a day for 5 days. The proliferation groups were killed on day 6 and the survival groups were killed on day 21. In brief, the rats were deeply anesthetized with isoflurane, transcardially perfused with buffered paraformaldehyde, the brains were post-fixed, cryoprotected and sectioned serially (coronal; 30 μ m thick) covering DG and SVZ. Brain sections were subjected to immunostaining using BrdU antibodies (1:50; biotin-conjugated mouse anti-BrdU; Molecular Probes, Eugene, OR, USA) and DCX antibodies (1:100; rabbit anti-DCX; Santa Cruz Biotechnology, Santa Cruz, CA, USA) essentially as described earlier (Dempsey et al., 2003; Yan et al., 2007). We stereologically counted the BrdU⁺ proliferating progenitors in the SVZ and DG of each rat with an established procedure that our lab used in several previous studies (Dempsey et al., 2003; Tureyen et al., 2004; Tureyen et al., 2005; Naylor et al., 2005; Park et al., 2007; Yan et al., 2006; Yan et al., 2007). In this method, sections were analyzed microscopically and the images were acquired using a CCD camera (Spot Camera, Diagnostic Instruments Inc., Sterling Heights, MI, USA). The BrdU⁺ cells reacted with DAB were quantified using 4 sections from each rat covering +1.1 to -0.2 mm from bregma as per the Rat Brain Atlas (Paxinos and Watson, 1998). In each case, the combined area of SGZ and the granular cell layer (GCL) of the DG was traced and measured in mm² using Image-J software (written by Wayne Rasband, NIH, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij>). The cells in the selected area were counted and divided by the area to obtain cells per mm². This method was chosen to compensate for variations in the area of the cell layers between sections. To count the BrdU⁺ cells in the SVZ, 3 vertical, partial images of the lateral ventricle were microscopically acquired. The images were then compiled to make a complete image of the lateral ventricle using Photoshop (Adobe, San Jose, CA, USA). Using Image-J software, the rostral blade of the subependymal layer was traced, measured in mm², the cells in the selected area were counted and divided by the area to obtain cells per mm². Several previous studies presented the progenitor cell proliferation data as cells/area (Arvidsson et al. 2002; Aberg et al., 2003; Nakatomi et al. 2002). Compared to the total number of cells present in the GCL and SZG, the number of newly formed cells is insignificant. Furthermore, Cameron and McKay (2001) have shown that DG is constantly undergoing proliferation, yet the total number of cells in the GCL does not increase and the volume remains same with proliferation and cell death being in balance.

4.3. Neural Progenitor Culture

Neurospheres were generated from the SVZ and DG of adult GK and WKY rats as described earlier (Kalluri et al., 2007; Yan et al., 2007). In brief, a rat were deeply anesthetized with isoflurane, the brain was removed and washed in ice-cold Dulbecco's phosphate buffered saline with 4.5 g/L glucose (DPBS/glu). The hippocampus and the lateral ventricle of SVZ were separately dissected under a microscope, and washed in fresh DPBS/glu. Tissues were minced in cold Hank's balanced salt solution without Mg²⁺ and Ca²⁺ (HBSS; Invitrogen) and treated with 0.01% papain (Worthington Biochemical Corporation, Lakewood, NJ, USA), 0.1% dispase II (Boehringer Mannheim, Indianapolis, IN, USA), 0.01% DNase I (Worthington) and 12.4 mmol/L MgSO₄ in HBSS at 37°C for 40 min. The cell suspension was triturated every 10 min, centrifuged, and the pellet was rinsed with Dulbecco's-modified Eagle's medium (DMEM)/F12 (1:1) (Invitrogen). The cells were suspended in neurobasal medium with B27 (without retinoic acid, Invitrogen), 2 mmol/L glutamine, 0.1 g/mL penicillin/streptomycin (Invitrogen), 2 g/mL heparin (Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL FGF2 (R&D Systems, Minneapolis, MN, USA), and seeded in T-25 culture flasks. The cultures were

maintained at 37°C in an incubator with 5% CO₂. Neurospheres were formed by 5 to 7 days of suspension in culture. Neural progenitors were passaged once a week by dissociating neurospheres into single cells with accutase. The neurosphere growth was estimated calorimetrically as described earlier (Kalluri et al., 2007; Yan et al., 2007). The cells were maintained for at least 15 passages. Cells (2×10^4) were suspended in 100 µL neurobasal medium and cultured in the presence and absence of IGF1 (20 ng/mL) and/or FGF2 (20 ng/mL) in a 96-well plate. After 3 days in culture, 20 µL of assay reagent (CellTitre 96 AQueous; Promega Inc., Madison, WI, USA) was added to the cell suspension and incubated at 37°C for 3 h to allow the conversion of tetrazolium salt into a formazan product. The optical density of the formazan product was measured at 490 nm in an ELISA plate reader. The same volume of medium without cells was used as blank.

4.4. Statistical analysis

The data are expressed as mean $\pm$ SD. Comparisons among groups were performed by one-way ANOVA followed by Tukey-Kramer multiple comparisons post-hoc test.

Acknowledgements

These studies were partially supported by grants from the United States National Institute of Health (RO1 NS044173 and RO1 NS049448) and the American Heart Association (Grant-in-Aid 0350164N).

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Fig. 1.

Blood sugar levels as a function of age in WKY and GK rats. We used 30 WKY and 30 GK rats. As needed by different experiments, at 7 weeks, 9 weeks, 18 weeks, 19 weeks and 21 weeks of age, we killed $n = 6$ rats/group. Hence, the blood sugar values presented in each group are mean $\pm$ SD of $n = 30$ at 6 weeks, $n = 18$ at 10 weeks and 16 weeks, and $n = 6$ at 20 weeks. Statistics: ^a $p < 0.05$ compared with the respective 6 weeks old value and ^b $p < 0.05$ compared with the age-matched WKY cohort (one-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 2.

At 6 weeks of age, WKY and GK rats showed similar pattern of neural progenitor proliferation in the SVZ and DG. Panel A shows the areas from which the images B to G were taken. The BrdU⁺ proliferating neural progenitors are shown in different areas (indicated with arrows) of SVZ of WKY (B to D) and GK (E to G) cohorts. Panels H and I show the BrdU⁺ proliferating neural progenitors in the DG of WKY and GK rats, respectively. Panels J and K show the DCX⁺ neural progenitors in the DG of WKY and GK rats, respectively. Similar results were observed in 6 rats/group.

Fig. 3.

At 18 weeks of age, the GK rats showed significantly higher number of proliferating neural progenitors in the SVZ and DG compared to WKY rats. Panel A shows the complete SVZ. The BrdU⁺ proliferating neural progenitors are shown in different areas of SVZ of WKY (B to D) and GK (E to G) cohorts. Panels H and I shows the BrdU⁺ proliferating neural progenitors in the DG of WKY and GK rats, respectively. Panels J and K shows the DCX⁺ neural progenitors in the DG of WKY and GK rats, respectively. Similar results were observed in 6 rats/group.

Fig. 4.

Number of proliferating neural progenitors in the WKY and GK rats. BrdU was injected twice a day for 5 days and the rats were killed on day 6 for assessing proliferation. Values are mean $\pm$ SD of $n = 6$ rats/group. In case of each rat, the number of BrdU⁺ cells was counted using 4 brain sections.

Fig. 5. Number of surviving neural progenitors in the WKY and GK rats. BrdU was injected twice a day for 5 days and the rats were killed on day 21 for assessing survival. Values are mean $\pm$ SD of $n = 6$ rats/group. In case of each rat, the number of BrdU⁺ cells was counted using 4 brain sections. Statistics: ^a $p < 0.05$ compared with the respective WKY group and ^b $p < 0.05$ compared with the respective proliferation group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 6.

The neural progenitors cultured from the DG of 18 week old WKY rats proliferated rapidly to form neurospheres (A). Addition of FGF2 alone (B) or FGF2 + IGF1 (C), but not IGF1 alone (D), significantly increased the WKY neurosphere proliferation. On the other hand, neural progenitors cultured from the DG of 18 weeks old GK rats proliferated at a similar rate to WKY in the absence of added growth factors (E), but showed a significantly curtailed response to FGF2 alone (F) or FGF2 + IGF1 (G) or IGF1 alone (H). The histogram shows the results of the cell proliferation assay in each of the groups. The bars are mean ± SD of n = 6 per group. Statistics: ^ap < 0.05 compared with the no growth factor added group and ^bp < 0.05 compared

with the respective GK group (one-way ANOVA followed by Tukey-Kramer multiple comparisons test).