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Effects of streptozotocin diabetes on the ultrastructure of astrocytes in the cervical enlargement of rat spinal cord

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A chronic complication of diabetes mellitus (DM), diabetic neuropathy, causes neurodegeneration leading to the development of neuropathic pain and sensation loss, particularly in the hands and arms. The cervical enlargement of the spinal cord sends the nerve supply to the hands and arms. Moreover, astrocytes effectively provide structural and functional maintenance of neurons in the spinal cord. Nevertheless, there is very little research concerning the ultrastructural changes of diabetic spinal astrocytes in the cervical enlargement. Thus, in this study, the ultrastructural changes of protoplasmic and fibrous astrocytes in streptozotocin (STZ)-induced diabetic and control rats using transmission electron microscopy (TEM) were examined and compared. Thirteen male diabetic and six male control rats were intraperitoneally administered with either STZ in citrate buffer or only the buffer solution, respectively. Rats were sacrificed at four (short term) and twenty-four weeks (long term) after induction of DM. Then, the cervical enlargements were collected for TEM processing. Under TEM, the morphologies of protoplasmic and fibrous astrocytes were similarly changed in each group. In the short-term DM model animals, hypertrophic nuclei with heterochromatin clumping were seen in both types of astrocytes. The rough endoplasmic reticulum (rER), Golgi complexes, and mitochondria were distended. Moreover, augmented glial fibrils were seen in astrocytic processes. In the long-term diabetes, increased heterochromatin clumping with severely disrupted nuclear membranes was seen in both types of astrocytes. The rER, Golgi complexes, and mitochondria were extremely degenerated. Disruptions of astrocytic processes were also observed. These results indicate that DM leads to abnormalities of both protoplasmic and fibrous astrocytes, which are possibly involved in the impairment of neuronal function that causes diabetic neuropathy. Thus, this basic knowledge can support better understanding of the pathogenesis in diabetic neuropathy.

Key words: Astrocytes, cervical enlargement, spinal cord, diabetes mellitus, streptozotocin.

INTRODUCTION

Diabetes mellitus (DM) involves abnormally high glucose levels in the bloodstream (Samreen, 2009). This leads to damage to a number of organs, including heart, liver and the superior colliculus (Lanlua et al., 2012; Sricharoenvej et al., 2012; Upachit et al., 2015). A chronic complication in diabetic patients is diabetic neuropathy, which leads to
degeneration of neurons, resulting in autonomic dysfunction, neuropathic pain, and sensation loss, especially in the hands and arms (Tesfaye et al., 2010; Morales-Vidalet et al., 2012). In fact, the primary neurons that supplies the upper limb is located in the cervical enlargement of the spinal cord. Moreover, the most abundant and largest glial cell type in the spinal cord is astrocytes. Even though the morphological structures of astrocytes are the same, these cells can be classified into two types: protoplasmic and fibrous astrocytes, which are located in gray and white matter, respectively (Sofroniew and Vinters, 2010; Molofsky et al., 2012). Astrocytes play effective roles in supporting neurons in terms of neuroprotection, transmission of neurotransmitters, and regulation of neurogenesis (Tsacopoulos and Magistretti, 1996; Goldman, 2003; Newman, 2003). Moreover, astrocytes maintain the blood-spinal cord barrier (BSCB), which is an important structure for controlling diffusion of oxygen, ions and other substances from capillaries to neurons (Bartanusz et al., 2011). In the white matter, the perinodcal processes of fibrous astrocytes envelope the nodes of Ranvier to stimulate action potential at the nodes of axons (Black and Waxman, 1988; Black et al., 1989). Several studies have been investigated the effects of DM on spinal astrocytes. The numbers of astrocytes in the gray and white matter of the spinal cord are increased in 4-week streptozotocin (STZ)-induced diabetic rats (Surcheva et al., 2009; Chookliang et al., 2014). Normally, platelet-derived growth factor (PDGF) is synthesized and released by astrocytes for neuroprotection after neuronal injury (Ridet et al., 1997). During diabetes, the level of PDGF in spinal astrocytes is significantly decreased, so the recovery of neurons is slow after spinal cord injury. Finally, DM leads to diabetic neuropathy (Wu et al., 2010). It has been demonstrated that there is thickening of the basement membrane in diabetic capillaries in the BSCB (Zhao et al., 2010). However, there is no data on the effect of DM on astrocytic end feet surrounding capillaries in the BSCB. Although the functions of astrocytes in STZ-induced diabetic rats have been examined, few studies have been focused on the ultrastructural alterations of diabetic spinal astrocytes in the cervical enlargement, which may have a role in the pain and loss of feeling in the hands and arms of diabetic patients. Consequently, the aims of this investigation were to examine and compare the ultrastructural changes of protoplasmic and fibrous astrocytes in the cervical spinal enlargements between short- and long-term STZ-induced diabetic and control rats using transmission electron microscopy (TEM). It was hypothesized that structural impairments of both types would be observed in the astrocytes of the cervical spinal enlargements in short- and long-term STZ-induced diabetic rats. Therefore, the dysfunctions of both types in astrocytes can lead to abnormalities of structure and function in neurons of cervical enlargement, that supply upper extremities. Ultimately, diabetic neuropathy occurs.

MATERIALS AND METHODS

Animal preparation and diabetic induction

Nineteen healthy male Sprague-Dawley rats aged 5 to 8 weeks, weighing 200 to 270 g, were used (National Laboratory Animal Center, Mahidol University, Thailand). In this study, the rats were handled according to the Mahidol University Council’s Guidelines for Care and Use of Laboratory Animals. After acclimatization for a week, the rats were fasted for at least 6 h. Subsequently, the glucose concentration in urine was determined using urinalysis control strips (Diabur Test 5000, Roche Ltd., Germany). Additionally, tail vein blood samples were assessed using a blood glucose monitoring system (OneTouch Ultra®, USA). If the levels of urine glucose concentration were 0 mg/dl, and blood glucose levels were <300 mg/dl, the rats could be used in this experiment. Rats were randomly divided into two groups. First, in the STZ-induced diabetic group (short term: n=5, long term: n=7), each rat was intraperitoneally induced with a single dose of 60 mg/kg body weight of STZ in citrate buffer (Acros Organics, Janssen Pharmaceuticals, Geel, Belgium). Second, in the control group (short term: n=5, long term: n=3), each rat was intraperitoneally injected with the same amount of buffer. After fasting for 10 to 12 h, urine glucose concentrations and body weights were measured daily. Moreover, blood glucose levels were determined for each rat as previously described (Lanlua et al., 2012; Sricharoenvej et al., 2012; Upachit et al., 2015). Thereafter, the rats were sacrificed at four (short-term) and twenty-four (long-term) weeks after induction.

Preparation for ultrastructural studies of protoplasmic and fibrous astrocytes in the cervical enlargements

Each rat was anesthetized by halothane inhalation. After that, 0.1 M phosphate buffer solution (PBS) was administered, followed by injection of 500 ml of 2.5% glutaraldehyde in 0.1 M PBS to preserve the tissues. Next, the cervical enlargements were carefully removed and cut into small pieces ~1 x 1 x 1 mm³, with samples of both gray and white matter. These specimens were prepared for TEM processing, as described elsewhere (Lanlua et al., 2012; Sricharoenvej et al., 2012 and Upachit et al., 2015). The ultrastructures of protoplasmic and fibrous astrocytes in the cervical enlargements were observed and photographed under TEM (JEOL JEM100S; JEOL Ltd., Tokyo, Japan).

RESULTS

TEM was used to examine and characterize two major distinct morphological phenotypes of astrocytes. In the normal condition, protoplasmic astrocytes had an oval or round nucleus with homogenous nucleoplasm. The heterochromatin was evenly distributed beneath the inner nuclear membrane. Numerous organelles occupied the
Figure 1. Transmission electron micrographs of protoplasmic astrocytes in the control (1A-B) and short- (1C) and long-term diabetic (1D) groups. Nucleus of protoplasmic astrocytes (Nu), rough endoplasmic reticulum (rER), Golgi complex (G), mitochondria (m), free ribosomes (r), dendrite (D), unmyelinated axon (Ax₁), dilated rER (a white arrow), enlarged mitochondria with disrupted cristae (black arrow), aggregation of polysomes (white arrowheads), long-sectioned glial fibrils (black arrowhead), oligodendrocyte (Ol), clear area and loss of organelles (black star), residual organelles in the perinuclear area (white asterisk), fragmented rER (small white arrow), and ruptured mitochondrial cristae with large vacuole (small black arrow).

cytoplasm. Short cisternae of rough endoplasmic reticulum (rER) studded with ribosomes were located near the outer nuclear membrane. Golgi complex and mitochondria were also observed. Moreover, free ribosomes were distributed throughout the cytoplasm (Figure 1A and B). Dendrites and unmyelinated axons appeared adjacent to protoplasmic astrocytes (Figures 1A and 2A).

Among the alteration of protoplasmic astrocytes, observed by electron microscopy during short-term DM, was a swollen nucleus with slight heterochromatin clumping at the nuclear periphery. Abnormal organelles were observed in an altered protoplasmic astrocyte. The mitochondria were enlarged and displayed disrupted mitochondrial cristae. Additionally, polysome aggregations were seen (Figure 1C). Increased glial
Figure 2. Transmission electron micrographs of the processes of protoplasmic astrocytes in the control (2A) and short- (2B) and long-term diabetic (2C) groups. Nucleus of protoplasmic astrocyte (Nu), rough endoplasmic reticulum (rER), astrocytic process (Asp), long-sectioned glial fibrils (f), mitochondria (m), dendrite (D), unmyelinated axon (Ax), increased long-sectioned (black arrowheads) and cross-sectioned (white arrowheads) glial fibrils, swollen mitochondria (black arrow), and disrupted astrocytic process (white asterisk) near a neuron with a shrunken nucleus (N).

fibrils (Figure 2B) and glycogen granules (Figure 3A) were noticed in astrocytic processes and astrocytic end feet around the capillaries, respectively.

In long-term DM models, protoplasmic astrocytes showed markedly intensified degenerative changes characterized by condensed heterochromatin in the nucleus and a disrupted nuclear membrane. The cytoplasm of one remarkably damaged protoplasmic astrocyte was almost completely empty with dispersed residual organelles, such as fragmented rER and disrupted cristae with a vacuolar structure in the mitochondria (Figure 1D). Moreover, astrocytic processes and astrocytic end feet in the BSCB were severely damaged, and were found near neurons that showed nuclear shrinkage and damaged organelles in the cytoplasm (Figures 2C and 3B).

Normal fibrous astrocytes were observed in the white matter. These had oval or round nuclei similar to protoplasmic astrocytes. The organization of the cytoplasm appeared similar to that in protoplasmic astrocytes with cisternae of the Golgi complex, rER, free ribosomes, and mitochondria (Figure 4A). Additionally, fibrous astrocytes contained more abundant glial fibrils than did protoplasmic astrocytes. Long glial fibrils were arranged parallel to the long axis of the astrocytic process (Figure 5A). At the nodes of Ranvier, the perinodal processes of fibrous astrocytes enclosed a thin layer of nodal axolemma and contained mainly mitochondria and glial fibrils (Figure 6A). Additionally, the astrocytic end feet of fibrous astrocytes were located close to capillaries.

Ultrastructural changes in fibrous astrocytes during
short-term DM did not differ from those in protoplasmic astrocytes. Swollen fibrous astrocytes with slightly condensed heterochromatin and abnormal organelles, such as dilated short cisternae of rER, enlarged mitochondria with degenerated cristae, large numbers of polysomes (Figure 4B), and dense bundles of glial fibrils (Figure 5B), were seen. Furthermore, augmented glial fibrils and swollen mitochondria were found in the perinodal processes of fibrous astrocytes near the nodes of Ranvier (Figure 6B). These characteristics remained in the astrocytic end feet that surrounded the capillaries (Figure 7A).

In long-term DM group, fibrous astrocytes showed increased heterochromatin clumping and reduced cellular organelles. A ruptured nuclear membrane was examined, and the outer nuclear membrane was seen to extend into the perinuclear cytoplasm. Moreover, large vacuoles filled the cytoplasm (Figure 4C). Fragmentation of glial fibrils was found along the astrocytic processes (Figure 5C) and near the nodes of Ranvier (Figure 6C). Additionally, the astrocytic end feet surrounding capillaries were degenerated and appeared empty (Figure 7B).
Figure 5. Transmission electron micrographs of glial fibrils in the processes of fibrous astrocytes in the control (5A) and short- (5B) and long-term diabetic (5C) groups. Nucleus of fibrous astrocyte (Nu), Golgi complex (G), free ribosomes (r), astrocytic process (Asp), long glial fibrils (f), mitochondria (m), myelinated axon (Ax₂), increased bundles of long glial fibrils (black arrowheads), and fragmentation of glial fibrils (white arrowheads).

Figure 6. Transmission electron micrographs of the perinodal processes of fibrous astrocytes surrounding the nodes of Ranvier in the control (6A) and short- (6B) and long-term diabetic (6C) groups. Node of Ranvier (NR), perinodal process (PN) of fibrous astrocyte, glial fibrils (f), mitochondria (m), myelinated axon (Ax₂), increased amount of cross-sectioned glial fibrils (white asterisks), swollen mitochondria (white arrowheads), fragmented glial fibrils (small black arrow), mitochondrial atrophy (black arrowhead), and clear area in the perinodal process (black star).

DISCUSSION

Hypertrophy of protoplasmic and fibrous astrocytes occurs during short-term DM. In the polyol pathway during DM, sodium ion/potassium ion ATPase activity decreases, resulting in ATP depletion (Brownlee, 2001; Obrosova, 2005). Hyperglycemia generates increased production of lactic acid through glycolysis, because of the decrease in the level of ATP. It has been indicated that the high level of lactic acid induces increased expression of aquaporin 4 (AQP4), a water channel on the cell membranes of astrocytic processes, via an unclear mechanism. A high level of AQP4 results in water influx into cells (Morishima et al., 2008). Moreover, the elevation of intracellular glucose can interrupt the mitochondrial electron transport chain at complex III to cause increased oxidation of molecular oxygen by reactive coenzyme Q. After that, increased production of hydrogen peroxide occurs, which finally results in augmented accumulation of reactive oxygen species (ROS) in astrocytes (Nishikawa and Araki, 2007). Then, ROS induce increased lipid peroxidation to remove hydrogen atoms from polyunsaturated fatty acids, the basis of the phospholipids in the cell membranes of cytoplasmic organelles, as well as the nuclear membrane. Next, hydrogen atoms react with peroxy...
Figure 7. Transmission electron micrographs of the astrocytic end feet surrounding capillaries in fibrous astrocytes of the short-(7A) and long-term diabetic (7B) groups. Astrocytic end feet (Asf), lumen of capillary (L), myelinated axon (Ax2), elevated number of cross-sectioned glial fibrils (white arrowheads), enlarged mitochondria (black arrowheads), disrupted astrocytic end feet with a clear area and loss of organelles (black star), and a nucleus of a fibrous astrocyte (Nu).

radicals to form hydroperoxides, which cause a disorganized membrane and deterioration of the pores crossing the double phospholipid layers. This results in increased permeability of cell membranes in cytoplasmic organelles and nuclei (Lipinski, 2001). Then, intracellular water freely enters into cytoplasmic organelles and nuclei to cause swelling of these structures. Furthermore, V-akt murine thymoma viral oncogene homologue activation and AMP-activated protein kinase inhibition during hyperglycemia stimulate mammalian target of rapamycin complex 1 (mTORC1) activity. Activation of mTORC1 directly phosphorylates protein translation p70S6 kinase and 4E binding protein 1. Next, these two regulators activate mRNA translation and protein synthesis, which leads to elevated polysomes in both types of astrocytes (Codeluppi et al., 2009; Lieberthal and Levine, 2012; Lv et al., 2014). Consequently, the swollen cytoplasm, cytoplasmic organelles, and nucleus, as well as the increased polysomes, enhance the hypertrophy of both types of astrocytes during short-term diabetes.

In short-term DM, glial fibrillary acidic protein (GFAP) was increased in the astrocytic processes of both types of astrocytes, similar to the finding of a previous study (Lebed et al., 2008). Liao et al. (2011) found that GFAP and interleukin (IL)-1β expressions in astrocytes, as well as the levels of phosphorylated NR1, a subunit of N-methyl-D-aspartic acid receptors (NMDARs) on neurons, are significantly higher in db/db mice than in db/+ mice. It has been indicated that astrocytic activation and increased IL-1β expression are crucial factors to induce mechanical allodynia in the spinal dorsal horn. Moreover, IL-1β may enhance spinal NMDAR phosphorylation in neurons to stimulate neuropathic pain. Consequently, the astrocytic activation in the cervical enlargement of short-term DM model animals in this study may be associated with the hypersensitization in the hands and arms of diabetic patients.

Glucose can react with proteins to generate advanced glycation end products (AGE) in the endothelium. Subsequently, increased AGE stimulates matrix metalloproteinase 9 (MMP-9) to degrade myelin basic protein, leading to demyelination (Chandler et al., 1995; Chen et al., 2011). Next, the debris from myelin lamellae activates microglia to secrete interleukin-1, which promotes astrocytic activation (Liu et al., 2011). Activated astrocytes produce and secrete ciliary neurotrophic factor (CNTF) (Clatterbuck et al., 1996; Hudgins and Levison, 1998). Then, CNTF activates the JAK pathway and induces myelin-specific genes to promote synthesis of myelin sheath in oligodendrocytes (Stankoff et al., 2002). Recently, it was shown that GFAP plays a role in the transportation of intracellular vesicles, such as those containing glutamate (Potokar et al., 2005; Kreft et al., 2009). The glutamate in the extracellular space is imported into astrocytes by binding of vesicles to two glial glutamate transporters: glutamate transporter 1 (GLT-1) and glutamate aspartate transporter (GLAST) (Danbolt, 2001). During short-term diabetes, an increase in extracellular glutamate concentration occurs, as well as increased expression of GFAP, GLT-1, and GLAST in astrocytes (Ward et al., 2005; Coleman et al., 2010). These findings suggest that the extracellular glutamate is carried into astrocytes by increased affinities for GLT-1 and GLAST. It may be possible that the increased GFAP in astrocytes during short-term DM functions in the transportation of glutamate vesicles in cells. Thus, the increased extracellular glutamate uptake into astrocytes might protect neurons from glutamate toxicity in short-
term diabetic models.

A large glycogen cluster was found in the cytoplasm of astrocytic end feet in the short-term diabetic rats. This result was supported by the research of Alaraj et al. (2004). They found clusters of glycogen granules in the brains of diabetic astrocytes. During hyperglycemia, an increase in the level of glucose transporter (GLUT) 1 is found in cerebral microvessels (Vannucci et al., 1997) and pyramidal and granule neurons in the hippocampus (Reagan et al., 2001). A similar process may occur in astrocytes of spinal cord during short-term DM. Thus, a high level of glucose is uptaken into astrocytes by binding to GLUT1. This leads to elevated synthesis of glycogen in astrocytes. An increase in the inactive form of glycogen synthase kinase 3 (GSK3) is observed during DM. Normally, active GSK3 inhibits glycogen synthase activity, leading to a decrease in glycogen synthesis. Therefore, decreased active GSK3 results in an increase in the inactive form GSK3, contributing to glycogen accumulation in astrocytes during diabetes (Khandelwal et al., 1979).

Increased heterochromatin condensation in the nuclei of both types of the astrocytes was observed in long-term DM models. ROS, which are elevated during diabetes, attack to protein thiol groups in the pores of the mitochondrial membrane, which causes the loss of mitochondrial membrane potential and depletion of ATP. This leads to increased mitochondrial permeability (Batandier et al., 2004). Then, apoptosis-inducing factor in the mitochondrial intermembrane space is translocated into the nucleus via independent caspase activation to induce large-scale DNA fragmentation and increased condensation of heterochromatin, resulting in astrocytic apoptosis (Daugas et al., 2000).

Decreased levels of GFAP in protoplasmic and fibrous astrocytes were found in long-term DM animals. The increased permeability of the cell membranes of cytoplasmic organelles, including the rER, Golgi complex, and mitochondria, as reported earlier, stimulates the release of calcium ions (Ca$^{2+}$) from intracellular stores in these organelles to the cytoplasm (Dolman and Tepikin, 2006). Then, the increased Ca$^{2+}$ stimulates S100B, a Ca$^{2+}$ receptor protein, to bind with phosphorylation sites on GFAP protein to block access to these sites for protein kinases. Ultimately, this leads to inhibition of GFAP polymerization to cause decreased GFAP in astrocytes (Frisco et al., 2004). Furthermore, elevated accumulation of glutamate in the extracellular space and low levels of GFAP are observed in long-term diabetic models (Li et al., 2000). GLT-1 and GLAST levels are decreased in GFAP-/- mice (Hughes et al., 2004; Sullivan et al., 2007; Nagayach et al., 2014). Low levels of GFAP and glutamate receptors may lead to lower levels of transportation of glutamate into astrocytes, which would cause increased accumulation of glutamate in the extracellular space. As a result, the increased glutamate in postsynaptic neurons leads to neuronal injury in long-term diabetes (Li et al., 2000). Therefore, the astrocytic damage in chronic hyperglycemia means that astrocytes cannot maintain functional and structural support for neurons, and finally, neurons in the cervical enlargement become injured and this affects the nerve supply to the hands and arms. As a result, the loss of sensation in the hands and arms, as well as the muscle weakness in the hands of diabetic patients, is progressive.

Conclusively, the results indicate that diabetes has effects on protoplasmic and fibrous astrocytes in both the short and long terms. The first detection of destruction of these cells might consequently be related to dysfunction of neurons, that finally causes the neuropathy in the diabetes. Therefore, the early detection of the types of cells can help to prognosis the symptoms of neuropathy.

**Conflict of interests**

The authors have not declared any conflict of interests.

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