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Kanokporn Plaengrit

Sirinush Sricharoenvej

Passara Lanlua

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Kanokporn Plaengrit*

Sirinush Sricharoenvej* Passara Lanlua*

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Background : *Diabetic microangiopathy strongly associates with the developing complication of the nervous system, as in the spinal cord. The developments of spinal cord infarction, which is relevant to physical mobility impairment and loss of sensation in diabetic patients, have been reported. Thus, an insight investigation on spinal microvascular changes needed to be concentrated.*

Objective : *This study aimed to investigate the alterations of spinal capillaries in streptozotocin (STZ)-induced diabetic rats by using light microscopy (LM) and transmission electron microscopy (TEM).*

Methods : *Seventeen male Sprague-Dawley rats, 200 - 270 g, 5-8 weeks, were used. The animals were divided into two groups, namely: control and STZ-induced diabetic groups. All rats were sacrificed at 24 weeks after the induction, and the rat spinal cords were removed and prepared for LM and TEM.*

Results : *During the diabetic stage, the characteristics of spinal capillaries were the same in all spinal cord levels. The several organelle deteriorations, indicating apoptotic features, were presented in both endothelial cells and pericytes. The cytoplasmic protrusion and disrupted tight junction of endothelial cells were observed. Remarkably, the basement membrane was marked thickness with increased collagen deposition in the diabetic vessel.*

*Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University

Conclusion : *This study indicates that diabetes induced morphological changes of the spinal capillaries. This knowledge is beneficial for early detection and prevention of further pathological progression in diabetic patients.*

Keywords : *Diabetic microangiopathy, spinal capillary, streptozotocin.*

Correspondence to: Sricharoenvej S. Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
E-mail: sirinush.sri@mahidol.ac.th

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เหตุผลของการทำวิจัย : *ภาวะแทรกซ้อนของเบาหวานในหลอดเลือดขนาดเล็ก มีความสัมพันธ์กับการพัฒนาไปสู่ภาวะแทรกซ้อนของระบบประสาท ดังเช่นในไขสันหลัง โดยมีรายงานการเกิดเนื้อไขสันหลังตาย ซึ่งนำไปสู่ความบกพร่องของระบบประสาทสั่งการ และระบบรับสัมผัสของร่างกาย ดังนั้นความเข้าใจถึงการเปลี่ยนแปลงที่เกิดขึ้น กับหลอดเลือดฝอยในไขสันหลังจึงจำเป็นอย่างยิ่ง*

วัตถุประสงค์ : *การศึกษานี้ต้องการตรวจสอบการเปลี่ยนแปลงของหลอดเลือดฝอยในไขสันหลังของหนูเบาหวานที่ถูกเหนี่ยวนำด้วยสาร streptozotocin (STZ) ภายใต้กล้อง light microscope (LM) และ transmission electron microscope (TEM)*

วิธีการทำวิจัย : *การวิจัยครั้งนี้ใช้หนู Sprague-Dawley เพศผู้ อายุ 5 - 8 สัปดาห์ น้ำหนักเฉลี่ย 200 - 270 กรัม โดยหนูจะถูกแบ่งออกเป็น 2 กลุ่ม ได้แก่ กลุ่มควบคุมและกลุ่มเบาหวานที่ถูกเหนี่ยวนำด้วยสาร STZ การเก็บตัวอย่างไขสันหลังจากหนูทั้ง 2 กลุ่ม เก็บในสัปดาห์ที่ 24 หลังจากการเหนี่ยวนำ และนำมาเตรียมชิ้นเนื้อเพื่อศึกษาภายใต้กล้อง LM และ TEM*

ผลการศึกษา : *ในภาวะเบาหวานพบว่ามีการเปลี่ยนแปลงของหลอดเลือดฝอยที่เหมือนกันในทุกระดับของไขสันหลัง โดยพบการเสื่อมสลายของโครงสร้างต่าง ๆ ภายในเซลล์ที่บ่งชี้ถึงการตายของ endothelial cell และ pericyte นอกจากนี้ยังพบการยื่นของ cytoplasm และการแยกตัวของ tight junction ใน endothelial cell และมีการหนาตัวของ basement membrane จากการสะสมเส้นใย collagen*

สรุป : *การศึกษาครั้งนี้ พบว่าภาวะเบาหวานเหนี่ยวนำให้เกิดการเปลี่ยนแปลงของหลอดเลือดฝอยในไขสันหลังของหนู ซึ่งจะเป็นความรู้พื้นฐานที่ประโยชน์สำหรับการประเมินเบื้องต้นในผู้ป่วยเบาหวานเพื่อป้องกันพยาธิสภาพที่รุนแรงขึ้นจากการดำเนินของโรค*

คำสำคัญ : *ภาวะแทรกซ้อนของเบาหวานในหลอดเลือดขนาดเล็ก, หลอดเลือดฝอยในไขสันหลังของหนู, สาร streptozotocin.*

A condition with hyperglycemia is called diabetes mellitus (DM), and the uncontrolled high glucose can contribute to the earliest complications of diabetic microangiopathy and diabetic neuropathy. Up to date, numerous evidences support the association of diabetic microangiopathy and diabetic neuropathy. Many studies concentrate mainly on lesions of the capillaries in the peripheral nerves and the brain in DM, without the spinal capillaries. [1, 2] The spinal cord, as a part of the central nervous system, is the main route to conduct sensory impulses from the peripheral organs to the brain and return of the motor signals to other organs of the body. Long-term type 2 diabetic patients suffer from severe deficit symptoms; moreover, there is the biphasic spinal cord infarction in thoracic level after medical imaging. Additionally, the infarct lesions of the spinal cord are found in young age type 1 DM with severe neurological symptoms, such as bilateral weakness, abnormalities and losses of sensation, and impaired anal sphincter control. [3, 4] Furthermore, the capillaries of the spinal cord consist of non-fenestrated endothelium linked with tight junctions, basement membrane, pericytes, and astrocytic foot processes. Its function acts as blood spinal cord barrier (BSCB) to provide proper homeostasis in the spinal cord. In the diabetes, however, tight junctions of BSCB have been altered in terms of protein composition and integrity. Therefore, alterations of spinal microvessel can lead to abnormal functions of the nerve cells, glial cells, and nerve fibers that lead to spinal cord injury and poor clinical outcomes. [5] Although many evidences support that diabetes is strongly related to microvascular abnormalities in the spinal cord, nevertheless, few studies have insight investigations on their morphological aspects of spinal microvessels. Therefore, the aim of this study was to investigate the alterations of spinal capillaries in streptozotocin (STZ)-induced diabetic rats by using light microscopy (LM) and transmission electron microscopy (TEM) in order to provide basic knowledge for clarifying and understanding of the relationship between diabetic vascular complications and neuropathy, which leads to early detection and prevention of further pathological progression in the diabetic patients.

Methods

Seventeen male Sprague-Dawley rats, 200 - 270 g, 5 - 8 weeks, were used. These animals were randomized from the National Laboratory Animal Center, Mahidol University, Thailand. This study was performed in accordance to the Guide for the Care and Use of Laboratory Animals by the National Research Council. The animals were divided into two groups, namely control (n = 6; LM = 3, TEM = 3) and diabetic (n = 11; LM = 4, TEM = 7) groups. All rats were sacrificed at 24 weeks after the induction, and their spinal cords were removed and cut into three levels: cervical enlargements, thoracic segments, and lumbosacral enlargements. According to the preparation, induction for DM, LM and TEM, the processes were done as in other studies. [6]

Results

Histological study of the spinal capillary

The characteristics of spinal cords in both the control and diabetic groups were same at all levels, i.e., cervical enlargements, thoracic segments, and lumbosacral enlargements. In normal condition, the spinal cord can be divided into the gray and white matter. The spinal capillaries traveled in both areas to supply the spinal cord. The circular lumen of spinal capillary was lined by a single layered-endothelial

cells, containing a basophilic flattened nucleus. In addition, a pericyte with a prominent nucleus directly contacted with the endothelial cell in the capillary (Figures 1A, 1C). In diabetic rats, however, the endothelial nuclei were marked by dark basophilic staining. The pericytes began to shrink. Their nuclei reduced in size as slender elongated-shape with pyknotic characterizing (Figures 1B, 1D). Additionally, the capillaries became irregular and unclear lumen, surrounded with coarse spinal neuropils in the gray matter (Figure 1B) and disorganized nerve fibers in the white matter (Figure 1D); therefore, clear areas were obviously seen in both matters of diabetic rats, compared to the control (Figures 1A-D).

Ultrastructural study of the spinal capillary

In the control group, the endothelial cell contained a flattened nucleus, enclosing a spherical capillary lumen (Figure 2A). It was completely encircled by basement membrane. Many organelles were located in the cytoplasm, such as mitochondria and slim cistern of rough endoplasmic reticulum (rER) with the dark spots of the ribosomes (Figure 3A). In the diabetic rats, however, the spinal capillary had an irregular shape lumen (Figure 2B). The endothelial cells showed apoptotic features, such as chromatin condensation, small mitochondria with few cristae and electron dense matrix (Figure 3B) as well as dilated rER (Figure 3C). Moreover, the phagolysosome formation was observed nearly a group of lysosomes which formed a large sphere with dense materials (Figure 3D). Numerous pinocytic vesicles were found at the rim around the lumen (Figure 3E) and the cytoplasmic protrusions were also observed at the luminal side of diabetic endothelial cells (Figures 2B, 3F).

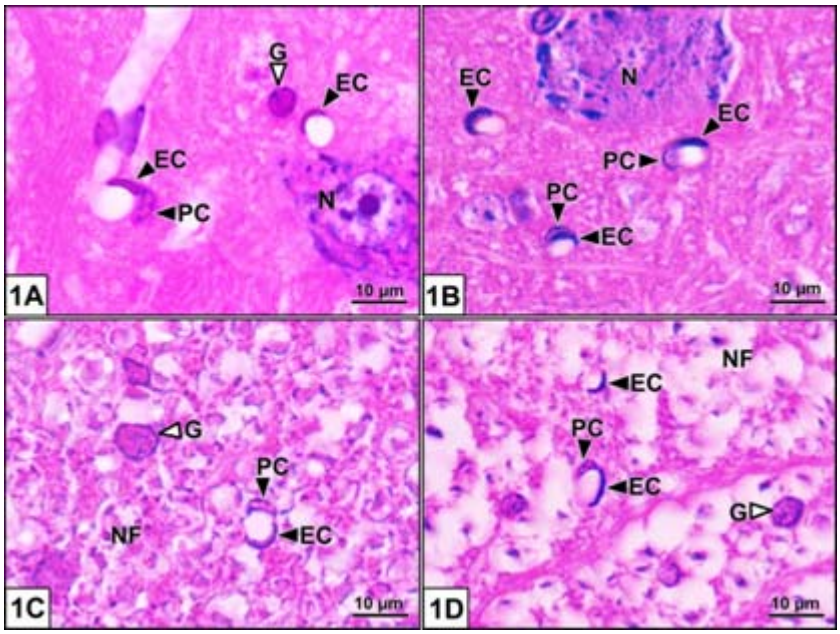

Figure 1. Light micrographs of the spinal capillaries in gray (1A, 1B) and white matters (1C, 1D) of spinal cord from control (1A, 1C) and diabetic (1B, 1D) rats. Endothelial cell (EC), pericyte (PC), neuron (N), neuroglia (G), and nerve fiber (NF).

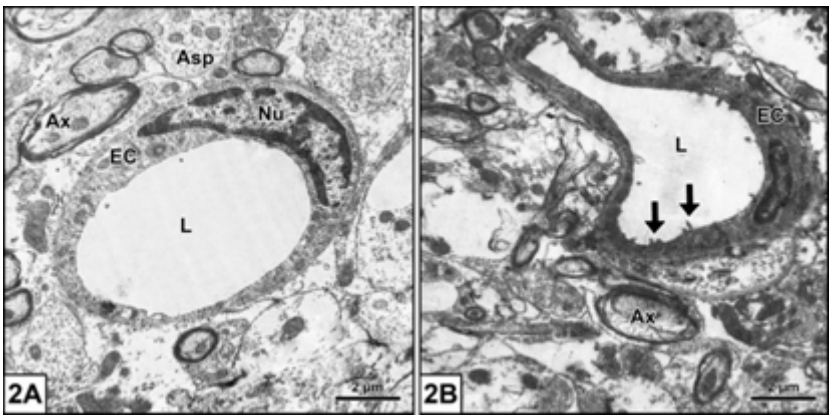

Figure 2. TEM images of spinal capillaries in control (2A) and diabetic (2B) rats. A single layered-endothelial cell (EC), nucleus (Nu), capillary lumen (L), axon (Ax), astrocytic foot process (Asp), cytoplasmic protrusion (black arrows).

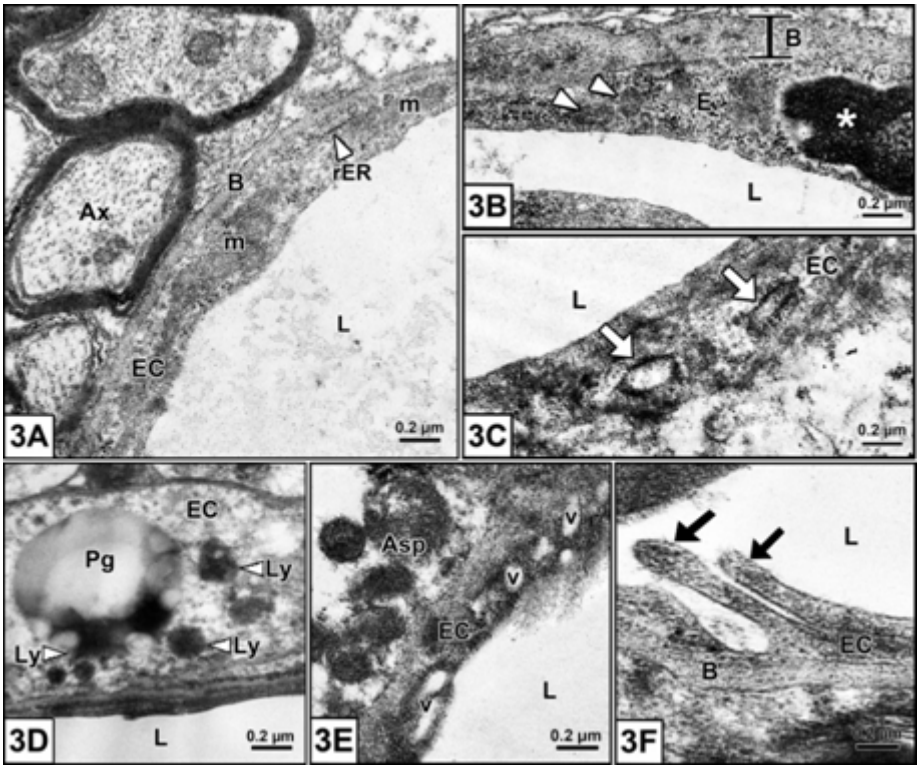

Figure 3. Ultrastructural analysis of endothelial cells in spinal capillaries of control (3A) and diabetic (3B-F) rats. Endothelial cell (EC), mitochondria (m), rough endoplasmic recticulum (rER), basement membrane (B), capillary lumen (L), axon (Ax), nuclear chromatin clumping (a white asterisk), small mitochondria with dense matrix (white arrowheads), capillary basement membrane thickening (I B), dilated rER (white arrows), phagolysosome (Pg), a group of lysosomes (Ly), a lot of vesicles (v), astrocytic foot process (Asp), and endothelial cytoplasmic protrusions (black arrows).

Normally, the basement membrane was seen as a thin fibrillar layer on the abluminal aspect of the endothelial cell (Figure 4A). In the diabetic rats, the basement membrane was marked thickness with increased collagen deposition. The large perivascular space was observed close to the unclear border of thickened basement membrane (Figure 4B). On the abluminal surface, the endothelial protrusions were also found in the diabetic capillary as a finger-liked structure. Moreover, the astrocytic foot processes surrounding the capillary underwent degeneration that were manifested by decreased glial fibrils, loss of organelles and large clear area (Figure 4C).

Concerning to the tight junction, it appeared in the control as the electron-dense area on the inner surface of endothelial plasma membranes to perfectly seal the paracellular clefts between the adjacent capillary endothelial cells (Figure 5A). In the diabetic rats, however, there was disrupted tight junction at the endothelial corner as evidenced by wide gap within the tight junction strand (Figure 5B). Furthermore, the electron-dense materials, that presented in the area of disruption, decreased (Figure 5C), compared with normal tight junctions.

The pericytes located on the abluminal side of endothelial cells to share a basement membrane reciprocally (Figures 6A-D). Normal pericytes contain many basic organelles, such as a prominent round nucleus, mitochondria, and microfilaments stretching along the cytoplasmic extension (Figure 6C). Remarkable changes of a pericyte also appeared in the diabetic rats. Their nuclear chromatin was denser than those in the control (Figures 6B, 6D). Moreover, the cytoplasmic area in a pericyte increased and contained large clear area with only few organelles (Figure 6D).

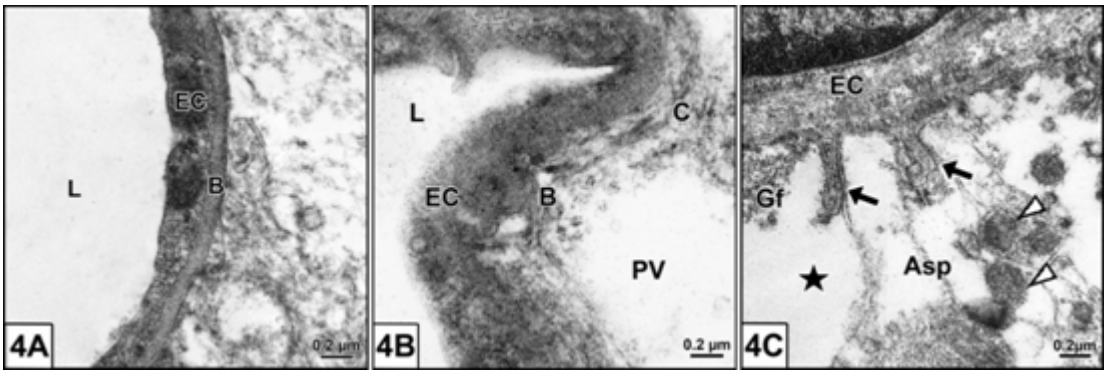

Figure 4. Transmission electron micrographs of spinal capillaries at abluminal side in control (4A) and diabetic (4B, 4C) rats. Endothelial cell (EC), basement membrane (B), capillary lumen (L), accumulation of the collagen fibers (C), a large perivascular space (PV), endothelial cytoplasmic protrusions at the abluminal surface of capillary (black arrows), astrocytic foot process (Asp), a large cytoplasmic clear area of astrocyte (a black star), glial fibrils (Gf), and small dense mitochondria (white arrowheads).

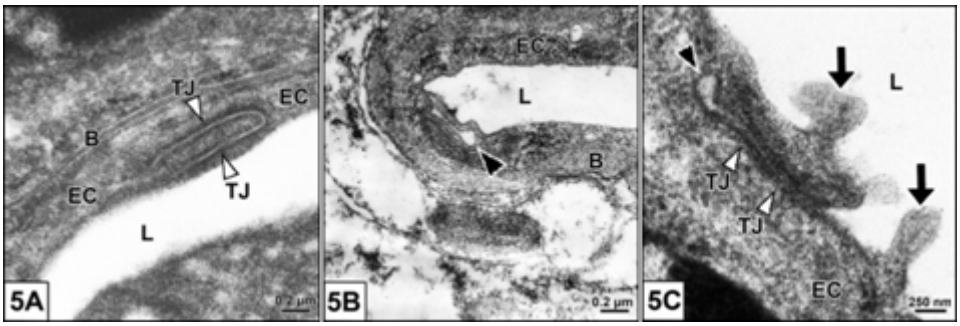

Figure 5. Transmission electron micrographs of tight junctions between endothelial cells of spinal capillary in control (5A) and diabetic (5B, 5C) rats. Tight junction (TJ), endothelial cell (EC), basement membrane (B), capillary lumen (L), a reduction of electron dense and detachment of tight junction (black arrowheads), and cytoplasmic protrusions of endothelial cell (black arrows).

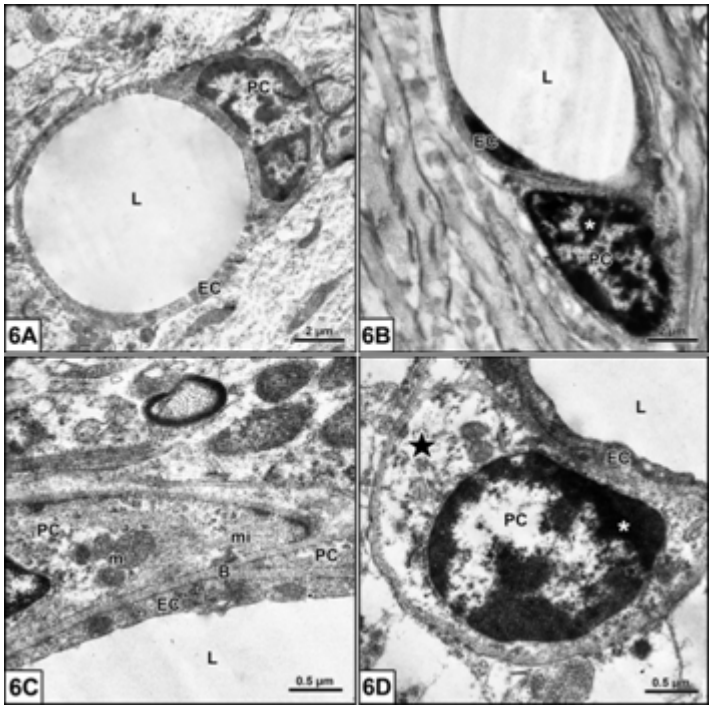

Figure 6. Transmission electron micrographs of pericytes in control (6A, 6C) and diabetic (6B, 6D) rats. Pericyte (PC), lumen (L), endothelial cell (EC), condensation of nuclear chromatin in the pericyte (white asterisks), basement membrane (B), mitochondria (m), microfilaments (mi), and swollen cytoplasm with numerous clear areas and destroyed organelles (a black star).

Discussion

After prolonged diabetic condition, there were changes in the capillaries of both the gray and white matters at all levels of the spinal cord: apoptotic endothelial cells, endothelial cytoplasmic protrusion, disrupted tight junction, and basement membrane with increased collagen deposition. The features of apoptotic cells were chromatin condensation, increased numbers of vesicles/vacuoles, phagolysosome, and destructive cell organelles. These events can be described by high calcium (Ca^{2+}) level in endothelial cells and pericytes. When the glucose level increases, free radicals, such as reactive oxygen species (ROS), are generated via multiple pathways. [7] After that, ROS causes oxidative degradation of unsaturated fatty acid in lipid peroxidation. As a result, the cell membrane and organelles are damaged. Because of impaired membrane of rER, intracellular Ca^{2+} leaks into the cytosol. Moreover, the damaged cell membrane leads to extracellular ion and fluid influx via numerous vesicles and vacuoles, which also add up to the intracellular Ca^{2+} concentration. High Ca^{2+}level induces cytochrome C to be released from mitochondria via calpain activation. The cytochrome C binds to apoptotic protease activating factor 1 to form apoptosome. Next, caspase 9 and 3 are respectively activated to stimulate caspase-activated DNase. Finally, DNA fragmentation occurs, that has been seen as chromatin condensation. Additionally, activated caspase 3 also upregulates protease to destroy cell organelles, which are fused with lysosomes to be phagolysosome. [8, 9] Finally, the apoptotic cell death in endothelial cells and pericytes induces as described above.

In a diabetic capillary, it was suggested that the endothelial cytoplasmic protrusions in both luminal and abluminal sides, indicating as a part of angiogenesis. In the DM, ROS can activate protein kinase C (PKC) pathway in hypoxic cells, in order to generate hypoxia-induced factor (HIF) 1α protein, which induce vascular endothelial growth factor (VEGF) released from neurons, glial cells, and vascular cells. [10] Next, VEGF being bound to its receptor on the endothelial cell can stimulate phosphoinositide 3 kinase via cell division control protein 42. Accordingly, the neuronal Wiskott-Aldrich syndrome protein is activated and binds to actin-related protein to induce actin polymerization for cytoplasmic protrusion during angiogenesis. [11]

Disrupted endothelial tight junction occurred in the diabetic spinal capillary, suggesting a reduction of transmembrane tight junction proteins. It was described before that hyperglycemia during diabetes causes increased VEGF and Ca^{2+} in endothelial cell. The VEGF activates PKC, moreover high Ca^{2+} undergoes c-Jun N-terminal kinase 2-c-Src signaling pathway. These two pathways promote tyrosine phosphorylation on occludin and zonula occludens (ZO), which are components of tight junction proteins. As a result, disassembly of endothelial tight junction is established by occludin internalization and prevents the binding of ZO to c-terminal tail of occluding. [12, 13] All these lead to the reduction of tight junction complexity causing the separation of tight junctions.

The thickening of basement membrane with collagen fiber deposition in the diabetic spinal capillary was demonstrated. In diabetic stage, transforming growth factor beta (TGF-β1) released from vascular cells and glial cells in interleukin-1-activated nuclear

factor kappa beta pathway. [14] TGF-β1 undergoes the pathway of Smad 3/Smad 4 complex that is translocated to the nucleus. Consequently, increased tissue inhibitors of metalloproteinases and decreased matrix metalloproteinases, extracellular matrix (ECM) degrading enzyme, prevent ECM degradation. [15] Therefore, ECM accumulation occurred in diabetic vessels.

Conclusion

Diabetic patients become neuropathy, which not only the neurons and glial cells but also capillary are destroyed. The features of capillaries were apoptotic endothelial cells with thick basement membrane, which cause hypoxia in the diabetic spinal cord. However, some endothelium became sprouting to maintain its function.

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