—**Original**—

Localization of Extracellular Matrix and Mitogen-Activated Protein Kinase (MAPK) in Aorta of Streptozotocin Treated *Mongolian* Gerbils

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Abstract

To evaluate the relationship among the extracellular matrix (ECM) and mitogen-activated protein kinase (MAPK) family for the vascular damages in hyperglycemia, we injected *Mongolian* gerbils intravenously with 150 mg/kg streptozotocin (STZ) and observed over the next one year the resulting aortic changes by immunohistochemical techniques.

After STZ treatment, hyperglycemia was confirmed. At 4 weeks after STZ administration morphological observation revealed increased stromal components among the vascular smooth muscle cells (SMCs). Immunohistochemically, extracellular matrices such as fibronectin and laminin were localized in the aorta at 4 weeks and one year after STZ administration. The reaction products of MAPK in vascular SMCs were more increased at one year than at 4 weeks after STZ administration.

After STZ administration, the increase of ECM and MAPK was observed in the aorta, which suggests these factors play important roles in the pathogenesis of macrovasculopathy in diabetes mellitus. (J Nippon Med Sch 2001; 68: 37—44)

Key words: fibronectin, laminin, mitogen-activated protein kinase (MAPK), macrovasculopathy, *Mongolian* gerbil

Introduction

Streptozotocin (STZ)-treated animal reveals hyperglycemia and have been used as a model for insulin-dependent diabetes mellitus[1]. As far as we know, we investigated vasculopathy in STZ-treated Mongolian gerbils for the first time[2,3].

Relative or absolute increase of advanced glycation end products (AGEs) and the receptor of AGEs (RAGE) after STZ treatment is considered to contribute to the vasculopathy associated with early diabetic vascular dysfunction[1,4]. AGEs are found not only in the extracellular matrix (ECM) but also in the foam cells originating from macrophages, endothelial cells and smooth muscle cells[5].

On the other hand, ECM such as fibronectin seems to play an important role in promoting the characteristic changes of vascular smooth muscle cells under persistent hyperglycemia[6]. And in the overproduction of fibronectin the mitogen activated protein kinase (MAPK) is considered to play a key role[7]. Transforming growth factor-beta (TGF-β) and epidermal growth factor (EGF) seem to stimulate fibronectin production through the intracellular signaling protein MAPK[8]. At the same time fibronectin is considered to induce the

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activation of MAPKs[9,10]. And a complex comprising laminin-5 and α3β1 integrin is multifunctional and contributes to the regulation of cell growth via a signaling pathway involving MAPK[11].

This study was undertaken to clarify the roles of ECM and MAPK in the pathogenesis of vasculopathy in STZ treated *Mongolian* gerbils.

Materials and Methods

This experimental study was approved by the Animal Experimental Committee of Nippon Medical School.

(1) Animals

Thirty *Mongolian* gerbils (10～12 weeks old) were injected with 150 mg/kg streptozotocin (STZ) into left femoral vein. Nine control *Mongolian* gerbils (10～12 weeks old) were injected intravenously with normal saline. The STZ treated animals showed hyperglycemia by 3 days after STZ administration.

Eighteen of the animals, including controls, were sacrificed at various time points (one week, 4 weeks and one year) after STZ or normal saline administration.

(2) Body weight and blood assay

Body weight was measured by ISHIDA CB-600 (Ishida Co, Japan). Blood sample was taken from tail of *Mongolian* gerbils and blood sugar concentration was measured by TIDEX (Bayer-Sankyo Co, Japan), the principle of which is glucose oxidase method. Blood sugar concentration was indicated as mean ± standard deviation.

(3) Specimen preparation

Aortic changes were observed chronologically by light microscopical techniques.

The aortic tissues were extirpated from the portions of thoracic aorta transected at the level the sixth costal artery branches out. Then they were immediately cut into small pieces and fixed in 4% paraformaldehyde solution for 6 hours at 4℃, embedded in paraffin, sectioned and stained with hematoxylin and eosin and Periodic Acid Shiff (PAS) for light microscopical observation.

For immunohistochemical observation, specimens were fixed in 4% paraformaldehyde solution for 6 hours at 4℃ and endogenous peroxidase was inhibited by treatment with 0.3% H_2O_2 in 100% methanol. The following antibodies diluted with phosphate buffer solution were used. ERK (extracellular responsive kinase), JNK (c-jun N-terminal kinase) and p38 (p38 mitogen-activated protein kinase) are mitogen-activated protein kinase (MAPK) family.

1) *Monoclonal* rabbit anti-fibronectin antibody (DAKO, Kyoto, Japan) at a dilution 1: 500, 2) Polyclonal rabbit anti-laminin antibody (E-Y Laboratories Inc. ,San Mateo, CA, USA) at a dilution 1: 200, 3) Monoclonal mouse anti-phosphorylated ERK antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution 1: 500, 4) Monoclonal mouse anti-phosphorylated JNK antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution 1: 800, 5) Monoclonal mouse anti-phosphorylated p38 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution 1: 300.

Sections were incubated with the primary antibodies in a moist chamber overnight at 4℃.

After incubation with the primary antibody, the sections were treated with affinity-purified peroxidase-conjugated F $(ab')_2$ fragment of goat anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc. ,West Grove, PA, USA) diluted 1: 50 for the polyclonal primary antibody and with peroxidase-conjugated F $(ab')_2$ fragment of goat anti-mouse IgG (DAKO, Kyoto, Japan) diluted 1: 1000 dilution for the monoclonal primary antibody for 30 minutes. The sections were then reacted with 0.02% diaminobenzidine tetrahydro-chloride containing 0.05% H_2O_2 for 5 min, counterstained with hematoxylin for 1 min, dehydrated, cleared and permanently mounted.

As a negative control for the immunohistochemical staining, tissue sections were treated with normal rabbit serum instead of a primary antibody.

Results

1. Body weight and blood assay

In the streptozotocin (STZ) treated *Mongolian* gerbils, body weight transiently decreased and blood sugar increased. The STZ treated animals showed hy-

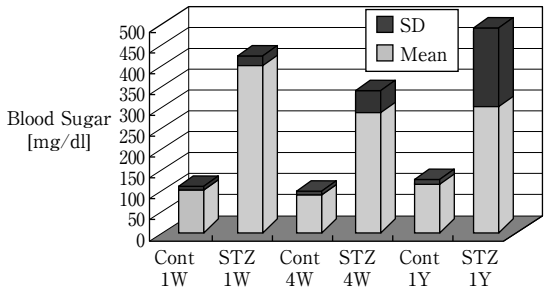

Fig. 1 Blood assay. The streptozotocin (STZ) administered *Mongolian* gerbils shows hyperglycemia by 3 days after STZ administration. The blood sugar consentration of STZ treated animals at one week, 4 weeks, and one year are 402.7 ± 22.2, 291.0 ± 51.0, and 304.0 ± 189.1 [mg/dl], respectively. However, control value at one week, 4 weeks, one year are 103.3 ± 8.3, 90.7 ± 9.0, and 118.3 ± 11.1 [mg/dl], respectively.

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2. Morphological observation

Light microscopically, no significant changes were observed in the endothelial and medial cells of the aorta in control *Mongolian* gerbils at one week after normal saline administration. At 4 weeks after normal saline administration, flattened endothelial cells and slight anisocytosis of smooth muscle cells were observed (**Fig. 2 a**). At 4 weeks after streptozotocin (STZ) administration, mild hypertrophy of endothelial cells and anisocytosis of swelled medial smooth muscle cells in the aortic wall where stromal components were slightly increased (**Fig. 2 b**). At one year after STZ administration, anisocytosis was markedly observed in the hypertrophic endothelial cells and smooth muscle cells of the aortic wall in which stromal components were increased (**Fig. 2 c**).

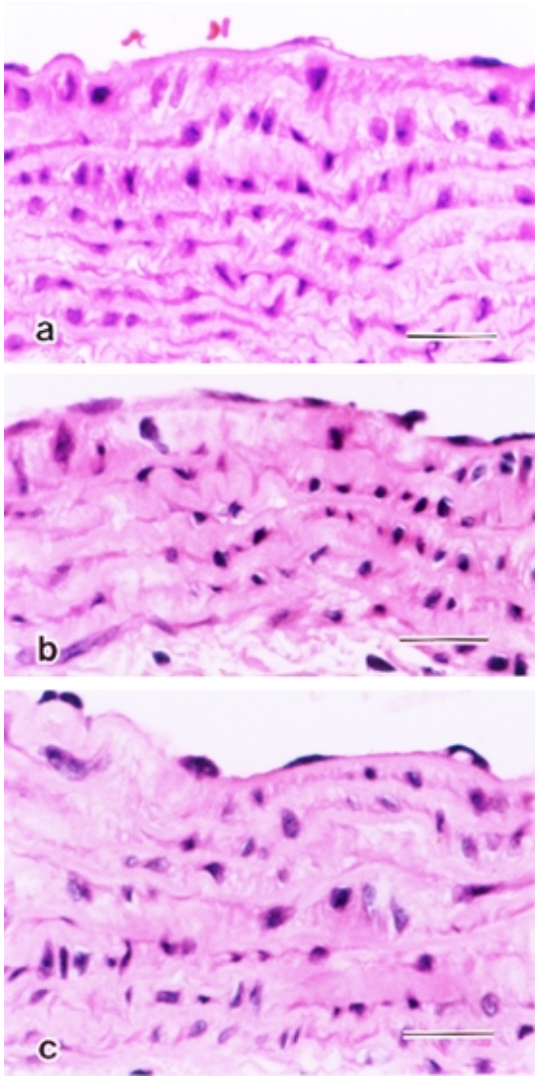

Fig. 2 Light micrographs of sections stained with hematoxylin and eosin in aorta from control and streptozotocin (STZ) administered *Mongolian* gerbils. a: At 4 weeks after normal saline administration, flattened endothelial cells and slight anisocytosis of smooth muscle cells are observed in the aortic wall. Magnification, ×200, Bar=20 μm. b: At 4 weeks after STZ administration, hypertrophy of endothelial cells, and anisocytosis of swelled smooth muscle cells are observed in the aortic wall where stromal components were slightly increased. Magnification, ×200, Bar=20 μm. c: At one year after STZ administration, anisocytosis is markedly observed in the endothelial cells and medial cells in the aortic wall. Magnification, ×200, Bar=20 μm

3. Immunohistochemical observation

In control animals at one week, 4 weeks and one year after normal saline administration and STZ-treated animals at one week, fibronectin was weakly localized in the pericellular spaces of aorta (**Fig. 3 a**). At 4 weeks and one year after STZ treatment fibronectin was irregularly localized in the pericellular spaces including endothelial cells, especially on elastic

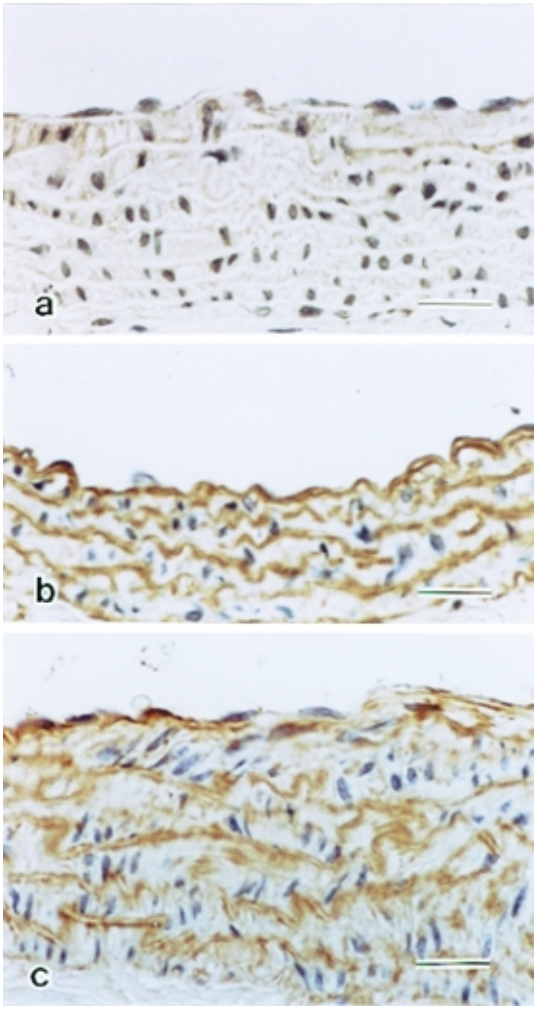

Fig. 3 Immunohistochemical staining for fibronectin in aorta of *Mongolian* gerbils. a: Immunohistochemically fibronectin is faintly localized in the pericellular spaces of medial smooth muscle cells of aorta at 4 weeks of control animals. Indirect method, Magnification, × 100, Bar=40 μm. b: At 4 weeks after STZ treatment the reaction products of fibronectin are irregularly localized in the pericellular spaces including endothelial cells of aorta. Indirect method, Magnification, × 100, Bar=40 μm. c: At one year after STZ administration fibronectin is localized in the endothelial cells and smooth muscle cells including pericellular spaces of aorta. Indirect method, Magnification, × 100, Bar=40 μm.

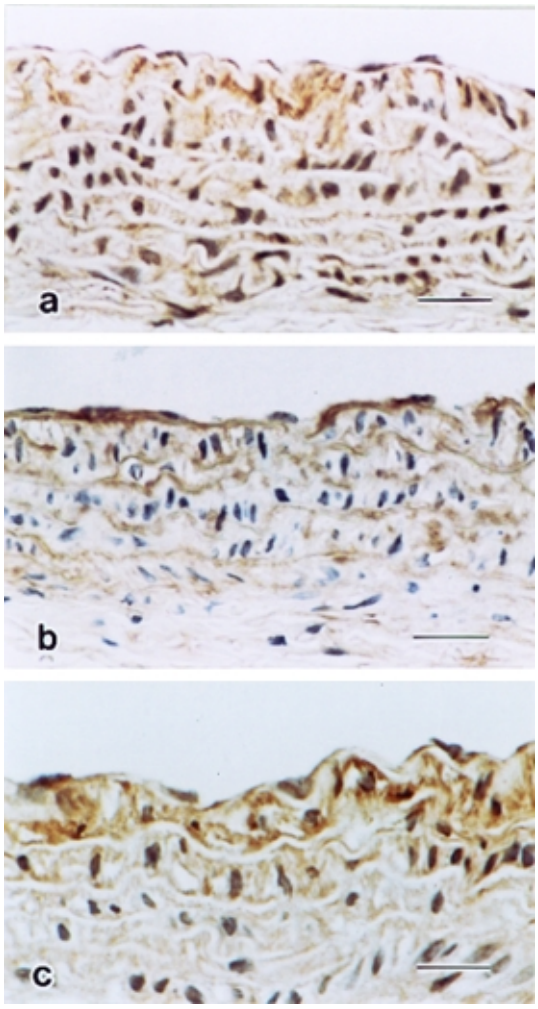

Fig. 4 Immunohistochemical staining for laminin in aorta of *Mongolian* gerbils. a: Immunohistochemically laminin is faintly localized in smooth muscle cells including pericellular spaces of aorta at 4 weeks of control animals. Indirect method, Magnification, × 100, Bar=40 μm. b: At 4 weeks after STZ treatment the reaction products of laminin are localized in the endothelial cells and smooth muscle cells including pericellular spaces of aorta. Indirect method, Magnification, × 100, Bar=40 μm. c: At one year after STZ administration the reaction products of laminin are observed in endothelial cells and smooth muscle cells of aorta. Indirect method, Magnification, × 100, Bar=40 μm.

fibers and in smooth muscle cells of media to compare with control animals (**Fig. 3 b, c**).

At 4 weeks in STZ-administered Mongolian gerbils, immunoreactivity of laminin increased in endothelial cells of aorta in comparison with control animals (**Fig. 4 a, b**). The localization of laminin was much more increased in the intracellular and pericellular spaces of media in aorta at one year than at 4 weeks after STZ treatment (**Fig. 4 c**).

There was no reaction products with anti-phosphorylated ERK in control and STZ treated animals at one week. But in control and STZ treated animals at 4 weeks, a small amount of reaction products with anti-phosphorylated ERK antibody was localized

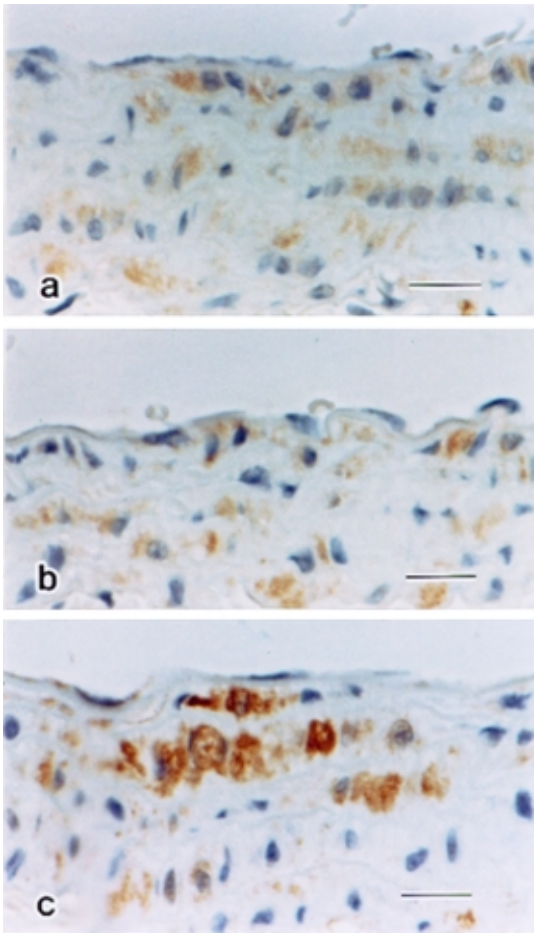

Fig. 5 Immunohistochemical staining for phosphorylated ERK in aorta of *Mongolian* gerbils. a: At 4 weeks of control animals, a small amount of reaction products with anti-phosphorylated ERK antibody is localized in the smooth muscle cells of aorta. Indirect method, Magnification, × 200, Bar=20 μm. b: At 4 weeks after STZ administration, the localization of phosphorylated ERK immunoreactivity is observed in the smooth muscle cells of aorta. Indirect method, Magnification, × 200, Bar=20 μm. c: The reaction products are much more localized in the nuclei and cytoplasm of smooth muscle cells of STZ treated animals at one year than at 4 weeks. Indirect method, Magnification, × 200, Bar=20 μm.

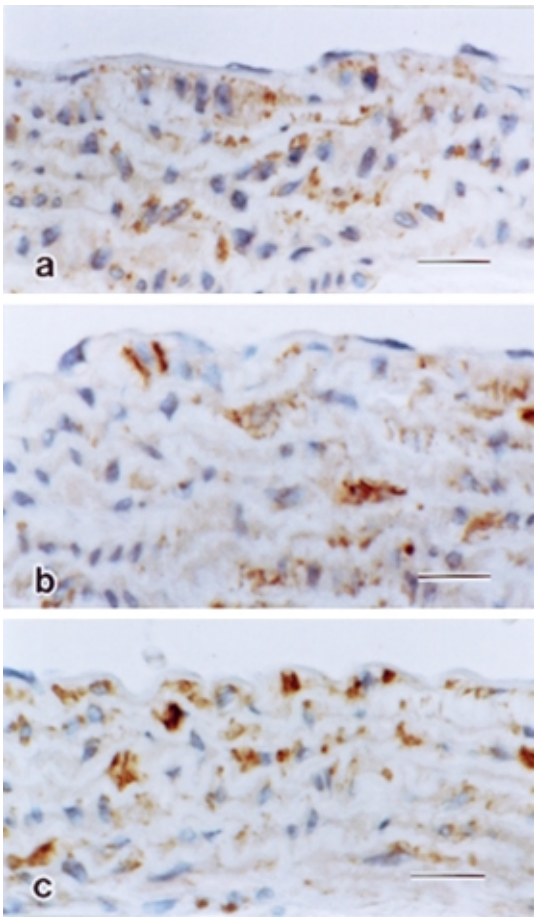

Fig. 6 Immunohistochemical staining for phosphorylated JNK in aorta of *Mongolian* gerbils. a: At 4 weeks of control animals the reaction products with anti- phosphorylated JNK are faintly localized in the smooth muscle cells of aorta. Indirect method, Magnification, × 200, Bar=20 μm. b: At 4 weeks after STZ administration, the localization of phosphorylated JNK immunoreactivity is much more encountered in the smooth muscle cells of aorta in comparison with these cells of control animals. Indirect method, Magnification, × 200, Bar=20 μm. c: In STZ treated animals at one year, the reaction products with anti-phosphorylated JNK are strikingly observed in the nuclei and cytoplasm of smooth muscle cells of aorta. Indirect method, Magnification, × 200, Bar=20 μm.

in medial smooth muscle cells of aorta. At one year after STZ administration, the localization of phosphorylated ERK immunoreactivity was increased in the nuclei and cytoplasm of smooth muscle cells of aorta in comparison to control and STZ treated animals at 4 weeks (**Fig. 5 a, b, c**).

In control and STZ-treated animals at one week, there was no reaction products with anti-phosphorylated JNK antibody in endothelial cells and smooth muscle cells of aorta. However, the irregular localization of phosphorylated JNK immunoreactivity was extensively encountered in the nuclei and cytoplasm of smooth muscle cells of aorta in control animals at 4 weeks, STZ-treated animals at 4 weeks and STZ-treated animals at one year (**Fig. 6 a, b, c**).

There was little reaction product with anti-phosphorylated p 38 antibody in the endothelial cells

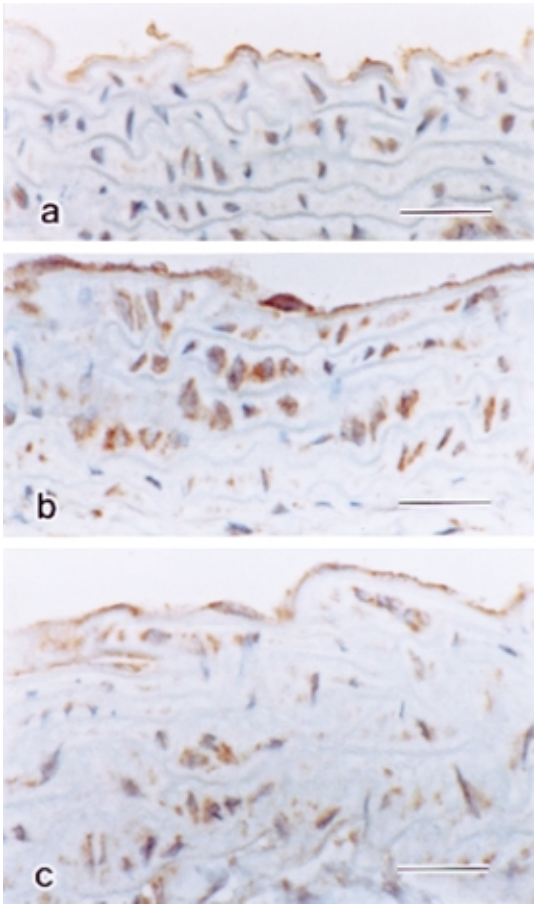

Fig. 7 Immunohistochemical staining for phosphorylated p 38 in aorta of *Mongolian* gerbils. a: At 4 weeks of control animals, phosphorylated p 38 is localized in the endothelial cells of aorta. Indirect method, Magnification, × 200, Bar=20 μm. b: At 4 weeks after STZ treatment, phosphorylated p 38 is much more localized in the nuclei and cytoplasm of endothelial and smooth muscle cells of aorta than control animals. Indirect method, Magnification, × 200, Bar=20 μm. c: At one year after STZ treatment, the reaction products with phosphorylated p 38 are observed in the nuclei and cytoplasm of endothelial and medial smooth muscle cells of aorta. Indirect method, Magnification, × 200, Bar=20 μm.

of aorta in control animals. But at 4 weeks after STZ treatment the localization of anti-phosphorylated p 38 antibody was observed in the nuclei and cytoplasm of endothelial and medial smooth muscle cells of aorta. The reaction products were much more localized in STZ-treated animals than in control animals at 4 weeks (**Fig. 7 a, b**). At one year after STZ treatment, the reaction products with anti-phosphorylated p 38 antibody were localized in the nucleus and cytoplasm of endothelial and smooth muscle cells (**Fig. 7 c**).

All control sections that were incubated with IgG showed no positive staining.

Discussion

In this study, morphologically, after STZ administration, the hypertrophy of endothelial cells, remarkable anisocytosis of SMCs and vacuolar degeneration were occasionally observed in the aortic wall where stromal components were increased.

Proliferation and phenotypic modulation of vascular smooth muscle cells (SMCs) play a major role in the pathogenesis of cardiovascular diseases including atherosclerosis. Phenotypic modulation of vascular SMCs is characterized by the loss of expression of the SMC-specific genes as well as a selective upregulation of the fetal/neonatal isoforms of the contractile proteins, extracellular proteins, growth factors, and their receptors[12].

The morphological changes of endothelial cells and phenotypical modulation of vascular SMCs are very important events for pathogenesis of diabetic micro- and macroangiopathy. Persistent hyperglycemia results in the glycation of many proteins including extracellular matrix (ECM) and intracellular proteins. Functional alterations may be induced by hyperglycation of protein including extracellular glycoprotein, such as fibronectin and laminin.

The alterative synthesis of growth factors and their receptors in vascular SMCs under persistent hyperglycemia may contribute to the proliferation of vascular SMCs in autocrine and paracrine patterns[13-15].

Activation of the aldose reductase in diabetic animals may play an important role in detrimental alterations of the cell-surface receptors for ECM proteins[16].

Fibronectin in STZ treated animals was irregularly localized in the pericellular spaces especially on elastic fibers, and medial SMCs of aorta at 4 weeks and one year after treatment.

ECM such as fibronectin is a large glycoprotein that has been implicated in a wide variety of cellular functions. These functions include cell adhesion, migration, spreading, and differentiation. It has been proved *in vitro* that fibronectin is synthesized by fibroblasts, smooth muscle cells, endothelial cells and macro-

phages[17]. And also hyperglycemia and insulin status influence laminin isoform expression[18]. In this study, laminin was localized in endothelial cells, medial SMCs and pericellular spaces of aorta under 4 weeks of hyperglycemia. Ability of SMCs to maintain a synthetic phenotype is correlated in part by a particular distribution of type IV collagen and laminin[19,20]. A complex comprising laminin-5 and a 3 b 1 integrin is multifunctional and contributes to the regulation of cell growth via a signaling pathway involving mitogen activated protein kinase (MAPK)[11].

The advanced glycation end products (AGEs) which accumulate on extracellular matrix proteins under persistent hyperglycemia are implicated in the micro- and macrovascular complications of diabetes mellitus and stimulate smooth muscle cell proliferation[2,3,21].

In this study, phosphorylated activation of extracellular signal- related kinase (ERK), c-jun N-terminal kinase (JNK) and p 38 mitogen activated protein kinase (p 38) were observed at 4 weeks and one year after STZ treatment. They trend to be more extensively expressed in the vascular cells of aorta in STZ-treated animals than control animals at 4 weeks.

After STZ administration, the immunoreactivity of phosphorylated ERK was increased in SMCs of aorta. It is reported that the 42- and 44-kD MAPKs, as ERK-1/2, are transiently activated by stretching vascular smooth muscle cells[22]. These kinases may be related to a novel function of urokinase-type plasminogen activator (UPA) on vascular smooth muscle cell proliferation and migration, and additional evidence for a role in the pathogenesis of atherosclerosis[23]. Fibroblast growth factor 2-induced ERK-1/2 activation promotes medial cell replication after injury[24].

And also the irregular intracellular immunoreactivity of phosphorylated JNK and phosphorylated p 38 were extensively encountered in smooth muscle cells of aorta after STZ administration.

The effects of phytoestrogens on MAPK activity inhibit mitogen-induced proliferation, migration and extracellular matrix synthesis and inhibit MAPK activity[25]. In this study the localization of ECM proteins and phosphorylated MAPKs of STZ treated animals was confirmed. Intranuclear localization of phosphorylated MAPKs, phosphorylated ERK, phosphorylated JNK and phosphorylated p 38 is thought to be released by MAPK kinase (MAPKK) activation from MAPKK-MAPK complex.

As for p 38, moderate hyperglycemia can activate p38 kinase by protein kinase C (PKC)-delta isoform-dependent pathway but glucose at extremely elevated levels can also activate p 38 kinase by hyperosmolarity via PKC-independent pathway[26]. The p 38 activity in endothelial cells is considered to be requsite for signal transduction of basic fibroblast growth factor (bFGF)[27]. The roles of p 38 and ERK MAPK homologs are not identical, but these kinases seem to work in a coordinated fashion. On the other hand p 38 pathway seem to convey the vascular endothelial growth factor (VEGF) signal to microfilaments inducing rearrangements of the actin cytoskeleton that regulate cell migration[28,29]. By modulating cell migration, p 38 may be an important regulator of angiogenesis.

The MAPK seems to play a key role in the overproduction of fibronectin[7]. Fibronectin is considered to play an important role in promoting the characteristic changes of vascular SMCs under persistent hyperglycemia[6].

Alterative synthesis of ECM proteins and transcriptional activation through phosphorylated MAPKs pathway in endothelial cells and SMCs of blood vessels under hyperglycemia would be important for the pathogenesis and progression of diabetic angiopathy.

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