—Originals—

Ultrastructural changes and immunohistochemical localization of nitric oxide synthase, advanced glycation end products and NF-κB in aorta of streptozotocin treated *Mongolian* gerbils

Ryutaro Nishigaki[1], Fang Guo[1], Munehiko Onda[1], Nobutaka Yamada[1], Munehiro Yokoyama[1], Zenya Naito[1], Goro Asano[1], Masumi Shimizu-Suganuma[2], Kazuhiro Shichinohe[2] and Takumi Aramaki[3]

[1]Department of Pathology, [2]Department of Laboratory Animal Science, [3]First Department of Internal Medicine, Nippon Medical School

Abstract

To evaluate the relationship among the induction of nitric oxide synthase (NOS), advanced glycation end products (AGEs) and NF-κB for vascular damage 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 and electron microscopical techniques.

After STZ treatment, hyperglycemia was confirmed and body weight transiently decreased.

Morphological observation revealed no remarkable changs in vascular endothelial cells or vascular smooth muscle cells in the aorta at one week after STZ administration. After 4 weeks increased collagen fibrils were observed in the pericellular spaces of media. At one year after STZ administration, increased collagen fibrils and thickened elastic fibers were found around the vascular smooth muscle cells with vacuolization and increased cytoplasmic organellae compared with non-treated animals of the same age. Immunohistochemically endothelial constitutive NOS (ecNOS) was localized in the endothelium of the aorta of *Mongolian* gerbils. At one year after STZ administration, the reaction products of iNOS, AGEs and NF-κB in vascular endothelial cells and smooth muscle cells were much more greatly increased than at one week and 4 weeks.

After STZ administration, the localization of NOS, AGEs and NF-κB was observed in the aorta, which suggests these factors play important roles in the pathogenesis of vasculopathy in diabetes mellitus. (J Nippon Med Sch 1999; 66: 166—175)

Key words: nitric oxide synthase (NOS), advanced glycation end products (AGEs), nuclear factor-kappa B (NF-κB), streptozotocin (STZ), *Mongolian* gerbil

Introduction

Streptozotocin (STZ) is a highly effective cytotoxic agent for pancreatic β cells, and STZ-treated animals have been used as a model for insulin-dependent diabetes mellitus[1]. Nitric oxide (NO) is an inorganic free radical gas[2]. STZ is considered as a nitric oxide (NO)-generation reagent, and NO-generation contributes to damage of β cells in islets of the pancreas, which leads to diabetogenesis.

Also hyperglycemia and NO are fully believed to play significant roles in the pathogenesis of vasculopathy in diabetes[3].

Mailing address; Ryutaro Nishigaki, Department of Pathology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

Glucose is considered to form chemically reversible early glycosylation products with protein. However, some of the early glycosylation products on collagen and other long-lived proteins of the vessel walls do not dissociate[4]. And it is suggested that diabetic complications are caused by the hyperglycemia-accelerated formation of nonenzymatic advanced glycation end products (AGEs) in tissue. AGEs-modified proteins in the arterial wall increase vascular permeability and are important modulators of nitric oxide activity and endothelium-dependent relaxation[5].

On the other hand, the accelerated formation of AGEs has been implicated in the pathogenesis of diabetic macrovascular complications[6]. Cell-bound AGEs can modulate cellular properties, as occurs after AGE-mediated activation of transcription factor, nuclear factor-κB (NF-κB). NF-κB proteins regulate transcription of a variety of cellular genes, including those involved in immune responses and growth control[7]. Activated NF-κB is believed to express inducible nitric oxide synthase (iNOS), by which NO is formed from L-arginine[2,8]. It is indicated that iNOS is induced in neointimal smooth muscle cells after arterial injury[9].

Morphologically, it is highly acceptable that AGEs promote the accumulation of collagen in atherosclerotic lesions through the insolubility of collagen and the inhibition of collagenase activity of smooth muscle cells[10].

This study was undertaken to clarify the roles of NOS, AGEs and NF-κB 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 the 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 killed at various time points (one week, 4 weeks and one year) after streptozotocin administration.

(2) *Specimen preparation*

Aortic changes were observed chronologically by light and electron microscopical techniques.

The aortic tissues were immediately cut into small pieces and fixed in 4% paraformaldehyde solution for 6 hours, embedded in paraffin, sectioned and stained with hematoxylin and eosin and Periodic Acid Shiff (PAS) for light microscopical observation.

For ultrastructural observation, aortic tissues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution (pH 7.4) over night, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for one hour, dehydrated through graded ethanol and embedded in Epok 812. Semi-thin sections were cut and suitable tissue areas were selected for electron microscopic observation. Ultrathin sections were cut with a diamond knife on an Ivan Sorvall MT-5000 ultramicrotome (Du Pont, Newtown, CT, USA). The sections were stained with uranyl acetate and lead citrate and some sections were stained with tannic acid. They were observed with a Hitachi H-7000 electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 75 kV. Photographs were taken at a magnification of × 2000 or × 5000.

For immunohistochemical observation, specimens were fixed in 4% paraformaldehyde solution for 6 hours and endogenous peroxidase was inhibited by treatment with 0.3% H_2O_2 in 100% methanol. The following antibodies diluted with BSA solution by overnight incubation at 4℃ were used: (a) monoclonal human anti-α-smooth muscle actin antibody (Dako, Glostrup, Denmark) at a dilution of 1 : 100, which reacts with the α-smooth muscle isoform of actin as indicated by immunoblotting and ELISA and does not react with actin from fibroblasts (β- and γ-cytoplasmic), striated muscle (α-sarcomeric) and myocardium (α-myocardial), (b) polyclonal rabbit anti-endothelial constitutive NOS antibody (Santa Cruz Biotechnology, USA) at a dilution of 1 : 500, for which a 20.4 kDa protein fragment corresponding to amino acids 1030～1209 of human ecNOS was used as an immunogen, (c) polyclonal rabbit anti-inducible NOS antibody (Santa Cruz Biotechnology, USA) at a dilution 1 : 1000, which has been successfully used to detect iNOS in Western blot experiments (d) anti-advanced glycation end products (AGEs) antibody (Panapharm Laboratories, Japan) at a dilution of 1 : 500, which was purified by Protein A chromatography from the cell line (6

Table 1 Blood sugar of *Monogolian* gerbils [mg/d*l*]

	1 week	4 weeks	1 year
STZ-treated	325.0 ± 75.3	267.0 ± 51.2	330.7 ± 96.4
non-treated	85.3 ± 21.2	98.3 ± 4.2	105.0 ± 2.6

STZ: Streptozotocin

D 12) with positive reaction to AGEs-human serum albumin but negative to BSA, (e) anti-Nuclear Factor-κB (anti NF-κB) antibody (Boehringer Mannheim, Germany) at a dilution of 1 : 1200, which recognizes an epitope overlapping the nuclear location signal of p 65 subunit of the NF-κB. After incubation with the primary antibody, the sections were treated with biotin-conjugated goat anti-rabbit IgGs antibody (Nichirei, Tokyo Japan) for the polyclonal primary antibody and with biotin-conjugated anti-mouse IgGs antibody (Nichirei) for the monoclonal primary antibody for 20 minutes. The sections were then reacted with 0.05% H_2O_2 in 0.02% DAB solution for 5 min, They were 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 hyperglycemia by 3 days after STZ administration. The blood sugar concentrations of STZ treated animals at one week, 4 weeks, and one year were 325.0 ± 75.3, 267.0 ± 51.2, and 330.7 ± 96.4 [mg/d*l*], respectively. However, control valus at one week, 4 weeks, one year were 85.3 ± 21.2, 98.3 ± 4.2, and 105.0 ± 2.6 [mg/d*l*], respectively (**Table 1**).

2. Morphological observation

Light microscopically, no significant changes were observed in the endothelial and medial cells of the aorta in *Mongolian* gerbils at one week after normal saline and STZ administration. At 4 weeks after streptozotocin (STZ) administration, mild endothelial swelling and anisocytosis in medial cells were observed compared with control animals. At one year after STZ administration, swelling of endothelial cells, remarkable anisocytosis of smooth muscle cells and vacuolar degeneration were occasionally observed in the aortic wall where elastic fibers were thickened. However, these morphological changes were not observed in the aorta at one year after normal saline administration (**Fig. 1 a, b**).

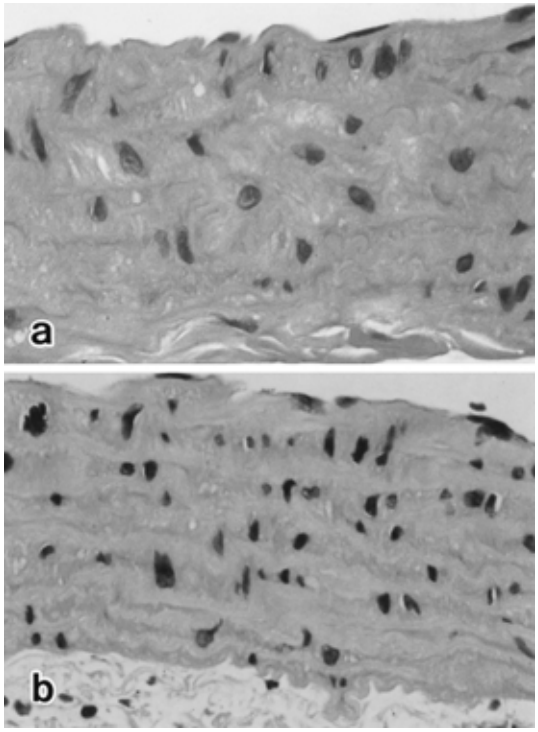

Fig. 1 Light micrographs of sections stained with hematoxylin and eosin in aortas from control and streptozotocin (STZ) administered *Mongolian* gerbils. a: At one year after saline administration, slight anisocytosis of smooth muscle cells is noted in the aortic wall. Magnification, ×400, b: At one year after STZ administration, mild swelling of endothelial cells and anisocytosis of smooth muscle cells are observed in the aortic wall. Magnification, ×400

Ultrastructurally there was no remarkable change in control animals at one week after normal saline administration. But at one week after STZ administration, endothelial cells were swollen and nuclei were enlarged. And in the smooth muscle cells of aorta irregular distribution of actin filaments were demonstrated in the cytoplasm (**Fig. 2 a**). After 4 weeks of STZ administration, in addition to endothelial changes, an increase of collagen fibrils and irregular thickening of elastic fibers were conspicuously observed in the pericellular spaces of hypertrophic smooth muscle cells in comparison to control animals at 4 weeks after

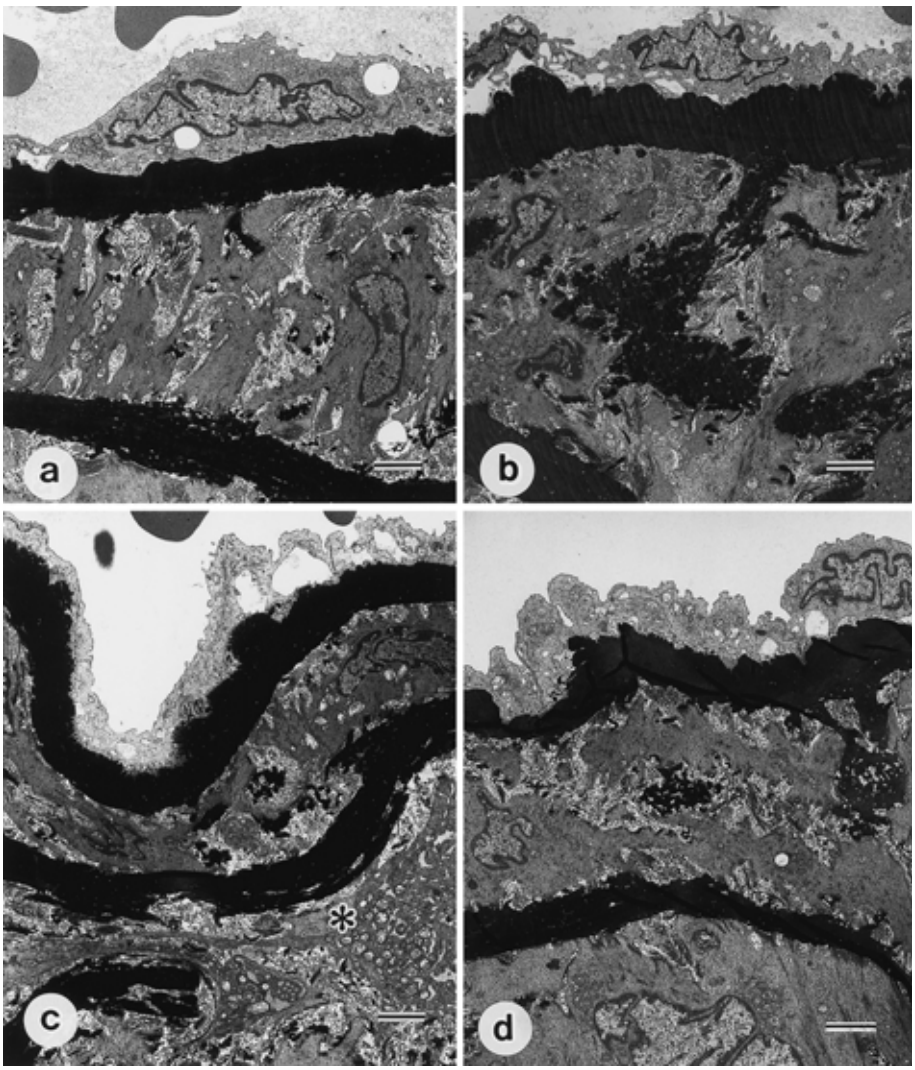

Fig. 2 Transmission electron photomicrographs of aortas from control and streptozotocin-treated *Mongolian* gerbils. a: Endothelial cells are enlarged and nuclear membrane is invaginated at one week after STZ administration. Tannic acid stain, Magnification, ×4,000 Bar: 2 μm. b: After 4 weeks of STZ administration, in addition to endothelial changes, increased collagen fibrils and irregular thickening of elastic fibers are observed in the pericellular spaces of enlarged smooth muscle cells. Tannic acid stain, Magnification, ×3,700 Bar: 2 μm. c: In STZ-treated animals at one year, increased collagen fibrils and irregular thickening of elastic fibers are observed in intercellular spaces. The smooth muscle cells reveal increased intracytoplasmic organellae (asterix). Tannic acid stain, Magnification, ×4,000 Bar: 2 μm. d: At one year after saline administration, enlarged nuclei in endothelial and smooth muscle cells are noted. Tannic acid stain, Magnification, ×4,000 Bar : 2 μm

normal saline administration (**Fig. 2 b**). At one year after STZ administration, collagen fibrils were increased and irregular thickening of elastic fibers were demonstrated and the smooth muscle cells were irregular in shape and contain increased intracytoplasmic organellae such as mitochondria and actin filaments. The smooth muscle cells occasionally revealed vacuolar degeneration (**Fig. 2 c**). However, in control animals at one year after normal saline administration, there was no remarkable change in elastic fibers, but medial smooth muscle cells were occasionally hypertrophic (**Fig. 2 d**).

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, α-smooth muscle actin was regularly localized in the smooth muscle cells of

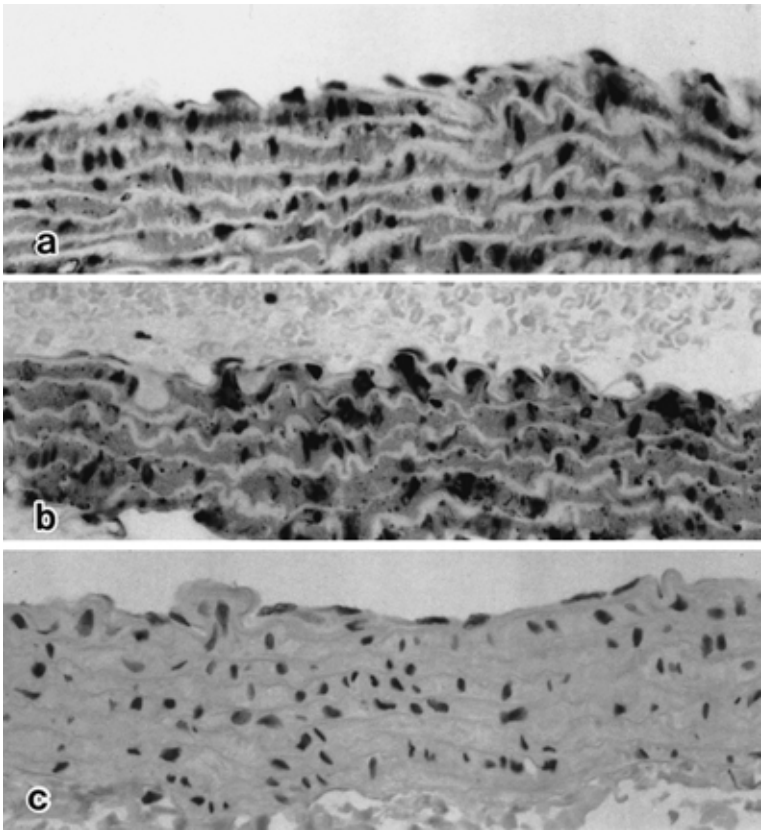

Fig. 3 Immunohistochemical staining for a-smooth muscle actin in the aorta of *Mongolian* gerbils. a: The reaction products of α-smooth muscle actin are irregularly localized in the medial smooth muscle cells of the aorta at 4 weeks after STZ administration. Indirect method, Magnification, ×400. b: At one year after STZ treatment the reaction products of a-smooth muscle actin are irregularly localized in the smooth muscle cells of the aorta. Indirect method, Magnification, ×400. c: There are no reaction products in smooth muscle cells of the aorta treated without anti-a-smooth muscle actin antibody at one year after STZ administration. Indirect method, Magnification, ×400

aorta. At 4 weeks and one year after STZ treatment the reaction products of α-smooth muscle actin were irregularly localized in hypertrophic medial cells as compared with control animals (**Fig. 3 a, b, c**).

At 4 weeks after STZ administration, ecNOS immunoreactivity increased in the endothelial cells of aorta in comparison to non-treated and one week treated animals. The localization of ecNOS was much more greatly increased in the swollen endothelial cells of the aorta at one year after STZ administration than in control animals at one year after normal saline treatment (**Fig. 4 a, b, c, d**).

There were no reaction products in control animals at one week. But at one week after STZ administration, a small amount of reaction products with anti-iNOS antibody was localized in endothelial cells of the aorta. At 4 weeks and one year after STZ administration, the localization of iNOS immunoreactivity was increased in swollen endothelial cells and smooth muscle cells of aorta in comparison to control animals at 4 weeks and one year (**Fig. 4 e, f, g, h**).

In control and STZ-treated animals at one week, there were no reaction products with anti-AGEs antibody in endothelial cells and smooth muscle cells of the aorta. However, irregular localization of AGEs immunoreactivity was encountered in swollen endothelial cells and smooth muscle cells of the aorta at 4 weeks and one year after STZ administration in comparison to these cells in non-treated control animals (**Fig. 5 a, b, c, d**).

There were no reaction product in the endothelial cells of the aorta in control animals. But at one week after STZ administration, the localization of nuclear factor-κB (NF-κB) was observed in the endothelial cells of the aorta. At 4 weeks and one year after STZ treatment, NF-κB was localized in the nucleus of inti-

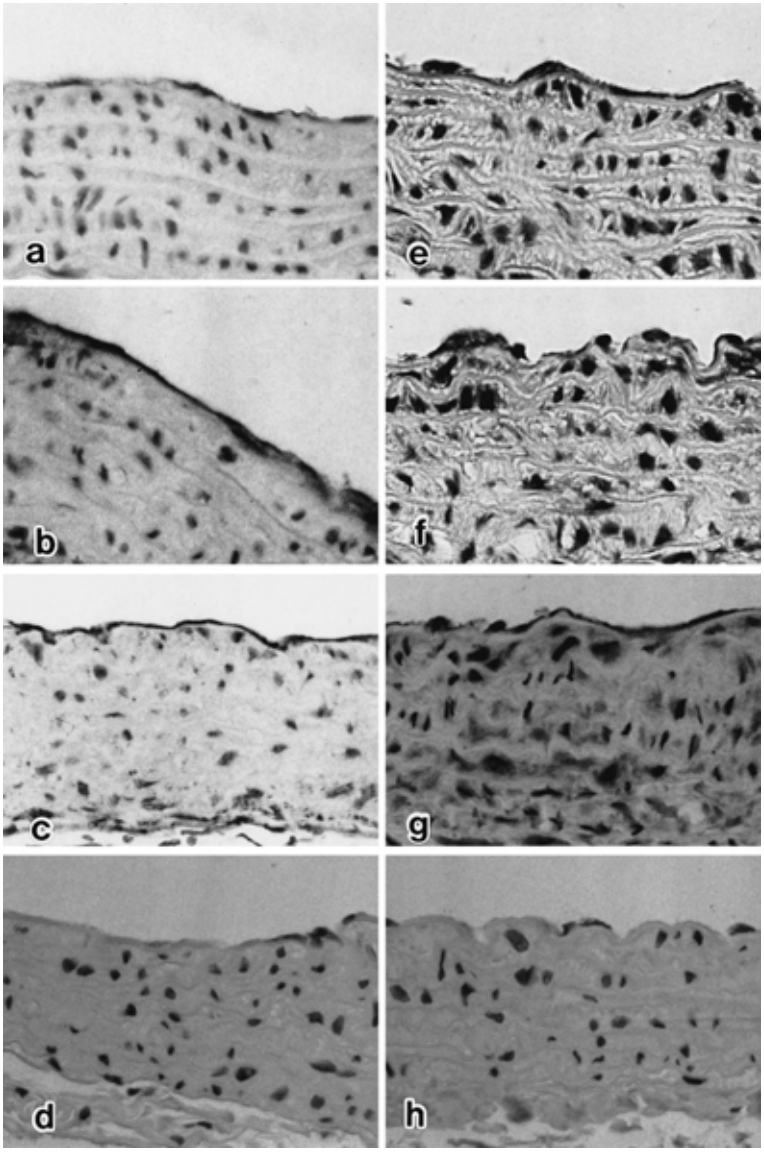

Fig. 4 Immunohistochemical staining for ecNOS and iNOS in the aorta of *Mongolian* gerbils. a and b: The ecNOS immunoreactivity is demonstrated in endothelial cells and smooth muscle cells of the aorta at one week (a) and at 4 weeks (b) after STZ administration. Indirect method, Magnification, ×400. c: The localization of ecNOS immunoreactivity is found in swollen endothelial cells at one year after STZ administration. Indirect method, Magnification, ×400. d: There are no reaction products in endothelial cells and smooth muscle cells of the aorta treated without anti-ecNOS antibody at one year after STZ administration. Indirect method, Magnification, ×400. e: The reaction products with anti-iNOS antibody are faintly localized in endothelial cells of the aorta at one week after STZ administration. Indirect method, Magnification, ×400. f and g: At 4 weeks (f) and one year (g) after STZ administration, the localization of iNOS immunoreactivity is slightly increased in swollen endothelial cells and found in smooth muscle cells of the aorta. Indirect method, Magnification, ×400. h: There are no reaction products in the vascular cells of the aorta in control specimen treated without anti-iNOS antibody at one year after STZ administration. Indirect method, Magnification, ×400

mal and medial smooth muscle cells including endothelium (**Fig. 6 a, b, c, d).** However no reaction products of NF-κB were localized in the vascular cells of non-treated control *Mongolian* gerbils at 4 weeks and one year.

No control sections that were incubated with IgG showed positive staining.

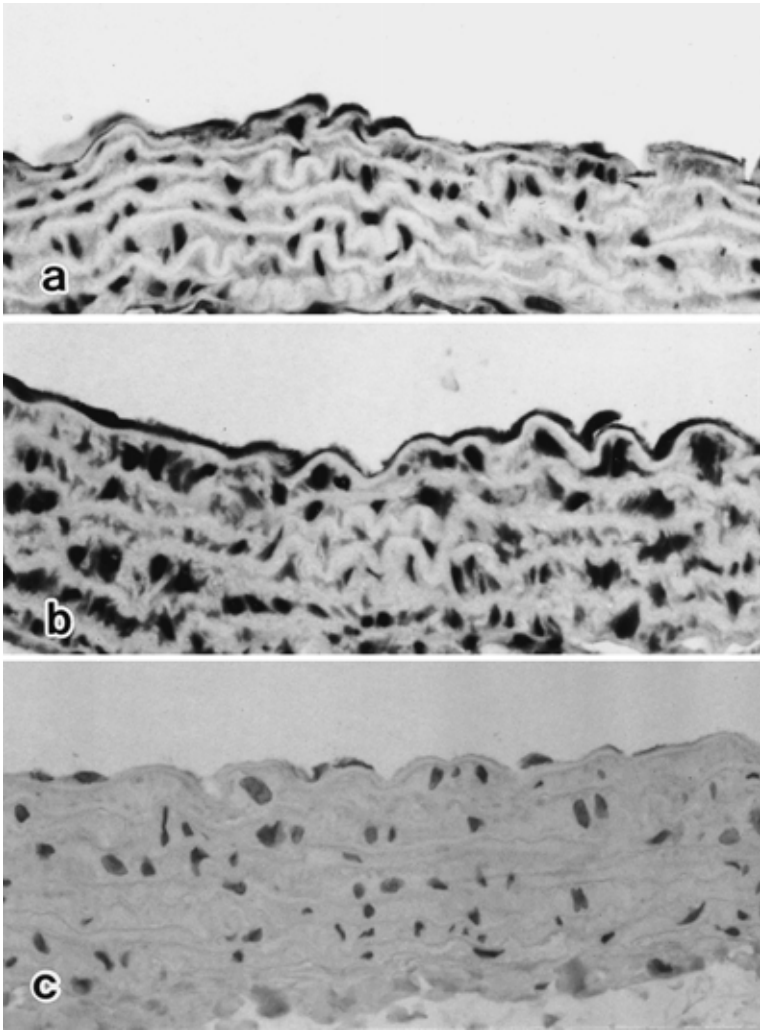

Fig. 5 Immunohistochemical staining for AGEs in the aorta of *Mongolian* gerbils. a: At 4 weeks after STZ administration the reaction products are localized in endothelial and some smooth muscle cells of the aorta. Indirect method, Magnification, ×400. b: Irregular localization of increased AGEs immunoreactivity is found in swollen endothelial cells, smooth muscle cells and intercellular spaces of the aorta at one year after STZ administration. Indirect method, Magnification, ×400. c: Immunohistochemical localization of AGEs is not detected in the aorta of control specimens treated without anti-AGEs antibody at one year after STZ administration. Indirect method, Magnification, ×400

Discussion

We developed a new model for diabetes mellitus by the injection of streptozotocin (STZ) into *Mongolian* gerbils. These original models revealed moderate hyperglycemia and nitric oxide (NO) generation in Langerhans islets of the pancreas, which is a diabetogenetic factor by STZ toxicity[3]. In this study the reaction products of nitric oxide synthase (NOS), including endothelial constitutive NOS (ecNOS) and inducible NOS (iNOS) increased in the aorta of *Mongolian* gerbils after STZ administration.

Prolonged exposure to high glucose increases ecNOS gene expression and NO release[11]. This explains impaired endothelial function and is important for diabetic vascular disease[3]. An increase in extracellular glucose led to a rapid dose-dependent increase in endothelial cell permeability via the activation of protein kinase C (PKC)[12].

And the polyol pathway is speculated to be related to contraction abnormality associated with ecNOS expression in the aorta from chronic streptozotocin-diabetic animals[5].

In addition to NOS, advanced glycation end products (AGEs) were increased in the aorta after STZ ad-

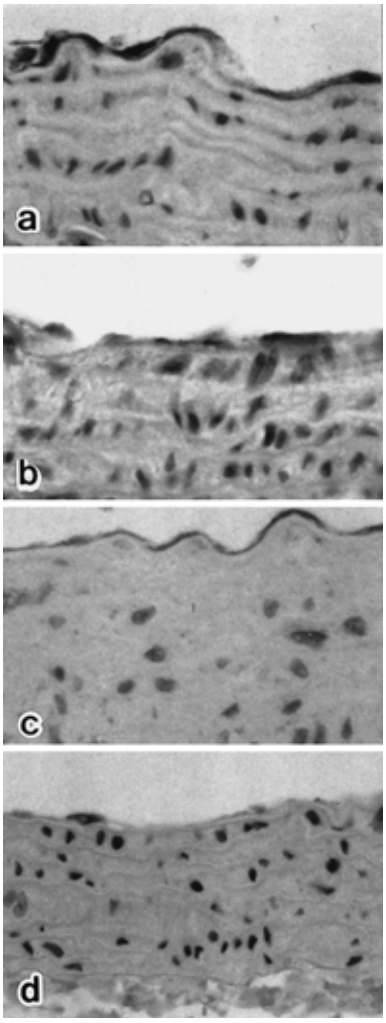

Fig. 6 Immunohistochemical staining for NF-κB in the aorta of *Mongolian* gerbils. a and b: The reaction products of NF-κB is detected in the endothelium of the aorta at one week (a) and 4 weeks (b) after STZ administration. Indirect method, Magnification, ×400. c: NF-κB is localized in the nuclei of swollen endothelial cells and smooth muscle cells of the aorta at one year after STZ administration. Indirect method, Magnification, ×400. d: Immunohistochemical localization of NF-κB is not detected in the aorta treated without anti-NF-κB antibody at one year after STZ administration. Indirect method, Magnification, ×400

ministration. The interaction of AGEs with cellular targets, such as endothelial cells may lead to oxidant stress resulting in changes in cellular proteins, potentially contributing to the development of vascular lesions[13]. Sun indicated that the ligation of AGEs by endothelial receptors for AGEs (RAGE) induces hyperpermeability and that AGE-RAGE interaction may promote atherosclerosis *in vivo*[14]. And AGE-RAGE interactions may predispose to angiogenesis and lead to the progression of diabetic angiopathies[15].

On the other hand, the AGEs, through the coordinate induction of cytokines and growth factors, play a role in tissue remodeling and enhance smooth muscle cell replication[16]. Smooth muscle cell replication with regulating collagen and proteoglycan synthesis is an important change in atherogenesis[17]. In fact, the incubation of smooth muscle cells with AGE-bovine serum resulted in significant cell migration[18].

In this study the reaction products of AGEs were localized and increased in endothelial and smooth muscle cells of the aorta after STZ treatment. Collagen fibrils also increased in the intercellular region of media.

The AGEs accumulate on extracellular matrix proteins and are implicated in the micro- and macrovascular complications of diabetes mellitus[19]. It is generally accepted that AGE-stimulated smooth muscle cell proliferation and matrix production are traced in hyperglycemia.

AGEs accumulation induces dysfunctional changes in extracellular matrix and alters the function of intracellular proteins and also AGEs immunoreactivity has been observed within human atherosclerotic plaques[20,21].

In this study, thickened elastic fibers with increased collagen fibrils were observed in the aorta of STZ treated *Mongolian* gerbils.

Glycosylation of collagen induces an increased rigidity and decreased susceptibility to collagenase digestion[22]. The nonenzymatic glycation of extracellular matrices, especially collagen, accelerates atherosclerosis by an insoluble deposition of collagen in the extracellular matrix in relation to diabetes mellitus[22,23]. Immunohistochemically the localization of NF-κB was increased in endothelial and smooth muscle cells of the aorta after STZ treatment. Binding of AGEs to the cellular surface receptor (RAGE) induces translocation of NF-κB into the nucleus and NF-κB-mediated gene expression[24].

The activated transcription factor NF-κB is considered to induce the expression of iNOS. The promotor including the NF-κB is functional and iNOS gene is transcriptionally regulated via NF-κB[8,25]. AGEs and NF-κB activation contributes to the increased expression of molecules such as VCAM-1 and MCP-1[26]. The endothelial adherence of monocytes to the aortas of

diabetic animals suggests altered endothelium in diabetes[27]. NF-κB also plays an important role in regulating smooth muscle cell gene expression and proliferation[26].

AGEs also play a role in the modulation of cellular communication and nitric oxide quenching may contribute to hyperglycemic changes[28].

These results suggest that a relative or absolute increase of NOS, AGEs and NF-κB contributes to the vasculopathy associated with early diabetic vascular dysfunction.

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