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Effects of an aldose reductase inhibitor, SNK-860, on the histopathological changes of retinal tissues in a streptozotocin-induced diabetic rat model.

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Abstract

In order to clarify the mechanism of retinal tissue damage in diabetes mellitus, the effects of the inhibition of aldose reductase on the pathologic changes in the retina of streptozotocin-induced diabetic (STZ-diabetic) rats were examined histologically and histochemically. The STZ-diabetic animals were maintained with and without peroral administration of an aldose reductase inhibitor, SNK-860, and their retinas were examined microscopically after 12 months. Several abnormal changes observed; folding and edema in the retina, loss of pericytes in the retinal capillary walls, and thickening of basement membranes in the retinal capillaries, were significantly inhibited by SNK-860. Some of these changes were similar to those that had been previously noted in diabetic and galactosemic rats. These data suggest that the enhanced polyol metabolism may be involved in the diabetic changes of the retina.

KEYWORDS: streptozotocim, diabetes mellitus, aldose reductase Inhibitor, snk-860, retinopathy

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Effects of an Aldose Reductase Inhibitor, SNK-860, on the Histopathological Changes of Retinal Tissues in a Streptozotocin-Induced Diabetic Rat Model

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In order to clarify the mechanism of retinal tissue damage in diabetes mellitus, the effects of the inhibition of aldose reductase on the pathologic changes in the retina of streptozotocin-induced diabetic (STZ-diabetic) rats were examined histologically and histochemically. The STZ-diabetic animals were maintained with and without peroral administration of an aldose reductase inhibitor, SNK-860, and their retinas were examined microscopically after 12 months. Several abnormal changes observed; folding and edema in the retina, loss of pericytes in the retinal capillary walls, and thickening of basement membranes in the retinal capillaries, were significantly inhibited by SNK-860. Some of these changes were similar to those that had been previously noted in diabetic and galactosemic rats. These data suggest that the enhanced polyol metabolism may be involved in the diabetic changes of the retina.

Key words : streptozotocin, diabetes mellitus, aldose reductase inhibitor, SNK-860, retinopathy

Diabetic retinopathy, which causes blindness by affecting the retinal vascular system, is now one of the most serious complications facing the diabetic patients (1, 2). As with many other systemic complications of diabetes, the enhanced polyol pathway is apparently involved in the etiology of the pathological changes in the eyes of patients with diabetes. Fortunately, noticeable amelioration of diabetic ocular damage can be expected if treated with an aldose reductase inhibitor (3). Aldose reductase (AR, E.C. 1, 1, 1, 21) converts glucose to sorbitol and is the rate-limiting enzyme in the sorbitol pathway. The intracellular hyperosmolality in the elevated sorbitol pathway has been also implicated as the cause of other diabetic complications (2–7). A number of aldose reductase inhibitors have been shown to be effective in treating diabetic and galactosemic retinopathy in rodent models (2, 8–11). Both *in vitro* and *in vivo* experiments showed that (2S, 4S)-6-fluoro-2', 5'-dioxospiro [chroman-4, 4'-imidazolidine]-2-carboxamide (SNK-860, Fig.1)

is a potent inhibitor of aldose reductase (12). In the paper, we report that SNK-860 noticeably suppresses histopathological changes in the retinal tissues of rats with streptozotocin (STZ)-induced diabetes.
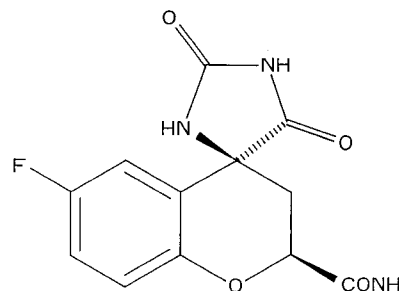

Fig. 1　The chemical structure of SNK-860, *(2S,4S)*-6-fluoro-2', 5'-dioxospiro [chroman-4, 4'-imidazolidine]-2-carboxamide.

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Materials and Methods

Animals. Thirty adult male Sprague-Dawley rats obtained from SLC Japan (Shizuoka, Japan), were used for the experiments after one week acclimatization period. Their body weight at the beginning of the experiments averaged 200 g. The animals were given food, CRF-1 (Charles River Japan, Kanagawa, Japan) and water *ad libitum* and were kept under a 12 h on/off light cycle.

The animals were divided into 3 groups of 10. Those in groups I and II were given an intravenous injection of STZ (40 mg/kg of body weight) dissolved in 0.05 M citrate buffer, pH 4.5 to induce diabetes. Those in group III served as controls and were given only the citrate buffer by intravenous injection. Four days after the streptozotocin injection, blood was collected from the left ocular vein using a heparinized syringe and blood plasma was obtained by centrifugation at 1700 × *g* for 10 min at 4 °C. The plasma glucose concentration was estimated by the enzymatic method using glucose oxidase and peroxidase (Glucose B-test Wako). For groups I and II, rats with glucose levels of 300 mg/dl or higher were selected for further study. Finally, 5 rats were selected for each of the 2 diabetic groups (Groups I and II) and 5 animals for the non-diabetic group (Groups III). Four days after the injection of STZ, 2 mg/kg of body weight of SNK-860 was administered orally to those in Group I once a day for 12 months. Non-diabetic rats in Group III and STZ-diabetic rats in the Group II were not given SNK-860. Both groups were given only arabic gum.

Tissue processing. After the last day of administration of SNK-860 to Group I, all the animals were killed by blood drawing, and the right eyeball of each animal in every group was removed rapidly and fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C for 60 min. After fixing, the eyes were opened at the anterior segment and incubated at 4 °C for 24 h, after which time they were transferred to a 0.05 M cacodylate buffer (pH 7.4) containing 0.2M sucrose. The tissues were dehydrated through graded ethanol and embedded in paraffin. Tissue sections (3–5 μm in thickness) were cut perpendicular to the retinal layers, and were stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS). For the immunohistochemical staining for albumin, the labelled streptavidin biotin method (LSAB kit system, DAKO)

was employed by using rabbit IgG against rat serum albumin (13–15). To evaluate the effects of SNK-860 on the retinal vasculature, the number of pericytic and endothelial cell nuclei of the retinal capillaries was counted from the outer plexiform layer to the inner nuclear layer, and from the inner plexiform layer to the nerve fiber layer on the total retinal layers on the PAS stained specimens and then on the albumin stained specimens.

The results were expressed as the mean ± standard error of the mean (SEM) and statistically analyzed for any significant differences using an unpaired Student's *t*-test.

Compounds. SNK-860 was prepared at Sanwa Kagaku Kenkyusho Co., Ltd. It was suspended in a 5 % solution of arabic gum and administered orally. Other chemicals were purchased from the following sources: streptozotocin (Sigma, St. Louis, MO, USA); Glucose B-test Wako (Wako, Osaka, Japan); rabbit IgG against rat serum albumin (Cappel, West Chester, PA, USA); LSAB Kit System 40 (DAKO, Carpinteria, CA, USA).

Results

The effects of SNK-860 on plasma glucose concentrations and body weight are shown in Table 1. The plasma glucose levels in the STZ-diabetic group (Group II) were about 2.5 times higher than those in the non-diabetic control group (Group III). Plasma glucose levels in the STZ-diabetic group treated with SNK-860 (Group I), while lower than those in the Group II, were about 2 times higher than those of the healthy control (Group III).

Light microscopic examinations of HE-stained tissue sections revealed pathological folding in the retina associated with retinal edema or cell dissociation in all STZ-diabetic rats (Group II) (Fig. 2b). There were distinct changes in one case. Retinal edema, folds, and other associated pathological changes were found neither in the SNK-860 treated STZ-diabetic rats (Group I) nor

Table 1 Body weight and plasma glucose levels after 12 months treatment

Group	Initial body weight (g)	Final body weight (g)	Initial plasma glucose levels (mg/dl)	Final plasma glucose levels (mg/dl)
I. Diabetic + SNK-860	225 ± 2	312 ± 11*	402 ± 20**	419 ± 41**
II. Diabetic	229 ± 5	382 ± 10*	480 ± 82**	502 ± 65**
III. Non-diabetic	246 ± 7	657 ± 36	82 ± 3	185 ± 20

*$p < 0.05$, **$p < 0.01$ *vs.* Non-diabetic control

in the non-diabetic healthy control (Group III) (Fig. 2a, 2c). In STZ-diabetic rats, light microscopic examinations were unable to detect any epiretinal membranes on the surface area of the pathologic retinal folds. The distortion of the retinal tissues caused by the formation of pathologi-

cal retinal folds appeared more distinct in the outer layer of the retina than in the inner layer. At the base of the folds, the retina was often dissociated from the retinal pigment epithelium, and showed a distinct positive reaction to albumin staining,   although no degenerative

Fig. 2   Light microscpe photographs of the retinal tissues of rats.
a: A retina from a healthy rat (Group III).
b: A retina from a STZ-diabetic rat (Group II) with folding. The folding involves the outer layer of the retina and shows some dissociation between the outer and inner nuclear layer.
c: A retina from a STZ-diabetic rat administered with SNK-860 for 12 months (Group I), showing a nearly normal structure. HE stain. $\times$ 304.
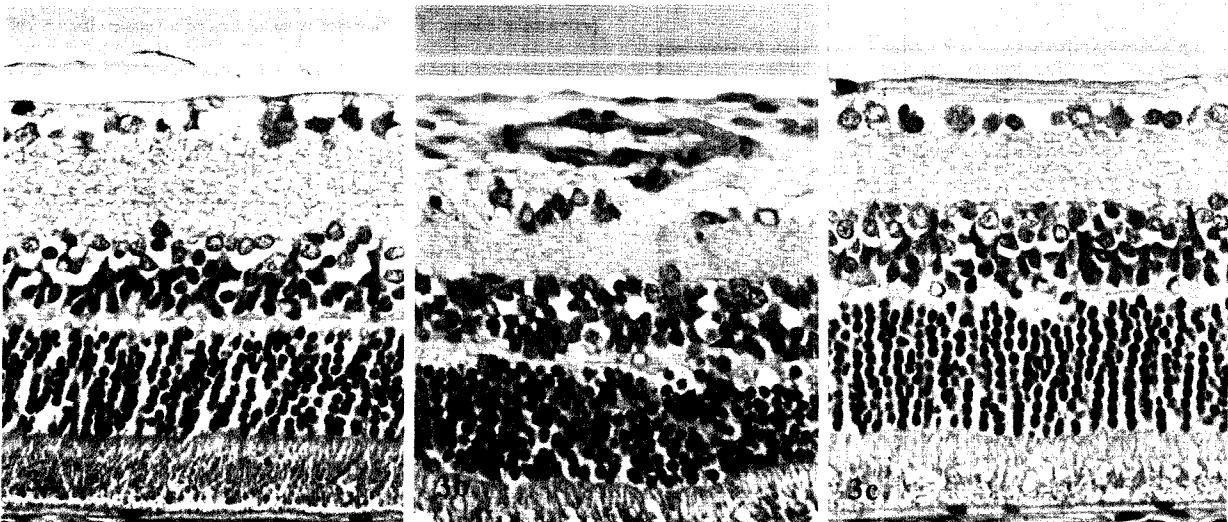

Fig. 3   Photographs of the cross sections of retinal capillaries.
a: A retina from a non-diabetic rat showing the normal structure of the capillaries.
b: A retina from a STZ-diabetic rat, which shows a thickening of the capillary's basement membrane and is stained deeply with PAS reaction. Some PAS positive granules appear in the spaces between the inner and outer nuclear layers (arrowhead).
c: A retina from a STZ-diabetic rat receiving SNK-860 treatment for 12 months, shows an almost normal structure. PAS stain. $\times$ 380.

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changes were found in retinal pigment epithelium. The edema, observed in all layers of the retina, was distinct in the area of the optic disc. These pathological changes found in the STZ-diabetic rats were not encountered in the SNK-860 treated diabetic rats and the healthy control. Apparently SNK-860 successfully inhibited edema in the ganglionic cell layer and inner plexiform layer.

STZ-diabetic rats showed a marked thickening of the basement membranes of the capillaries in the inner and the outer layer of the retina, and in some cases, gave a more intense PAS stain than the non-diabetic rats (Fig. 3a, 3b). In SNK-860 treated diabetic rats, the retinal capillary basement membranes appeared generally to be thinner than those of the non-treated diabetic rats (Fig. 3c). The pericyte loss, indicated by the ratio of the number of nuclei of pericyte to that of endothelial cell, was distinct in the outer plexiform layer to the inner nuclear layer in STZ-diabetic rats, although it was not distinct from the inner plexiform layer to the nerve fiber layer. The SNK-860 treated diabetic rats showed a nearly normal pericyte/endothelial cell ratio, with a significant reduction in the loss of pericytes in the outer plexiform layer to the inner nuclear layer (Fig. 4). The development of microaneurysms was observed in the retinal capillaries of STZ-diabetic rats (Fig. 5), but not in the SNK-860 treated diabetic rats or in the non-diabetic control. The microaneurysms, located in the outer plexiform and inner
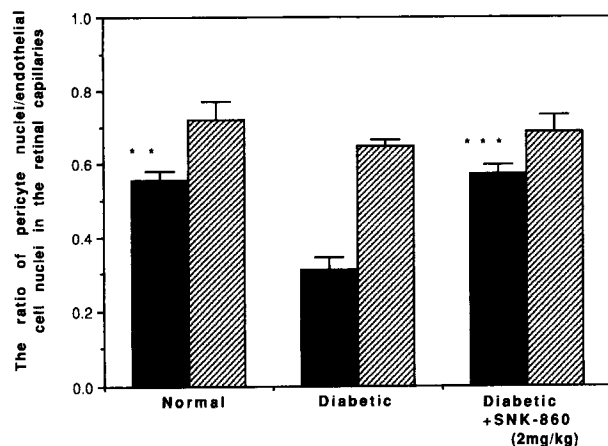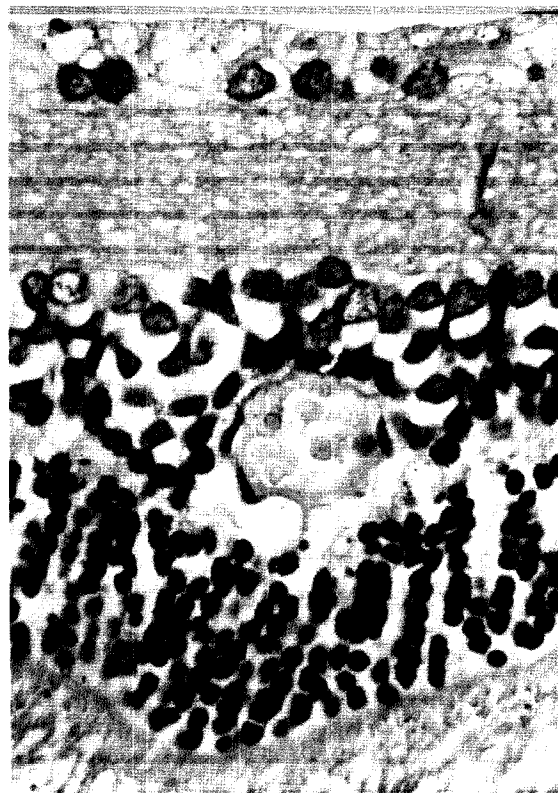

Fig. 5 A retina from a STZ-diabetic rat, showing a microaneurysm. A microaneurysm is located in the inner nuclear layer. The microneurysm is an enlarged space filled with red cells, lined by endothelium showing distinct perivascular edema. The diameter is about that of a venule (16). × 760.

Fig. 4 Effect of SNK-860 on the retinal capillaries. The ratio of pericyte nuclei/endethelial cell nuclei in the retinal capillaries in the outer plexiform layer to the inner nuclear layer (solid bar) and in the inner plexiform layer to the nerve fiber layer (hatched bar) was compared in each group by PAS-stained specimens and albumin-stained specimens. **$p < 0.01$, ***$p < 0.001$ vs. Non-diabetic control.

nuclear layer of the retina, appeared as a large, blood filled spaces lined by endothelium, with the diameter of a venule (16). Some PAS positive granules appeared occasionally in the outer plexiform layer of STZ-diabetic animals (Fig. 3b), which were not encountered in the SNK-860 treated diabetic animals and the healthy control.

Immunohistochemical staining of the tissues indicated some leakage of albumin from the retinal blood vessels in the area under the retinal folds in STZ-diabetic rats.

All the STZ-diabetic rats developed severe cataracts within 3 months of the STZ injection. SNK-860 treatment delayed the onset of cataracts and 6 months after the STZ injection, the animals still had not developed any grossly visible cataracts. However, they did begin to show some indications of cataract development between 6 and 12 months after STZ injection.

Discussion

Certain difficulties may be expected in maintaining rats with uncontrolled diabetes for 12 months. But, we found that the intravenous administration of STZ (40 mg/kg body weight) induces a moderately severe diabetic state with the development of retinopathy in which the animals can survive for 12 months under proper conditions. The STZ injection (40 mg/kg body weight) caused the rats to become diabetic and develop distinct glycemia and retinal lesions comparable to human diabetic retinopathy. These changes included pathological retinal folds, edema, preferential loss of pericytes, a thickening of capillary basement membranes, and the formation of microaneurysms (17-22).

SNK-860 treatment was not associated with any distinct improvement in the plasma glucose levels or body weight of the diabetic rats, but it did appear to effectively suppress the pathological changes, seen in the retina and choroid capillaries, and cataract development. Thus, in diabetic rats we were able to show that SNK-860 is capable of suppressing the development of diabetic retinopathy by inhibiting the activity of aldose reductase. According to previous studies SNK-860 (2 mg/kg of body weight) treatment of STZ-diabetic rats for 2 weeks or 25 weeks resulted in a significant lowering of sorbitol levels (23, 24). Accumulated sorbitol in the sciatic nerve of STZ-diabetic rats were reduced by 91 % and 98 %. These reports lack data about the effect of this treatment upon the concentrations of sorbitol levels in the retinas of diabetic rats. However, a subsequent short-term experiment showed that the sorbitol levels in the retina were reduced by 55 % after SNK-860 treatment (2 mg/kg of body weight) for 6 weeks (data not shown).

It has also been reported that similar morphological changes in the retina of the experimental STZ-diabetic rats were encountered in rats that were kept on a galactose diet and have galactosemia (8, 25-29). These types of retinal damage are comparable to the pathologic changes in human diabetes mellitus (1). This suggests that abnormalities in the pathway of hexose metabolism are common to these 3 different diseases.

A striking finding in STZ-diabetic rats was the formation of folds in the retina, which was not observed in non-diabetic or STZ-diabetic animals receiving SNK-860. Unlike the retinal foldings or wrinkling reported to occur in human diabetes mellitus (30) or in other experimental models (31), associated epiretinal membranes could not

be detected, and the distortion was more pronounced in the outer retina. Folding of this type has been also reported in galactosemic rats (8) and occurs spontaneously in several strains of rats including Sprague-Dawley (32, 33). The spontaneous folds occur much more frequently in the retinal periphery (32), but we have never encountered the folds in any of the healthy Sprague-Dawley rats used for present observations. Lai and Rana noted fewer Müller cells per unit area within spontaneous folds, suggesting that the folding may occur as a result of age-related loss of Müller cells (32). Based upon the present study, in which we found a much higher incidence of posterior retinal folds in STZ-diabetic rats, one might postulate an accelerated drop in Müller cells in STZ-diabetic rats. These studies have demonstrated detectable levels of aldose reductase in Müller cells in non-diabetic animals (6, 34). The absence of similar folds in diabetic BB rats may be related to the small number of Müller cells in this strain (25), because differences in the occurrence of spontaneous folds have been noted between the strains (32).

Types of retinal damage similar to those seen in STZ-diabetic rats can be seen in galactosemic rats, but are more severe in the latter group. Galactose is a substrate more susceptible to aldose reductase than glucose and the resultant polyol (galactitol) is not further metabolized as is sorbitol by sorbitol dehydrogenase, leads to potentially greater intracellular polyol accumulation (35). All these findings suggest that the enhanced polyol metabolism may play a significant role in the development of the observed pathological changes.

In conclusion, this study described several types of light microscopical changes that occurred in the retinas of STZ-diabetic rats. Many of the changes were similar to those seen in galactosemic rats and were totally or partly inhibited by SNK-860, suggesting a possible role of aldose reductase in the development of retinopathy.

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