

Studies on the Effects of Growth Hormone on the Retinal Vascular Elements of Streptozotocin Induced Diabetic Rats

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The morphologically characteristic changes were irregular thickening of the basement membrane in the streptozotocin induced diabetic group (*S rats*) and more striking irregular hypertrophy of the same membrane in the group further administered with growth hormone (*S+BGH rats*). In the outer basement membrane surrounding the pericytes of *S rats* and *S+BGH rats* retinal capillary, and deposit of elastine could be suggested, which were seen as electron lucid, were proved by tannic acid staining to be substances with high electron density. Biochemical assay disclosed that the characteristic change was a marked increase in hydroxyproline in the group with diabetes and an increase in hexosamine and also in sialic acid in the group further administrated with growth hormone.

In view of the above finding, it was considered that substances of the collagen group are involved in the thickening of the basement membrane in diabetes, and that the loading with growth hormone is influential on glycoproteins deposition.

INTRODUCTION

It was described in one of our previous papers²²⁾ that diabetic retinopathy, one of the serious complications in diabetes mellitus, was improved by pituitary ablation. However, there remain not a few aspects yet to be elucidated in the mechanism of the effect of the surgery, and moreover little is known about the effects of growth hormone on the retinal capillaries in diabetes mellitus. For the purpose of elucidating the nature of diabetic retinal angiopathy and to reveal the effects of growth hormone, we have recently made morphological and biochemical studies of pathological and biochemical studies of pathological changes in the retinal blood vessels of rats with the streptozotocin-induced diabetes, and obtained some interesting findings.

MATERIALS AND METHODS

Female Wister rats, weighing 180–210 g, were injected intravenously through the caudal vein with 65 mg/kg of streptozotocin (The Upjohn Co., U. S. A.) dissolved in 0.3 ml of a citrate buffer (pH 4.5).

One injection was sufficient to induce diabetes. The animals were weighed, and their blood sugar levels were measured every 3 weeks after the injection and their urine sugar levels every 10 days. A simplified blood sugar measuring equipment "The Ames Reflectance Meter" (Ames Co., U. S. A.) (glucose-oxidase method) was used for the measurement of blood sugar levels, and the Tes-Tape (Eli Lilly and Co., U. S. A.) for the test of urine sugar. The rats with blood sugar levels of not less than 150 mg/dl and with persistent positive urine sugar 10 days after the injection were classified as diabetic (*S*), and these rats were bred for the following 6 months. The group treated with growth hormone (*S*+BGH) consisted of the *S* rats that had been reared for six months, then injected intraperitoneally with 3 mg of BGH (Miles Labs., U. S. A.) (activity: 0.8–1.0 U. S. P. units/mg) daily for 10 days, and observed for one month prior to their use for experimental purposes.

1. For the preparation of electron-microscopic specimens, the eyeballs were enucleated from the animals under ether anesthesia while 3 % glutaraldehyde-phosphate buffer (pH 7.3) solution was being applied dropwise; each eyeball was immediately fixed in the same 3% glutaraldehyde solution for 3 hours; its retinal tissue was cut into sections, fixed in 1% osmium tetroxide for 2 hours, dehydrated with ethanol, and embedded with the Epon 815 resin. Part of the embedded material was stained with 0.1 % toluidine blue, and, after the presence of small blood vessels in a histochemical specimen was confirmed, ultrathin sections were prepared with the Porter-Blum model MT₁ ultramicrotome, double stained with uranium acetate and lead citrate, and examined under the JEM model 7 electron microscope. Tannic acid staining⁸⁾ was performed to locate that part of the basement membrane which was less electron dense.
2. For the biochemical study, several units of 30 eyeballs each of the rats bred by the aforementioned method were assigned to the respective experimental groups. After fixation in 10% formaldehyde solution, the retina was separated and immersed in 3% trypsin solution (by Kuwabara-Cogan's method¹¹⁾) for 8 hours to obtain the retinal blood vessels. The retinal blood vessels thus obtained were homogenized in a Teflon homogenizer, washed and dehydrated with anhydrous acetone, and dried over P₂O₅ in a vacuum desiccator, and dry weight was measured. Part of the sample thus prepared was assayed for hydroxyproline, hexosamine and sialic acid.

1) For the assay of hydroxyproline, part of the dried sample was sealed in a test tube together with 1 ml of 6 N hydrochloric acid, and allowed to be hydrolyzed 100°C for 16 hours, after the hydrochloric acid was removed, the hydrolysate was made up to a given volume with 2 ml of distilled water, and assayed by Neuman-Logan's method

2) For the assay of hexosamine, part of the dried sample was added with 1 ml of 6 N hydrochloric acid. The mixture was sealed in a test tube and hydrolyzed at 100 °C for 6 hours. After removal of the hydrochloric acid, the hydrolysate was made up to a given volume with 2 ml of distilled water, and assayed for hexosamine by Rondle-Morgan's method¹⁷⁾.

3) For the assay of sialic acid, part of the dried sample was digested with papain at 65° for 1 hour. The digest was added to 1 ml of 0.1 N sulfuric acid, and hydrolyzed at 80°C for 1 hour. The hydrolysate was centrifuged and the supernatant was assayed for sialic acid by the thiobarbituric acid method¹⁸⁾.

RESULTS

1 Biological behavior of rats

1) Body weights and blood sugar levels of BGH-treated rats

While normal rats weighed 192 ± 16 g (mean \pm S. D.) and had the blood sugar levels of 89 ± 9 mg/dl, the animals one month after daily treatment with 3 mg of BGH for 10 days weighed 230 ± 11 g and had the blood sugar levels of 114 ± 13 mg/dl. Thus, the latter presented increased body weights and increased blood sugar levels, though the urine sugar remained negative. These findings proved BGH to be diabetogenic.

2) Body weights and blood sugar levels of *S*-rats.

The *S*-rats that had been reared for 6 months prior to BGH administration weighed 207 ± 31.5 g and had the blood sugar levels of 531 ± 39.6 mg/dl.

3) Body weights and blood sugar levels of S+BGH rats.

The above-mentioned S rats were administered daily with 3 mg of BGH for 10 days; and their body weights and blood sugar levels were measured, 15 days and 30 days after the end of the administrations. The animals weighed 227 ± 17 g, and had the blood sugar levels of 452 ± 26 mg/dl at termination of the administration, 246 ± 17 g and 527 ± 46 mg/dl 15 days after the administrations, and 259 ± 24 g and 32 mg/dl 30 days after the administrations.

The maximum blood pressure measured with the automatic blood pressure recorder model USM-105 (Ueda Electronic Works) at the end of each experiment was 102 ± 14 mmHg remaining within normal range.

2. Electron-microscopical findings (Figs. 1–5)

Electron-microscopical observation was made for retinal capillaries of which the basement membrane surrounding the endothelium was in direct contact at least in part with the surrounding glia tissue. The capillary plexuses of the retina in the posterior pole were divided into 2 layers; the plexuses in the nerve fiber layer and the ganglion cell layer were defined as the superficial layer ones, and those in the inner nuclear layer to the outer plexiform layer as the deep layer ones.

1) Retinal capillaries of normal rats (Fig. 1)

There was no morphologic difference between the superficial and deep layer capillaries. The retinal capillary lumen consisted of a thin monolaminar endothelium without fenestration. The endothelial cells were tightly interconnected. Their nuclei were shaped irregularly and their cytoplasm contained a few mitochondria and poly-somes and a relatively large number of pinocytotic vesicles in contact with the surface of the internal cavity. The outer basement membrane contacting the pericytes was 500–2500 Å thick, and the inner basement membrane contacting the endothelium 500–2000 Å thick. It consisted of amorphous substances with mostly consistent electron density (Fig. 1).

2) Retinal capillaries of *S rats* (Fig. 2)

Localized irregular thickening of the basement membrane was remarkable so as to measure 700–7500 Å in both the inner and outer basement membranes. Electron-lucid, almost homogeneous substances were present in the thickened basement membrane. The pericytes showed a decrease of cytoplasmic organelles associated with a marked decrease in electron density.

Changes in the endothelial cells were far less than those in the basement membrane and pericytes, but pinocytotic vesicles were increased.

3) Retinal capillaries of *S+BGH rats* (Fig. 3–5)

The basement membrane of deep capillaries was irregularly and prominently thickened in some localities, being even as thick as 8000 Å at some sites.



Fig. 1 Cross section of retinal capillary of normal rat (x 6800)



Fig. 2 Cross section of retinal capillary of *S* rat, shows an irregular thickening of the basement lamina (x 5800)

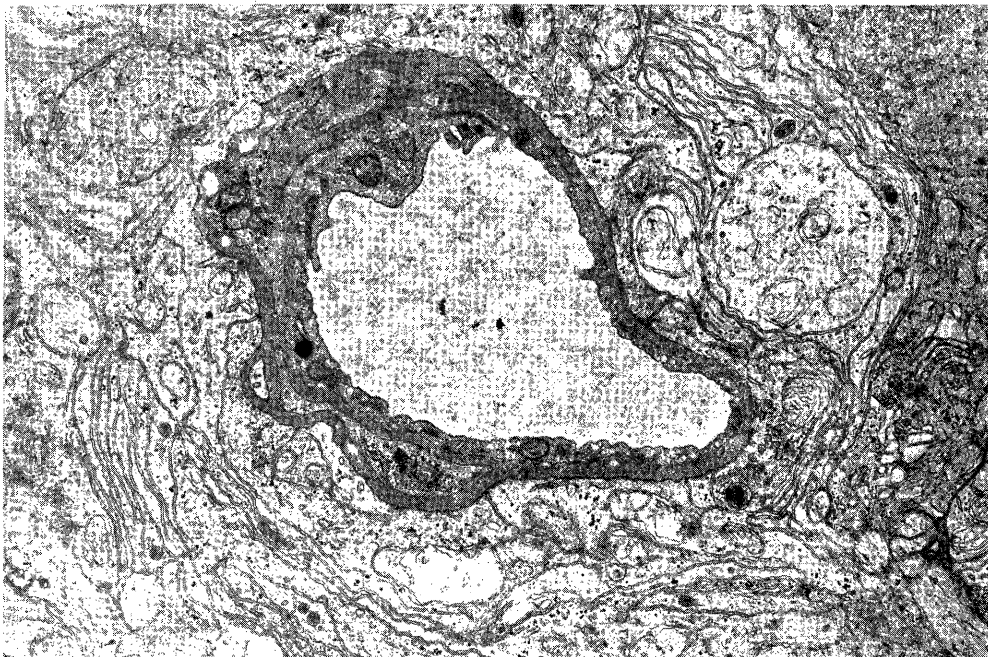


Fig. 3 Cross section of retinal capillary of *S+BGH* rat deposit of low electron density substance in outer basement lamia (x 10000)

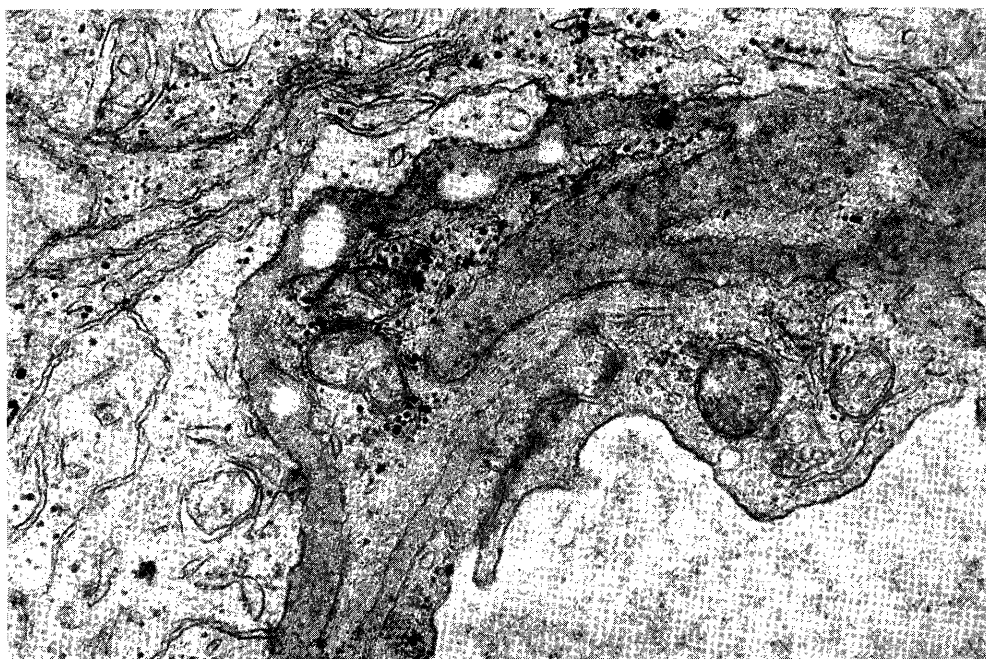


Fig. 4 High magnification of Fig. 3 (x 47000)

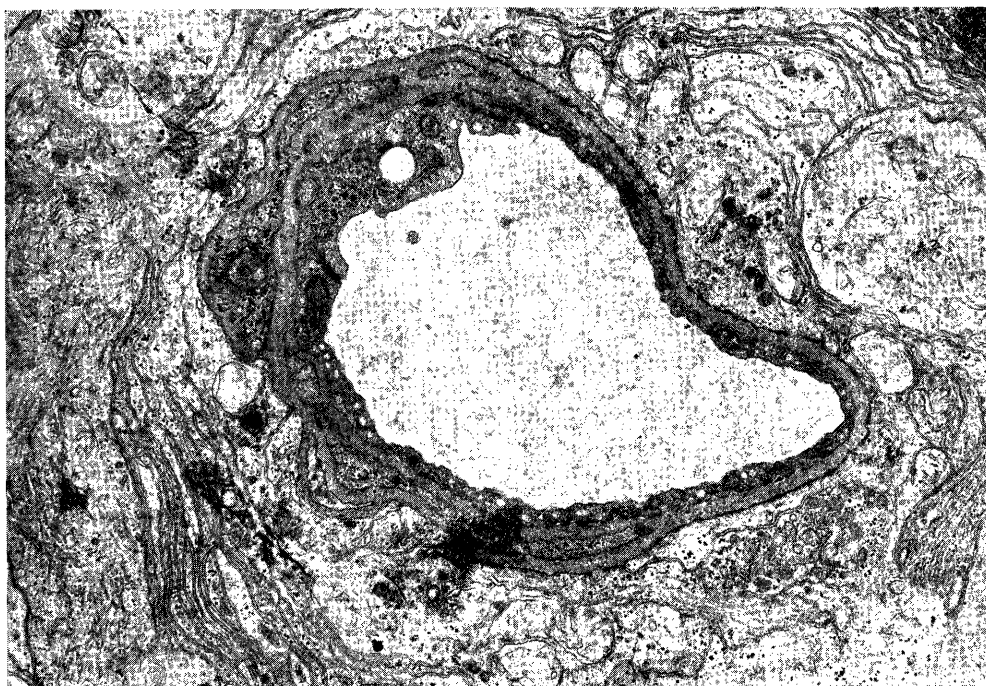


Fig. 5 Cross section of retinal capillary of *S+BGH* rat the electron lucid materials was showed as high electron density substances by tannic-acid staining (x 12000)

In the outer basement membrane surrounding the pericytes of the deep capillaries, there were some parts which were as electron-lucid as those found in the retinal capillaries of *S rats*, and which were proved by tannic acid staining to be substances with high electron density (Fig. 3, 4 and 5, 6).

3. Biochemical analysis of retinal blood vessels

For the biochemical study, 30 eyeballs each from *normal rats*, *S rats* and *S+BGH rats* collected as a unit were preserved in 10% formaldehyde solution; after washing the eyeballs, the retinas were separated and the retinal blood vessels were isolated by Kuwabara-Cogan's trypsin digesting method (8 hours) for use as materials. In order to know the degrees of damages to the blood vessels, preliminary experiments were made by electron-microscopic observation of the retinal blood vessels after the digestion. Further several units of 30 normal retinas were assayed at different times for hydroxyproline, hexosamine, and sialic acid to confirm their contents to be mostly constant.

The results of assays of 30 retinas each from the three groups after 8-hour digestion were as shown in Table 1.

Table 1. Hydroxyproline, Hexosamine and Sialic acid content of *normal*, *S* and *S+BGH rats* retinal vascular system

	Hydroxyproline	Hexosamine	Sialic Acid
Normal rats	10.40±0.31	12.38±0.43	10.26±0.31
S rats	18.90±1.20	21.60±1.20	18.90±1.12
S+BGH rats	19.10±0.64	27.60±0.75	23.40±0.70

mg/g dry weight tissue ± S.E.

DISCUSSION

Ever since streptozotocin, a broad spectrum antibiotic produced from *Streptomyces achromogenes*, was reported by Rakieten¹⁹⁾ in 1963 to be diabetogenic, this compound has been frequently used in animal experiments.

A single administration of streptozotocin is considered to induce diabetes with very high frequency, and selectively destroy the β cells of the islands of Langerhans. On the other hand, it has been proved that growth hormone is greatly influential on sugar metabolism, its major actions being (1) hypoglycemic action, (2) sugar transport and glycolysis, (3) glycogen retaining action, and (4) diabetogenic action.

The relationship between diabetic retinopathy and growth hormone administration was described for the first time by POULSEN¹⁶⁾ in 1936, and LUFT et al¹⁴⁾ in 1955 demonstrated that pituitary ablation is effective in treatment of the retinopathy. Many reports have been published on this theme ever since.

There are reports by LEUEMBERGER et al^{[12)(13)}, SOSULA et al^{[21)}, KOJIMA et al^{[10)}, TANIGUCHI et al^{[23)}, WATANABE^{[24)}, and BABEL et al^{[3)}, on electron-microscopic studies of the retinal capillaries in experimental diabetes. LEUEMBERGER et al^{[12)(13)}, showed the disposition of a basement membrane-like substance in the retinal capillary basement membrane and fibrous substance-containing thickening of the membrane of *S rats* that had been bred for not less than 6 months; and BABEL et al^{[3)}, also, observed irregular thickening of the basement membrane of *S rats* that had been bred for 6–12 months.

TANIGUCHI et al^{[23)}, obtained a finding similar to vacuolation within the basement membrane. However, the electron lucid part in the middle of the basement membrane found in our present study was not surrounded by the limiting membrane, and thus it could not be considered as vacuolation.

Studies on experimental diabetic retinopathy with growth hormone were made by HAUSLER^{[6)} in 1936, by ENGERMAN^{[5)} in 1965, by AGARWAL^{[1)} in 1965, and by BLOODWORTH^{[4)} in 1969. In our electron microscopy of the retinal capillaries of BGH-treated rats, however, capillary hemorrhage, microaneurysm, acellularity, and disappearance of mural cells which had been described by the aforementioned authors were not found.

Thus BGH appeared to play little or no role as a retinopathy-inducing factor. Therefore, from the point of view that it might be a retinopathy-accelerating factor, the retinal blood vessels of rats treated with STZ+BGH were studied.

There are few literature on the administration of growth hormone to animals in experimental diabetic condition nor are there any published data from biochemical studies. The electron lucid parts similar to those noted in STZ-treated rats were also observed in part of the basement membrane of deep capillaries, especially in the outer membrane surrounding pericytes. This finding is what is commonly called vacuole cavitation or "Swiss cheese." ASHTON^{[21)} in 1974 described it as lipid inclusion. However, we morphologically consider it as representing the presence of elastin, for the reasons (1) that the central area was electron lucid, (2) that the central area was surrounded by substances with high electron density, (3) that such structures occurred within the basement membrane, and (4) that the electron-lucid areas stained with tannic acid.

There are two ways of thinking about the mechanism of thickening of the basement membrane, i.e., lack of native enzyme^{[7)} and a acceleration of mucopolysaccharide composition ability^{[20)}. However, there appears to be no objection to the concept that the thickening is caused by the deposition of mucopolysaccharides in the basement membrane.

In the endothelial cells increased transport activity was observed. Such morphologic changes were more frequent in the deep capillaries than in the superficial ones, and this finding was consistent with the opinion that changes in the blood capillaries due to diabetic retinopathy are severe in the area close to the inner granular layer^{[9)}. There were, however, no apparent morphologic differences, from the rats treated with

STZ.

Few biochemical studies have been made of diabetic retinal angiopathy. As the results of this study in which the retinal blood vessels were separated by trypsin digestion from the retina of normal men and diabetics, and assayed for hydroxyproline, hexosamine and sialic acid, HEATH⁷⁾ in 1967 described that hydroxyproline was most increased in the diabetic eyeballs, followed by the increase in hexosamine, while sialic acid was not increased on the whole. Biochemical changes in the glomeruli in diabetic nephropathy are already known to a considerable extent. Based on the concept that pathologic changes in the glomerular basement membrane arise from abnormal sugar and protein metabolisms, SPIRO²⁰⁾ showed that the increase in glycerylgalactosyl-hydroxylysine in the basement membrane due to insulin shortage is the one and only characteristic change.

Alterations in the assays that were noted in the 3 groups, i.e., the group of normal rats, the group of rats treated with STZ and the group treated with STZ+BGH were considered to have resulted from the diabetic conditions in the respective groups and also from the direct or indirect effects of BGH.

Hydroxyproline, a characteristic amino acid of fiber protein, was strikingly increased in diabetes, and the administration of growth hormone markedly increased hexosamine and sialic acid which represent mucopolysaccharides or sial glycoproteins.

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