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Early Glomerular Changes in Streptozotocin Diabetes of the Guinea Pig *

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Summary. This work was to study—using morphometric methods—whether glomerular alterations are demonstrable in the guinea pig kidney even in streptozotocin diabetes of only short duration.

In 25, 50, 100 and 150-days diabetes was investigated the blood sugar, the glucose tolerance test, histological and morphometric studies.

Storage of glycogen in the kidney was to be found in numerous treated animals whereby it was evident that the cells of the macula densa showed no storage. The morphometric studies performed under blind conditions have shown that with a duration of diabetes of 50 and 100 days the number of cells in the mesangium is increased and that an increase of the mesangial matrix is demonstrable.

Hence the mesangium is in the foreground of the initial alterations in diabetes mellitus.

In numerous studies in the experimental animal (Bloodworth, 1965; Gibbs *et al.*, 1966; Shirai *et al.*, 1967; Østerby-Hansen *et al.*, 1967; Bergstrand *et al.*, 1968; Mancini *et al.*, 1969; Wehner *et al.*, 1970; Andreev *et al.*, 1970; Camerini-Davalos *et al.*, 1970; Ditscherlein, 1970; Rosenmann *et al.*, 1971; Like *et al.*, 1972; Wehner *et al.*, 1972) and in investigations in human diabetics (Hatch *et al.*, 1961; Geiler, 1966; Fukuhara, 1968; Kawano *et al.*, 1969; Kuhlmann *et al.*, 1969; Ireland, 1969; Fischer, 1969; Wehner and Anders, 1970; Olsen, 1971; Strauss *et al.*, 1971; Balodimos *et al.*, 1971; Olsen, 1972) one has attempted to elucidate the pathogenesis and the character of diabetic glomerulosclerosis. These authors have discussed many and varied factors which are said to be involved in the aetiology of diabetic glomerular alterations such as the diabetic metabolic state, the duration of diabetes, immunological and genetic processes or a combination of these three. Some authors have performed quantitative morphological studies on the glomeruli whereby it was demonstrated that both in man (Kawano *et al.*, 1969; Wehner and Anders, 1970; Kimmelstiel, 1970; Iidaka *et al.*, 1968) and in the experimental animal (Wehner *et al.*, 1972) diabetic glomerulosclerosis is associated with widening of the mesangium and proliferation of mesangial cells. Other authors consider changes of the basement membrane to be mainly responsible for the lesion (Østerby, 1973). However the studies mentioned above deal with the glomerular structure in diabetes of long standing or in freshly diagnosed diabetes but unknown duration of the prediabetic state in the human and the experimental animal and hence they do not furnish any reliable evidence as to the initial morphologically detectable alterations.

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Consequently the purpose of the present work was to study—using morphometric methods—whether glomerular alterations are demonstrable in the guinea pig kidney even in diabetes of only short duration. We employed streptozotocin as the diabetogenic substance (Rudas, 1972) since using the method of Brosky and Logothetopoulos (1969) it is possible to induce diabetes mellitus with this agent in the guinea pig.

Material and Methods

1. Experimental Animals

We employed a total of 68 guinea pigs from our own breed weighing between 465 and 560 g. The animals had free access to food up to the day before the streptozotocin injection. The kidneys of 28 surviving animals were employed for the morphometric studies (see below for details).

2. Streptozotocin Solution

Purified crystalline streptozotocin (The Upjohn Co., Kalamazoo, Michigan, U.S.A.) was dissolved in a buffered citrate solution (pH 4.5) immediately before the injection and used within a period of 5 minutes.

3. Experimental Groups, Dose, Injection

a) 25-Days Diabetes. Twenty-six animals with an average weight of 500 g were employed. The animals were deprived of food for 24 hours before the injection. The animals of this group also received 10 units of regular insulin subcutaneously 2–3 hours before the injection. The dose consisted of 150 mg per kg of body weight whereby 17 animals received the substance under ether anaesthesia as an i.v. injection into the dorsal vein of the penis. This did not succeed in 9 animals and the injection was given intraperitoneally. Eighteen animals succumbed within 2 days and 2 further animals after 8 days. Six surviving animals were killed after an experimental period of 25 days.

b) 50-Days Diabetes. The five animals of this group with an average weight of 560 g received 100 mg streptozotocin per kg of body weight intravenously after fasting for 24 hours and they were killed 50 days after this injection.

c) 100-Days Diabetes. Twenty animals weighing on average 560 g received 100 mg streptozotocin per kg of body weight intravenously after fasting for 24 hours. The 13 animals surviving after 7 weeks were given a second injection at this point of time of 50 mg per kg intraperitoneally. After the second injection 4 of the 13 animals had died by the end of the experiment. The remaining 9 animals were sacrificed after an experimental period of 100 days.

d) 150-Days Diabetes. Seventeen animals with an average weight of 465 g received 150 mg streptozotocin per kg of body weight after fasting for 24 hours; in 13 animals this was injected intravenously and in 4 animals intraperitoneally. After 8 days a total of 9 animals had died. The surviving 8 animals were killed 150 days after the injection.

4. Control Group

Five untreated normal animals of the same breed weighing between 400 and 500 g were used as controls.

5. Blood Sugar Determinations

The mean blood sugar value in the normal guinea pigs was calculated from individual measurements performed in the animals of group II+III before the injection of streptozotocin.

During the period of the experiments the blood sugar was determined in the animals at 7 to 14 day intervals and blood sugar curves were derived for some of the animals. The determinations were carried out with the glucose UV test using the hexokinase method (Boehringer Mannheim). Blood for the determinations was drawn from capillaries of the ear.

6. Glucose Tolerance Test

A glucose tolerance test according to Straub-Traugott was performed in 4 treated animals and 4 control animals in such a way that after fasting for 24 hours and an initial blood sugar determination the animals received 3 g of glucose by intraperitoneal injection. The blood sugar was determined again one and two hours after the administration of glucose.

7. Histological Methods

Six animals were killed by exsanguination under ether anaesthesia 25 days after commencement of the study, 5 animals after 50 days, 9 animals after 100 days and 8 animals after 150 days. Both kidneys were excised and fixed in 4 per cent buffered formalin. The pancreas was fixed in Bouin, the liver in alcohol and partly in Gendre, a segment of the kidney was fixed in the same manner for demonstration of glycogen. The pancreas was stained with aldehyde-fuchsin-trichrome for demonstration of the beta-cell granules; the liver and part of the kidney-glycogen staining after Best. Otherwise, PAS-reaction, HE and vG stainings. The renal tissue from cortical segments near the surface to be used for the quantitative studies was fixed again for 2 hours in 2 per cent buffered osmic acid and embedded in plexiglass. The sections 0.5–1 μ in thickness were argentated after Movat.

8. Morphometric Methods

In a blank experiment—i.e. without any knowledge of the group from which the material to be evaluated originated—58–81 different glomerular sections were studied per animal, on average 73. The following values were determined:

a) The surface area of each glomerular section (limited by Bowman's capsule) and the surface area of the mesangium contained therein using the point counting technique employing a Reichert Visopan (object lens plan 63/0.75 160/0.17; scale after measurement with object micrometer 770:1; point distance (net value)=9.1 μ).

b) The percentage fraction of the mesangial surface area of the total glomerular surface area.

c) The number of glomerular cells as a total and as a differential cell count of epithelial cells, mesangial and endothelial cells as well as the percentage distribution of the cells. These studies were likewise performed using a Reichert Visopan with object lens 63 and in difficult cases with oil immersion (object lens 100).

d) The total cell density in the glomerulus as a total cell count in 1000 μ^2 glomerular surface area and the mesangial cell density in the mesangium as a mesangial cell count in 100 μ^2 mesangial surface area.

Since according to the investigations by Kawana and assoc. (1971) and Hanberg-Sörensen (1972) the structures located in the middle half of a glomerulus are representative for the whole glomerulus in regard to morphometric parameters we have not evaluated sections whose total surface area was less than 250 μ^2. Further, renal corpuscles were not evaluated in the case of which the diameter of the free space between glomerular tuft and Bowman's capsule was larger than 1/5 of the diameter of the glomerulus in order to reliably exclude artificially altered or markedly tangentially cut glomeruli.

9. Statistical Methods

The statistical evaluation was performed using Student's t-test. We chose 2 $p<0.05$ (5%) as the limiting value of the error probability. Such results are regarded as being significant.

Results

1. Metabolic Behaviour

The mean normal blood sugar for the guinea pig is 63.9 ± 20.6 mg%. As compared with this value a significant increase in blood sugar is to be observed for all

Table 1. Behaviour of the blood sugar in the various groups and mean blood sugar levels in normal animals. (Values in brackets = number of animals studied. In groups I + IV determinations at intervals of 7 to 14 days, in groups II + III at 14 day intervals)

Experimental group	Number of blood sugar determinations								Mean value of all measurements
	1	2	3	4	5	6	7	8	
I.	153 (7)	90 (6)	102 (6)	112 (6)					123
II.	137 (5)	95 (5)	90 (5)	82 (5)					104
III.	119 (5)	78 (5)	85 (5)	122 (5)	145 (5)	115 (5)	85 (5)	60 (5)	100
IV.	81 (15)	93 (13)	114 (18)	105 (8)	99 (8)	68 (8)			98
Normal				64 (25)					

Table 2. Results of the glucose tolerance test

	n	BS before glucose injection	BS 1 hour after glucose injection	BS 2 hours after glucose injection
Normal	4	86 ± 17	99 ± 26	85 ± 10
Diabetes	4	65 ± 5	130 ± 15	106 ± 8

the treated groups which is marked particularly during the first 2 to 4 weeks but which diminishes in the course of time. Furthermore, the blood sugar level is related to the dorsage and the mode of administration whereby in our experiments the double administration of smaller streptozotocin doses was shown to be favourable since with a lower mortality rate relatively high blood sugar levels were achieved. In some of the animals during the later phases of the study and particularly in group III+IV the blood sugar level had partly returned to the normal range again (Table 1). The mean values of all measurements in the various groups, however, are significantly higher than the mean normal value (Table 1). The diabetic metabolic situation is also evident from the results of the glucose tolerance test (Table 2). It is evident from the blood glucose curve of individual animals that the blood sugar level in the treated animals fluctuated considerably in which differences of 100 mg % were partly to be observed at different points of time when determinations were performed.

2. Pancreas

The morphological expression of this metabolic behaviour in the sense of unstable diabetes are the changes in the islets of Langerhans. We observed marked degranulation of the beta-cells in the treated animals.

3. Liver

The expression of increased deposition of glycogen in the liver was the evidence of fine-granular storage of glycogen in the liver epithelia of the treated animals.

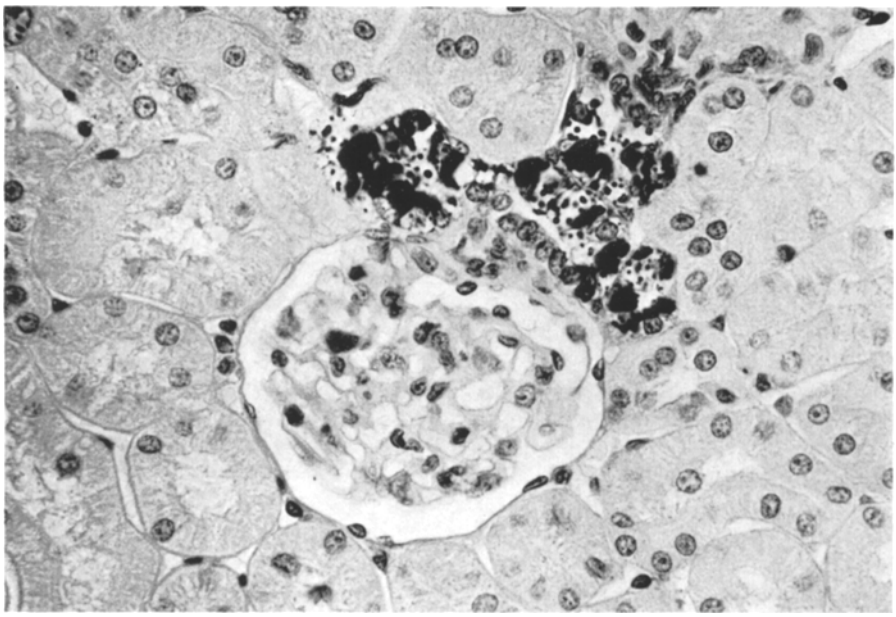

Fig. 1. Glycogen deposition in the middle segment epithelia, practically no storage in the cells of the macula densa (guinea pig, 50-days streptozotocin diabetes, carmine staining after Best, ×280

4. Kidney

a) Storage of Glycogen

Storage of glycogen in the kidney was to be found in numerous treated animals whereby it was evident that the cells of the macula densa showed no storage at all or only a very slight one whereas opposite epithelia of the middle segment showed marked deposition (Fig. 1).

b) Quantitative Morphology of the Glomeruli

Normal Animals. The mean surface area of the glomerular sections studied was $6610 \pm 534\ \mu^2$, that of the mesangial section was $485 \pm 51\ \mu^2$. This corresponds to $7.42 \pm 1.28\%$ of the total surface area. The cell count with a glomerular total cell count within a section of 30.1 ± 2.6 showed on average 10.7 ± 0.9 (=35.7%) epithelial cells, 11.5 ± 2.0 (=38.0%) endothelial cells and 7.9 ± 1.0 (=26.3%) mesangial cells. Consequently a normal cell density within a glomerular section of 4.59 ± 0.6 total cells /1000 μ^2 of total glomerular surface area and a mesangial cell density of 1.63 ± 0.1 mesangial cells/100 μ^2 mesangial surface area (Table 3) results.

Treated Group I. The average surface area of the glomerular section in these 25-days diabetic animals of $5648 \pm 486\ \mu^2$ and the average mesangial surface area of $372 \pm 62\ \mu^2$ were just significantly lower than the normal values. Correspondingly the total glomerular cell count of 26.8 ± 2.6 cells is significantly lower

Table 3. Summary of the morphometric parameters in the various groups of animals ($\bar{x} \pm s$ = mean value $\pm$ standard deviation, $2p < x$ = error probability as compared with normal values)

	Duration of study in days				Normal animals
	25 $\bar{x} \pm s$ ($2p < x$)	50 $\bar{x} \pm s$ ($2p < x$)	100 $\bar{x} \pm s$ ($2p < x$)	150 $\bar{x} \pm s$ ($2p < x$)	
No. of Epithelial cells	10.0 ± 0.9 (0.3)	9.7 ± 0.9 (0.2)	9.7 ± 0.8 (0.2)	10.1 ± 0.9 (0.2)	10.7 ± 0.9
No. of Endothelial cells	9.5 ± 1.4 (0.1)	12.8 ± 1.0 (0.3)	12.0 ± 1.8 (0.7)	11.4 ± 2.6 (0.975)	11.5 ± 2.0
No. of Mesangial cells	7.3 ± 1.0 (0.4)	9.3 ± 2.0 (0.3)	8.3 ± 0.9 (0.7)	9.6 ± 0.9 (0.02)	7.9 ± 1.1
Total no. of cells	26.8 ± 2.6 (0.1)	31.7 ± 1.8 (0.2)	30.0 ± 2.8 (0.95)	31.1 ± 2.5 (0.5)	30.1 ± 2.6
Epithelial cell % of total cells	37.4 ± 3.3 (0.5)	30.7 ± 3.8 (0.1)	32.3 ± 2.0 (0.2)	32.6 ± 3.1 (0.2)	35.7 ± 4.2
Endothelial cell % of total cells	35.3 ± 2.9 (0.3)	40.2 ± 2.7 (0.4)	39.9 ± 2.6 (0.4)	36.4 ± 5.3 (0.6)	38.0 ± 3.8
Mesangial cell % of total cells	27.3 ± 1.4 (0.4)	29.1 ± 5.0 (0.3)	27.7 ± 2.9 (0.5)	29.9 ± 4.7 (0.2)	26.3 ± 2.2
Total area (sq. μ)	5648 ± 486 (0.02)	7278 ± 1610 (0.5)	6791 ± 280 (0.6)	6567 ± 259 (0.9)	6610 ± 534
Total cells/1000 sq. μ of total area	4.8 ± 0.4 (0.7)	4.5 ± 0.7 (0.8)	4.4 ± 0.4 (0.6)	4.8 ± 0.5 (0.6)	4.6 ± 0.6
Mesangial area % of total	6.7 ± 1.4 (0.4)	6.8 ± 1.8 (0.4)	6.3 ± 0.6 (0.2)	7.8 ± 2.7 (0.8)	7.4 ± 1.3
Mesangial area (sq. μ)	372 ± 62 (0.01)	459 ± 136 (0.7)	426 ± 28 (0.1)	515 ± 180 (0.8)	485 ± 51
Mesangial cells/100 sq. μ of mesangial area	2.1 ± 0.5 (0.2)	2.1 ± 0.3 (0.02)	2.0 ± 0.2 (0.025)	2.0 ± 0.6 (0.2)	1.6 ± 0.1

than normal. On the other hand the relative comparative values of $37.4 \pm 2.2\%$ epithelial cells, $35.3 \pm 2.9\%$ endothelial cells, $27.3 \pm 1.4\%$ mesangial cells and $6.7 \pm 1.4\%$ mesangial surface area are not significantly different from the normal values. Likewise the glomerular cell density (4.75 ± 0.43 total cells/1000 μ^2 glomerular surface area) and the mesangial cell density in the mesangium (2.1 ± 0.5 mesangial cells/100 μ^2 mesangial surface area) are within the normal range (Table 3).

Treated Group II. In the 50-days diabetic animals the mesangial cell density in the mesangium of 2.1 ± 0.2 cells/100 μ^2 mesangial surface area is significantly increased as compared with the normal value. None of the other values shows any significant difference as compared with the normal values (Table 3).

Treated Group III. Likewise in the 100-days diabetic animals the mesangial cell density of 2.0 ± 0.2 mesangial cells/100 μ^2 mesangial surface area is the only parameter which is altered as compared with the normal values (Table 3).

Treated group IV. In the 150-days diabetic animals the number of mesangial cells of on average 9.6±0.9 cells in the glomerular section is significantly increased as compared with the normal value. Furthermore, the mesangial surface area and percentage mesangial fraction of the total surface area (515±180 μ^2 and 7.8%) show the highest values of all investigated groups. However these values are not adequately statistically significant. Likewise the remaining parameters are not statistically significantly changed as compared with the normal values (Table 3).

Discussion

If we first consider the results in regard to the disturbance of metabolism then it is apparent that by i.v. or i.p. injection of 100–150 mg streptozotocin per kg of body weight it is possible to induce a diabetic metabolic state in the guinea pig as well. In contrast, Kushner *et al.* (1969) did not consider that this was inducible whereas Brosky and Logothetopoulos (1969) and Losert *et al.* (1971) have already published findings similar to our own. The decrease in the blood sugar levels parallel to the duration of the study was also reported by Losert *et al.* (1971) which was first explained hypothetically by particular regenerative properties of the beta-cells of guinea pigs. To summarize one can say that in streptozotocin-treated guinea pigs a diabetic metabolic state was demonstrable over the entire period of the investigations which is characterized by marked fluctuations of the blood sugar level. According to Frösch (1971) this factor in particular promotes the development of a diabetic microangiopathy. Hence it appears that this model of diabetes is very suitable for studies on the connections between the diabetic metabolic disturbance and glomerular alterations (Cameron *et al.*, 1973).

Deposition of glycogen in the region of the median epithelial segments with the special feature that the cells of the macula densa are practically free from glycogen was also reported by Torhorst (1971) in alloxan diabetic rats. Hence this finding too suggests a particular (functional) differentiation of the macula densa as compared with the other epithelia of the median segment.

The morphometric studies performed under blind conditions have shown that:

1. With a duration of diabetes of 50 and 100 days the number of cells in the mesangium is increased in a comparable surface area as compared with the non-diabetic animals, i.e. at this point of time there is a proliferation of mesangial cells.

2. This was also reflected in 150-days diabetic animals in an absolute increase in the mesangial cells.

3. The fraction of the mesangium of the glomerular surface area is increased after diabetes of 150 days duration, i.e. at this point of time an increase of the mesangial matrix is demonstrable.

Hence the mesangium is in the foreground of the initial alterations in diabetes mellitus. This is in general acknowledged as far as advanced forms of diabetic glomerulosclerosis is concerned (Kimmelstiel and Wilson, 1936; Kimmelstiel, 1968; Ditscherlein *et al.*, 1970) and it has also been confirmed by quantitative morphological findings (Wehner *et al.*, 1972; Kawano *et al.*, 1969; Wehner and Anders, 1970; Iidaka *et al.*, 1968). Apart from the diffuse thickening of the basal membrane Kimmelstiel (1968) and Ditscherlein (1969) consider that the mesangial

cell proliferation with increased formation of mesangial matrix constitutes the commencement of the diffuse mesangial thickening, i.e. they are of the opinion that the proliferation of mesangial cells occurs at the commencement of the glomerular processes in diabetes mellitus. Hoet (1973) also described marked glomerulosclerosis even in diabetes of short duration. Likewise our own findings can be interpreted in this manner since we were able to demonstrate mesangial cell proliferation in the animals with diabetes of only short duration. If one assumes that a cell proliferation in the glomerulus is an expression of an inflammatory process then our findings suggest that diabetic glomerulosclerosis is of an inflammatory origin.

The investigations by Østerby (1972, 1973) are not in consistence with our conception that the initial alterations take place in the mesangium; she considers that the thickening of the basement membrane is the first lesion. She demonstrated a thickening of the glomerular basement membrane in diabetes mellitus of long standing which however is said not to commence primarily in the juxtamesangial segments of the basal membrane. According to her investigations the mesangial region is unchanged at the commencement of diabetes (Østerby, 1973 b). She considers that the first alterations is an increase in basement-membrane-like material in the mesangium. This increases parallel to the thickness of the basement membrane. According to her electron microscopic studies the cell counts are the same in diabeticd during the first five years of the disease and in non-diabetics (Østerby, 1973 c). Since deposition of basement-membrane-like material in the mesangium, which has also been demonstrated by Østerby, must result in widening of the mesangium, the finding that the mesangial region is unchanged is incomprehensible. The reason for the diverging results is evidently related to the method employed for quantification and especially as in studies by Østerby arbitrary limitation of the mesangial region renders the planimetry possible. It has not yet been clarified whether this can replace a hit method which is based on the principle of chance of the measured points and which consequently can be evaluated statistically (point counting technique). The different number of the investigated glomeruli which is naturally smaller employing electron microscopic quantitative methods also plays a role.

On the other hand there is no doubt that the first changes after short-lasting diabetes mellitus are discrete. Cameron *et al.* (1973) in streptozotocin diabetic rats did not find a borderline-statistical thickening of the basement membrane in the glomeruli as compared with non-diabetic controls until after 6 months' duration of diabetes. On the other hand Salazar *et al.* (1973) in streptozotocin-diabetic monkeys found no differences in the thickness of the basement membrane as compared with normal animals after 6 to 15 months' duration of diabetes. In dogs with alloxan diabetes of 7 years' duration the changes were unequivocal; there was glomerulosclerosis with proliferation of the mesangial cells and depositon of basement-membrane-like material and thickening of the basement membrane (Bloodworth *et al.*, 1973). No thickening of the basement membrane was found in KK mice as compared with control animals during the first few months of life. On the other hand marked increase of the mesangial matrix was found in 3-month old KK mice (Ehrenreich *et al.*, 1973) and in mice with virus-induced diabetes mellitus-like disease (Kanich *et al.*, 1973). These findings show that the

development of the glomerular alteration in diabetes mellitus is a very slow process and in early phases only discrete findings are to be expected which can be detected by quantitative methods. As far as the aetiology of these changes is concerned we are in agreement with Østerby (1972). We too consider that the metabolic disorder is the causative factor since in our assay system only the parameter of hyperglycaemia was given. However we would like to suggest that the duration of the metabolic disorder is of greater importance than its severity (Camerini-Davalos *et al.*, 1970; Kuhlmann *et al.*, 1969; Ireland, 1969; Kimmelstiel, 1968).

A direct toxic effect of streptozotocin on the glomeruli has not as yet been described (Rudas, 1972), likewise in histological studies of the kidneys we have not observed any alterations which would support such a possibility.

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