Irreversibility of Glomerular Basement Membrane Accumulation Despite Reversibility of Renal Hypertrophy with Islet Transplantation in Early Experimental Diabetes

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SUMMARY

A quantitative morphologic study of the glomeruli in rats after 4 wk of streptozotocin-induced diabetes showed a number of glomerular changes, as previously described. Of particular interest was the increase in the total amount of glomerular basement membrane material [from 0.94 ± 0.13 (SD) mm^3 to 1.26 ± 0.14 mm^3 per kidney]. This parameter did not change after 4 wk of normoglycemia following islet cell transplantation (1.19 ± 0.17 mm^3), nor was the total glomerular volume normalized. The contralateral kidney was weighed and used for estimating the total amounts of protein, RNA, and DNA. Four weeks of diabetes expectedly resulted in a 50% increase in kidney weight, and islet cell transplantation diminished this to 15% in excess of normal.

The average cell size (protein/DNA ratio) paralleled the kidney size after diabetes and following transplantation. The average amount of RNA per cell (RNA/DNA) increased significantly after induction of diabetes and was totally normalized after transplantation. Kidney protein concentration (mg protein/mg kidney) remained constant throughout the experiment. Considering that a few weeks of diabetes provokes a large increase in basement membrane material, it is especially noteworthy that 1 mo of normoglycemia is quite insufficient to reverse the accumulation.

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Very early in the course of diabetes, preceding evidence for microangiopathy such as basement membrane (BM) thickening, there are marked changes in the kidney that include renal and glomerular hypertrophy with enlargement of the surface area of the glomerular capillaries.[1–4] For the study of these early renal changes, the diabetic rat has proven to be a useful model.[5] As early as 4 days after induction of diabetes, there is a significant increase in kidney weight (20%), in glomerular volume (30%), and in the total amount of basement membrane material (BMM) (40%).[6,7]

The aim of the present study was to investigate whether the acutely accumulated BMM can be removed during comparatively short periods after islet cell transplantation. The renal hypertrophy was examined by measuring the total amounts of protein, RNA, and DNA before and after transplantation.

MATERIAL AND METHODS

Highly inbred female Lewis rats, weighing 150–190 g, were allocated into three groups: normal control animals (C), diabetic animals (D), and diabetic transplanted animals (T). Diabetes was induced by streptozotocin (70 mg/kg i.p.). Only rats with a nonfasting blood glucose between 250 mg/dl and 450 mg/dl and with a weight loss of less than 30 g after 4 wk of diabetes were included. Blood glucose was measured twice weekly throughout the experimental period (Ames Reflectance Meter). The experimental protocol is outlined in Figure 1.

Transplantation. After 4 wk of diabetes, islet transplantation was performed intraportally by the method of Matas et al.[8] with minor modifications. The donors (4–6 per recipient) were 3–8 days old. After decapitation, the pancreata were excised and finely minced in 5 ml of cold Hanks' solution. Collagenase (25 mg) (Calbiochem. Inc.) was added and the tube was placed in a shaking waterbath for 20 min at 37°C. The tissue was then washed four times in 10 ml cold Hanks' solution and centrifuged for 1 min (110 × *g*). The final sediment was suspended in 0.5 ml Hanks' solution and injected. The two control groups, C and D, were sham operated after 4 wk and given an intraportal injection of 0.5 ml of Hanks' solution. Blood glucose was normalized 1–3 wk after the transplantation (see Figure 2), and thereafter, all the transplanted animals remained normoglycemic. [Blood glucose was 104 ± 17 (SD) mg/dl versus 100 ± 16 mg/dl in the nondiabetic control animals.] At the time of death, transplanted

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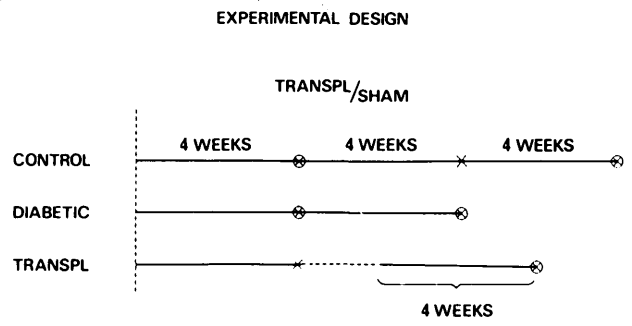


FIGURE 1. Experimental protocol. At the start of the experiment the animals were allocated into three groups: controls, diabetics, and diabetics to be transplanted. Four weeks later, the diabetic group T was transplanted while groups C and D were sham operated. Animals from groups C and D had their kidneys removed at points of times indicated by ⊗. Transplanted animals were killed after 4 wk of normoglycemia. Because of the varying time span from the transplantation to the appearance of normoglycemia, this was done 5–7 wk after the transplantation.

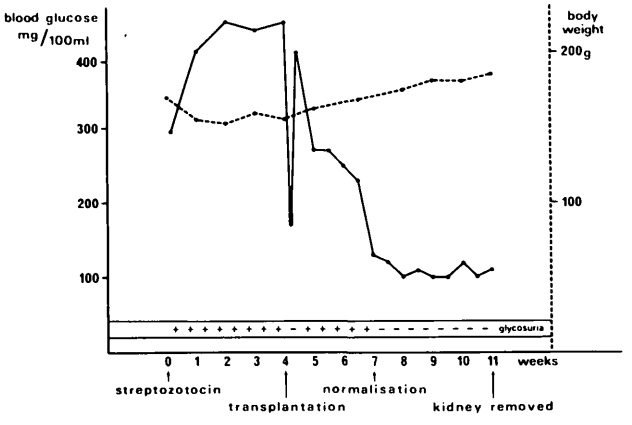
animals had reached the body weight of the normal controls.

Biochemical determination. At termination, the animals were anesthesized with pentobarbital, the right renal pedicle was ligated, and the kidney was removed. The kidney was trimmed free of capsule and fat, weighed, and immersed immediately in ice-cold water. The kidney was homogenized, and RNA and DNA were extracted by a Schmidt-Tannhaüser technique according to Munro and Fleck.[9] RNA was determined by ultraviolet spectrophotometry at 260 nm (assuming that an absorbance of 1000 corresponds to 32 μg of RNA) and DNA was determined with diphenylamine.[10] The protein concentration of kidney homogenate was determined by the method of Lowry et al.[11] with bovine albumin as a standard.

Tissue preparation. After removal of the right kidney, the left kidney was fixed by perfusion in situ at a constant pressure of 120 mm Hg. Kidneys that failed to blanch immediately after the start of the perfusion were discarded. The differences in sample sizes between the biochemical and morphologic estimations are partly explained by this fact and partly by the easy access of animals for biochemical studies. The perfusion medium, prepared shortly before the experiment, consisted of 1% glutaraldehyde in a modified Tyrode's buffer containing 22.5 g/L dextran T40.[12] The osmolality of the medium was checked each time and varied from 336 to 354 mosm/L. The kidney was postfixed in the perfusion medium for 1½ h and stored in Tyrode's buffer for 5 days. It was then cut into a series of slices of alternating thickness (3 and 0.8 mm) by use of a set of fixed razor blades with an arbitrary position with respect to the kidney. All thick slices were embedded in paraffin. PAS stained sections from three levels of each slice, spaced about 300 μm apart, were used for the light microscopic determination of glomerular volume.

Blocks 1 mm in diameter were systematically punched out of the thin slices and embedded in Vestopal. Thin sections were cut from three strictly randomly selected glomerular cross-sections. They were photographed in toto with a Jeol 100 C electron microscope to provide a final magnification of 3200× (Low Magnification Electron Microscopy, LMEM), as shown in Figure 3. A systematic, independently positioned sample comprising an average of 10 micrographs per cross-section was photographed to provide a final magnification of 19,500× (High Magnification Electron Microscopy, HMEM). For further details of the above sampling procedures, see Østerby and Gundersen.[13]

FIGURE 2. Effect of transplantation. Nonfasting blood glucose, body weight, and glycosuria in one of the transplanted animals.



MORPHOMETRY

LIGHT MICROSCOPY

By use of standard stereologic techniques,[14] the volume fraction of glomeruli was estimated from the paraffin sections in systematically selected, independently positioned fields of vision using a magnification of 340×. A glomerulus was defined as the minimal convex figure enclosing the glomerular tuft. As it has been shown that the density of kidney structures equals 1.0 g/cm³ in diabetic as well as in control rats,[7] the total glomerular volume in the left kidney V(glom.) equals V_V(glomeruli/kidney) · kidney wt.

ELECTRON MICROSCOPY

Relative structural quantities. On LMEM micrographs, the minimal convex figure enclosing the epithelial side of the BM was drawn (string polygon, see Figure 3), and all other compartment quantities were expressed in relation to this glomerular reference space. The "naked" tuft volume, delineated by the bases of the epithelial foot processes and consisting of the combined volume of mesangial regions, endothelium, peripheral BM, and capillary lumen, was the reference volume employed in the quantitative analysis of the HMEM micrographs.

On the LMEM micrographs the individual tuft volume fractions, V_V(tuft/glomerulus), were estimated by point counting. The surface density (S_V) (e.g., the peripheral BM per unit glomerular volume) was estimated by

$$S_V \text{ (peripheral BM/glomerulus)} = \frac{2 \cdot I \text{ (peripheral BM)}}{k \cdot P} \text{ mm}^2/\text{mm}^3$$

where I(peripheral BM) is the total number of intersections between the surface trace of peripheral BM and the test lines, P is the number of test points hitting the string-polygon, and k is the real distance at the tissue level in mm between two adjacent test points. The length density (L_V) of glomerular capillaries per unit glomerular volume was esti-

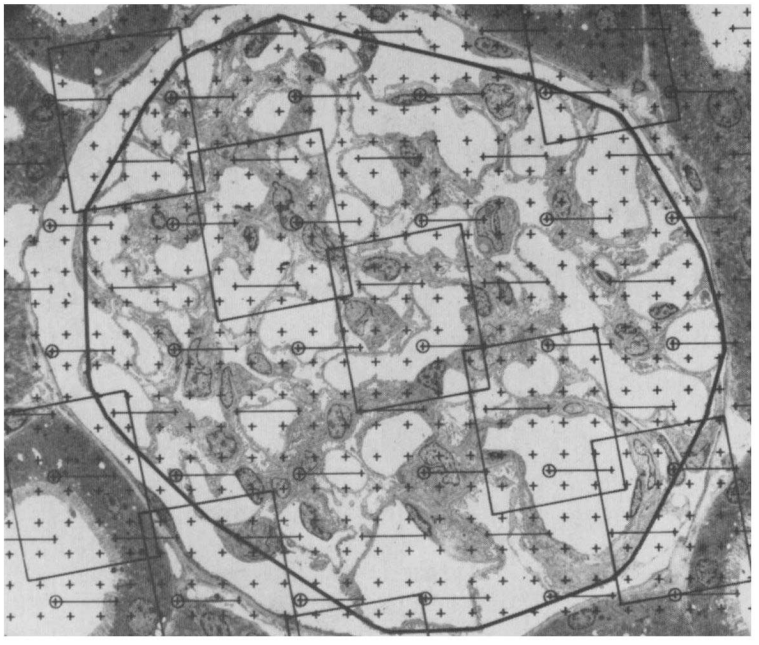


FIGURE 3. Low Magnification Electron Micrograph (LMEM) with grid superposed. The so-called string polygon is delineated on the micrograph with a solid line. The rectangular areas photographed at high magnification (HMEM) are shown.

mated by

$$L_V = \frac{2 \cdot Q\ (\text{lumina})}{k^2 \cdot P\ (\text{glomerulus})}\ \text{mm/mm}^3$$

where Q(lumina) is the total number of luminal profiles, i.e., the isolated blood space transections.[15]

On HMEM micrographs, the fractional volumes of epithelium, endothelium, capillary lumen, mesangial region, peripheral BM, and basement membrane-like material in the mesangium were estimated by point counting with the tuft as reference volume.

Absolute structural quantities. BM thickness was determined by measuring orthogonal BM intercepts. The empirical distribution of reciprocal orthogonal intercept length, L_0, was then transformed into the expected distribution of the true BM thickness, t, from which the harmonic and arithmetic mean thicknesses, t_h and t_a, respectively, were calculated.[16] The harmonic mean BM thickness is given in Table 1.

The total volume of peripheral capillary BMM was estimated by

$$V(BM) = S(\text{peripheral BM}) \cdot \bar{t}_a(BM)\ \text{mm}^3$$

as described elsewhere.[6]

By multiplying all the relative structural quantities defined above with the appropriate absolute reference volumes, absolute individual quantities are obtained, e.g.,

$$V(\text{lumen}) = V_v(\text{lumen/tuft}) \cdot V_v(\text{tuft/glomerulus}) \cdot V(\text{glom.})\text{mm}^3$$

The reproducibility of the above stereologic techniques and the contribution of the methodologic variation to the overall variation of the results are described in detail elsewhere.[13,17]

STATISTICS

Student's *t* test was used for estimating the significance of the change in BMM after transplantation. A 5% level of sig-

TABLE 1
Morphologic parameters in one left-sided kidney

	C N = 7	D N = 11	T N = 5	D − C	T − D
Glomerular volume (mm³)	28.5 ± 3.8	38.7 ± 3.8	34.5 ± 3.8	10.2 ± 1.3	−4.2 ± 1.8
Volume of PBM (mm³)	0.94 ± 0.13	1.26 ± 0.14	1.19 ± 0.17	0.32 ± 0.07	−0.07 ± 0.08
Surface area of peripheral BM (cm²)	59.2 ± 9.4	77.7 ± 8.9	75.6 ± 14.8	18.4 ± 4.4	−2.1 ± 5.8
Surface of mesangial regions towards the urinary space (cm²)	24.5 ± 3.7	32.0 ± 8.3	27.0 ± 7.8	7.5 ± 3.4	−5.0 ± 0.5
Length of capillaries (m)	387 ± 85	482 ± 36	420 ± 104	95 ± 34	−62 ± 48
Volume of capillary lumen (mm³)	10.2 ± 1.5	13.6 ± 2.9	12.0 ± 2.3	3.4 ± 1.2	−1.6 ± 1.5
Volume of endothelium (mm³)	1.81 ± 0.23	2.22 ± 0.33	1.83 ± 0.54	0.41 ± 0.14	−0.39 ± 0.25
Volume of epithelium (mm³)	5.7 ± 1.5	8.2 ± 1.7	6.3 ± 0.6	2.5 ± 0.8	−1.9 ± 0.8
Volume of mesangium (mm³)	3.8 ± 0.6	5.3 ± 1.1	4.2 ± 0.9	1.5 ± 0.4	−1.1 ± 0.6
Volume of mesangial BM material (mm³)	1.57 ± 0.39	2.12 ± 0.54	1.98 ± 0.62	0.55 ± 0.24	−0.14 ± 0.32
Thickness of PBM (nm)	145 ± 7	144 ± 5	149 ± 13	−1 ± 3	5 ± 4

C indicates normal control animals, D diabetic animals, T diabetic transplanted animals, and n the number of animals in each group. The mean and SD are given for each group, and for estimating the effect of diabetes (D − C) and transplantation (T − D), respectively, the differences of means and their SEM are indicated.

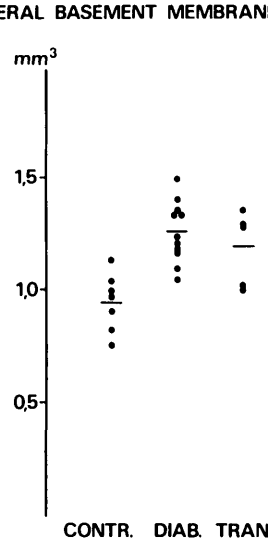


FIGURE 4. Total amount of peripheral basement membrane material (mm³/left kidney). C indicates normal control animals, D diabetic animals, and T diabetic transplanted animals.

nificance was used for evaluating the a priori formulated null hypothesis.

RESULTS

The various control and diabetic subgroups as defined in Figure 1 showed no systematic variation within their respective groups, and the results were therefore pooled into one control and one diabetic group.

Four weeks of diabetes induced a pronounced increase in the total amount of peripheral BMM [from 0.94 ± 0.13 (SD) mm³ to 1.26 ± 0.14 mm³ per kidney], as shown in Figure 4. After transplantation, this amount did not change (1.19 ± 0.17 mm³), 2P = 0.85. BM thickness was not increased after 4 wk of diabetes. Substantial changes of the other glomerular morphologic parameters were observed in the diabetic animals (Table 1). After transplantation, they were not reversed, although there was a general tendency toward normalization.

A 50% increase in kidney weight was found in diabetes. Islet cell transplantation followed by 4 wk of normoglycemia resulted in a significant reversal of this hypertrophy, although the kidneys from the transplanted animals were still 15% larger than those from the controls, as shown in Table 2.

Total protein and RNA content followed the changes in kidney weight. After transplantation, the increase was reversed but not totally normalized. The DNA content showed an increase after 4 wk of diabetes and the amount in the transplanted group was intermediate between controls and untreated diabetics. The average cell size, indicated by the protein/DNA ratio and the average amount of RNA per cell (RNA/DNA), showed the same changes following diabetes and transplantation as the kidney weight.

DISCUSSION

As expected, 4 wk of streptozotocin diabetes in the rat induced a number of changes in the kidney: renal and glomerular hypertrophy with enlargement of the filtration surface. Of particular interest is the finding of increased amount of BMM, which is in accordance with our earlier findings.[6] Transplantation and the ensuing 1 mo of complete normoglycemia had very little effect on this parameter. This should be seen in relation to the fact that the renal hypertrophy could be reversed, although not completely normalized, and that some of the other glomerular parameters tended to revert.

Some recent reports on the reversibility of the diabetic glomerulopathy in experimental diabetes conclude that the changes in the mesangium can be reversed. Bretzel et al. reported a quantitative light microscopic study showing a marked reduction in mesangial volume fraction after transplantation.[18] However, their results are left uninterpretable by the fact that transplanted animals were heminephrectomized at the time of transplantation, a procedure which leads to a number of changes in the contralateral kidney.[6] In biopsy studies, Steffes et al.[19] used quantitative measurements at the EM level, and they showed a reverse of mesangial enlargement after transplantation. Their animals had blood glucose levels considerably higher than the rats in our study, and this might explain why the glomerular volume did not increase significantly after 9 mo of diabetes.[5] This together with differences in methodology could explain our apparently different results. Our results do not, however, exclude the possibility that an earlier intervention and/or a more prolonged period of normoglycemia could normalize the changes in BMM.

In this study with short-term diabetic animals, the thickness of the peripheral BM was not increased. Rasch has shown that after 6 mo of streptozotocin-induced diabetes, there is a significant BM thickening that is preventable by

TABLE 2
Biochemical parameters. Kidney values are for one right-sided kidney

	C (N = 23)	D (N = 12)	T (N = 8)	D − C	T − D
Blood glucose (mg/100 ml)	100 ± 16	362 ± 41	104 ± 17	262 ± 13	−258 ± 13
Body wt. (g)	179 ± 10	165 ± 10	184 ± 8	−14 ± 3.4	19 ± 4.3
Kidney wt. (mg)	540 ± 47	808 ± 75	623 ± 33	268 ± 32	−185 ± 35
Kidney protein (mg)	71.8 ± 5.9	97.4 ± 5.0	81.5 ± 4.0	25.6 ± 2.0	−15.9 ± 2.4
Kidney RNA (mg)	2.24 ± 0.72	2.84 ± 0.55	2.43 ± 0.48	0.60 ± 0.05	−0.41 ± 0.08
Kidney DNA (mg)	3.20 ± 0.29	3.52 ± 0.21	3.36 ± 0.48	0.32 ± 0.09	−0.16 ± 0.16
Protein/kidney (mg/mg)	0.129 ± 0.008	0.124 ± 0.008	0.127 ± 0.008	−0.005 ± 0.002	0.003 ± 0.003
Protein/DNA (mg/mg)	22.7 ± 1.3	27.8 ± 2.0	25.0 ± 3.1	5.1 ± 0.6	−2.8 ± 1.1
RNA/DNA (mg/mg)	0.70 ± 0.05	0.81 ± 0.03	0.70 ± 0.08	0.11 ± 0.02	−0.11 ± 0.03

Symbols and abbreviations as in Table 1.

strict insulin treatment.[20] Steffes et al. have investigated the reversibility of BM thickening by islet cell transplantation in diabetic rats.[21] After 7 mo of diabetes, they found BM thickening, but when transplanted animals were followed for a further 6 mo, no tendency to normalization was seen. These findings together with those of the present study indicate that in the streptozotocin-diabetic rat, there is an early accumulation of peripheral BM as the glomerulus enlarges, later followed by a small but significant increase in BM thickness. The accumulation of BM is preventable, but the transplantation studies have shown that it is not reversible. This implies that the turnover of BM constituents, which biochemically has been shown to be very slow in normal rats,[22] is not significantly accelerated after normalization of the blood glucose (i.e., once the material has been laid down it is hard to remove).

Earlier studies have shown that the kidney weight is increased after 36 h of streptozotocin-induced diabetes.[23] The weight gain continues at a declining rate for at least 4 wk.

The protein concentration (mg protein/mg kidney) remained unchanged, a finding that discounts the possibility of significant changes in water content. At moderate degrees of diabetes, there is a significant correlation of kidney growth with the blood glucose level, but in animals having blood glucose above 500 mg/dl, there is no renal hypertrophy, probably because they are very sick and show marked weight loss.[5] The pattern of kidney growth has been studied previously by measurements of protein content and the total amount of RNA and DNA,[23] with results identical to our findings. The tubules, constituting about 80% of the kidney, clearly account for most of the described changes.[6] However, in isolated glomeruli, there is also an increase of RNA (about 12%) after 2 days of diabetes.[24]

Again, strict insulin treatment can prevent the renal hypertrophy.[25] The present study shows that after islet transplantation followed by persistent normoglycemia, the kidney enlargement can be reversed. This stresses the significance of the diabetic metabolism in regulating the size of the kidney.

The precise relationship between the findings in these animal studies and the conditions in human diabetes mellitus is not known. However, the early glomerular and renal changes described so far in the rat model have also been shown to exist in early human diabetes.[1–4,26] Our animal study has shown that the BM accumulation is not reversible by even complete normoglycemia, a condition rarely obtainable in insulin-dependent diabetic patients. If this holds true also for human diabetes, then the whole discussion about the significance of good control of the diabetic state has underestimated the problem: even a few days of bad control may induce changes in the glomerular basement membrane that cannot be compensated for by a longer period of good control.

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