Amelioration of Long-Term Renal Changes in Obese Type 2 Diabetic Mice by a Neutralizing Vascular Endothelial Growth Factor Antibody

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Diabetic nephropathy in type 2 diabetic patients is a frequent complication associated with increased morbidity and mortality. Various growth factors and cytokines have been implicated in the pathogenesis of diabetic kidney disease, including vascular endothelial growth factor (VEGF). To explore a role for VEGF in renal changes in type 2 diabetes, we examined the renal effects of a neutralizing murine VEGF antibody in the diabetic *db/db* mouse, a model of obese type 2 diabetes. One group of *db/db* mice was treated for 2 months with a VEGF antibody, while another *db/db* group was treated for the same period with an isotype-matched irrelevant IgG. A third group consisting of nondiabetic *db/+* mice was treated with the same isotype-matched IgG for 2 months. Placebo-treated *db/db* mice showed a pronounced increase in kidney weight, glomerular volume, basement membrane thickness (BMT), total mesangial volume, urinary albumin excretion (UAE), and creatinine clearance (CrCl) when compared with nondiabetic controls. In VEGF antibody–treated *db/db* mice, increases in kidney weight, glomerular volume, BMT, and UAE were attenuated, whereas the increase in CrCl was abolished. VEGF antibody administration tended to reduce expansion in total mesangial volume. These effects in diabetic animals were seen without impact on body weight, blood glucose, insulin levels, or food consumption. In conclusion, chronic inhibition of VEGF in *db/db* mice ameliorates the diabetic renal changes seen in type 2 diabetes. *Diabetes* 51:3090–3094, 2002

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BMT, basement membrane thickness; CrCl, creatinine clearance; LM, light microscopy; STZ, streptozotocin; TGF, transforming growth factor; UAE, urinary albumin excretion; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

The incidence of type 2 diabetes is increasing worldwide. The development of diabetic nephropathy is seen in 30–40% of type 2 diabetic individuals, with an associated increased morbidity and mortality. Accordingly, diabetic nephropathy is the most common cause of end-stage renal failure in the Western world. Mechanisms underlying the development of diabetic kidney disease in type 2 diabetes are complex. Among the many potential pathogenic mechanisms responsible for the development of diabetic kidney disease, growth factors have been suggested to be important players. Accordingly, growth hormone/IGFs and transforming growth factor (TGF)-β have been shown to have measurable effects on the development of diabetic kidney changes in animal models of type 1 diabetes (1). Recently, the vascular endothelial growth factor (VEGF) system has been proposed to play a role in the development of diabetic renal changes in animal models of type 1 diabetes (1–4); the potential role of the VEGF system in renal complications of type 2 diabetes remains unknown.

The aim of the present study was to explore the role of VEGF in the development of renal changes in type 2 diabetes. Accordingly, a specific neutralizing murine VEGF antibody was administered for 2 months in *db/db* mice, a genetic model of type 2 diabetes characterized by obesity, sustained hyperglycemia, hyperinsulinemia, lack of ketonuria, and progressive renal kidney disease (5–8).

RESEARCH DESIGN AND METHODS

Animals. Adult female *db/db* mice (C57BLKS/J-*lepr^{db}*/*lepr^{db}*) and their age-matched nondiabetic *db/+* littermates (C57BLKS/J-*lepr^{db}*/+) (M&B, Ry, Denmark) were used. Nondiabetic *db/+* mice had a body weight of 19–20 g, and the *db/db* mice had an initial weight of 39–41 g. Intervention with VEGF antibody administration was initiated at 8 weeks of age because 100% of the *db/db* mice become frankly hyperglycemic from week 7–8 (8). The *db/db* mice were included in the study 1–2 weeks after development of diabetes, at the age of 8 weeks. The mice were housed six to eight per cage in a room with a 12:12 h artificial light cycle (7:00 A.M. to 7:00 P.M.), a temperature of 21 ± 1°C, and a humidity of 55 ± 5%. The animals had free access to standard chow (Altromin no. 1324; Altromin, Lage, Germany) and tap water throughout the experiment. The study complied with Danish regulations for care and use of laboratory animals.

Study design. The *db/db* mice were randomized into two groups of 12 per group. One group of *db/db* mice was treated with intraperitoneal injections of a neutralizing VEGF antibody, and the other group was treated with an isotype-matched irrelevant IgG, as were the nondiabetic *db/+* mice (n = 6). The VEGF antibody and irrelevant IgG were administered intraperitoneally in an initial bolus dose of 300 μg, followed by doses of 100 μg three times weekly. The VEGF antibody and irrelevant IgG were dissolved in 0.154 mol/l

TABLE 1
Mean body weight, blood glucose, and food consumption at day 0 and 60 in placebo-treated controls, placebo-treated diabetic *db/db* mice, and VEGF antibody–treated diabetic *db/db* mice

	Day 0			Day 60		
	Body weight (g)	Blood glucose (mmol/l)	Food consumption (g/24 h)	Body weight (g)	Blood glucose (mmol/l)	Food consumption (g/24 h)
Control, placebo	19.6 ± 0.4	5.4 ± 0.3	5.5 ± 0.6	21.3 ± 0.2	5.5 ± 0.3	5.9 ± 0.8
Diabetic, placebo	41.4 ± 0.7*	18.6 ± 1.0*	8.5 ± 0.7*	47.3 ± 1.0*	19.8 ± 1.4*	9.1 ± 1.1*
Diabetic, VEGF antibody	40.6 ± 0.8*	18.4 ± 1.0*	8.8 ± 0.8*	46.2 ± 0.9*	18.8 ± 2.0*	8.9 ± 0.9*

Data are means ± SE (n = 6–12 in each group). *$P < 0.01$ vs. nondiabetic controls.

NaCl and injected in a volume of 0.5 ml. A full characterization of the VEGF antibody used has been described elsewhere (2,4). Briefly, 8-week-old female Balb/C mice were immunized by repeated intraperitoneal and subcutaneous injections of 50 μg rhVEGF$_{165}$, which was emulsified with complete Freund's adjuvant for the primary immunization and incomplete Freund's adjuvant for the subsequent immunizations. Mice with the highest serum titer to VEGF$_{165}$ received an additional injection of 30 μg VEGF$_{165}$ in PBS, and 3 days later, spleen cells were harvested for production of hybridomas to rhVEGF$_{165}$. Two hybridoma cell lines with the highest antibody titer and neutralizing activity were cloned three to four times in microplates and injected intraperitoneally (10^7 cells). Ascites fluid was collected, and purified IgG was prepared by protein A chromatography, with a further characterization of the neutralizing activity as described previously (2).

Body weight, food consumption, and blood glucose were determined at initiation of the experiment and every 2 weeks. Blood glucose was measured in tail-vein blood as described below. After 8 weeks, mice were placed in metabolic cages to collect 24-h urine samples for urinary albumin excretion (UAE) and urinary creatinine determinations. At sacrifice, mice were anesthetized with pentobarbital (50 mg/kg i.p.) and nonfasting blood samples were drawn from the retro-orbital venous plexus using heparinized capillary tubes. Serum samples were stored at −80°C until analysis was performed. In all animals, the right and left kidneys were removed and weighed. The middle piece of the right kidney (including the papilla) was fixed in 4% paraformaldehyde for determination of glomerular volume by light microscopy (LM) (see below). The middle piece of the left kidney (including the papilla) was fixed in 0.1 mol/l cacodylate buffer with 1% glutaraldehyde and 2% paraformaldehyde for later determination of basement membrane thickness (BMT) and mesangial fraction by electron microscopy (see below). In addition, liver and heart were removed, weighed, and snap frozen in liquid nitrogen.

Determination of blood glucose and serum insulin. Blood glucose was measured at day 0 and every 2 weeks in tail-vein blood by Precision Xtra Plus (Abbott Laboratories, MediSence Products, Bedford, MA), and urine was tested for glucose and ketone bodies by Combur^5 Test D (Roche Diagnostics, Mannheim, Germany). Serum insulin was measured by an ultrasensitive rat insulin enzyme-linked immunosorbent assay (DRG Diagnostics, Marburg, Germany). Semilog linearity of mouse serum and rat insulin was found at multiple dilutions, indicating antigen similarity between mouse and rat insulin. The intra- and interassay coefficients of variation were <5% and <10% for the insulin assays.

Determination of UAE and creatinine clearance. The urinary albumin concentration was determined by radioimmunoassay as previously described (9) using rat albumin antibody and rat albumin standard. Semilog linearity of mouse urine and rat albumin (in the standard) was found at multiple dilutions, indicating antigen similarity between mouse and rat albumin. Urine samples were stored at −20° C until assay was performed. Serum and urinary creatinine concentrations were measured by an automated technique adapted from the method of Jaffé and corrected for the prevailing glucose content due to interference in the Jaffé reaction. The creatinine clearance (CrCl) was expressed in milliliters per hour. The intra- and interassay coefficients of variation were <5% and <10% for both assays.

Estimation of glomerular volume. The middle part of the right kidney (containing the papilla) was embedded in paraffin for LM examination. Two micron-thick sections were cut on a rotation microtome and stained with *p*-aminosalicylic acid and hematoxylin. The mean glomerular tuft volume (V_G) was determined from the mean glomerular cross-sectional area (A_G) at a magnification of 400×, as previously described (10–12). The areas were determined with a two-dimensional version of the nucleator (CAST; Olympus, Copenhagen, Denmark) (12) by LM as the average area of a total of 40–50 glomerular profiles (tuft omitting the proximal tubular tissue within the Bowmann capsule). V_G was calculated as $V_G = \beta/k \times (A_G)^{3/2}$, where $\beta = 1.38$, which is the shape coefficient for spheres (the idealized shape of glomeruli), and $k = 1.1$, which is a size distribution coefficient (10–12).

Estimation of mesangial fraction, total mesangial volume, and BMT. The middle part of the left kidney (containing the papilla) was embedded in Epon 825 for electron microscopy examination. Thin sections were cut on a Reichert Ultracut (Leica, Vienna, Austria) and stained with uranyle acetate and lead citrate. From an electron microscope (Tecnai 12; Phillips, Enthoven, Holland), images covering the whole glomerular profile were recorded with a MegaView video camera (Soft Imaging System, Münster, Germany) onto a monitor. Measurements of mesangial regions were performed at a final magnification of 3,200×. Four to six glomeruli were measured from two blocks. Mesangial fractions were determined by point counting of mesangial regions as fraction of the tuft. The total mesangial volume was calculated by multiplying the mesangial fraction by the total glomerular volume. For measurements of BMT, randomized fields were recorded at a magnification of 30,000× from the same sections described above. BMT was measured, applying the orthogonal intercept method as previously described (13). About 60 measurements were performed per glomerulus, and BMT is given as a harmonic mean.

Statistical analysis. For repeated measurements, ANCOVA was used to evaluate differences with Student's t test for unpaired comparisons. A P value <0.05 was considered statistically significant. For data not following a normal distribution, the Mann-Whitney rank-sum test was used. All data are expressed as means ± SE, with n indicating the number of mice studied. Statistical analysis was performed using SPSS for Windows.

RESULTS

Body weight, blood glucose, food consumption, and serum insulin. The *db/db* mice had a greater body weight than the nondiabetic *db/+* mice, as was also the case for food consumption (Table 1). Mean blood glucose levels were 18–19 mmol/l in *db/db* mice throughout the study, and 5–6 mmol/l in *db/+* animals (Table 1). The *db/db* mice had severe hyperinsulinemia (Table 2). VEGF antibody administration did not affect any of the above parameters in *db/db* mice throughout the study duration (Tables 1 and 2).

Kidney weight, glomerular volume, BMT, and mesangial volume. Placebo-treated *db/db* mice showed an in-

TABLE 2
Mean serum insulin, liver weight, and heart weight at day 60 in placebo-treated controls, placebo-treated diabetic *db/db* mice, and VEGF antibody–treated diabetic *db/db* mice

	Day 60		
	Serum insulin (μg/l)	Liver weight (mg)	Heart weight (mg)
Control, placebo	2.86 ± 0.29	1,136 ± 36	103 ± 4
Diabetic, placebo	18.82 ± 1.60*	2,272 ± 88*	109 ± 3
Diabetic, VEGF antibody	16.85 ± 1.67*	1,891 ± 65†	103 ± 3

Data are means ± SE (n = 6–12 in each group). *$P < 0.01$ vs. nondiabetic controls; †$P < 0.05$ vs. the two other groups.

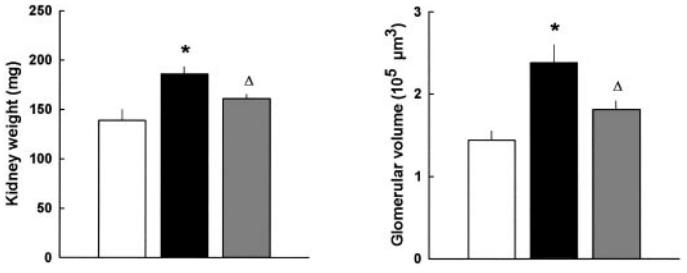

FIG. 1. Mean right kidney weight and glomerular volume on day 60 in nondiabetic controls (□), placebo-treated diabetic *db/db* mice (■), and VEGF antibody–treated diabetic *db/db* mice (▨). Values are means + SE (n = 6–12 in each group). *P < 0.01 vs. nondiabetic controls; ΔP < 0.05 vs. nondiabetic controls and placebo-treated *db/db* mice.

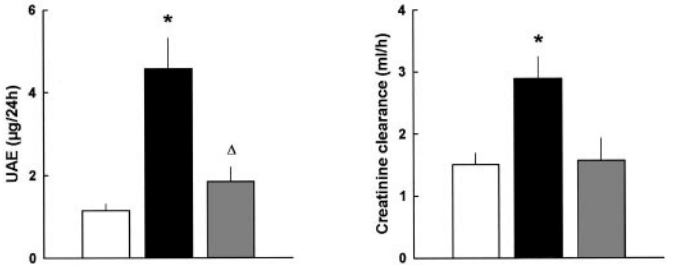

FIG. 3. Mean 24-h UAE and CrCl on day 60 in nondiabetic controls (□), placebo-treated diabetic *db/db* mice (■), and VEGF antibody–treated diabetic *db/db* mice (▨). Values are means + SE (n = 6–12 in each group). *P < 0.01 vs. nondiabetic controls; ΔP < 0.05 vs. nondiabetic controls and placebo-treated *db/db* mice.

crease in kidney weight of 34% at day 60 (187 ± 7 vs. 139 ± 11 mg, $P < 0.01$) when compared with nondiabetic *db/+* controls (Fig. 1). In VEGF antibody–treated *db/db* mice, a significantly smaller increase in kidney weight was observed versus placebo-treated *db/db* mice (161 ± 4, $P < 0.05$), although the kidney weight was higher than that seen in nondiabetic controls ($P < 0.01$). The same pattern of changes was seen in glomerular volume (Fig. 1). Total glomerular volume increased by 65% in placebo-treated *db/db* mice compared with nondiabetic controls (2.38 ± 0.22 vs. 1.44 ± 0.11 10^5 μm^3, $P < 0.01$). VEGF antibody treatment in *db/db* mice partially prevented the increase in glomerular volume versus placebo-treated *db/db* mice (1.81 ± 0.11 10^5 μm^3, $P < 0.01$). The glomerular volume was, however, still elevated above that of nondiabetic controls ($P < 0.05$). BMT increased by 18% in placebo-treated *db/db* mice when compared with nondiabetic controls (176 ± 6 vs. 149 ± 4 nm, $P < 0.05$), while an insignificant increase was seen in VEGF antibody–treated *db/db* mice (160 ± 6 nm, NS), with a value significantly lower than that of placebo-treated *db/db* mice ($P < 0.05$) (Fig. 2). Both diabetic groups had a significant increase in mesangial fraction ($P < 0.05$, data not shown), and total glomerular mesangial volume tended ($0.05 < P < 0.10$) to be lower in the VEGF antibody–treated *db/db* group (Fig. 2).

UAE and CrCl. A pronounced increase in UAE was observed in placebo-treated *db/db* mice at day 60 versus nondiabetic *db/+* controls (4.57 ± 0.75 vs. 1.15 ± 0.16 μg/24 h, $P < 0.01$), with a considerably lower level in *db/db* mice treated with the VEGF antibody (1.85 ± 0.34 μg/24 h, $P < 0.01$ vs. placebo-treated *db/db* mice) (Fig. 3). Placebo-treated *db/db* mice showed a pronounced increase in CrCl when compared with nondiabetic controls (1.51 ± 018 vs. 2.89 ± 0.35 ml/h, $P < 0.05$), with normalization in the VEGF antibody–treated *db/db* mice (1.57 ± 0.36 ml/h) (Fig. 3).

Liver and heart weight. The placebo-treated *db/db* mice had greater liver weights than the *db/+* mice, whereas VEGF antibody–treated *db/db* mice had less liver weight gain (Table 2). There were no significant differences in heart weight among the three groups.

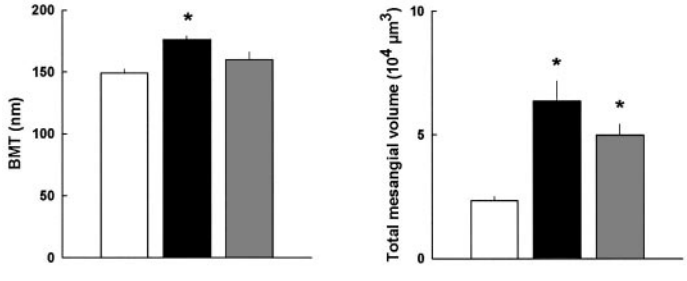

FIG. 2. BMT and total mesangial volume on day 60 in nondiabetic controls (□), placebo-treated diabetic *db/db* mice (■), and VEGF antibody–treated diabetic *db/db* mice (▨). Values are means + SE (n = 6–12 in each group). *P < 0.05 vs. nondiabetic controls and VEGF antibody–treated diabetic *db/db* mice.

DISCUSSION

The *db/db* mouse, which expresses a leptin receptor defect in the hypothalamus, is a genetic model of type 2 diabetes characterized by obesity, sustained hyperglycemia, hyperinsulinemia, and lack of ketonuria. Previously, this model has been shown to present with robust diabetic renal changes characterized by increased renal/glomerular volume, BMT, UAE, and mesangial volume within 2 months of diabetes (5–8).

The major new finding of the present study is an amelioration of diabetes-induced renal changes in *db/db* mice by VEGF antibody administration. Accordingly, antibody administration attenuated the increase in renal/glomerular volume, BMT, and UAE and abolished the increase in CrCl. These effects were seen without affecting metabolic control, insulin levels, body weight, or food consumption, indicating that VEGF plays a causal role in the development of late renal changes in a model of type 2 diabetes.

The VEGF system consists of different isoforms of homodimeric glycoproteins (14–21). Furthermore, at least two high-affinity VEGF receptors (VEGFR-1 and -2) have been described (17). VEGF has pronounced angiogenic actions (15,18–20) and causes vasodilation and increased vascular permeability (14,18). The expression of VEGF was initially described to be markedly increased in highly vascularized rapidly growing tumors (22), and VEGF has been shown to be a potent mitogenic factor for endothelial cells (20,21). The two VEGFRs (VEGFR-1 and -2), also known as the fms-like tyrosine kinase and fetal liver kinase 1, are high-affinity transmembrane tyrosine kinase receptors (17). Both VEGF and the two VEGFRs are expressed in the kidney (3,23–27). VEGF expression and specific VEGF binding have been described in rat (23) and human kidney (24–26). VEGF has been localized to epithelial glomerular cells (i.e., podocytes) (3,26,27), distal tubules, and renal collecting ducts (3,25). Furthermore, VEGFR-2 has been localized mainly to glomerular endo-

thelial cells and cortical interstitial fibroblasts (3). Mesangial cells, glomerular endothelial cells, vascular smooth muscle cells, and proximal and distal tubular cells are capable of producing VEGF in vitro (27–29). High glucose has been shown to stimulate VEGF expression in vascular smooth muscle cells (30). Also, in a recent study in OLETF rats (an experimental rat model of type 2 diabetes), renal VEGF mRNA and glomerular VEGF immunoreactivity were reported to be elevated over a diabetes duration of 9–68 weeks (31). In another study, changes in renal VEGF levels were described in streptozotocin (STZ)-induced diabetic rats (a rat model of type 1 diabetes) with a diabetes duration of 3 and 32 weeks (3). VEGF mRNA and protein were mainly localized to the glomerular epithelial cells and VEGFR-2 mRNA mainly to glomerular endothelial cells (3). VEGF mRNA and peptide were increased in diabetic animals at both time points examined, whereas the expression of VEGFR-2 and VEGFR binding were increased only at 3 weeks (3).

Although the area of identifying and developing specific antagonists of a pathophysiologically enhanced VEGF system in oncology and different eye diseases has attracted increasing interest (32), no studies have appeared on the effect of VEGF antagonists in diabetic kidney disease of type 2 diabetes. Direct evidence for a role of VEGF in the early renal changes observed in a model of type 1 diabetes (i.e., STZ-induced diabetic rats) has been published using the same VEGF antibody (4). Six weeks treatment with the VEGF antibody abolished the diabetes-associated hyperfiltration and partially blocked the increase in UAE (4). VEGF antibody administration in nondiabetic control rats had no impact on any renal parameters, indicating a diabetes-specific effect of VEGF antibody administration in diabetes (4). In the present study, using a mouse model of type 2 diabetes, administration of the VEGF antibody was shown to ameliorate both the classical early features of diabetic kidney disease, i.e., renal/glomerular hypertrophy and hyperfiltration (measured as CrCl), and more importantly, late renal changes (i.e., BMT), with a tendency to reduce total mesangial volume. The *db/db* mouse has previously been reported to develop decreased CrCl within 2 months after the onset of diabetes, suggesting a progressive diabetic kidney disease with loss of kidney function (8). In the present study, however, several lines of evidence indicated that placebo-treated *db/db* mice presented with renal hyperfunction, which was partially or fully normalized by VEGF antibody treatment, i.e., partial effect on kidney weight, glomerular volume, UAE, and normalization of elevated CrCl. The reason for this discrepancy is unknown, but may be explained by a variable susceptibility to diabetes in subbreedings of the *db/db* mouse strain.

The observation that VEGF antibody treatment abolished the increase in BMT and renal hyperfiltration and partially blocked the increase in UAE is interesting in view of the well-known actions of VEGF on vascular permeability (14,18) and the anatomical localization of the VEGF system in the glomerulus (i.e., podocytes and glomerular endothelial cells) (3,25–27). These results indicate that administration of a specific, neutralizing VEGF antibody in *db/db* mice fully or partly restores the abnormally increased albumin permeability in the diabetic kidney, which is believed to be caused by abnormalities in the filtration barrier due to increased membrane pore size and reduced anion charge. Although VEGF expression has been described in glomerular epithelial cells (3,26), VEGF antibody administration only tended to reduce total mesangial volume in the present study. These results suggest that the primary role of VEGF in the diabetic renal changes in type 2 diabetes is linked to the diabetes-associated permeability changes, while the role of VEGF in mesangial expansion, if any, seems to be secondary. In this context, it is interesting that administration of a neutralizing TGF-β antibody in *db/db* mice has been shown to ameliorate diabetes-associated glomerular matrix expansion without affecting either elevated UAE or renal VEGF expression (33).

Although currently unproven, several potential pathways involved in diabetes-induced vascular changes (1) may involve VEGF as a downstream cytokine. In vitro, VEGF has been shown to be stimulated by IGF-I (34), and furthermore, IGF-I receptor blockade in an ischemia-induced retinopathy model has been shown to reduce the intracellular VEGF-mediated mitogen-activated protein kinases along with ameliorating retinal neovascularization (35). Blockade of protein kinase C β activity with a specific inhibitor (LY333531) suppresses the VEGF-induced alterations in retinal leakage, retinal blood flow, and ischemia-induced retinal neovascularization (36). In addition, ACE inhibition in diabetic rats has been shown to reduce diabetes-associated retinal changes in VEGF expression and vascular permeability (37). Also, in the study described above in a rat model of type 2 diabetes (31), it was shown that long-term administration of an advanced glycation end product inhibitor (OPB-9195) abolished the enhanced renal VEGF mRNA and glomerular VEGF immunoreactivity along with renoprotection, in terms of normalization of diabetes-induced renal collagen IV accumulation and a reduction of the rise in UAE (31).

In conclusion, the present data strongly support the hypothesis that VEGF is an important pathogenetic factor in the development of long-term renal changes in type 2 diabetes. Further studies are warranted to fully elucidate the role of VEGF as a downstream mediator for some of the well-known pathways leading to diabetic renal damage.

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