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INCREASED EXPRESSION OF HEPARIN BINDING EPIDERMAL-GROWTH-FACTOR-LIKE GROWTH FACTOR mRNA IN THE KIDNEY OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

Yau-Jiunn Lee, Shyi-Jang Shin, Shiu-Ru Lin, Mian-Shin Tan, and Juei-Hsiung Tsai*

Graduate Institute of Medicine, Kaohsiung Medical College, Kaohsiung 80317 Taiwan, Republic of China

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SUMMARY: Heparin binding epidermal growth factor (HB-EGF), a new member of the EGF family, is a potent mitogen for smooth muscle cells, fibroblasts, and mesangial cells. To study whether the HB-EGF is involved in the development of diabetic nephropathy, we measured the expression of the HB-EGF gene in the kidney tissues of streptozotocin-induced diabetic rats by Northern blot analysis. The mean kidney weight of diabetic rats without strict blood sugar control was significantly increased as compared to that of the control group. Renal HB-EGF mRNA expression was also increased in diabetic rats without strict blood sugar control at 7 days after induction of diabetes and remained elevated for the entire 3-month study period. Strict insulin treatment abolished the elevation of HB-EGF mRNA expression and kidney growth. As HB-EGF is a mitogen for mesangial cells, our results suggest that HB-EGF may be involved in the development of diabetic nephropathy.

Heparin binding-epidermal growth factor like growth factor (HB-EGF), a potent mitogen for vascular smooth muscle cells and fibroblasts, was purified from conditioned medium in which macrophage-like U-937 cells had been cultured (1). Recently, we have found that the HB-EGF gene is expressed in a highly regulated manner by protein kinase C (PKC) activators in cultured rat mesangial cells (RMC). Furthermore, we transfected COS cells with HB-EGF expression plasmid and the culture medium from the transfected COS cells increased [^{3}H]-thymidine incorporation in RMC in a dose-dependent

*To whom correspondence and reprint requests should be addressed at Department of Internal Medicine, Kaohsiung Medical College, No 100, Shih-Chuan 1st Rd, Kaohsiung, 80317, Taiwan, Republic of China. Fax: 011-886-7-3212062.

Abbreviations: HB-EGF, heparin binding-epidermal growth factor; STZ, streptozotocin.

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manner. These results suggest that HB-EGF may have an autocrine function in the proliferation of mesangial cells and play some role in glomerulosclerosis (2).

Renal hypertrophy and hyperfunction in diabetes have been suggested as initiating or accelerating late diabetic nephropathy, although the exact mechanism is unknown (3, 4). In the last decade, there is considerable evidence suggesting the involvement of growth factors with diabetic nephropathy, mainly because it has been possible to isolate and produce pure and new factors and because modern molecular and cellular techniques have been developed (5-9). Growth factors produced by the kidney can act locally as mediators of kidney growth in both an autocrine and paracrine manner (10). Some growth factors are well recognized as playing a role in the renal hypertrophy or hyperplasia of diabetic nephropathy (5-9). Yet, no such factors have been identified as the initiating or sustaining factor in diabetic hypertrophy. In an attempt to clarify whether HB-EGF is involved in the development of diabetic nephropathy, we measured the renal HB-EGF gene expression in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Animal experiment: Male Wistar rats (200 to 250 gm) were induced to diabetes by a single intraperitoneal injection of 60 mg/kg body weight STZ (Sigma, St. Louis, MO) dissolved in citrate buffer (pH 4.2). Control rats were injected with the same volume of citrate buffer. All rats were allowed free access to food and water and housed in individual metabolic cages. Rats with random plasma glucose levels higher than 350 mg/dl 24 hours after STZ injection were chosen for study. After the development of diabetes was confirmed, rats were randomly divided into two groups: 1) diabetic group without strict blood sugar control; 2) diabetic group with strict blood sugar control. Heat-treated bovine insulin ultralente (Novo Nordisk A/S, Bagsvaerd, Denmark) was used to control blood sugar by single daily injection. The appropriate dose of insulin was determined according to the sugar level measured by blood samples taken from the tail each day. Random blood sugar was controlled to under 200 mg/dl in the strict control group. The blood sugar level of the group without strict blood sugar control was usually greater than 400 mg/dl. Blood glucose was determined with Dextrostix reagent strips (Eiken, Tokyo, Japan). Blood glucose levels were monitored daily in all diabetic rats and occasionally in nondiabetic rats. Rats were sacrificed 4, 7, 14, 21, 28, 35, 42, 56, and 84 days after STZ injection to obtain the kidneys for renal HB-EGF mRNA analysis. Tissues were frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction and Northern blot hybridization: Total RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (11) and quantified by its absorbance at 260 nm. Total RNA (40 μg/lane) was separated by formaldehyde/1% agarose gel electrophoresis and transferred to a nylon membrane (Schleicher & Schuell GmbH, Dassel,

Germany). After baking, the RNA immobilized on the membrane was hybridized with a [^{32}p]-labeled 473 bp *Ecor* RI-*Kpn* I fragment of the HB-EGF cDNA in the presence of 50% formamide at 42°C over 16 hours according to the standard technique (2, 12). The membranes were washed first in 2XSSC/0.1% sodium dodecyl sulfate (SDS) at room temperature, then in 0.2XSSC/0.1% SDS at 65°C, and autoradiographed with an intensifying screen at -80°C. Membranes were subsequently rehybridized with β-actin cDNA probe to determined the relative amount of RNA content. The signals on the autoradiography were scanned with a 100S Molecular Dynamic computing densitometer and MD Image Quant software release version 3.22 (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis: Analysis of variance (ANOVA) was used to compare the mean blood sugar levels, body weight changes, and kidney weight among the control and the two diabetic groups.

RESULTS

The mean blood sugar levels and kidney weight of diabetic group without strict blood sugar control were significantly increased as compared to those of the control group. Strict blood sugar control restored the kidney weight to a level comparable with the control group (Table 1). Diabetic rats with hyperglycemia failed to gain body weight, and when the diabetic rats were controlled with insulin to near normoglycemia, normal weight gain was observed (Table 1). Renal HB-EGF mRNA expression increased progressively with age in diabetic rats without strict blood sugar control at 7 days after induction of diabetes (Fig. 1). The elevated renal HB-EGF mRNA expressions in diabetic rats without strict blood sugar control were observed for 3 months (Fig. 2). Figure 2 also shows that the enhancement of HB-EGF mRNA expression was ameliorated by strict blood sugar control with insulin.

Table 1. The mean blood sugar levels, body weight changes, and kidney weight in control and diabetic rats with (DM-Ins) or without insulin (DM) treatment at the day of sacrifice

	Control n=13	DM n=22	DM-Ins n=10
Blood sugar (mg/dl)	139.8 ± 27.3	488.0 ± 11.7**	85.3 ± 2.6
Change in body weight (%)	16.6 ± 3.2	-7.9 ± 2.8**	18.1 ± 2.8
Kidney weight (g)	0.99 ± 0.05	1.20 ± 0.04*	1.09 ± 0.07
Kidney weight/ body weight ($\times 10^{-3}$)	3.32 ± 0.15	5.04 ± 0.07**	3.63 ± 0.11

Values are mean ± SEM. *$p<0.05$ as compared to the control group; **$p< 0.01$ as compared to the control group and diabetic rats with insulin treatment by ANOVA and Scheffe's *F* test.

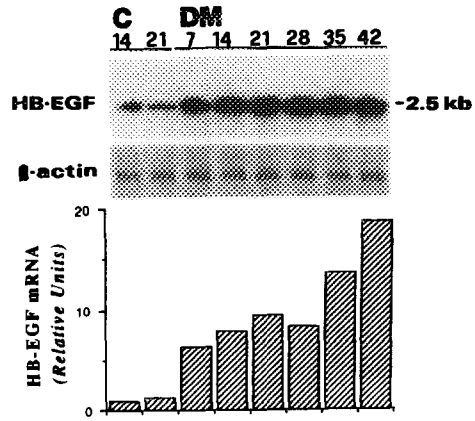

Fig.1. Serial changes of renal HB-EGF mRNA expression of control and diabetic rats. Total cellular RNA (40 μg/lane) was electrophoresed, transferred to nylon membrane, and then hybridized with a specific rat HB-EGF cDNA probe as described in Methods. The optical density of the autoradiography signals was quantified by densitometry and calculated as the ratio of HB-EGF to β-actin mRNA. The mRNA level of a control rat was assigned the number 1.0, and all other values were divided by that of controls and presented as relative units. A 2.5-kb transcript of HB-EGF gene was expressed in the kidney of control (C) and diabetic (DM) rats without strict blood sugar control. The renal HB-EGF mRNA was elevated progressively with age in the diabetic kidney 7 days after induction by streptozotocin.

DISCUSSION

In this study, we demonstrate that the HB-EGF gene is expressed in the rat kidney, and renal HB-EGF gene expression is enhanced in diabetic rats with hyperglycemia. The elevated HB-EGF gene expression returned to the control level by strict blood sugar control with insulin. HB-EGF is a more potent smooth muscle cell mitogen than EGF, and its potency is similar to that of platelet-derived growth factor (PDGF)(1). Our recent study has shown that HB-EGF is also a mitogen for mesangial cells (2). Considerable evidence suggests that peptide growth factor stimulates mesangial cell proliferation as well as matrix accumulation (10). Though the effect of HB-EGF on mesangial matrix production needs to be confirmed by further experimentation, our results may indicate that HB-EGF is involved in the pathogenesis of diabetic nephropathy.

Renal hypertrophy and hyperfiltration are early manifestations of human and experimental diabetes (3, 4). Even in long-standing diabetes with evidence of advancing nephropathy, the hypertrophy persists (13). Thus, alterations of control of renal growth exist in the diabetic kidney and may be related to the pathogenesis of the disease state. The current study shows that the expression of the HB-EGF gene is enhanced at 7 days after the

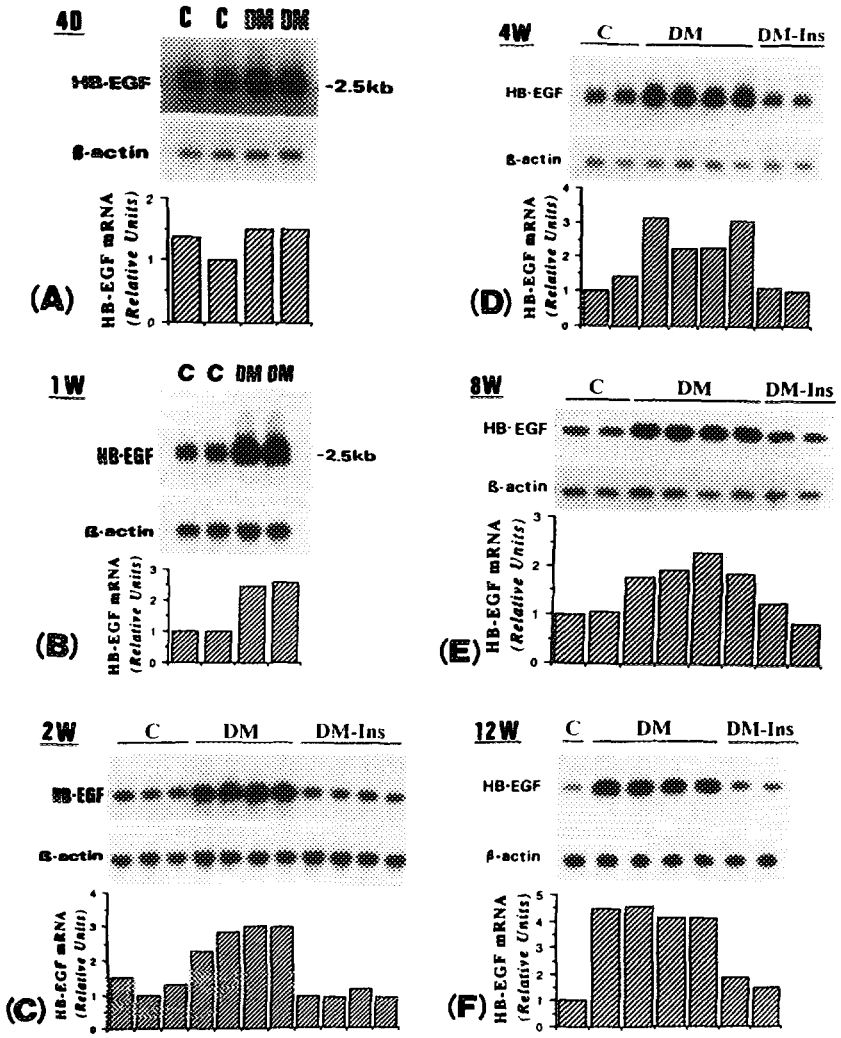

Fig. 2. The relative renal HB-EGF mRNA expression of the control (C), diabetic rats with (DM-Ins) and without (DM) insulin treatment at 4[A], 7[B], 14[C], 28[D], 56[E], and 84[F] days after the injection of streptozotocin or citrate buffer. Note significant expression of renal HB-EGF mRNA at 7 days after induction of diabetes mellitus which remained elevated for 3 months. The enhancement of HB-EGF mRNA expression was ameliorated by strict blood sugar control with insulin.

induction of diabetes and the elevation persists for at least 3 months. These results suggest that HB-EGF may not only be involved in the genesis but also the progression of diabetic nephropathy. However, a causal relationship cannot be deduced from the present data.

Mesangial expansion and/or increased mesangial cell numbers are the prominent features of diabetic nephropathy. Within 36 hours of the onset of

diabetes, kidney weight starts to increase and is 15% above baseline within 3 days (7). That the present results fail to show significant elevation of HB-EGF gene expression at day 4 after induction of diabetics may imply that HB-EGF is not the only factor involved in the pathogenesis of renal hypertrophy. The well-known growth factors such as PDGF, basic fibroblast growth factor, transforming growth factor-β, proliferating cell nuclear antigen, tumor necrosis factor, insulin-like growth factor I, EGF, etc. are recognized to contribute to diabetic nephropathy (5-9, 14). The current study adds a new factor, HB-EGF, to the list. Our results imply that the mechanism of diabetic nephropathy may be multifactorial.

The enhanced renal HB-EGF gene expression of STZ-diabetic rats could occur through several mechanisms. Our recent work had indicated that HB-EGF gene expression was highly regulated by the activity of PKC signal pathway (2), and hyperglycemia was known to be able to increase renal PKC activity (15). We propose that expression of the HB-EGF gene may be enhanced by elevated PKC activity activated by hyperglycemia. HB-EGF gene expression was also known to be stimulated by cytokines and vasoactive peptides such as tumor necrosis factor and endothelin-1 (ET-1) (2, 12). The elevated production of cytokines, growth factors, and ET-1 found in kidney of STZ-induced diabetic rats (9, 14) may also contribute to enhanced renal HB-EGF gene expression.

In summary, our results indicate that the HB-EGF gene is expressed in the normal rat kidney. The expression of HB-EGF mRNA in the diabetic rat kidney is enhanced 7 days after DM induction by STZ injection and the enhancement of HB-EGF mRNA expression in the diabetic kidney persisted for at least 3 months. Strict insulin treatment abolishes both the elevation of HB-EGF mRNA expression and the growth of the kidney. Our results suggest that HB-EGF may have a pathophysiological role in the development of diabetic nephropathy.

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