

Heparin-binding EGF-like growth factor contributes to reduced glomerular filtration rate during glomerulonephritis in rats

Lili Feng,¹ Gabriela E. Garcia,¹ Young Yang,² Yiyang Xia,¹ Francis B. Gabbai,³ Orjan W. Peterson,³ Judith A. Abraham,⁴ Roland C. Blantz,³ and Curtis B. Wilson¹

¹Department of Immunology, The Scripps Research Institute, La Jolla, California 92037, USA

²Robert Wood Johnson Pharmaceutical Research Institute, San Diego, California 92121, USA

³Division of Nephrology-Hypertension, University of California–San Diego School of Medicine and Veterans Affairs Medical Center, La Jolla, California 92161, USA

⁴Scios Inc., Mountain View, California 94043, USA

Address correspondence to: Curtis B. Wilson, Department of Immunology (IMM 5), The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA. Phone: (858) 784-8056; Fax: (858) 784-8558.

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Heparin-binding epidermal growth factor–like growth factor (HB-EGF), a member of the epidermal growth factor (EGF) family, is expressed during inflammatory and pathological conditions. We have cloned the rat HB-EGF and followed the expression of HB-EGF in rat kidneys treated with anti-glomerular basement membrane (anti-GBM) antibody (Ab) to induce glomerulonephritis (GN). We observed glomerular HB-EGF mRNA and protein within 30 minutes of Ab administration and showed by *in situ* hybridization that glomerular HB-EGF mRNA expression was predominantly in mesangial and epithelial cells. Expression of HB-EGF correlated with the onset of decreased renal function in this model. To test the direct effect of HB-EGF on renal function, we infused the renal cortex with active rHB-EGF, prepared from transfected *Drosophila melanogaster* cells. This treatment induced a significant decrease in single nephron GFR (SNGFR), single nephron plasma flow, and glomerular ultrafiltration coefficient and an increase in the glomerular capillary hydrostatic pressure gradient. In addition, anti-HB-EGF Ab administered just before anti-GBM Ab blocked the fall in SNGFR and GFR at 90 minutes without any change in the glomerular histologic response. These studies suggest that HB-EGF expressed early in the anti-GBM Ab GN model contributes to the observed acute glomerular hemodynamic alterations.

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Introduction

Growth factors contribute to renal injury/repair in several ways including proliferative responses, leading to regeneration after acute tubular injury, and to reparative, often sclerotic, outcomes after other forms of glomerular and tubular renal injury (1, 2). Growth factors, such as epidermal growth factor (EGF), may also have glomerular hemodynamic consequences, as infusion of EGF is reported to acutely reduce glomerular filtration rate (3). Renal EGF expression, however, is rapidly diminished in several forms of renal injury including acute glomerular injury caused by administration of anti-glomerular basement membrane (GBM) antibody (Ab) (4), so that it is unlikely to contribute to the altered pathophysiology encountered in anti-GBM Ab glomerulonephritis (GN). In contrast, expression of another EGF family member, heparin binding EGF-like growth factor (HB-EGF), which is also a ligand for the EGF receptor family, was rapidly increased after anti-GBM Ab induced glomerular injury in our preliminary studies (5) and may play a role in the attendant glomerular hemodynamic change (1, 6).

HB-EGF was first purified from conditioned medium of macrophages and macrophage-like cells (7). A membrane-bound form of HB-EGF (pro-HB-EGF) in humans functions as a diphtheria toxin receptor and has juxtacrine mitogenic activity (8–12). The sites of HB-EGF expression and its regulation suggest a role for this growth factor in development, inflammatory processes, wound healing, and pathological conditions such as atherosclerosis, diabetes mellitus, and kidney injury (13–19). HB-EGF is a potent mitogen for vascular smooth muscle cells (SMCs), fibroblasts, and keratinocytes (7, 20, 21). It is expressed by vascular smooth muscle and endothelial cells, T lymphocytes, and mesangial cells and is found in wound fluid (22–26). HB-EGF transcription can be upregulated in vascular endothelial cells by cytokines (IL-1 β , TNF- α), shear stress, and lysophosphatidylcholine (22, 27, 28). It is enhanced by phorbol ester and angiotensin II (AII) in SMCs (29). Oxidized low-density lipoprotein stimulates HB-EGF expression in monocytes, mesangial cells, and endothelial cells (30, 31).

HB-EGF is normally expressed in the S3 segment of the nephron tubular epithelial cells and is ubiquitously present in epithelial cells of the proximal tubules and the

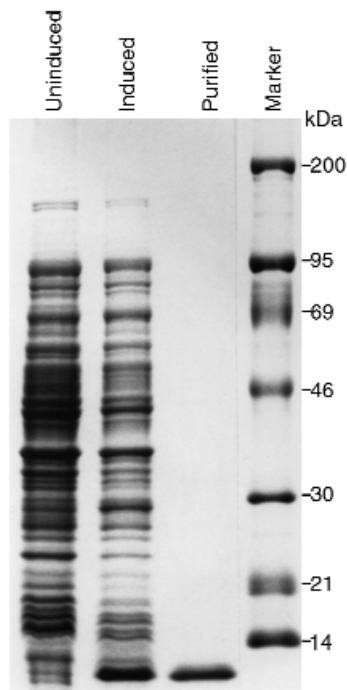


Figure 1
Expression of mature, carboxyl his-tagged HB-EGF, purified by nickel column. Cell lysates and purified protein from transformed *E. coli* were subjected to SDS and 7–20% polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The lanes are marked and represent uninduced cell lysates, induced cell lysates 4 hours by IPTG, 1 μ g affinity-purified HB-EGF (9.5 kDa), and markers.

arterial SMCs (32). It is not detected normally in glomeruli or other parts of the kidney. After acute injury in rat kidney, HB-EGF may play a juxtacrine role in tubular survival (33–35). It is also expressed in mesangial cells and glomerular epithelial cells in the anti-Thy-1 Ab model of mesangial cell injury, in glomerular epithelial cells before the onset of proteinuria in passive Heymann nephritis and puromycin models of glomerular injury, and in glomerular epithelial cells in models of focal glomerulonephritis (36–38). HB-EGF is produced in the kidney by both mesangial and epithelial cells, which also have EGF receptors. These cells play a central role in the inflammatory response of the glomerulus to injury and in the associated changes in glomerular hemodynamics.

The expression of HB-EGF in anti-GBM Ab GN was determined. Active recombinant HB-EGF (rHB-EGF) was expressed in *Drosophila melanogaster* cells, and the ability of the molecule to alter glomerular microcirculatory dynamics using glomerular micropuncture methodology was demonstrated by infusion of rHB-EGF directly into the renal cortex. In addition, the modulation of anti-GBM Ab-induced changes in glomerular hemodynamics was shown using a neutralizing anti-Hb-EGF Ab.

Methods

PCR cloning and generation of an expression construct of Rat HB-EGF. Two sets of primers were used to amplify rat HB-EGF for expression constructs. The first set of

primers covered only the mature HB-EGF (for sense, 5'-dGCGCTGCAGGACTTGGGAAGGGACCGAT-3', which contained a PstI site and 18 nucleotides of specific rat HB-EGF sequence from the 5' end of the mature rat HB-EGF; for antisense, 5'-dCGGCATCC-TAGGGTCAGCCCATGACA-3', which contained a BamHI site and 18 nucleotides of rat-specific HB-EGF sequence at 3' end of EGF domain). An in vivo excised rat macrophage cDNA library was used for PCR for 30 cycles. The PCR amplified fragments were sequenced by an ABI 373A automated DNA sequencer (Applied Biosystems, Foster City, California, USA). HB-EGF was subcloned into pET-M1, an *Escherichia coli* expression vector (39). pET-M1 was modified from pET-11a by adding the sequence coding for 6 \times histidines into pET-11a (40). The recombinant plasmid was transformed into BL21 (DE3) and induced by isopropylthiogalactoside (IPTG). The cell pellet was harvested and was lysed for purification by nickel column (Invitrogen, San Diego, California, USA). The recombinant peptide was injected into rabbits for antibody production. After 2 boosters, the serum was obtained and used for Western blot and immunoprecipitation.

The second set of primers, sense and antisense, covered the sequences for the signal peptide, the heparin-binding domain, and the EGF domain (for sense, 5'-dCGGGATCCACCATGAACTGCTGCCGTCG-3', which contained a BamHI site and Kozak structure [underlined] and 18 nucleotides of specific rat HB-EGF sequence; for antisense, 5'-dCTAGATATCAGT-GTGGTCATATGTGTACAGG-3', which contained an EcoRV site and 22 nucleotides of specific rat HB-EGF

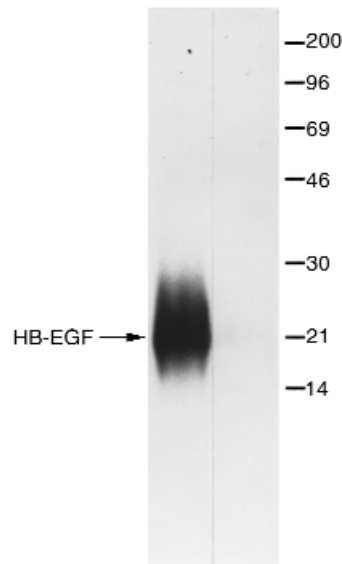


Figure 2
Expression of HB-EGF in *D. melanogaster* cells (SC2). Western blot demonstrated that rabbit anti-HB-EGF Ab generated against *E. coli*-derived HB-EGF recognized glycosylated 21-kDa HB-EGF in the supernatant of the sense (left lane), but not the mock (right lane), transfectants. The molecular weight was higher than recombinant HB-EGF expressed in *E. coli* due to the glycosylation process.

sequence before transmembrane domain). The PCR product amplified by the second set of primers was fused with the sequence coding for 6× histidines and was subcloned into pRM-Ha for expression in *Drosophila melanogaster* cells (SC2) (41). The recombinant pRM-Ha, pRM-HB-EGF, was transfected into SC2 cells by phosphate methods. Permanent SC2 transfects were selected by G418. The supernatant was saved for affinity purification. Ni-NTA affinity resin (Invitrogen) was used for purification following the manufacturer's directions with some modifications, as described previously (40). The supernatant was assayed by Western blot for specificity, and cell proliferation in AKR-2B cells, for functional activity.

Cell proliferation assay. The activity of recombinant HB-EGF expressed in SC2 cells was determined by cell proliferation assay in AKR-2B cells. The proliferation of AKR-2B cells was assayed by measuring the incorporation of [³H]thymidine into DNA, as described previously (42). Growth factor assays were performed in triplicate, and background was subtracted. One unit of growth factor activity is defined as the amount required to stimulate half-maximal DNA synthesis in AKR-2B cells.

Anti-GBM GN. Anti-GBM Ab was prepared by immunizing New Zealand White rabbits with rat GBM as described previously (4, 43). Normal rat serum (NRS) was obtained by bleeding naive unimmunized rabbits. Thirty Lewis rats (The Scripps Research Institute Breeding Colony, La Jolla, California, USA) weighing 150–175 g were divided into 2 groups, with group 1 (*n* = 15) receiving anti-GBM Ab (0.5 mL) intravenously. Group 2 (*n* = 15) rats received 0.5 mL NRS intravenously. Three rats from each group were sacrificed at 30 minutes and at 1, 3, 6, and 24 hours after injection.

Assessment of HB-EGF mRNA and protein expression

Total RNA preparation. Glomeruli were isolated by sequential sieving through nos. 60 and 100 mesh wire screens (Small Parts Inc., Miami, Florida, USA), as

described previously (44). After washing, the glomeruli were homogenized in 4 M guanidine isothiocyanate with a sonicator (Heat Systems-Ultrasonics, Inc., Plainview, New York, USA). The RNA was prepared by a single-step method (45), quantitated by its absorption at 260 nm, and then frozen at -70°C.

Riboprobe preparation and RNase protection assay. The Sac I fragment of rat HB-EGF (from bp 412 to 665) was subcloned into pGEM4Z, and the clone with the 3' end toward the SP6 promoter was selected. BamHI was used to linearize the recombinant plasmid for transcription of an antisense riboprobe with incorporation of [³²P]UTP. A 114-bp probe prepared from rat GAPDH cDNA was used as a housekeeping gene. The RNase protection assay was performed as described previously, using a radioanalytic imaging system (AMBIS Inc., San Diego, California, USA) (44, 46).

In situ hybridization. In situ hybridization was carried out using frozen sections from kidneys perfused and prefixed with 4% paraformaldehyde (44). A cDNA fragment amplified by PCR, from bp 31 to 905 (stu I site), was subcloned into BamHI (5' end) and SmaI (3' end) sites of Pbluescript. The antisense riboprobe was synthesized by T₇ after linearization with BamHI, and the sense riboprobe was synthesized by T₃ after linearization with Eco RI. The probes were synthesized with incorporation of [³⁵S]UTP.

Immunoprecipitation and Western blot. The expression of HB-EGF protein in glomeruli was done by immunoprecipitation to enrich the HB-EGF, followed by Western blot. In brief, the glomeruli were homogenized in PBS with protease inhibitors. After centrifugation, the supernatant was quantitated for protein content by the Bradford assay. A total of 500 μg of protein from each sample was used for immunoprecipitation with rabbit anti-HB-EGF Ab using Pansorbin (40) (Calbiochem, La Jolla, California, USA). The immunoprecipitated samples were subjected to SDS-PAGE and followed by electroblotting to a nitrocellulose membrane. After blot-

Table 1
Glomerular hemodynamics during basal and during vehicle or rHB-EGF infusion

	MAP mmHg	GFR	SNGFR nL/min	SNPF	SNFF	PG mmHg	P _{US}	Δp	AR ×10 ⁹ dyn/s per cm ⁻⁵	ER	LpA nL/s per mmHg	APR nL/mm	FR	πA mmHg	πE
Control group															
Basal	107.8 ± 5.0	1.40 ± 0.1	31.2 ± 4.0	87.8 ± 11	0.36 ± 0.1	43.6 ± 0.6	13.2 ± 0.7	30.4 ± 0.7	33.3 ± 3.0	19.3 ± 3.0	0.082 ± 0.01	13.7 ± 1.0	0.45 ± .02	16.3 ± 0.6	31.5 ± 0.7
Vehicle	107.6 ± 4.0	1.30 ± 0.1	34.8 ± 2.0	107.0 ± 14	0.34 ± .03	43.1 ± 0.6	13.3 ± 0.6	29.8 ± 0.7	29.1 ± 3.0	16.0 ± 2.0	0.083 ± 0.01	14.3 ± 1.0	0.40 ± .03	15.7 ± 0.7	29.2 ± 2.1
Experimental group															
Basal	103.7 ± 2.0	1.10 ^A ± 0.1	34.2 ± 4.0	107.5 ± 22.0	0.36 ± 0.04	40.0 ^A ± 0.5	12.4 ± 0.3	27.6 ^A ± 0.7	32.8 ± 6.0	16.5 ± 3.0	0.082 ± 0.01	16.2 ± 2.0	0.46 ± 0.03	13.7 ± 0.4	27.3 ± 2.2
rHB-EGF	100.0 ^A ± 1.0	1.00 ± 0.1	23.2 ^{A,B} ± 3.0	53.2 ^{A,B} ± 7.0	0.46 ^{A,B} ± 0.02	44.2 ^B ± 0.4	12.1 ± 0.3	32.1 ^{A,B} ± 0.5	55.5 ^{A,B} ± 9.0	40.2 ^{A,B} ± 7.0	0.044 ^{A,B} ± 0.005	9.3 ^{A,B} ± 1.0	0.40 ± 0.04	13.3 ± 0.4	33.5 ± 1.95

^AP < 0.05 control versus experimental group by Tukey test. ^BP < 0.05 basal versus experimental (vehicle or rHB-EGF) period by Student's *t* test. All values are expressed as mean ± SEM.

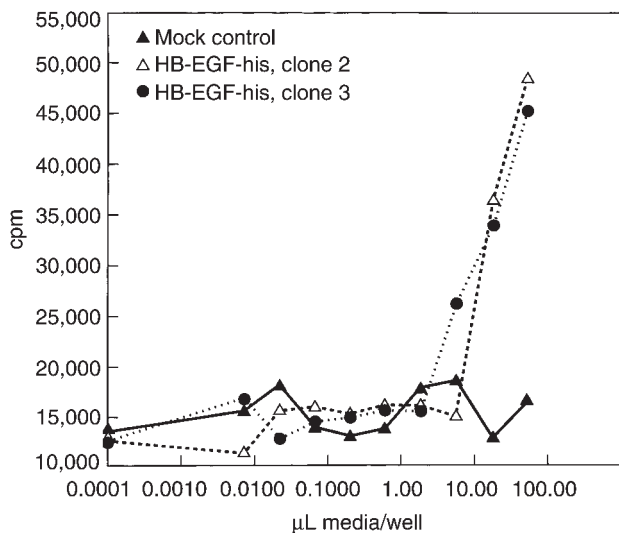


Figure 3
Functional analysis of SC2-derived HB-EGF by cell proliferation assay [H³]thymidine incorporation in AKR-2B cells stimulated with recombinant HB-EGF. Two different clones of histidine-fused rHB-EGF (nos. 2 and 3) were functionally active. Data presented are means \pm SD of triplicate determinations.

ting, the membrane was incubated with biotinylated anti-HB-EGF Ab. Alkaline phosphatase conjugate and the substrate were purchased from Vector Laboratories (Burlingame, California, USA), and color was developed according to the manufacturer's instruction.

Glomerular hemodynamic effects of rHB-EGF. Renal cortex infusion was used to focus the effect of rHB-EGF on the kidney following approaches validated by Knox, Roman, Cowley, and co-workers for acute and chronic studies (47–50). In these studies, infusion of a saline vehicle did not affect renal hemodynamics or renal interstitial hydrostatic pressure at rates less than 2 mL/h. Renal cortical infusion catheters were constructed by fusing a short tip of PE-10 into a PE-50 tubing. A small hole was made in the renal cortex with an electrocautery needle to minimize bleeding. The PE-10 tip was then inserted into the hole and secured in place with 01 910 adhesive (Permapond International, Englewood, New Jersey, USA).

A pilot study measuring only glomerular filtration rate (GFR) was done in Munich-Wistar rats. After a dose-response study, a dose of 120 ng rHB-EGF/kg per minute infused at a rate of 1.5 mL/h was selected. After 15 minutes of equilibration, two 15-minute clearances were done during which the NaCl-NaHCO₃ vehicle (V) was infused. This was followed by 2 additional clearances, during which either the V infusion was continued or V plus rHB-EGF was substituted. Under the conditions used, rHB-EGF produced a decrease in GFR.

To investigate the mechanisms by which rHB-EGF altered the glomerular hemodynamics, a micropuncture study was performed. Twelve male Harlan-Wistar, 250–330 g, rats (Simonsen Laboratories, Gilroy, California, USA) were anesthetized with Inactin (BYK, Konstanz, Germany), 10 mg/100g body weight intraperi-

toneally and placed on a temperature-regulated micropuncture table. The left kidney was exposed and placed in a Lucite cup as described previously (51). A PE-10 cortical infusion catheter was placed into the renal cortex as already described here. The surface of the kidney, illuminated with a fiber-optic light source, was bathed with heated (37°C) NaCl-NaHCO₃ solution. A femoral artery catheter was used for periodic blood samples and for monitoring of the mean arterial pressure (MAP) with a transducer (Model P23db; Statham Instruments, Gould Division, Inc., Hato Rey, Puerto Rico) and recorder on a Statham (Statham Instruments) chart recorder. All studies were performed using a euvoletic protocol (51). All rats received an additional infusion of NaCl-NaHCO₃ containing [³H]inulin at a rate of 100 μ Ci/h in a volume of 1.5 mL/h. Both infusions were initiated at the end of the surgical preparation and were maintained throughout the experiment. All determinations of glomerular ultrafiltration and nephron filtration were measured after 40 minutes of equilibration.

Two period euvoletic studies were conducted in each rat. In the first control (basal period), V infusion was used, and in the second or experimental period, either rHB-EGF (120 ng/kg per minute; $n = 7$) or V ($n + 5$) was infused into the renal cortex at a rate of 0.6 mL/h rather than the 1.5 mL/h used in the pilot study to reduce volume.

Hydrostatic pressures in the glomerular capillaries (stop-flow pressure; SFP), urinary space (P_{US}), and efferent arterioles were measured with a glass micropipette (1–3 μ m in external tip diameter, filled with 1.2 M saline) in series with a servo-nulling pressure measurement device (IPM, San Diego, California, USA), as described previously (51). Glass pipettes of 13–16 μ m in external tip diameter were used to collect 3 efferent arteriolar blood samples for determination of efferent protein concentration (C_E), and these were bracketed by 2 collections from the femoral artery for measurement of afferent protein concentration (C_A). Late surface segments of proximal tubules were identified by intratubular injection of diluted FD&C dye contained in a pipette of 3–5 μ m outer diameter. Five timed tubular fluid collections (2.5 minutes) were randomly obtained from late proximal segment with the use of an injected oil block from these sites. All measurements required less than 60 minutes. Urine was collected in preweighed containers under oil, and plasma samples were obtained for [³H]inulin concentration to compute whole kidney GFR, superficial nephron GFR (SNGFR), and the ratio of tubular fluid/plasma inulin activity (TF/P).

[³H]inulin activity in plasma, urine, and tubular fluid was monitored on a Model 2425 Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, Illinois, USA). SNGFR, GFR, TF/P, fractional reabsorption (FR), and absolute proximal reabsorption (APR) were determined as described in previous studies from this laboratory (52). Protein concentration in systemic and efferent arteriolar plas-

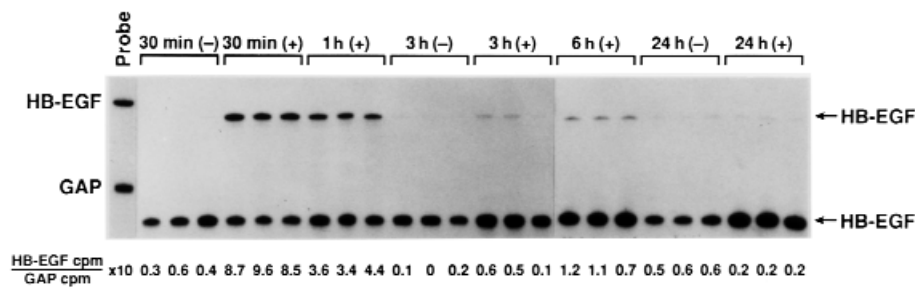


Figure 4

RNase protection assay analysis of mRNA for HB-EGF and GAPDH in glomeruli after anti-GBM Ab administration. The data are presented as a ratio of specific mRNA/GAPDH mRNA to ensure a constant quantity of RNA for each sample. The protected HB-EGF and GAPDH bands are shorter than the respective probes because the unhybridized polylinker regions in the cRNA probes are digested by RNase.

ma samples was measured by a microadaptation of the Lowry method (53). Superficial nephron filtration fraction (SNFF), single nephron plasma flow (SNPF), afferent arteriolar resistance (AR), efferent arteriolar resistance (ER), ultrafiltration coefficient (LpA), and oncotic pressure (π) from protein concentration were calculated as described previously (52, 54).

Effects of anti-HB-EGF Ab administration on renal function in rats with Anti-GBM GN. Two period micropuncture experiments were used to test the effects of rabbit anti-HB-EGF Ab in rats treated with anti-GBM Ab. Whole-kidney GFR and SNGFR were measured before and after anti-GBM Ab administration in rats treated with anti-HB-EGF Ab (group anti-HB-EGF; $n = 6$) or NRS (group NRS; $n = 6$). Male Wistar rats weighing 250–300 g were prepared for micropuncture as described earlier in this section, with the exception that no catheter was implanted in the renal cortex. Fifteen minutes after starting the equilibration period, rats received 0.4 mL of rabbit anti-HB-EGF Ab (group anti-HB-EGF) or equivalent volume of NRS (group NRS) intravenously. After a 60-minute equilibration period during which rats received rat plasma and NaCl-NaHCO₃ with [³H]inulin at the rates already described here, whole-kidney GFR and 5 SNGFR samples were collected. After completing the first period measurements, anti-GBM Ab (0.8 mL) was administered intravenously in all rats. Fifteen minutes later, another dose of NRS or anti-HB-EGF Ab (0.4 mL) intravenously was given. Ninety minutes after anti-GBM Ab administration, a new set of GFR and SNGFR values was obtained. The left kidney was then harvested for histologic evaluation. The numbers of neutrophils found in 30 hemisections of glomeruli were determined. The glomerular capillary lumens contained variable amounts of weakly PAS-positive material previously identified as fibrin. These deposits were semiquantitated in the 30 glomeruli using a 0–4 scale in which 0 = no deposit; 1 = 10–20% of capillary loops with deposits; 2 = 50% of loops; 3 = almost all loops; and 4 = in addition material in Bowman’s space. The amount of PAS-positive protein reabsorption droplets in proximal tubules was also semiquantitated using a 0–4 scale in the high-power

fields adjacent to the 30 glomeruli in which 0 = no droplets; 1 = small numbers of droplets; 2 = increased droplets; 3 = increased and larger droplets; and 4 = massive amounts of droplets with or without intraluminal proteinaceous casts.

Statistical analysis. Statistical analysis was performed using either paired or unpaired *t* test, depending on whether multiple or single values were obtained per period, for comparison between the control and experimental period (55). Tukey multiple comparison analysis was also performed to obtain statistical significance between groups of animals. The level of statistical significance was defined at a value of $P < 0.05$. All results are expressed as mean \pm SEM.

Results

Expression of HB-EGF protein in *E. coli* and Ab production. HB-EGF was expressed in the *E. coli* for Ab production. The cell lysate from transformed BL21 (D3) cells with or without induction by IPTG was examined by SDS-PAGE. After Coomassie blue staining, the expected size of recombinant HB-EGF peptide coding the mature HB-EGF was approximately 9.5 kDa (Figure 1). The purified recombinant peptide was used to immunize a rabbit and to obtain rabbit anti-rat HB-EGF Ab. The specificity of anti-HB-EGF Ab was confirmed by its ability to recognize HB-EGF from the supernatant of SC2 transfectants, but not from the mock transfectants (Figure 2).

Expression of HB-EGF in *D. melanogaster* cells. The molecular mass of the 6 \times his-fused HB-EGF peptide expressed in *D. melanogaster*, SC2, cells and purified by nickel column was around 20 kDa (Figure 2), a higher molecular weight than the *E. coli*-derived form (Figure 1), which presumably is due to the glycosylation process. The band was smeared in the supernatants of pRM-HB-EGF-transfected SC2 cells, indicating that fly cells cause posttranslational modification of HB-EGF (Figure 4). Supernatants from 2 clones of pRM-HB-EGF transfectants showed mitogenic activity detected as their ability to incorporate [³H]thymidine in AKR-2B cells (Figure 3).

Expression of HB-EGF during anti-GBM GN. The expression of HB-EGF mRNA in glomeruli was increased by 8- to 10-fold as early as 30 minutes after

Table 2

Effect of anti-HB-EGF antibody administration in rats with anti-GBM Ab glomerulonephritis

		MAP	Hct	GFR	SNGFR
		mmHg	%	mL/min	nL/min
NRS	Control	112 ± 6	44.3 ± 0.5	1.14 ± 0.1	33.7 ± 2.3
	AGBM-Ab	119 ± 6.6 ^A	42.7 ± 1	0.82 ± 0.1 ^A	26.2 ± 2 ^A
Anti HB-EGF Ab	Control	127 ± 5	45 ± 1	1.24 ± 0.1	35.5 ± 1.6
	AGBM-Ab	121 ± 4	45 ± 1	1.11 ± 0.2	32.6 ± 3

^AP < 0.05 versus control.

the administration of anti-GBM Ab (Figure 4). The expression persisted at 1 hour and declined thereafter. The maximum induction was at 30 minutes. As shown in Figure 5, the HB-EGF protein level correlated with the HB-EGF mRNA expression. The expression of HB-EGF also corresponded in time with the impaired glomerular hemodynamic changes found in the acute phase of anti-GBM Ab GN (43).

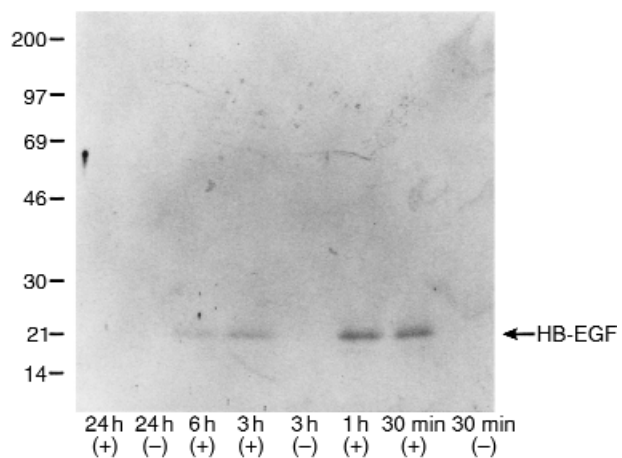
Expression of HB-EGF occurring during anti-GBM Ab GN was localized by in situ hybridization using a ³⁵S-labeled antisense riboprobe prepared from rat HB-EGF cDNA. In situ hybridization, 1 hour after anti-GBM Ab administration, revealed a strong signal over the glomeruli using an antisense riboprobe for HB-EGF; a less intense hybridization signal for HB-EGF was evident in tubular areas (Figure 6a). The cells of the glomerular capillary wall and mesangium, rather than intraluminal inflammatory cells, appeared to be the site of the expression. The specificity of the hybridization was confirmed by the lack of any hybridization in sections in which a sense riboprobe was substituted for the antisense probe (Figure 6b).

Hemodynamic effects of rHB-EGF infusion. In the pilot study, no significant changes were found in GFR during cortical V infusion; however, the rHB-EGF infusion resulted in 29% reduction in GFR.

During the micropuncture study, BW, Hct, and UV did not differ between the 2 groups during either basal or experimental periods. In the micropuncture basal period, MAP was not different between the control (V) and experimental (rHB-EGF) groups (107.8 ± 5 and 103.7 ± 2 mmHg, respectively; Table 1). GFR was slightly, but significantly, lower in the experimental group (1.1 ± 0.1 mL/min) compared with the control group (1.4 ± 0.1 mL/min). During the basal period, no differences were observed for SNGFR, SNPF, SNFF, AR, ER, C_E, APR, or FR between the groups. Because efferent oncotic pressure was not significantly different from ΔP, denoting the presence of filtration pressure equilibrium, minimal values of LpA were calculated. The basal ΔP was significantly lower in the experimental group (27.6 ± 0.7 mmHg) compared with the control group (30.4 ± 0.7 mmHg). The lower ΔP in the experimental group was due to a significantly lower C_A (4.5 ± 0.1 g% vs. 5.1 ± 0.1 g%).

During the experimental period, MAP was not modified during rHB-EGF infusion compared with V; however, SNGFR significantly decreased from 34.4 ± 3.7 to 23.2 ± 2.7 nL/min (*P* < 0.016). The reduction in SNGFR was due to a significant decrease in SNPF from 107.5 ± 22 to 53.2 ± 7 nL/min (*P* < 0.04) (Table 1 and Figure 7). SNPF did not change in the same proportion as SNGFR (33% vs. 51%); therefore, a significant increase was observed in SNFF (0.36 ± 0.04 vs. 0.46 ± 0.02; *P* < 0.016). The decrease of SNPF was dependent on both afferent and efferent vasoconstriction (*P* < 0.02 and *P* < 0.01 vs. control period, respectively). The increase in efferent glomerular resistance was higher than the afferent glomerular resistance, consequently leading to increased glomerular hydrostatic pressure and ΔP. A significant reduction in LpA (0.044 vs. 0.082 nL/s per mmHg in the basal period) also contributed to the reduction in SNGFR. Because the increase in ΔP could minimize the decrease in SNPF, the critical event leading to reduction in SNGFR was a major reduction in LpA. A reduction in APR was observed during rHB-EGF infusion without significant change in FR; thus the reduction in APR was likely secondary to the reduction in SNGFR.

The decrease in whole-kidney GFR during the cortical infusion of rHB-EGF (seen in the pilot study) did not reach statistical significance during the micropuncture study, suggesting that higher volume infusion (1.5 mL/h during the pilot vs. 0.6 mL/h during micropuncture) may have extended the renal effects of rHB-EGF to juxtamedullary as well as cortical glomeruli. That is, perhaps the lower infusion during

**Figure 5**

Expression of HB-EGF protein in glomeruli analyzed by immunoprecipitation and Western blot. A total of 500 μg of total glomerular lysate from each of the samples was used. After immunoprecipitation and electroblotting, biotinylated rabbit anti-HB-EGF Ab was incubated with the membrane, and subsequently alkaline phosphatase-conjugated goat anti-rabbit IgG was used before color development. The experiment was performed as described in the Methods. A band, about 21 kDa, appeared in the lysates from the rats with anti-GBM GN (+), but not from the controls (-).

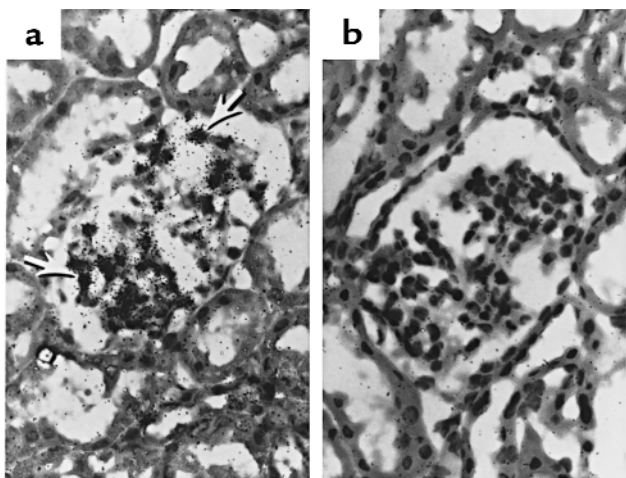


Figure 6
(a) In situ hybridization analysis of HB-EGF mRNA in kidney tissue 1 hour after anti-GBM Ab administration. The antisense HB-EGF probe hybridized with glomeruli structures, a less intensive signal, was seen in tubules. Glomerular capillary wall and mesangial cells, but not intraluminal inflammatory cells, were the site of mRNA expression (arrows). **(b)** No signal was found using a sense HB-EGF probe.

micropuncture allows a hemodynamic compensation by deeper nephrons. Previous studies in our laboratory have clearly demonstrated that superficial nephrons are quite sensitive to changes in adrenergic and AII activity, anesthesia, and extensive surgery, as required for micropuncture studies, and are associated with increased neuroadrenergic activity (56, 57). A higher sensitivity of the superficial nephrons can also explain the dissociation between the decrease in SNGFR during rHB-EGF infusion in the absence of change in whole-kidney GFR. Previous studies in our laboratory have demonstrated similar findings with reduction in superficial single-nephron GFR in the absence of any change in whole-kidney GFR (58–60).

Effects of anti-HB-EGF Ab administration on renal function in rats with anti-GBM Ab GN. Table 2 presents the results obtained in both the anti-HB-EGF Ab group and the NRS group. No significant differences were found in mean arterial pressure or hematocrit between the 2 groups, either in control (first period) or after anti-GBM Ab administration. GFR and SNGFR values were similar in control conditions in the 2 groups of rats, demonstrating that administration of anti-HB-EGF antibody does not modify renal function. Anti-GBM Ab reduced both SNGFR and GFR in the NRS group as previously shown by our laboratories (43). In contrast, no changes in GFR and SNGFR were observed in anti-HB-EGF Ab-treated rats after anti-GBM Ab administration, demonstrating that anti-HB-EGF Ab prevented the early changes in renal function after induction of anti-GBM Ab GN. The modulation of the SNGFR and GFR produced by anti-HB-EGF Ab did not appear to be related to changes in the glomerular inflammatory response to the anti-GBM Ab. In the anti-HB-EGF ($n = 5$) and

NRS ($n = 6$) groups, respectively, the numbers of neutrophils per 30 glomeruli were 397 ± 39 and 362 ± 35 (NS); the amounts of capillary lumen deposits related to fibrin were 39.7 ± 14.2 and 55.4 ± 12.7 (NS); and the proximal tubular protein reabsorption droplets were 39.1 ± 6.1 and 47.2 ± 6 (NS).

Discussion

The current state of knowledge of the mechanisms leading to glomerular immune injury is largely based on studies that have examined the very early phases of glomerular hemodynamic alterations after infusion of anti-GBM Ab. This experimental model provides an advantage over other models of immune induced nephritis in that events occur quickly after the administration of the Ab, probably related to the accessibility of the antigen and the proximity to proinflammatory elements circulating through the capillary lumen. Neutrophils are attracted within minutes and nephron filtration rate is markedly reduced within 30 minutes. The contribution of a variety of neurohumoral pathogenic factors has been delineated using this acute injury model. The question remains as to the initiating factors that produce the extremely early pathophysiological events, including the accumulation of neutrophils that can displace endothelial cells and alter patterns of blood flow, decreasing the glomerular ultrafiltration coefficient. Is there an early transcription of factors within glomeruli that is critical in the initiation of the pathophysiological changes? The current study has attempted to address the role of 1 candidate molecule, HB-EGF. We found HB-EGF to be transcribed and expressed in high levels in glomeruli in both epithelial and mesangial cells within 30 minutes after administration of anti-GBM Ab coincident with the onset of the glomerular hemodynamic alterations. In addition, HB-EGF has mitogenic activity and also stimulates DNA synthesis, suggesting HB-EGF, much as EGF, might exhibit renotropic functions during reparative phases of renal injury.

The early and transient expression of HB-EGF in glomeruli correlated with the impaired glomerular hemodynamic changes observed in the acute phase of anti-GBM Ab GN (43), which led us to examine the acute hemodynamic effects of recombinant HB-EGF. The cloning and expression of an active form of rHB-EGF was accomplished using the *D. melanogaster* SC2 fly cell line, which allowed the highly efficient expression of this eukaryotic protein with posttranslational modifications. The active rHB-EGF, in turn, made it possible to study the pathophysiological effects by direct infusion into the renal cortex. In addition, administration of neutralizing anti-HB-EGF Ab prevented the fall in SNGFR in the anti-GBM Ab model.

The early glomerular hemodynamic alterations in anti-GBM Ab GN include a significant decrease in SNGFR, SNPF, and LpA and an increase in the glomerular capillary hydrostatic pressure gradient due to a greater efferent vasoconstriction and the reduction

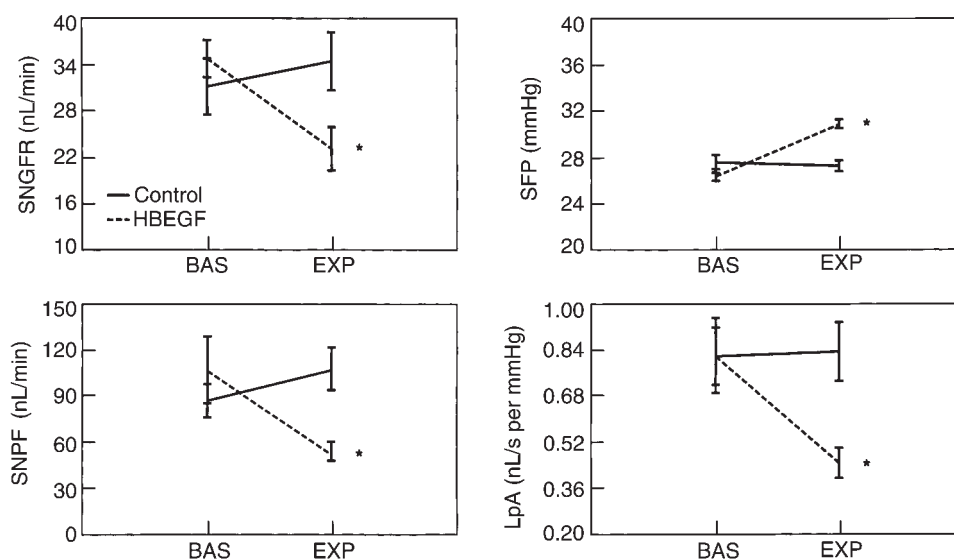


Figure 7

Glomerular hemodynamic effects in control rats (solid lines) and rats infused with HB-EGF (dashed lines). SNGFR, SNPF, and LpA significantly decreased in the HB-EGF group. HB-EGF infusion was associated with a significant decrease in SF. * $P < 0.05$ versus basal period. BAS, basal. EXP, experimental.

of LpA (6). Several factors have been implicated in the hemodynamic events of the anti-GMN Ab model of GN: (a) complement activation with a role in the early phase acting as a vasoconstrictor via C5a (61), chemotaxis of neutrophils, and perhaps cell damage via the terminal complement pathway, C5b-C9; (b) neutrophils that contribute to reduction in LpA by occupying capillary wall surface, as well as obstructing flow through some conduits, can further reduce filtration surface; (c) α -adrenergic activity on migration and/or attachment of neutrophils to the capillary wall; and (d) arachidonic acid metabolites (reviewed in refs. 1, 6). In the present study, the ability of anti-HB-EGF Ab to modulate the fall in SNGFR in the absence of alterations in the inflammatory infiltrate appear to exclude physical effect on the inflammatory response as a mechanism for the beneficial change.

A role for HB-EGF now must be considered as well, as cortical infusion produced a decrease in SNGFR, SNPF, and LpA identical to glomerular microcirculatory dynamics changes observed during anti-GBM Ab GN. It has been demonstrated that EGF is able to decrease renal function during infusion through the renal artery (3). However, expression of EGF mRNA declines acutely during anti-GBM Ab GN (4).

The expression of HB-EGF by glomerular mesangial and glomerular capillary cells after administration of anti-GBM Ab in this study suggests that these cells may contribute via EGF receptors (EGF-Rs) and could respond in autocrine and/or juxtacrine fashions to produce the observed pathophysiological effects. Glomerular mesangial cells among other renal cells express EGF-Rs that increase in GN (62). The mechanism(s) contributing to the pathological effects might include the contraction of glomerular cells due to actin poly-

merization induced during dimerization and autophosphorylation of 1 or more members of the EGF-R family (Erb-1 to Erb-4). HB-EGF is reported to bind to Erb-1 and Erb-4 with differing results in terms of chemotaxis and proliferation (63). The EGF-R dimerization and autophosphorylation lead to activation of several signaling pathways including RAS/Raf, signal transducers and activation of transcription (STATs), and mitogen activated protein kinases (MAPKs), leading to activation of other pathways including phospholipase A2. Subsequently, arachidonic acid is released that is converted to prostaglandins and leukotrienes. In addition to their direct physiological and pathophysiological effects, the arachidonic acid metabolites can act as second messengers to produce morphological effects on actin reorganization with induction of cytoskeletal changes (64–66).

The fact that the hemodynamic effects of HB-EGF strongly resemble those induced during the systemic infusion of angiotensin II (AII) (67) suggests that other factors may be involved in the mechanism(s) by which HB-EGF affects renal function. Furthermore, it has recently been demonstrated that EGF upregulated the expression of the AII cell surface receptor type I (68). It is known that AII upregulates HB-EGF in SMCs (29) and that the α -adrenergic agonist phenylephrine upregulates it in cardiac myocytes (69). HB-EGF upregulates its own transcription level (23), and it can upregulate bFGF in SMCs (70). The possible effect of HB-EGF on AII or on nitric oxide, the physiological antagonist of AII (71), points to interesting issues for investigation. It is noteworthy that the promoter of both HB-EGF and endothelin-1 (ET-1) contains AP-1 and GATA consensus sequences (72); ET-1 may also participate in the mediation of the acute glomerular hemodynamic alterations found during anti-GBM Ab GN.

In summary, HB-EGF is expressed and produced by intrinsic glomerular cells in anti-GBM Ab GN at the time in which a decline in renal function is observed. The infusion rHB-EGF into the renal cortex produces effects that resemble the hemodynamic effects seen during anti-GBM Ab GN, suggesting a role for this novel growth factor in the renal dysfunction observed in this model of GN. These effects were confirmed when anti-HB-EGF Ab was shown to modulate the hemodynamic effects without altering the morphological response to the anti-GBM Ab.

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