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Activation of Glomerular Mesangial Cells by Hepatocyte Growth Factor Through Tyrosine Kinase and Protein Kinase C

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ABSTRACT. Hepatocyte growth factor (HGF) induces mitogenesis, chemotaxis, and tubule formation in renal epithelial cells. This study examined the effects of wortmannin and protein kinase C (PKC) inhibitors on HGF-mediated changes in metabolic activity in glomerular mesangial cells and renal epithelial carcinoma A498 cells. The extracellular acidification rate of transformed mouse glomerular mesangial cells and A498 cells was measured as an index of metabolic activity with a microphysiometer. HGF increased the acidification rate of mesangial cells and A498 cells in a concentration-dependent fashion that was inhibited completely by the tyrosine kinase inhibitor tyrphostin-23 (100 μM). The PKC inhibitors RO-32-0432 and SKF-57048 also inhibited HGF-induced acidification. The IC_{50} values for SKF-57048 were 59 ± 2 and 20 ± 10 nM in mesangial cells and A498 cells, respectively ($P < 0.05$). 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), a phorbol ester that activates PKC, increased acidification in mesangial and epithelial cells similar to HGF. Wortmannin, an inhibitor of phosphatidylinositol (PI) 3-kinase (IC_{50} value 1–10 nM), inhibited HGF-induced acidification with an IC_{50} of 93 ± 31 and 9 ± 1 nM in mesangial and A498 cells, respectively ($P < 0.05$). In contrast, there was no significant difference in the IC_{50} value of wortmannin for epidermal growth factor (EGF)-induced acidification between mesangial and A498 cells (23 ± 9 vs 14 ± 1 nM, respectively). Because the IC_{50} value for wortmannin in inhibiting HGF but not EGF-induced acidification was an order of magnitude higher in mesangial cells than in epithelial A498 cells, a wortmannin-sensitive PI 3-kinase pathway may not be involved in HGF-mediated acidification in mesangial cells. BIOCHEM PHARMACOL **55**;2:227–234, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. hepatocyte growth factor; microphysiometer; wortmannin; kidney; mesangial cells; epithelial cells

HGF§ is a heterodimeric molecule derived from a prepro-precursor of 728 amino acids, which is proteolytically processed to form mature HGF [1]. A role for HGF in renal development and growth is suggested by *in vitro* studies and animal models of renal injury. *In vitro*, HGF stimulates growth of renal epithelial cells (mitogen), enhances the motility of epithelial cells (motogen), and induces renal epithelial tubule formation (morphogen) [2–4]. HGF plays a role in renal injury because HGF and its receptor (tyrosine kinase) c-met are increased in the kidney following nephrectomy and ischemia [5]. HGF administration also accelerates recovery of renal function following ischemia or $HgCl_2$-induced renal injury [6–8]. In addition, the embryonic kidney expresses HGF primarily in the metanephric mesenchyme, while the receptor c-met is in the developing renal tubular epithelial cells [9, 10]. Thus, HGF may be involved in the repair of the kidney by enhancing epithelial remodeling.

The signal transduction pathways that are activated by HGF include PLCγ, PKC, and PI 3-kinase [11, 12]. The mechanism by which HGF induces epithelial cell proliferation is thought to occur through PI 3-kinase [11, 13]. While HGF has robust proliferative effects on epithelial cells, it has no effect on the proliferation of glomerular mesangial cells [14]. However, recent data have indicated that glomerular mesangial cells can also express c-met mRNA and respond to HGF [15], although the second messenger systems by which HGF acts on mesangial cells are not known. The purpose of this study, therefore, was to

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§ *Abbreviations:* A498, renal epithelial carcinoma A498 cells; DMEM, Dulbecco's Modified Eagle's Medium; EGF, epidermal growth factor; FBS, fetal bovine serum; HGF, hepatocyte growth factor; HRPTEC, human renal proximal tubule epithelial cells; MMC, mouse mesangial cells; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; RT–PCR, reverse transcription–polymerase chain reaction; and TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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evaluate the metabolic effects of HGF on renal mesangial cells and the involvement of PI 3-kinase and PKC in this process.

MATERIALS AND METHODS

Cell Culture

MMC cultures were established from glomeruli obtained from the kidneys of 8- to 10-week-old SJL/J ($H\text{-}2^s$) mice [16], grown in DMEM containing 2 mM L-glutamine and 180 mg/dL glucose and supplemented with 10% FBS and 100 U/mL penicillin/streptomycin at 37° in 5% CO_2. Cells were subcultured by rinsing with PBS and then incubating with 0.05% trypsin supplemented with 20 mM EDTA. MMC were transformed with noncapsid forming SV-40 virus to establish a permanent cell line [16]. These cells exhibit many features of differentiated mesangial cells [16]. When the MMC were transferred to SmithKline Beecham Pharmaceuticals, the passage number was arbitrarily designated as passage 1. Experiments reported here used cells from passages 10–15. Cryopreserved human mesangial cells and HRPTEC were purchased from the Clonetics Corp. and grown in Clonetics Mesangial Cell Growth Medium (MsGM) supplemented with 5% FBS, 50 μg/mL Gentamicin, and 50 ng/mL Amphotericin-B and Clonetics HRPTEC medium, respectively. Human mesangial cells and HRPTEC were grown according to the methods provided by the Clonetics Corp. and were used in these experiments between passages 6 and 8. Human A498 cells, which have epithelial morphology, were acquired from the American Tissue Culture Collection and were used at passages 37–41. Rat glomerular epithelial cells were obtained from the kidney cortex of 55–70 g rats (Sprague–Dawley, Charles River). Isolated glomeruli were incubated for 10 min at 37° in collagenase (750 U/mL) and then plated in flasks in growth medium composed of RPMI 1640 medium supplemented with 0.6 U/mL insulin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 15% FBS. Cells were grown at 37° in 5% CO_2. Cells were subcultured by rinsing with Ca^{2+}- and Mg^{2+}-free PBS, and then incubated with 0.05% trypsin supplemented with 20 mM EDTA. A colony of epithelial cells was isolated using a cloning ring. Epithelial cells were characterized by morphology using transmission electron microscopy: cells display prominent cell junctions and microvilli and do not possess characteristics of mesangial cells or fibroblasts [17].

RT–PCR

The presence of c-met mRNA was determined by reverse-transcribed cDNA subjected to PCR using primers to a species-conserved region of c-met. (Sense: 5′-GG GAATCTGC CTGAGGAGTG AA-3′, bases 3677–3698 of human c-met; antisense: 5′-GATATCCTGG AGAC CAGTTC GGAAAA-3′, bases 4236–4261 of human c-met.) Total RNA from human A498 cells, MMC, and rat glomerular epithelial cells (REC) was extracted by acidified phenol/chloroform extraction [18]. Four micrograms total RNA was used in a reverse transcription reaction with 200 U MMLV reverse transcriptase (Life Technologies) for 1 hr at 42° to generate cDNA. PCR was performed with 4 μL of the cDNA product with 1 U *Taq* polymerase (Perkin–Elmer), at 96° for 1 min, 48° for 1 min, 72° for 1 min for 35 cycles. PCR products were resolved on a 1% agarose gel and transferred to nylon membrane with 0.4 N NaOH. c-Met-specific PCR product was visualized by a Southern blot using a cDNA probe made from human c-met (bases 3677–4261).

Western Blot

Cell membranes from MMC and A498 carcinoma cells were prepared according to Kjelsberg *et al.* [19]. Membrane proteins were fractionated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The blots were incubated with either anti-human or anti-mouse c-met (Santa Cruz Biotechnology Inc.) overnight at 4° at 1:1000 dilution. The secondary antibody (anti-rabbit conjugated with horseradish peroxidase) was incubated for 1 hr at room temperature at 1:10,000 dilution and detected with an ECL light detection kit (Amersham). Blots were exposed to X-ray film for 10 min.

Microphysiometry

The cytosensor microphysiometer is based on a pH-sensitive silicon sensor that is part of a microvolume flow chamber in which cells are immobilized [20]. MMC were subcultured with trypsin from 150 cm^2 flasks into capsule cups (Molecular Devices) having a polycarbonate membrane of 3 μm pore size at a density of 300,000 cells/cup. Cells were allowed to attach for 24 hr in the medium specified for each cell type. After spacer rings and insert cups were fitted into the capsule cups, the assembled units were transferred to the sensor chamber and perfused at 100 μL/min with bicarbonate-free RPMI 1640 medium (Molecular Devices) in the microphysiometer at 37°. The acidification rate was measured as a change in pH over time, which was determined when the pumps were turned off for 30 sec at 2-min intervals. Cells were treated with human recombinant HGF (Collaborative Biomedical Products) in the presence or absence of inhibitor for PKC, SKF-57048 (SmithKline Beecham), inhibitor for PI 3-kinase, wortmannin (Sigma), and inhibitor for tyrosine kinase, tyrphostin 23 (Sigma). TPA was purchased from Sigma. The protein kinase C inhibitor RO-32-0432 [21] was synthesized by A. Krog (SmithKline Beecham).

Mitogenesis Assay

The mitogenic effect of HGF was measured as the amount of [^{3}H]thymidine incorporated into newly synthesized DNA. Cells were subcultured into 24-well dishes (2.5×10^3 cells/well) and incubated in growth medium for 72 hr.

Subconfluent cultures were made quiescent by placing them in DMEM containing 2 mM L-glutamine and 100 mg/dL glucose and supplemented with 3% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin for 48 hr. HGF was diluted in unsupplemented DMEM and then added to triplicate wells for 24 hr. Cells were pulsed with [^{3}H]thymidine during the last 4 hr of the 24-hr incubation period. Cells were washed with PBS, and then 5% trichloroacetic acid was added to precipitate proteins and nucleic acids and to remove unincorporated [^{3}H]thymidine. Then the precipitate was dissolved by adding 0.5 mL of 0.5 N NaOH. Aliquots (400 μL) were added to scintillation fluid and counted on a Taurus liquid scintillation counter (ICN Biomedicals, Inc.). Statistical significance was determined by ANOVA (SuperANOVA software, Abacus Concepts).

RESULTS

c-Met Expression

The presence of the HGF receptor, c-met, in MMC and A498 cells was established by RT–PCR and western blot. Both cell lines contain an mRNA that can be reverse transcribed and amplified with c-*met* primers. No amplification product was seen in the absence of reverse transcriptase (Fig. 1A). Protein expression of c-met was verified with species-specific antibodies to c-met. Membranes from A498 cells had strong c-met immunoreactivity with the anti-human c-met. Membranes from MMC had weaker but detectable c-met immunoreactivity with the anti-mouse c-met (Fig. 1B). The antibodies were species specific and did not cross-react (Fig. 1B).

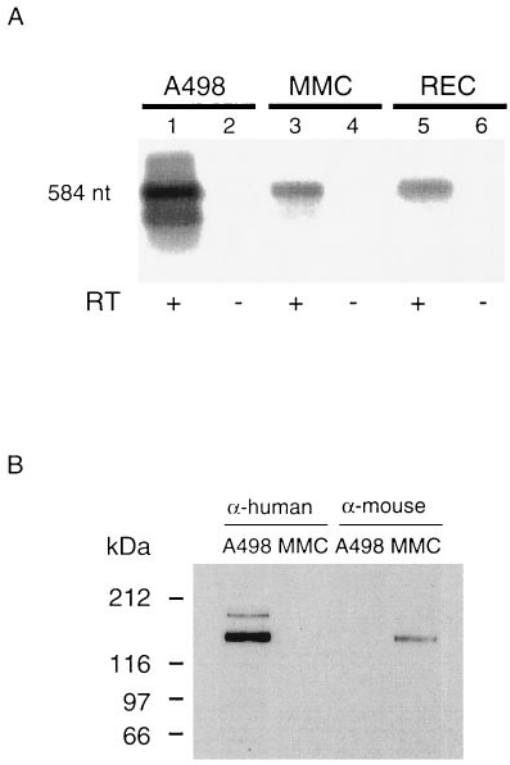

FIG. 1. (A) Determination of the presence of the HGF receptor c-met in A498 cells, MMC, and rat glomerular epithelial cells (REC), by RT–PCR. RT–PCR identified the expected molecular weight PCR product of 584 nucleotides (nt) in A498 cells, MMC, and REC. Samples without reverse transcriptase (RT -) had no PCR product. (B) Western blot of membrane proteins from MMC and A498 probed with anti-human or anti-mouse c-met. The species-specific antibodies recognized the beta chain of c-met (140 kDa). Due to the very high expression of c-met in A498 cells, an additional larger (unprocessed) form was detected.

Microphysiometry

Because the rate data (change in current/pH over time) are dependent on seeding density and seeding uniformity, acidification rates must be expressed as a percent of baseline such that data from different experiments can be equated [20]. The optimal duration of HGF exposure was determined by treating MMC with 100 ng/mL HGF for 2, 5, 10, and 15 min. While the 5-, 10-, and 15-min exposure times all gave similar robust peak responses in acidification rate, the 5-min exposure time was chosen for further experiments because of rapid recovery after this duration of exposure (data not shown).

The HGF concentration responses were established by treating A498 cells and MMC for 5 min with 3, 10, 30, or 100 ng/mL HGF. HGF induced a concentration-dependent increase in acidification rate in both A498 cells and MMC. The peak response in A498 cells was 20% above baseline with 100 ng/mL HGF, whereas in MMC it was 30% above baseline (Fig. 2). EGF increased acidification rates in MMC and A498 cells in a bell-shaped response. The optimal concentration for stimulating acidification in A498 cells was 10 ng/mL with a maximal response of 20% above baseline. In MMC, the optimal concentration of EGF was 30 ng/mL, which increased the acidification rate to 75% above baseline (Fig. 3). In comparison, transforming growth factor-β1 had no effect on acidification rates of either MMC or A498 cells at 10 ng/mL (data not shown). Because the HGF receptor is the tyrosine kinase c-met, the effect of the tyrosine kinase inhibitor tyrphostin 23 was examined on the HGF-mediated response. Tyrphostin 23 inhibited HGF-induced acidification in both MMC and A498 cells with IC_{50} values of 21 ± 6 and 5 ± 2 μM, respectively (Fig. 4).

HGF-induced proliferation of epithelial cells has been shown to be mediated by PI 3-kinase [11]. Wortmannin, a fungal metabolite, has been shown to be a potent inhibitor of PI 3-kinase [22]. The effect of wortmannin on HGF-stimulated acidification rate was evaluated with a 20-min pretreatment of the cells with increasing concentrations of wortmannin followed by a 5-min exposure to 50 ng/mL HGF. Wortmannin inhibited the HGF-induced acidification in A498 cells with an IC_{50} value of 9 ± 1 nM. However, in MMC, the IC_{50} value for wortmannin was 10-fold higher at 93 ± 31 nM ($P < 0.05$; Fig. 5). In contrast, EGF-stimulated acidification was inhibited equally by wortmannin in MMC and A498 cells (23 ± 9 vs 14 ± 1 nM, respectively; Fig. 5).

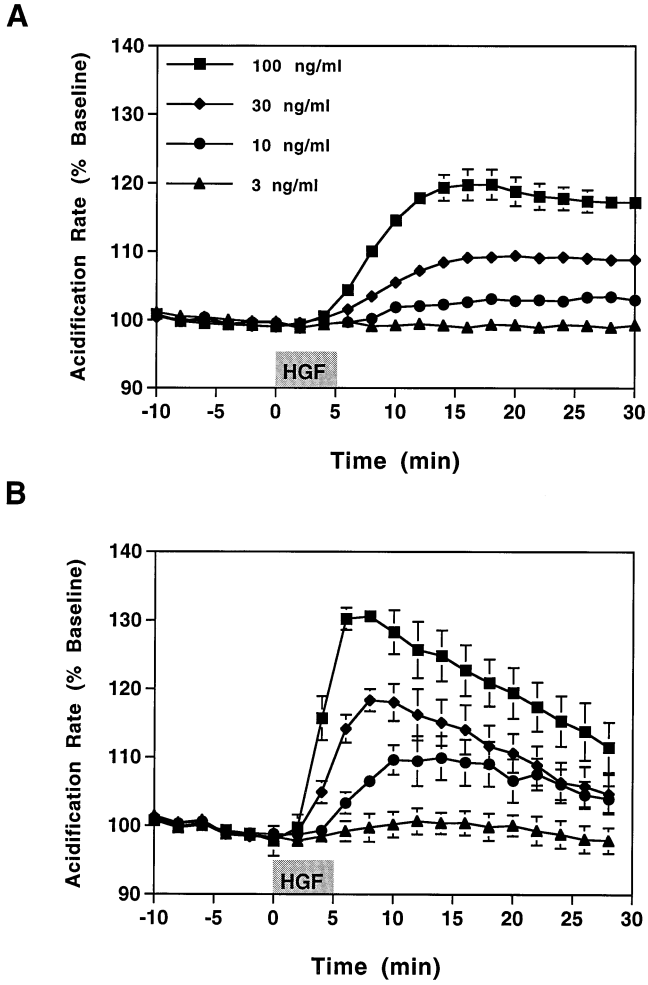

FIG. 2. HGF-induced acidification (3–100 ng/mL) of human A498 renal carcinoma cells (A) and MMC (B). Traces show acidification rates expressed as percent of baseline of 3–4 experiments (mean ± SEM). Cells were exposed to HGF for 5 min.

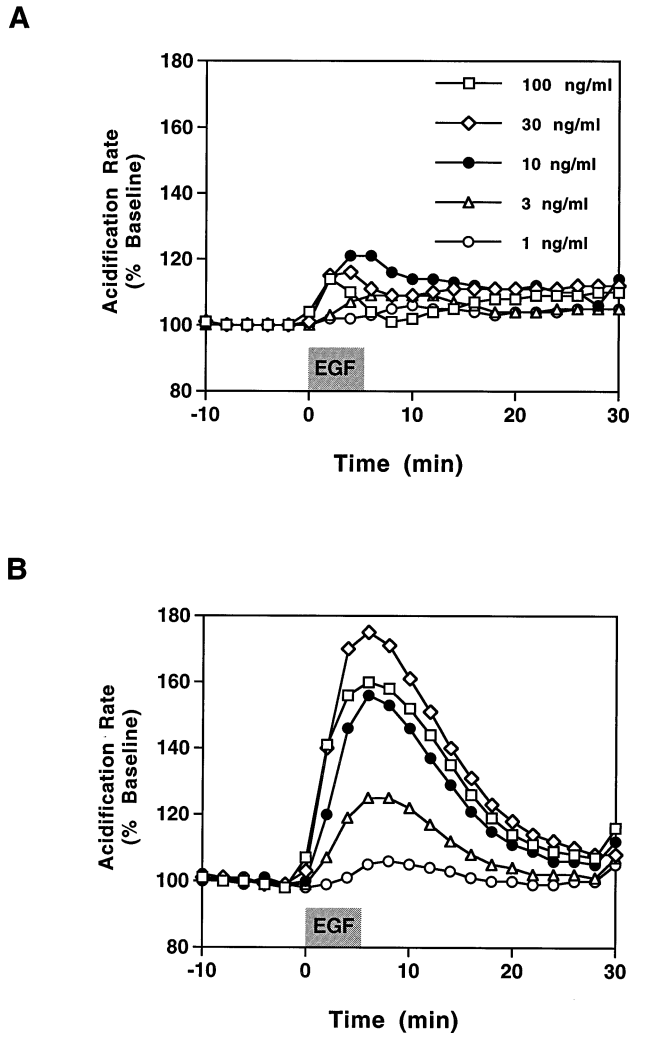

FIG. 3. EGF-induced acidification (1–100 ng/mL) of human A498 renal carcinoma cells (A) and MMC (B). Traces show acidification rates expressed as percent of baseline of 4 experiments. Cells were exposed to EGF for 5 min.

To determine if PKC is involved in HGF-mediated metabolic changes, the effects of PKC inhibitors on HGF-induced acidification were evaluated. SKF-57048 has been identified as a regulatory site inhibitor of PKC because it inhibited [^{3}H]phorbol dibutyrate binding and PKC activity of PKCβ2 with IC_{50} values of 3 and 10 μM, respectively. A 20-min pretreatment of MMC and A498 cells with SKF-57048 inhibited HGF-induced acidification with IC_{50} values of 59 ± 2 and 20 ± 10 nM, respectively ($P < 0.05$; Fig. 6). Involvement of PKC in HGF-mediated response was further confirmed by using RO-32-0432, a PKC selective inhibitor [21], and the phorbol ester TPA. Pretreatment of MMC with 1, 3, or 5 μM RO-32-0432 for 30 min followed by HGF (50 ng/mL) for 5 min resulted in a concentration-dependent decrease of the HGF response (Fig. 7). Human renal proximal tubule epithelial cells treated with either TPA (10^{-7} M) or HGF (50 ng/mL) responded with similar increases in acidification rates (Fig. 8). MMC also responded to TPA with an increase of 80% above baseline (data not shown).

Mitogenesis

HGF-mediated proliferation of primary human and immortalized MMC and primarily HRPTEC was evaluated by [^{3}H]thymidine incorporation. Human cells or MMC were serum starved for 48 hr or grown in 3% serum, respectively, and then were treated with increasing concentrations of HGF up to 100 ng/mL for 24 hr. HGF failed to change [^{3}H]thymidine incorporation at all concentrations tested in both normal human and immortalized MMC. In contrast, HGF increased proliferation of HRPTEC in a concentration-dependent fashion (Fig. 9).

DISCUSSION

Microphysiometry is a rapid and reliable method to study the activation of cell surface receptors by measuring changes in metabolic activity through extracellular acidification rates [20, 23]. This study showed that HGF changed

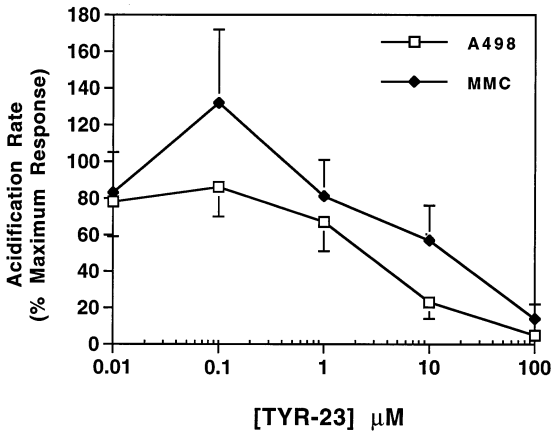

FIG. 4. Effect of the tyrosine kinase inhibitor tyrphostin-23 on HGF-induced acidification in A498 cells and MMC. The IC_{50} value in A498 cells was 5 ± 2 μM and in MMC 21 ± 10 μM. Traces show means ± SEM from 3–4 experiments.

metabolic activity in renal carcinoma A498 cells and MMC at concentrations known to induce proliferation in epithelial cells [3]. Because the tyrosine kinase inhibitor tyrphostin 23 inhibited HGF-induced acidification in both the epithelial A498 cells and MMC, HGF is likely to mediate metabolic changes in these cells through the HGF tyrosine kinase receptor, c-met. The identification of c-met by RT–PCR and western blot in mesangial cells supports this hypothesis (this report and Ref. 15). Although A498 cells had a higher expression of c-met, which is common for renal carcinomas [24], MMC had a much higher acidification response than A498 cells. This was true for both HGF and EGF and suggests that acidification rates of A498 cells are less responsive to agonist-induced changes than acidification rates of MMC.

This report also indicates that HGF-induced acidification may be mediated through the activation of PKC. SKF-57048, which was discovered as a regulatory site inhibitor of PKC, was able to inhibit HGF-induced acidification in both A498 cells and glomerular mesangial cells. Moreover, treatment of cells with TPA increased acidification similarly to HGF. A role for PKC in mediating the

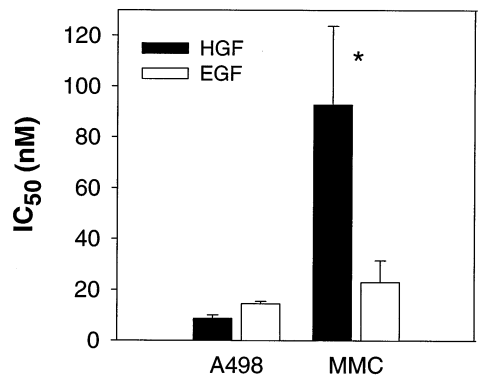

FIG. 5. Effect of the PI 3-kinase inhibitor wortmannin on HGF- and EGF-induced acidification of A498 cells and MMC. Bars show means ± SEM from 3–4 experiments. Key: (*) $P < 0.05$.

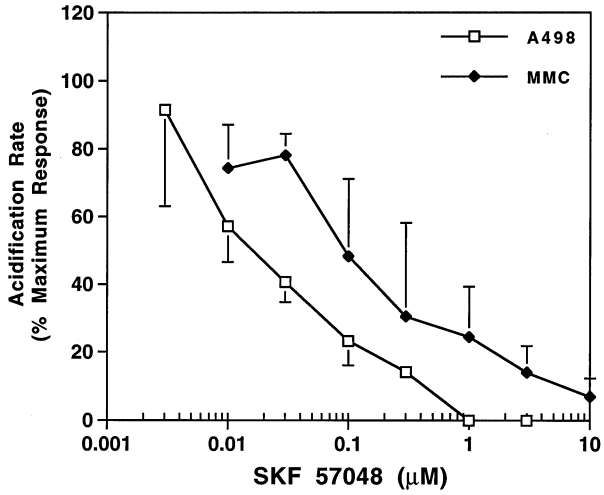

FIG. 6. Effect of the PKC inhibitor SKF-57048 on HGF-induced acidification of A498 cells and MMC. The IC_{50} values were significantly different ($P < 0.05$; 20 ± 10 and 59 ± 2 nM, respectively). Traces show means ± SEM from 3–4 experiments.

effects of HGF is supported by direct association and activation of PLCγ by c-met [25–27]. PLCγ, in turn, hydrolyzes phosphoinositides, resulting in the formation of diacylglycerol, which activates PKC. Much evidence suggests that subsequent to tyrosine kinase activation in epithelial cells, PKC activation is downstream of several signaling events including activation of PI-3 kinase (see review [28]).

However, the PI 3-kinase second messenger system appears to be different in glomerular mesangial cells compared with renal epithelial cells. HGF-induced morphogenesis, motogenesis, and mitogenesis in epithelial cells are thought to occur through PI 3-kinase activation [11, 13]. Wortmannin, an inhibitor of PI 3-kinase (IC_{50} 1–10 nM), inhibits HGF-induced mitogenesis in epithelial cells [13] and HGF-induced acidification in A498 cells. Because

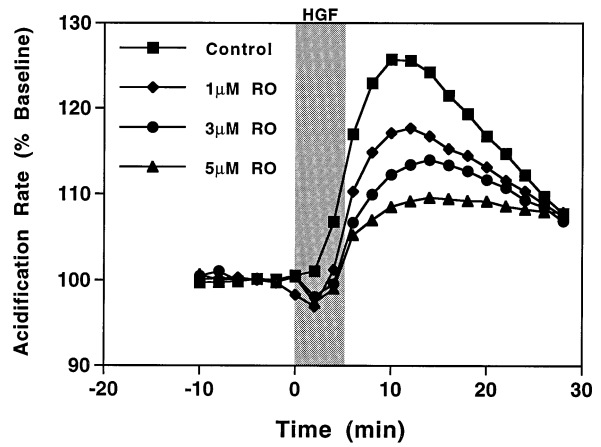

FIG. 7. Extracellular acidification rates of MMC treated with HGF (50 ng/mL). Cells were perfused with control medium or medium containing different concentrations of the PKC inhibitor RO-32-0432.

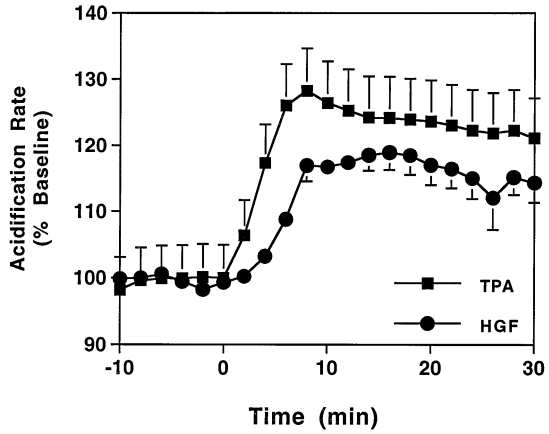

FIG. 8. Extracellular acidification rates of HRPTEC treated with HGF (50 ng/mL) or TPA (10^{-7} M). Traces show means ± SEM from 4 experiments.

EGF-induced acidification was equally inhibited by wortmannin in both MMC and A498 cells in the 10–20 nM range, the PI 3-kinase system appears to be intact in MMC. In contrast, HGF did not induce mitogenesis in mesangial cells, and concentrations of wortmannin greater than 100 nM were required to inhibit HGF-induced acidification in MMC. At these concentrations, wortmannin also inhibits other kinases and phospholipases [29–32]. Thus, c-met may not be coupled to PI 3-kinase in mesangial cells and could explain why HGF does not induce proliferation in mesangial cells. While the physiological role of HGF on glomerular mesangial cells remains to be determined, it is clear that HGF may have distinct effects on glomerular mesangial cells and epithelial cells in normal or diseased kidney.

A role for HGF in renal failure is suggested by findings that serum levels of HGF are elevated in patients with

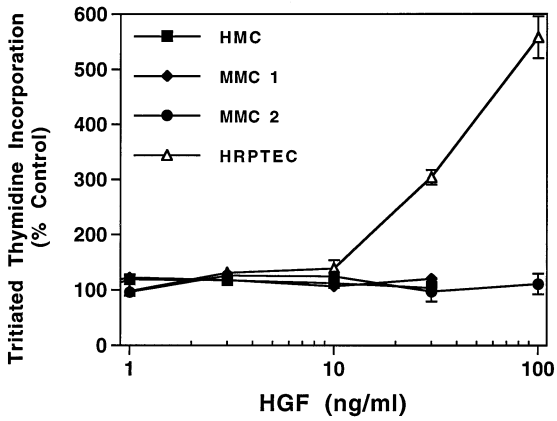

FIG. 9. [^{3}H]Thymidine incorporation of normal human mesangial cells (HMC), two different transformed MMC lines (MMC 1 and MMC 2), and human renal proximal tubule epithelial cells (HRPTEC) treated with different concentrations of HGF for 24 hr. Values are means ± SEM expressed as percent of thymidine incorporation of untreated cells (N = 3). Control [^{3}H]thymidine incorporation values for HMC, MMC 1, MMC 2, and HRPTEC were: 824 ± 79, 8,500 ± 260, 20,030 ± 1,230, and 98 ± 8 cpm, respectively.

chronic renal failure [33]. In addition, HGF mRNA is increased in the hypertrophic remnant kidney and non-injured kidney of sham-operated rats [5]. In contrast, the mRNA for c-met is increased specifically in the remnant or injured kidney after nephrectomy [5, 34]. Moreover, the serine protease that converts prepro HGF to mature HGF [1] is also specifically induced in the injured kidney [35]. Therefore, HGF-mediated pathways can be activated specifically in the injured kidney during chronic renal failure.

Chronic renal failure is the progressive loss of functional renal mass, accompanied by compensatory growth and remodeling. The molecular and cellular events that take place during chronic renal failure include release of growth factors, proliferation of glomerular mesangial cells, and expansion of extracellular matrix [36–38]. The components of the extracellular matrix that change during chronic renal failure include collagen, fibronectin, and laminin [38–40]. In the renal ablation model, immunocytochemical staining of fibronectin and collagen αI is elevated in areas of increased cellularity of glomeruli. This increase in fibronectin and collagen was shown at 2 and 6 weeks after 5/6 nephrectomy, when chronic renal failure is established [41]. Several protein factors have been implicated in stimulating mitogenesis of mesangial cells or extracellular matrix production during chronic renal failure. At least one report suggests that HGF decreases fibronectin immunoreactivity in mesangial cells [15]. Thus, HGF may antagonize matrix production induced by other growth factors in renal injury.

In summary, HGF changes the metabolic activity in glomerular mesangial cells as measured by an increase in extracellular acidification rate. The mechanisms by which HGF mediates this response appear to involve c-met tyrosine kinase and PKC activation. However, the reduced effectiveness of wortmannin in blocking HGF- but not EGF-induced acidification in mesangial cells compared with A498 cells suggests that PI 3-kinase is not coupled to c-met in glomerular mesangial cells.

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