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Gastrin-releasing peptide promotes the growth of HepG2 cells via EGFR-independent ERK1/2 activation

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Abstract. Gastrin-releasing peptide (GRP) plays an important role in regulating tumor growth and migration. However, little is known about its role in human hepatocellular carcinoma (HCC) cells. This study explored the effect of GRP on the growth of HCC HepG2 cells and the underlying mechanisms. Expression of GRP and its cognate receptor (GRPR) were detected by immunocytochemisty, reverse transcription-PCR and Western blotting and compared between two human HCC cell lines (HepG2 and MHCC97H) and a normal hepatic cell line (HL-7702). The effects of GRP on cell proliferation and signaling pathways were examined by Western blotting, MTT assay and flow cytometry. Both GRP and GRPR were overexpressed in HepG2 and MHCC97H cells. GRP activated MAPK/ERK1/2 in HepG2 cells, leading to enhanced proliferation, reduced apoptosis and accelerated cell cycle progression. The effect of GRP on ERK1/2 was effectively attenuated by the GRPR antagonist PD176252 or MEK inhibitor U0126, but not by the TNF-α protease inhibitor TAPI-1 or the EGFR tyrosine kinase inhibitor PD153035. The effect of GRP on the growth of HepG2 cells was significantly attenuated by PD176252 or U0126. GRP serves as a mitogen for HepG2 and MHCC97H cells. GRP promotes the growth of HepG2 cells through interaction with GRPR co-expressed in tumor cells, and subsequently activates MAPK/ERK1/2 via EGFR-independent mechanisms.

Introduction

Accumulating evidence has indicated that neuropeptides play important roles in regulating tumor growth and migration. For example, extensive studies have shown that mammalian bombesin-like gastrin-releasing peptide (GRP) binds to its cognate receptor (GRPR) and exerts various biological effects in tumors (1,2). In particular, GRP serves as a potent mitogen for various types of tumor including small cell lung, pancreatic, prostate, renal, breast and colon cancers (1-7). Furthermore, treatment with GRP antibody was found to lead to significant anti-proliferative effects, indicating that GRP is an autocrine growth factor and GRPR may be a drug target for tumor imaging and anti-tumor therapy (8-14).

Several studies have demonstrated a stimulatory role of GRPR for the mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (MAPK/ERK1/2) signaling pathway in a number of cancer cells (15-18). The mechanisms through which GRPR activated MAPK/ERK1/2 in tumor cells were not fully clarified, but appeared to vary in different types of tumor cells. For example, GRP was found to interact with GRPR in tumor cells to activate the TNF-α convertase (TACE), which in turn switched on the MAPK/ERK1/2 signaling pathway by shedding ligands for the epidermal growth factor receptors (EGFR) (17,18).

Experimental animal studies demonstrated that rat hepatocellular tumor cells also produced functional GRP and GRPR molecules that appeared to promote hepatocarcinogenesis (19). However, little is known about the role of GRP and GRPR in human hepatocellular carcinoma (HCC). HCC is a highly aggressive and the most common malignancy in liver with poor prognosis. Previously, it has been suggested that the poor prognosis of HCC may be associated with elevated MAPK/ERK1/2 activities in tumor cells (20-22). In the present study, we compared the expressions of GRP and GRPR in human hepatoma HepG2 and MHCC97H with those in normal hepatic HL-7702 cells. Furthermore, we investigated the influence of GRP on the growth of HepG2 cells in correlation with GRPR and the intracellular activity of MAPK/ERK1/2. Our observations provide important insights into the function and mechanism of GRP in the regulation of tumor cell growth.

Materials and methods

Chemicals and antibodies. Human GRP (sequence: VPLPAGGGTVLTKMYPR GNHWAVGHLM-NH2) was obtained from Sigma-Aldrich (St. Louis, USA). Antibodies

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against GRP, GRPR, TACE and phospho-TACE (T735) were obtained from Abcam (Cambridge, UK). Antibodies against EGFR and phospho-EGFR (Tyr1086) were obtained from Upstate Biotech (NY, USA). Antibody against p44/42-MAPK (ERK1/2) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibody against phospho-ERK1/2 (T202/Y204) was obtained from R&D Systems (USA). Specific secondary antibodies, EGFR tyrosine kinase inhibitor PD153035, and MEK inhibitor U0126 were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). The non-peptide gastrin releasing peptide receptor antagonist (S)-N-[[1-(5-Methoxy-2-pyridinyl)cyclohexyl]methyl]-a-methyl-a-[[[-(4-nitrophenyl)amino]carbonyl]amino-1H-indole-3-propanamide (PD 176252) was obtained from Tocris Bioscience (Bristol, UK). The TNF-α protease inhibitor-1 (TAPI-1, N-(R)-[2-(Hydroxyaminocarbonyl) methyl]-4-methylpentanoyl-L-naphthylalanyl-Lalanine, 2-aminoethyl Amide) was obtained from Merck Biosciences (UK).

Cell culture and treatment. Human normal liver cell line HL-7702 and human hepatoma cell line HepG2 were obtained from ATCC (Rockville, MD, USA), and the highly invasive human hepatocellular carcinoma cell line MHCC97H was obtained from Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China). HL-7702 and MHCC97H cells were maintained and propagated in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 15% fetal bovine serum (HyClone, USA). HepG2 cells were maintained and propagated in RPMI-1640 supplemented with 10% fetal bovine serum. All cultured cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2 and experiments were done using 70-80% confluent cultures.

Immunocytochemistry. The expression of GRP and GRPR in the afore-mentioned three cell lines was assessed by immunocytochemisty. Cells plated on glass cover slips were fixed by 4% paraformaldehyde, and permeabilized with 0.5% (v/v) Triton. Cells were washed with PBS, treated with ice-cold methanol for 20 min, and pre-blocked for 30 min with sheep serum at 37˚C. Cells were subsequently washed twice with PBS, and incubated with anti-GRP and anti-GRPR (1:100) overnight at 4˚C. After PBS wash, cells were incubated with secondary goat anti-rabbit immunoglobulin conjugated with biotin for 60 min at room temperature, followed by incubation with streptavidin-peroxidase complex for 30 min. The peroxidase reaction products were visualized by incubation with 0.05 M Tris-HCl buffer (pH 7.6) containing 20 mg 3,3-diaminobendizine (DAB). The slides were counterstained for nuclei with hematoxylin and mounted with gum.

Reverse transcription-PCR. Total cellular RNA of HL-7702, HepG2 and MHCC97H cells was isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen, USA). RNA concentration was measured by GeneQuant II (Pharmacia, Uppsala, Sweden) at 260 nm. Equal amount of total RNAs were used for reverse transcription reaction and cDNA synthesis in accordance with the manufacturer's instructions (Invitrogen). The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The molecular weight was derived based on a molecular weight marker and ß-actin was used as the loading control. Results were quantitated using densitometry and Image J software (version 1.34s; by Wayne Rasband, NIH, USA). The following primers were used to determine the mRNA expression for GRP, GRPR and ß-actin. GRP forward, 5'-GGACCGTGCTGACCAAGATG-3'; reverse, 5'-AAGTTGCTGCTATCCTCTGAATCC-3'; GRPR forward, 5'-GCCTGTTCGCCTTCTGCTG-3'; reverse, 5'-TCCTTCC AGTGCTGTGAGACC-3'; ß-actin forward, 5'-GTCCTCTC CCAAGTCCACAC-3'; reverse, 5'-GGGAGACCAAAAG CCTTCAT-3'. The PCR conditions were: 94˚C for 2 min followed by 35 cycles (94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 1 min), and a final extension at 72˚C for 10 min.

Western blot analysis. Cells were washed twice in PBS, lysed in ice-cold radioimmune precipitation (RIPA) buffer, and then centrifuged for 10 min at 4˚C. Supernatants were collected and protein concentrations were determined and adjusted to 2 mg/ml using a Bio-Rad protein assay kit (Bio-Rad, USA). Cell lysates were mixed with 3X Laemmli buffer and heated for 5 min at 95˚C. Sample proteins were then resolved by SDS-PAGE (8 or 10% polyacrylamide gels), transferred to PVDF membrane (Immobilon™ ; Millipore) by electroblotting. The membrane was blotted with 10% non-fat milk, washed in TBS/Tween and incubated with primary rabbit polyclonal antibodies overnight at 4˚C. After washing with TBS/Tween, they were incubated with secondary antibody (horseradish peroxidase conjugated IgG) for 60 min at room temperature. Membranes were washed again with TBS/Tween before detection using the ECL detection system (Amersham Pharmacia, USA). The dilutions for the primary antibodies were as follows: anti-GRP, 1:400; anti-GRPR, 1:400; anti-TACE, 1:800; anti-phospho-TACE, 1:800; anti-EGFR, 1:800; anti-phospho-EGFR, 1:800; anti-ERK1/2, 1:800; anti-phospho-ERK1/2, 1:800; anti-ß-actin, 1:800; Secondary antibody was used at a dilution of 1:10000. Results were quantitated using densitometry and Image J software (version 1.34s; by Wayne Rasband, NIH, Bethesda, MD, USA).

Cell proliferation assay. Proliferation of HL-7702, HepG2 and MHCC97H cells was investigated through MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; Sigma] assay. Briefly, $1x10^4$ cells per well were plated into flat-bottomed 96-well plates (Costar, Corning, USA). After 24 h, cells were serum-starved overnight. The next day, cells were stimulated by different concentrations of GRP in the absence or presence of inhibitors for different times. Control wells were treated with 0.1% dimethyl sulphoxide (DMSO) alone. Subsequently, 20 ml MTT (5 g/l) was added to each well and incubated for an additional 4 h. Plates were centrifuged for 5 min at 1000 rpm, and the medium was carefully discarded. The formed formazan crystals were dissolved in 100 μl of DMSO, and absorbance (A) was read at 570 nm using a spectrophotometer. The percentage of viable cells was calculated as follows: (A of experimental group/A of control group) x 100%.

Cell cycle and apoptosis analysis. HepG2 cells were serum-starved overnight. The synchronized cells were then treated

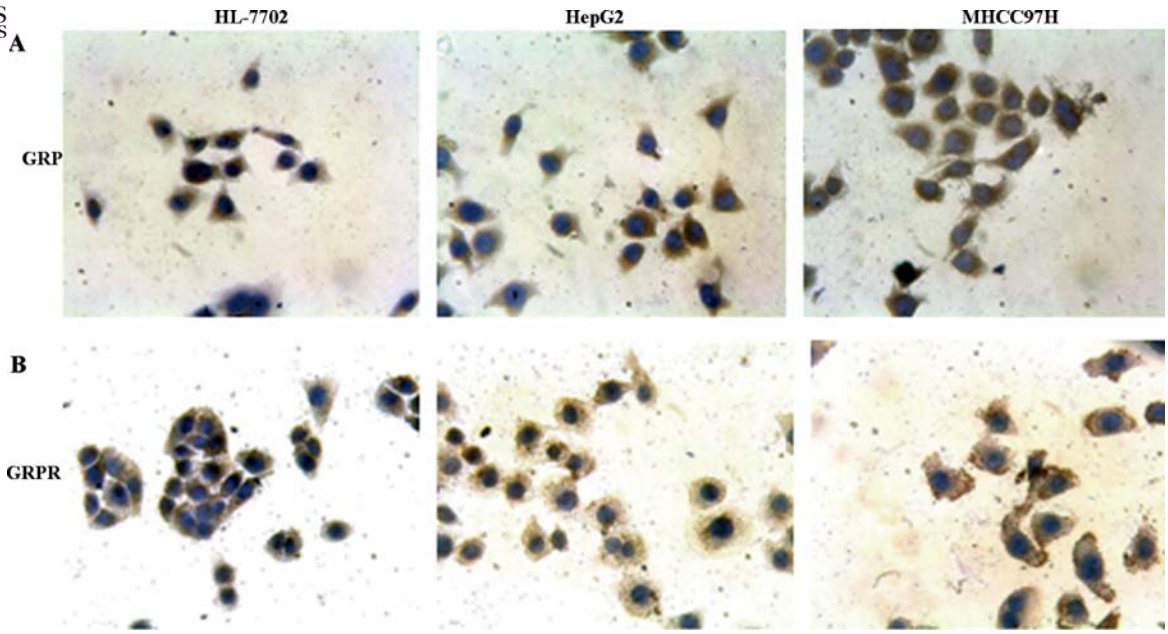

Figure 1. Immunochemistry analysis of the expression of gastrin-releasing peptide (GRP) and its cognate receptor (GRPR) in HL-7702, HepG2 and MHCC97H Cells. The expression of GRP and GRPR in HL-7702, HepG2 and MHCC97H cells was assessed by immunocytochemisty. Similar results were obtained in three independent experiments and representative results are shown. Both GRP and GRPR expressed at higher levels (luteotestaceously stained) in the cytoplasma and at the plasma membrane of HepG2 and MHCC97H cells than in HL-7702 cells.

for the indicated times, and harvested by trypsinization. PBS washed cells were fixed in ice-cold 80% ethanol, washed, and resuspended in 1 ml PBS. Samples were treated with 10 μl RNase A (21 mg/ml), and stained with 5 μl propidium iodide (PI) at 1 mg/ml for 30 min at room temperature in the dark. The stained cells were analyzed by flow cytometry (BD LSRII System, BD Biosciences, USA). To evaluate the cell cycle distribution of apoptotic cells, treated cells were washed in PBS, and resuspended in 100 ml binding buffer containing FITC-conjugated annexin V (Becton-Dickinson, USA) and PI to discriminate apoptotic cells from live cells and necrotic cells. Annexin V(+)/PI(-) and Annexin V(+)/PI(+) represented cells in early apoptosis and late apoptosis/necrosis, respectively.

Statistical analysis. Results were expressed as the mean ± SEM of three separate experiments and analyzed by the computer program SPSS 13.0 for Windows (SPSS Inc., USA), using one-way analysis of variance (ANOVA) followed by the Tukey's t-test. P-values <0.05 were considered statistically significant.

Results

Overexpression of GRP and GRPR in HepG2 and MHCC97H cells. We first examined GRP and GRPR expression in both tumor and normal cell lines. As shown in Fig. 1, immunocytochemisty analysis revealed readily detectable GRP and GRPR expression in the three cell lines HL-7702, HepG2 and MHCC97H. Importantly, elevated expression was observed in the two tumor cell lines HepG2 and MHCC97H compared to the human normal liver cell line HL-7702 (Fig. 1). Likewise, RT-PCR analysis demonstrated that both GRP mRNA and GRPR mRNA levels were elevated in the two tumor cell lines compared to that in HL-7702. Specifically, GRP mRNA showed 3.21-fold higher levels in HepG2 and 3.70-fold in MHCC97H versus HL-7702, and GRPR mRNA was 3.52-fold higher in HepG2 and 3.61-fold in MHCC97H than that of HL-02 (Fig. 2A). Overexpression of GRP and GRPR in tumor cells was further confirmed at the protein level by Western blotting. Levels of GRP peptides and GRPR proteins were enhanced in HepG2 (1.71- and 2.10-fold, respectively) and in MHCC97H (1.82- and 2.92-fold, respectively) versus HL-7702 (Fig. 2B).

Stimulatory effects of GRP on the proliferation of HepG2 and MHCC97H cells. Next we investigated the physiological significance of the elevated expression of GRP in tumor cells. MTT assays were carried out to investigate the effects of GRP on cell proliferation. The proliferation of HL-7702 cells was not significantly influenced; however, GRP appeared to markedly enhance the proliferation of HepG2 and MHCC97H cells in a dose-dependent manner (Fig. 3A). Specifically, stimulatory effects on proliferation of HepG2 and MHCC97H cells was first observed with 0.1 nM of GRP (115±6% and 117±3% of control, respectively; $P<0.05$) and peaked at 100 nM of GRP with the maximum increase of proliferation (167±8% and 173±4% of control, respectively; $P<0.01$). No significant influence of GRP was detected at concentrations lower than 0.1 nM, and no greater effect of GRP on the proliferation of HepG2 and MHCC97H cells was observed at concentrations higher than 100 nM. To further investigate the time-dependence of GRP effects, we incubated cells with 100 nM of GRP for 0, 12, 24, 48 and 96 h. Whereas GRP exerted virtually no effect on the proliferation of HL-7702 cells, time-dependent stimulatory effects on HepG2 and

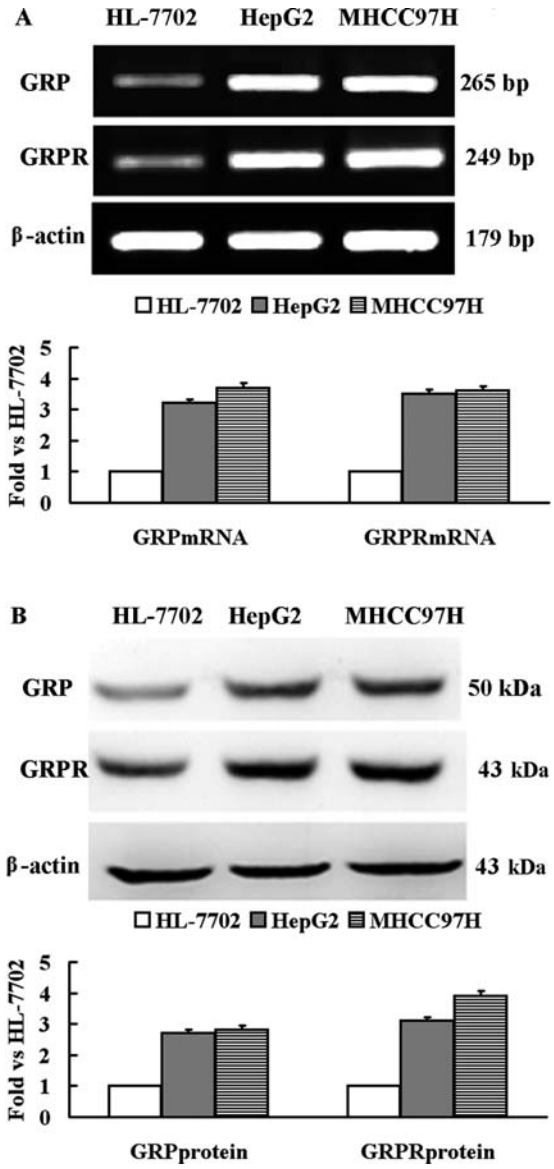

Figure 2. Reverse transcription-PCR and Western blotting analysis of the expression of GRP and GRPR in HL-7702, HepG2 and MHCC97H cells. Results for three independent experiments are shown. The normalized data against the control (HL-7702) are presented as mean ± SEM. (A) GRP and GRPR mRNA expression detected by RT-PCR. (B) GRP and GRPR protein expression detected by Western blotting.

MHCC97H cell proliferation by GRP were observed (Fig. 3B). From 12 h (126±8% and 132±6% of control, respectively; $P<0.01$) to 48 h (163±3% and 172±2% of control, respectively; $P<0.01$), increasing stimulatory effects of GRP on HepG2 and MHCC97H cells were observed, and longer incubation time did not lead to greater stimulatory effect.

GRP activated MAPK/ERK1/2 in HepG2 cells. Previous evidence indicated that human HCC exhibited high levels of MAPK/ERK1/2 and enhanced activity of MAPK/ERK1/2 in tumor cells predicted poor prognosis (21,22). To explore a possible correlation of GRP with the activity of MAPK/ERK1/2 in HepG2 cells, we examined the stimulatory effects of GRP on ERK1/2. GRP (100 nM) was added to HL-7702 and HepG2 cells and the levels of phosphorylated-ERK1/2 (pERK1/2) were determined by Western blotting. The addition

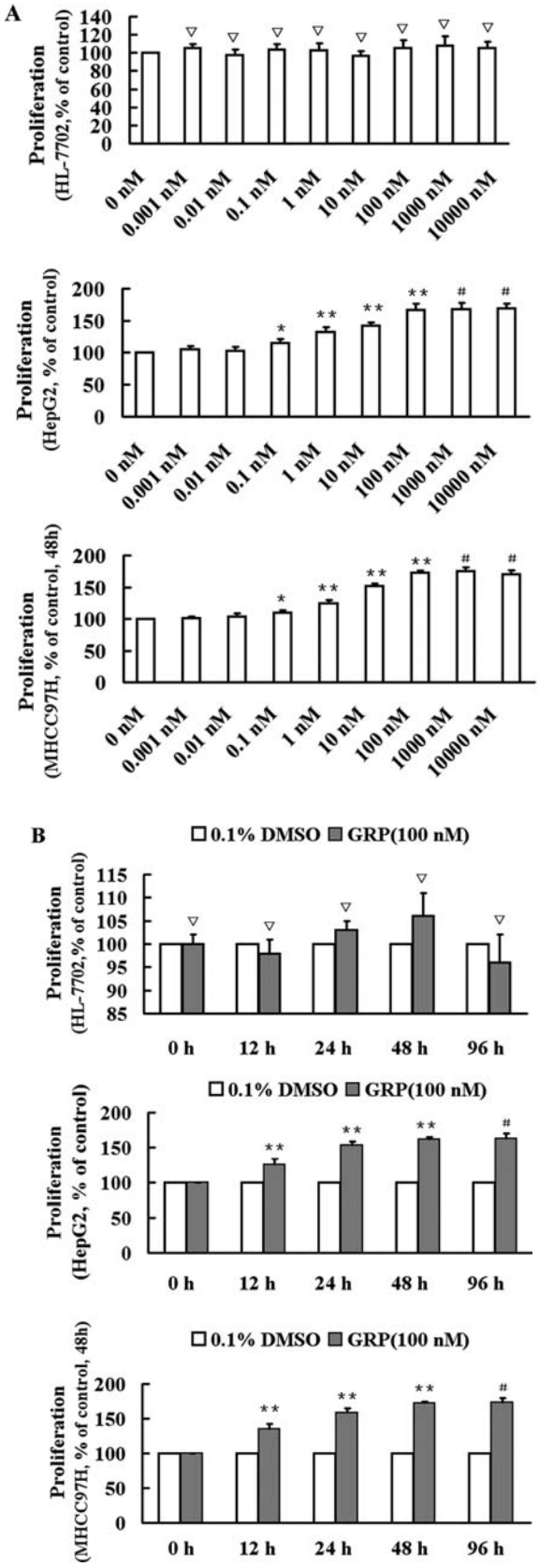

Figure 3. Proliferation-promoting effects of GRP on HepG2 and MHCC97H cells. HL-7702, HepG2 and MHCC97H cells were serum-starved overnight before treatment and the cell proliferation was measured via the MTT method. Results from three independent experiments are presented as mean percent of control ± SEM. (A) Cells were stimulated by different concentrations of GRP for 48 h. $^{\nabla}P>0.05$ vs. 0 nM. $^{*}P<0.05$, $^{**}P<0.01$ vs. control (0 nM). $^{\#}P>0.05$ vs. 100 nM. (B) Cells were stimulated by 100 nM of GRP for 0, 12, 24, 48 and 96 h. $^{\nabla}P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$ vs. control (0.1% DMSO). $^{\#}P>0.05$ vs. 48 h.

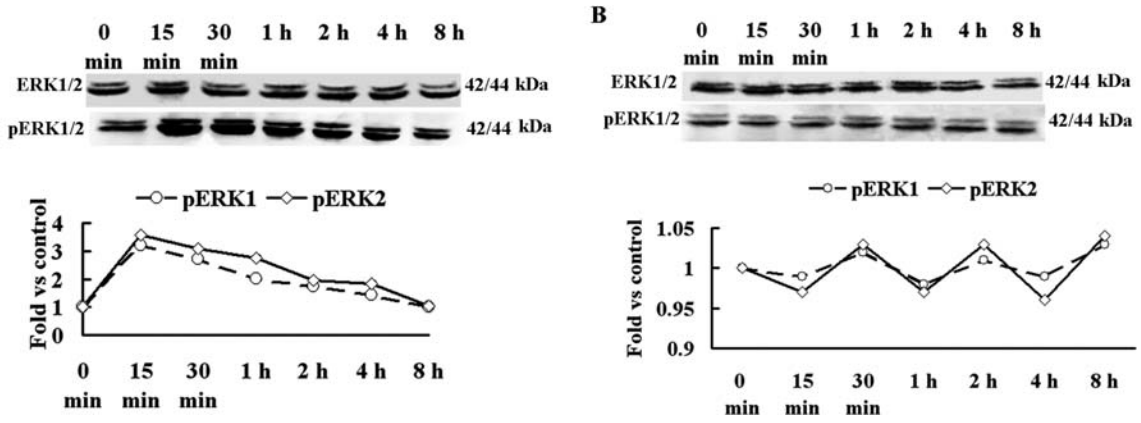

Figure 4. GRP transiently activated MAPK/ERK1/2 in HepG2 cells. HL-7702 and HepG2 cells were serum-starved overnight and then incubated with 100 nM of GRP for 15 and 30 min, and 1, 2, 4 and 8 h. The levels of ERK1/2 and phosphorylated-ERK1/2 were determined by Western blotting using specific antibody against ERK1/2 and phosphorylated-ERK1/2. Results from three independent experiments are shown. The results are presented as fold changes versus control (0 min). (A) The expression of ERK1/2 and phosphorylated-ERK1/2 in HepG2 cells. (B) The expression of ERK1/2 and phosphorylated-ERK1/2 in HL-7702 cells. Similar results were obtained in three independent experiments.

of GRP to HepG2 cells resulted in an immediate activation of ERK1/2. As early as 15 min after the administration of GRP, pERK1/2 was increased by an average of 2.21- and 2.56-fold. In contrast, HL-7702 cells, which weakly express pERK1/2, were not affected by GRP. However, the activation of ERK1/2 in HepG2 cells was transient, and gradually declined to baseline level within 8 h (Fig. 4).

MAPK/ERK1/2 activation by GRP in HepG2 required GRPR, but not EGFR. To confirm the stimulatory effect of GRP on ERK1/2 was mediated by GRPR, cells were treated with the non-peptide gastrin-releasing peptide receptor antagonist PD176252 or the MEK inhibitor U0126 before GRP treatment. To rule out the possibility that GRPR activation may lead to TACE-dependent transactivation of EGFR which may in turn activate ERK1/2 (17,18), the TACE inhibitor TNF-α protease inhibitor-1 (TAPI-1) and the inhibitor of EGFR tyrosine kinase PD153035 were also applied before GRP administration (17,21,22). HepG2 cells were first stimulated with either 0.1% DMSO (control), or 100 nM of GRP with or without 1 μM of TAPI-1. The levels of pTACE, pEGFR and pERK1/2 were determined by Western blotting. As shown in Fig. 5A, the levels of pTACE, pEGFR, pERK1 and pERK2 were all increased by 100 nM of GRP (1.98-, 1.55-, 3.21- and 3.56-fold over control, respectively). The presence of TAPI-1 attenuated the effect of GRP on pTACE and pEGFR, but not on pERK1/2 (Fig. 5A). We next stimulated HepG2 cells with GRP (100 nM) with or without PD176252 (5 μM), PD153035 (25 μM) or U0126 (10 μM). GRP-induced phosphorylation of ERK1/2 was attenuated by PD176252 and U0126, but not by PD153035 (Fig. 5B).

The growth-promoting effect of GRP was mainly mediated by GRPR-induced activation of MAPK/ERK1/2. To confirm the ERK1/2-dependent effect of GRP on HepG2 cells, we incubated HepG2 cells with 100 nM of GRP in the presence or absence of the GRPR antagonist PD176252 (5 μM) or the MEK inhibitor U0126 (10 μM). Cell proliferation was evaluated by MTT assay, and the apoptotic cells and cell cycle progression were analyzed by flow cytometry. GRP-

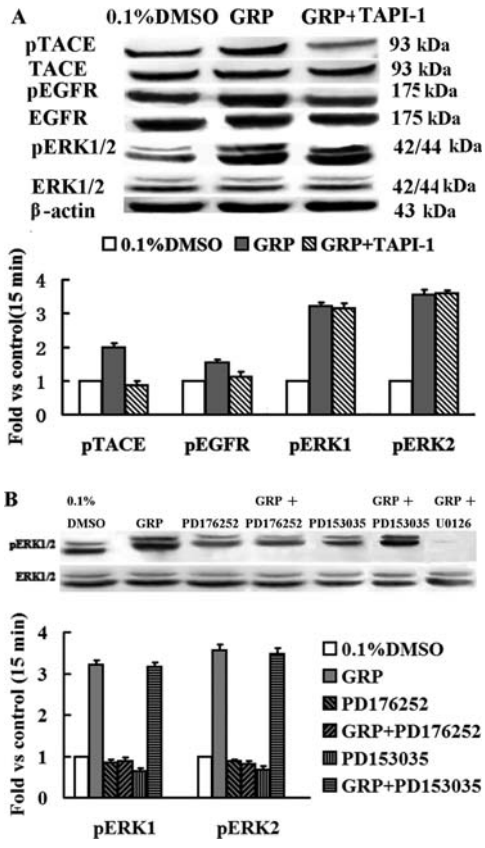

Figure 5. MAPK/ERK1/2 activation by GRP required GRPR, but not EGFR. HepG2 cells were serum-starved overnight and then treated with GRP (100 nM) in the presence or absence of different inhibitors. Results from three independent experiments are shown. (A) HepG2 cells were stimulated by 0.1% DMSO (control), GRP (100 nM), GRP (100 nM) + TAPI-1 (1 μM). TAPI-1 was added 30 min before GRP. At 15 min after GRP administration, the expressions of TACE, phosphorylated-TACE, EGFR, phosphorylated-EGFR, ERK1/2, phosphorylated-ERK1/2 were detected by Western blotting using specific antibodies. The results were presented as folds of control. (B) HepG2 cells were stimulated by 0.1% DMSO (control), GRP (100 nM), PD176252 (5 μM), GRP (100 nM) + PD176252 (5 μM), PD153035 (25 μM), GRP (100 nM) + PD153035 (25 μM) and GRP (100 nM) + U0126 (10 μM), respectively. PD176252, PD153035 and U0126 were added 30 min before adding GRP. At 15 min after GRP stimulation, the expression of ERK1/2 and phosphorylated-ERK1/2 were detected by Western blotting using specific antibodies. The data are presented as folds of control.

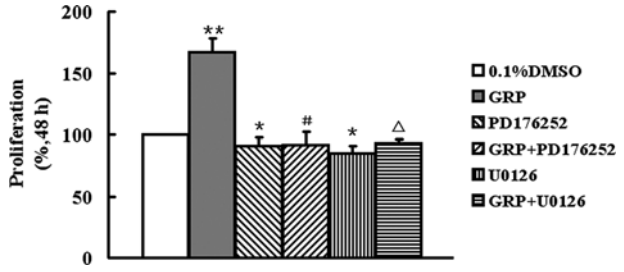

Figure 6. GRP promotes the growth of HepG2 cells by a MAPK/ERK1/2-dependent mechanism. HepG2 cells were serum-starved overnight and then stimulated by 0.1% DMSO (control), GRP (100 nM), PD176252 (5 μM), GRP (100 nM) + PD176252 (5 μM), U0126 (10 μM) and GRP (100 nM) + U0126 (10 μM), respectively. PD176252 and U0126 were added 30 min before adding GRP. At 48 h after GRP administration, cell proliferation was evaluated using the MTT assay and the results are presented as percent of control. *P<0.05, **P<0.01 vs. control (0.1% DMSO). $^{\#}$P>0.05 vs. PD176252. $^{\Delta}$P<0.05 vs. U0126.

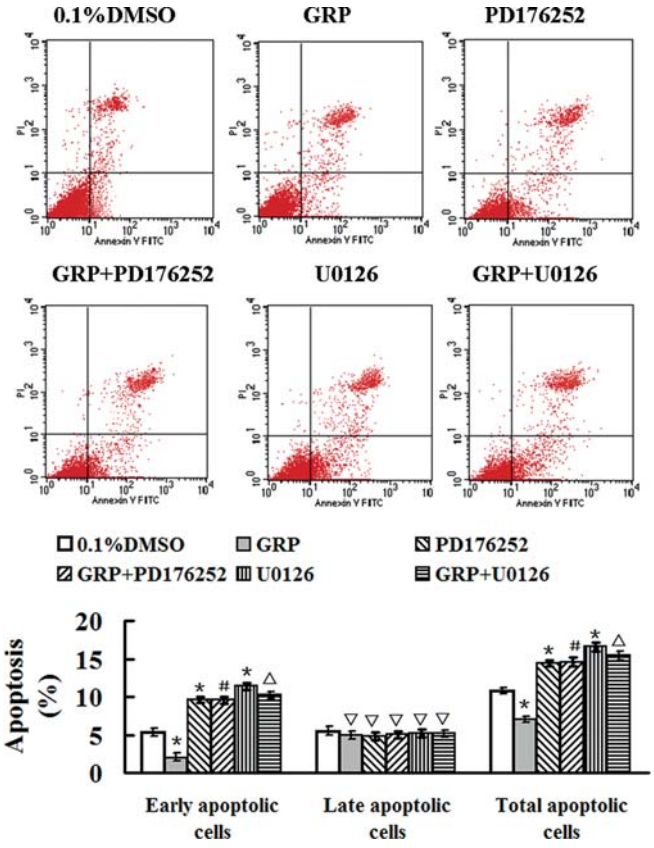

Figure 7. GRP inhibits the apoptosis of HepG2 cells. HepG2 cells were serum-starved overnight and then stimulated by 0.1% DMSO (control), GRP (100 nM), PD176252 (5 μM), GRP (100 nM) + PD176252 (5 μM), U0126 (10 μM) and GRP (100 nM) + U0126 (10 μM), respectively. PD176252 and U0126 were added 30 min before adding GRP. At 24 h after GRP treatment, apoptotic cells were evaluated by flow cytometry. Annexin V(+)/PI(-) and Annexin V(+)/PI(+) represent the cells in early apoptosis and late apoptosis/necrosis, respectively. The results are presented as percent of control. *P<0.01 vs. 0.1% DMSO. $^{\#}$P>0.05 vs. PD176252. $^{\Delta}$P<0.05 vs. U0126. $^{\nabla}$P>0.05 vs. 0.1% DMSO.

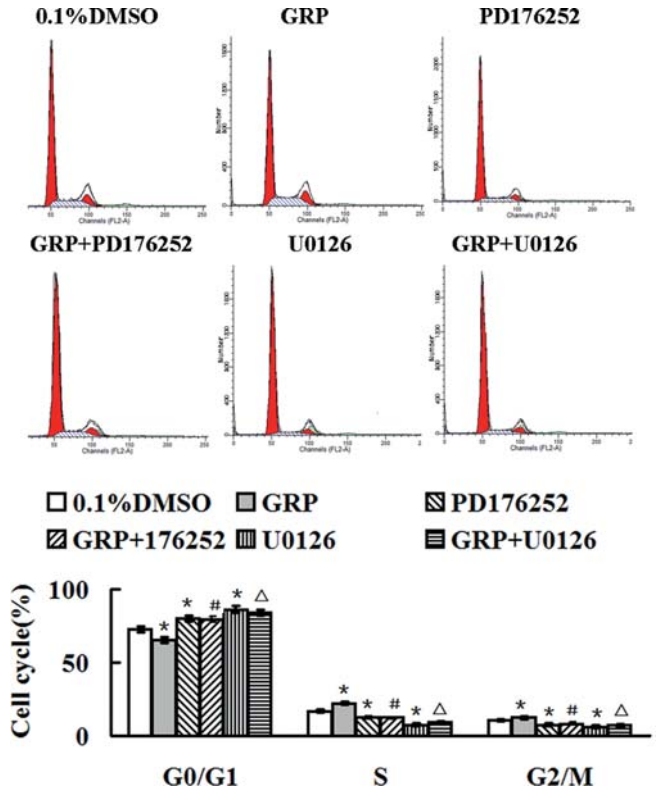

Figure 8. Stimulatory effects of GRP on cell cycle progression of HepG2 cells. HepG2 cells were serum-starved overnight and then stimulated by 0.1% DMSO (control), GRP (100 nM), PD176252 (5 μM), GRP (100 nM) + PD176252 (5 μM), U0126 (10 μM) and GRP (100 nM) + U0126 (10 μM), respectively. PD176252 and U0126 were added 30 min before adding GRP. At 48 h after GRP treatment, cells were stained with propidium iodide at 1 mg/ml for 30 min. Cell cycle distributions were also evaluated by flow cytometry and the results are presented as percent of control. *P<0.05 vs. control (0.1% DMSO). $^{\#}$P>0.05 vs. PD176252. $^{\Delta}$P<0.05 vs. U0126.

enhanced proliferation of HepG2 cells was abolished by PD176252 (GRP+PD176252 vs. PD176252: 92±10% vs. 91±7% of control; P>0.05) and significantly compromised by U0126 (GRP+ U0126 vs. U0126, 85±6% vs. 93±3% of control; P<0.05) (Fig. 6). Furthermore, GRP significantly reduced the early and total apoptosis (AP) of HepG2 cells (GRP vs. 0.1% DMSO: early AP: 2.01±0.55% vs. 5.33±0.52%; total AP: 6.98±0.48% vs. 10.42±0.39%; P<0.01). The percentage of apoptotic cells was significantly increased in the samples stimulated by PD176252 or U0126 (early and total AP; P<0.01); conversely, GRP-dependent apoptosis inhibition was abolished by PD176252 (early and total AP: GRP+PD176252 vs. PD176252; early AP: 9.59±0.43% vs. 9.62±0.41%; total AP: 14.60±0.55% vs. 14.40±0.42%; P>0.05) and significantly compromised by U0126 (early and total AP: GRP+U0126 vs. U0126, early AP: 10.21±0.48% vs. 11.39±0.49%, total AP: 15.40±0.53% vs. 16.56±0.58%; P<0.05) (Fig. 7). Finally, GRP also markedly increased the percentages of cells in S phase and G2/M phase (GRP vs. 0.1% DMSO: S phase, 22.04±0.98% vs. 16.90±1.03%; G2/M phase, 12.57±0.98% vs. 10.48±0.56%; P<0.05) and reciprocally decreased the percentages of cells in G0/G1 phase (GRP vs. 0.1% DMSO: 65.38±2.13% vs. 76.12±2.12%; P<0.05). The percentage of cells in G0/G1 phase was significantly increased by PD176252 or U0126 (P<0.05). Concordantly, GRP-mediated stimulation of cell cycle progression was abolished by PD176252 (GRP+PD176252 vs. PD176252, G0/G1 phase, S phase, G2/M phase: 79.64±2.15%, 12.38±0.98%, 7.98±0.88% vs. 80.02±2.03%,

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96%, 7.56±0.89%; P>0.05) or significantly compromised by U0126 (GRP+U0126 vs. U0126, G0/G1 phase, S phase, G2/M phase: 84.09±2.05%, 9.21±1.02%, 7.22±0.96% vs. 86.21±2.18%, 7.51±0.69%, 6.28±0.98%; P<0.05) (Fig. 8).

Discussion

GRP is an autocrine/paracrine growth factor for various human cancer cells (1-7). At present, several GRP analogues and GRPR antagonists are in preclinical trials for tumor imaging and anticancer therapy (8-14). Although it has been reported that GRP played a role in hepatocarcinogenesis in rat (19), no data are currently available confirming the contribution of GRP to human HCC. In the present study, we carried out pilot studies to examine the roles of GRP and GRPR in promoting the growth of HepG2 and MHCC97H human HCC cells and to delineate the possible mechanisms.

We first examined the expression of GRP and GRPR in normal hepatic cell line HL77-02 and human HCC cell lines HepG2 and MHCC97H. While both normal hepatic cell line and the two human HCC cell lines expressed detectable GRP and GRPR protein and mRNA, much higher levels of GRP and GRPR were detected in HepG2 and MHCC97H cells than in HL77-02 cells. These results indicated that GRP and GRPR were coexpressed in the two human HCC cell lines, consistent with previous observations with other tumor cells (3-7). Such a general coexpression pattern may suggest important biological effects, thus prompting us to further observe the influence of GRP on the growth of HepG2 cells with elevated GRP and GRPR expressions.

Our studies subsequently revealed enhanced stimulatory effects of GRP on the proliferation of HepG2 and MHCC97H cells compared with HL-7702 cells. Furthermore, the stimulatory role of GRP in the proliferation of HepG2 and MHCC97H cells was dose- and time-dependent, with the maximum proliferation rate at the dose of 100 nM and at the time-point of 48 h. Moreover, we found that GRP transiently (within 8 h) increased the levels of phosphorylated-ERK1/2 of HepG2 cells. The MAPK/ERK1/2 signaling pathway is a critically important signaling pathway promoting proliferation and migration of tumor cells, and activation of ERK1/2 in HepG2 cells might lead to the transcription of multiple genes required for growth and invasion (21,22). Thus, our results are consistent with an important role of GRP in the growth of liver cancer cells as observed in other tumor cells (1-7,15-18).

In addition, we found that the GRP-induced activation of ERK1/2 was mainly mediated by GRPR overexpression in HepG2 cells; conversely, application of the GRPR antagonist PD176252 blocked the stimulatory effect of GRP on ERK1/2. Previously, GRP was found to interact with GRPR in tumor cells, consequently resulting in the activation of TACE. TACE belongs to the MMP (matrix metalloproteinase) super-family that function to modulate the growth and invasion of cancer cells through ECM (extracellular matrix) cleavage and activation of the MAPK/ERK1/2 signaling pathway by shedding ligands for EGFR (17,18). However, we found that pre-incubation of HepG2 cells with the TACE inhibitor TAPI-1 or the EGFR tyrosine kinase inhibitor PD153035 did not diminish GRP-induced phosphorylation of ERK1/2. Thus, our data indicated that GRP activated ERK1/2 in HepG2 cells through GRPR-dependent and TACE- and EGFR-independent mechanisms.

Finally, we demonstrated that GRP promoted the growth of HepG2 cells concurrently stimulating proliferation, inhibiting apoptosis and accelerating cell cycle progression. The stimulatory effect of GRP on tumor cell growth was mainly mediated by GRPR-dependent activation of ERK1/2. However, the MEK inhibitor U0126 did not completely abolish GRP-dependent enhancement of HepG2 cell growth, suggesting that ERK-independent mechanisms were also involved in mediating the effect of GRP on HepG2 cells.

In conclusion, GRP serves as a mitogen for HepG2 cells through interaction with GRPR, and subsequently activates the MAPK/ERK1/2 signaling pathway via EGFR-independent mechanisms.

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