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Additive and Inhibitory Effects of Simultaneous Treatment with Growth Factors on DNA Synthesis through MAPK Pathway and G1 Cyclins in Rat Hepatocytes

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Several growth factors play an important role in liver regeneration. Once hepatic injury occurs, liver regeneration is stimulated by hepatocyte growth factor (HGF), transforming growth factor (TGF)-α, and heparin-binding epidermal growth factor-like growth factor (HB-EGF), whereas TGF-β1 terminates liver regeneration. In this study, we analyzed the effect of a combination of HGF and epidermal growth factor (EGF) on mitogen-activated protein kinase (MAPK) activity and G1 cyclin expression in primary cultured rat hepatocytes. Treatment with a combination of HGF and EGF, in comparison with that of either HGF or EGF, induced tyrosine phosphorylation of both c-Met and EGF receptor (EGFR) independently and additively stimulated MAPK activity and cyclin D1 expression, resulting in additive stimulation of DNA synthesis. On the other hand, although TGF-β1 treatment did not affect tyrosine phosphorylation of c-Met and EGFR, MAPK activity, and cyclin D1 expression, which were stimulated by HGF and EGF, DNA synthesis was completely inhibited through a marked decrease in cyclin E expression. These results indicate that potent mitogens, such as HGF, TGF-α, and HB-EGF, could induce the additive enhancement of liver regeneration cooperatively through an increase in Ras/MAPK activity followed by cyclin D1 expression, and that TGF-β1 suppresses the growth factor-induced signals between cyclin D1 and cyclin E, resulting in the inhibition of DNA synthesis.

Key Words: **hepatocyte growth factor; epidermal growth factor; transforming growth factor β; liver regeneration; signal transduction.**

In adult animals, hepatocytes are highly differentiated and rarely divide under normal conditions. However, under certain physiopathological stress situations, such as a partial hepatectomy, viral infection or toxic injury, hepatocytes are able to divide in response to liver mass loss (1, 2). Recent studies in the 70% partial hepatectomy model have offered a clearer picture of events that promote entry into the cell cycle (3). Immediately after a partial hepatectomy, hepatocytes enter into a state of prereplicative competence before they fully respond to growth factors. This priming step is an initiating event characterized by a transition from the G0 to the G1 phase of the cell cycle and progression through the early G1 phase and is mediated by cytokines, including tumor necrosis factor-α and interleukin-6, and in part by changes in the extracellular matrix (4, 5). However, progression of these initiated cells through the late G1 phase to the S phase is thought to require growth factors and involve activation of cyclin:cyclin dependent kinase (cdk) complexes. Therefore, a growth factor-dependent restriction point is precisely localized in the mid-late G1 phase in primary cultured rat hepatocytes (6), and it is mediated by transforming growth factor (TGF)-α, heparin binding-epidermal growth factor-like growth factor (HB-EGF) and mainly by hepatocyte growth factor (HGF).

HGF is known to be one of the major agents promoting the proliferation of hepatocytes. It was originally purified from the plasma of patients with fulminant hepatic failure (7, 8) and rat platelets (9). This protein is characterized as a pleiotropic factor acting as a mi-

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Abbreviations used: HGF, hepatocyte growth factor; TGF, transforming growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphatidyl inositol-3 kinase; Jak, Janus kinase; STAT, signal transducer and activator of transcription; pRb, retinoblastoma protein; CDK, cyclin dependent kinase; PCNA, proliferating cell nuclear antigen.

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togen, motogen and morphogen for a variety of cultured cells through binding to its receptor (c-Met) on the cell membrane (10). c-Met has a tyrosine kinase domain in its intracellular region. TGF-α (11) and HB-EGF (12) also stimulate DNA synthesis in primary cultured rat hepatocytes through binding to EGF receptor (EGFR), which has a tyrosine kinase domain (13). These growth factors are induced in the liver during liver regeneration, and both c-Met and EGFR transmit signals into the nucleus, mainly through the Ras/mitogen-activated protein kinase (MAPK) pathway (14). Following Ras/MAPK activation, cyclin D1 protein is expressed in the nucleus and drives the cell cycle from the G1 to the S phase and initiates DNA synthesis (15). These growth factors additively stimulate DNA synthesis in hepatocytes (14, 16, 17), and it is also thought that these factors act in concert during liver regeneration *in vivo*.

TGF-β1 is a growth inhibitory factor for hepatocytes; it completely inhibits the effect of HGF, TGF-α and EGF on DNA synthesis in hepatocytes (18). As is well known, TGF-β terminates liver regeneration in the late phase. Activin, which is expressed in normal rat liver, is another growth inhibitory factor for hepatocytes (19). After a partial hepatectomy, activin expression transiently decreases but recovers within 24 h. Activin may act as an inhibitory factor for liver regeneration, although details about how it does so are not yet fully understood.

HGF, TGF-α, HB-EGF and TGF-β act in concert to regulate liver regeneration; however, the interaction among these growth factors in intracellular signal transduction for DNA synthesis, such as the phosphorylation of their receptors, MAPK activation and induction of cyclin D1 expression, is not well understood. Therefore, the purpose of this study is to clarify whether combined treatment with HGF and EGF stimulates the Ras/MAPK system and cyclin D1 expression additively and to determine where TGF-β1 inhibits the growth factor-induced signal transductions in primary cultured rat hepatocytes.

MATERIALS AND METHODS

Animals. Seven-week-old male Wistar rats were obtained from Kyudo Co., Ltd. (Kumamoto, Japan). The rats were maintained under a constant room temperature (25°C) and provided with free access to a standard diet and tap water throughout, in accordance with institutional guidelines, and the study was approved by the ethical committee of Miyazaki Medical College (Miyazaki, Japan).

Primary cultured rat hepatocytes. Freshly isolated hepatocytes were prepared by perfusion of collagenase through the portal vein of anesthetized rats, as described previously (20). The culture medium was William's medium E containing 5% fetal bovine serum (FBS), penicillin-streptomycin (100 IU/ml) (Gibco BRL, Rockville, MD), insulin (1×10^{-7} M) and dexamethasone (1×10^{-7} M) (Sigma, St. Louis, MO).

Analysis of DNA synthesis. DNA synthesis was measured by [^{3}H] thymidine incorporation into DNA. After 4 h incubation with William's medium E containing 5% FBS, primary cultured rat hepatocytes were treated with various concentrations of growth factors for 36 h followed by incubation with 15 μCi/ml of methyl-[^{3}H]-thymidine for 2 h. Incorporated radioactivities were determined by liquid scintillation counting.

Tyrosine phosphorylation of c-Met and EGFR. Tyrosine phosphorylation of c-Met and EGFR was evaluated by Western blot analysis. The cells were treated with 10 ng/ml of HGF (Mitsubishi Kasei Co., Yokohama, Japan) and/or 10 ng/ml of EGF (Genezyme, Cambridge, MA) for 5 min and then incubated for a further 5 min in the presence or absence of 1 ng/ml of TGF-β1 (Becton–Dickinson Labware, Bedford, MA). The cells were solubilized, and the supernatants were mixed with anti-c-Met or anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) binding to protein A-Sepharose beads by rotation at 4°C for 6 h. After the beads were pelleted by centrifugation, c-Met or EGFR was eluted in gel-loading buffer. The samples were subjected to 8% polyacrylamide gel electrophoresis and blotted to nitrocellulose filters. To detect phosphotyrosine, filters were incubated with anti-phosphotyrosine-horseradish peroxidase (HRP) antibody and then subjected to enhanced chemiluminescence Western analysis (Amersham, Buckinghamshire, England).

Measurement of MAPK activity. We determined the MAPK activity in cell extracts using the p42/p44 MAPK enzyme assay system (Amersham) according to the manufacturer's instructions. Hepatocytes were treated with HGF (10 ng/ml) and/or EGF (10 ng/ml) for 5 and 10 min at 37°C and then incubated for 5 min in the presence or absence of TGF-β1 (1 ng/ml) for 5 min at 37°C. After the cells were solubilized, the supernatants were incubated with specific substrate and [γ-^{32}P]ATP at 30°C for 30 min. The radioactivity of ^{32}P-labeled proteins was counted using liquid scintillation.

Northern blot analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate method (21). To obtain rat cyclin D1 and cyclin E cDNA probe, total RNA was isolated from primary cultured rat hepatocytes, and reverse transcription-polymerase chain reaction (RT-PCR) was performed using two sets of primers, 5′-CTGGAGCCCCTGAAGAAGAGC-3′ and 5′-GAAAGTGCGTTGTGCGGTAGC-3′ for cyclin D1 and 5′-CCACTTCCCGTCTTGAATTGG-3′ and 5′-TGGTAAGAATTTCATCCCCGG-3′ for cyclin E under the following conditions: denaturation at 94°C for 30 s, annealing at 58°C for 15 s and extension at 72°C for 30 s (30 cycles). The PCR products of 424-bp cyclin D1 and 376-bp cyclin E cDNA were cloned into the pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced. The nucleotide sequences of the PCR products were identical with those of rat cyclin D1 and cyclin E cDNA previously deposited in the GenBank/EMBL Data Bank under Accession Nos. D14014 and D14015, respectively (22).

RESULTS

Effect of simultaneous treatment with growth factors on DNA synthesis in primary cultured rat hepatocytes. First, we confirmed the stimulatory effect of HGF and EGF on DNA synthesis and the inhibitory effect of TGF-β1 on growth factor-induced DNA synthesis in primary cultured rat hepatocytes. The DNA synthesis in hepatocytes was increased by either HGF or EGF treatments in a dose-dependent manner, and it reached a plateau at a concentration of 5 to 20 ng/ml of HGF or EGF (Fig. 1A). Furthermore, treatment with a combination of HGF and EGF increased DNA synthesis additively when compared to treatment with either HGF or EGF. On the other hand, TGF-β1 treatment completely inhibited the DNA synthesis induced by HGF, EGF and a combination of both (Fig. 1B).

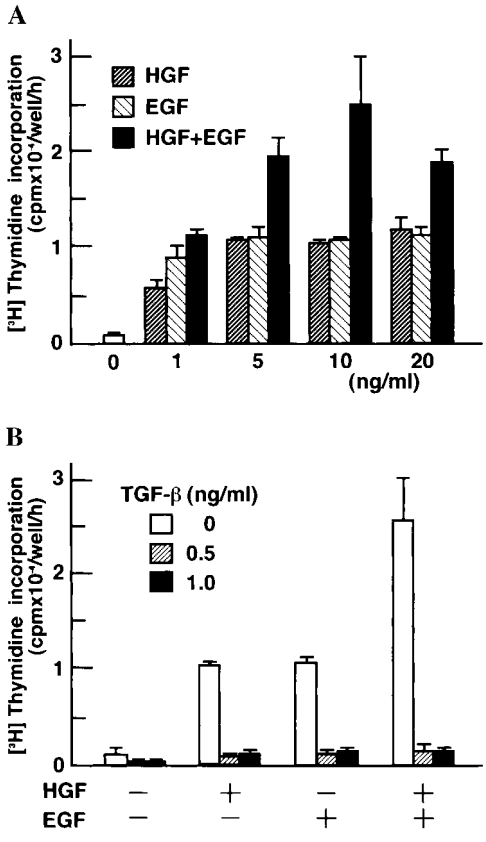

FIG. 1. DNA synthesis in primary cultured rat hepatocytes. (A) The effect of HGF and/or EGF on DNA synthesis. Hepatocytes were plated and stimulated by various concentrations of HGF and/or EGF as described under Materials and Methods. (B) The effect of TGF-β1 on DNA synthesis in rat hepatocytes stimulated by HGF and/or EGF. Hepatocytes were plated in the absence or presence of HGF (10 ng/ml) and/or EGF (10 ng/ml) and were treated with various concentrations of TGF-β1 simultaneously. DNA synthesis represents the mean [^{3}H]thymidine uptake (n = 3); bars, SE.

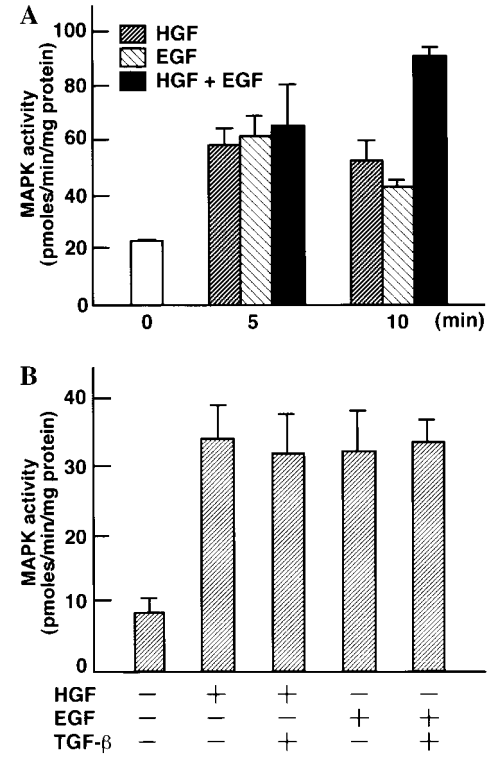

FIG. 3. MAPK activity in primary rat hepatocytes. (A) The effect of HGF and/or EGF on MAPK activity. Hepatocytes were stimulated with HGF (10 ng/ml) and/or EGF (10 ng/ml) for 5 or 10 min. (B) The effect of TGF-β1 on MAPK activity in rat hepatocytes stimulated by HGF or EGF. Hepatocytes were plated in the absence or presence of HGF (10 ng/ml) or EGF (10 ng/ml) and were treated with TGF-β1 (1 ng/ml) simultaneously. MAPK activity was determined by the p42/p44 MAPK enzyme assay system, as described under Materials and Methods. Each column represents the mean p42/44 MAPK activity (n = 3); bars, SE.

Tyrosine phosphorylation of c-Met and EGFR. We examined tyrosine phosphorylation of c-Met and EGFR in rat hepatocytes treated with HGF and/or EGF by Western blot analysis (Fig. 2). Treatment with HGF or EGF induced phosphorylation of each specific receptor, c-Met or EGFR, respectively. Although treatment with a combination of both HGF and EGF induced phosphorylation of their receptors, the intensity of phosphorylation of each receptor was not increased. On the other hand, TGF-β1 did not affect the HGF- and/or EGF-induced phosphorylation of c-Met and EGFR.

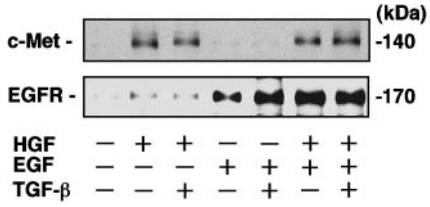

FIG. 2. Tyrosine phosphorylation of c-Met and EGFR by HGF and/or EGF, and the effect of TGF-β1 in rat hepatocytes. Primary cultured rat hepatocytes were stimulated by HGF (10 ng/ml) and/or EGF (10 ng/ml) or by each of them and TGF-β1 (1 ng/ml) for 5 min and solubilized with lysate buffer containing protease inhibitors. The cell lysates were immunoprecipitated with anti-c-Met or anti-EGFR antibody and blotted with anti-phosphotyrosine antibody.

MAPK activity. To analyze the downstream portion of phosphorylated receptors, we measured the activity of two highly related mammalian MAPKs, p44 and p42, in rat hepatocytes treated with HGF and/or EGF (Fig. 3). HGF and/or EGF treatment stimulated MAPK activity three times as much as did the control (no addition of growth factors) (Fig. 3A). Moreover, 10 min after treatment with a combination of HGF and EGF, MAPK activity continued to be 4- and 2-fold times as great in comparison with the control and with either HGF or EGF treatment, respectively. However, TGF-β1 did not affect the HGF- or EGF-induced MAPK activity in rat hepatocytes (Fig. 3B).

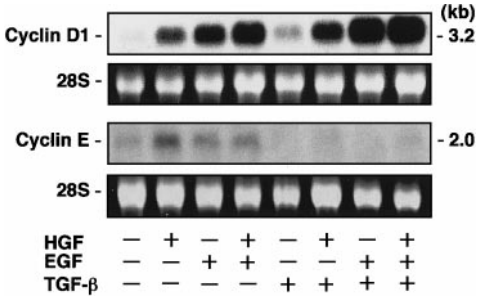

FIG. 4. Northern blot analysis of cyclin D1 and E transcripts. Total RNAs (10 μg) from unstimulated and HGF (10 ng/ml)- and/or EGF (10 ng/ml)-stimulated hepatocytes in the absence or presence of TGF-β1 (1 ng/ml) were analyzed as described under Materials and Methods using cyclin D1 or cyclin E cDNA as a probe and 28S as a control of the total amounts of RNAs in each lane.

Northern blot analysis of cyclin D1 and cyclin E. Since growth factor-induced signals for cell proliferation ultimately cause the expression of G1 cyclins, which leads to a mitogen-dependent progression of hepatocytes to the S phase, we evaluated the expression of cyclin D1 and cyclin E transcripts by Northern blot analysis. As shown in Fig. 4, when rat hepatocytes were treated with HGF or EGF alone for 24 h, the expression of cyclin D1 was increased, and it was additively enhanced by treatment with a combination of both. Moreover, the cyclin D1 expression induced by HGF and/or EGF was not affected by TGF-β1 treatment. In contrast, although the expression of cyclin E mRNA was also induced by HGF and/or EGF treatment and reached a maximum level after 36 h, TGF-β1 strongly inhibited the cyclin E expression induced by HGF and/or EGF (Fig. 4).

DISCUSSION

Hepatocyte proliferation requires growth factors such as HGF, TGF-α and HB-EGF, which lead to the activation of regulatory proteins that control the transition through the G1 phase of the cell cycle. These growth factors are induced during liver regeneration *in vivo*, such as liver injury and partial hepatectomy, and an additive effect of TGF-α and HGF on DNA synthesis in primary cultured hepatocytes has been reported (23, 24). In response to growth factors, Ras/MAPK, phosphatidyl inositol-3 kinase (PI3K) (25) and Janus kinase (Jak)/signal transducer and activator of transcription (STAT) (26) are activated in hepatocytes. In those pathways, the Ras/MAPK system plays a central role and is mainly associated with cyclin D1 expression, which regulates the progression of the G1 phase in the cell cycle (15). Therefore, with this study we intended to clarify the interaction of growth factors on their receptors' phosphorylation, MAPK activity and expression of G1 cyclins using primary cultures of normal rat hepatocytes.

We showed in the present study that treatment with a combination of HGF and EGF induced additive stimulation of MAPK activity and cyclin D1 expression, resulting in additive stimulation of DNA synthesis in primary cultured rat hepatocytes. Although tyrosine phosphorylation of c-Met and EGFR was also stimulated by the combined treatment, the intensity of phosphorylation was not affected. These results suggest that growth factors induced during liver regeneration act cooperatively through their receptors' phosphorylation and additive stimulation of the Ras/MAPK pathway followed by additive enhancement of cyclin D1 expression.

Up-regulation of cyclin D1 is widely believed to play an important role during the G1 phase in many types of cells (27, 28), and previous studies have suggested that this protein is involved in the regulation of hepatocyte proliferation. Cyclin D1 is also induced in rodent liver after a partial hepatectomy and is up-regulated in regenerating human liver (29). Recently, Albrecht *et al.* have shown that cyclin D1 expression induced by a recombinant adenovirus was sufficient to trigger the transition through the G1-S interval in a manner comparable to growth factor stimulation in primary hepatocytes (30).

The effect of treatment with a combination of HGF and EGF on signal transduction and cyclin D1 expression shown in this study was additive rather than synergistic. When rat hepatocytes were labeled with proliferating cell nuclear antigen (PCNA), combined treatment with HGF and EGF increased the number of PCNA-positive cells, when compared to the number noted with either HGF or EGF (data not shown). These results indicate the possibility that some populations of hepatocytes are responsive to either HGF or EGF. Therefore, the effect of treatment with a combination of HGF and EGF would be partly dependent on the number of stimulated cells, resulting in the effect being additive.

On the other hand, TGF-β, which is also induced in regenerating rat liver beginning 24 h after a partial hepatectomy (31), is well known as an inhibitory growth factor in hepatocytes that mediates the arrest of the G1 phase. We confirmed that TGF-β1 completely inhibited DNA synthesis stimulated by HGF and EGF in rat hepatocytes, as described previously (18). Moreover, we showed that TGF-β1 treatment did not affect the HGF- and/or EGF-stimulated receptors' phosphorylations, MAPK activity or cyclin D1 expression, while the cyclin E expression induced by HGF and/or EGF was completely inhibited by TGF-β1. These results indicate that TGF-β1 suppresses the growth factor-induced signals between cyclin D1 and cyclin E. In some cell types, cyclin E:cdk2 activation is triggered by cyclin D1 expression through the E2F-mediated transcription of cyclin E (32, 33) or as a result of p27 (Kip1), one of the major cdk inhibitors, sequestration by cyclin

D1 (34). In this study, since cyclin E expression was strongly inhibited by TGF-β1 treatment, TGF-β1 could inhibit the activation of the E2F transcription factors.

The E2F transcription factors normally interact with the retinoblastoma protein (pRb) to arrest cells in G1 (35). Once pRb is phosphorylated by cyclin D:CDK4/6 complexes, E2F is released from pRb and functions as a transcriptional factor regulating genes whose products are essential for DNA replication and cell cycle control (32, 33, 35, 36–39). Therefore, cyclin E exists downstream of pRb phosphorylation induced by cyclin D:CDK4/6 complexes. Cyclin D:CDK4/6 complexes previously have been shown to be specifically targeted by TGF-β signaling by either induction of $p15^{INK4b}$, a negative regulator of CDK4/6, a decrease in the activity of the G1/S phosphatase CDC25A, which may be required for the activation of CDK4/6, or down-regulation of CDK4 protein levels (40, 41, 42). Recently, Nagahara *et al.* described that TGF-β treatment resulted in the specific inactivation of cyclin E:CDK2 complexes caused by the inhibition of CDK2 activating kinase activity in HepG2 human hepatoma cells (43). In this study, we used primary cultures of normal rat hepatocytes and showed that HGF- and/or EGF-induced cyclin E expression was strongly inhibited in TGF-β1-treated hepatocytes. Therefore, although we did not examine the effect of TGF-β1 on the phosphorylation of pRb in rat hepatocytes, we believe that TGF-β1 inhibits cyclin D1:CDK4/6 complexes through an inactivation or a down-regulation of CDK4/6, resulting in the inhibition of pRb phosphorylation.

In conclusion, we showed that HGF and EGF additively stimulated DNA synthesis through the activation of the Ras/MAPK pathway and expression of cyclin D1 and cyclin E in primary cultured rat hepatocytes, and that TGF-β1 inhibited DNA synthesis by inhibiting growth factor-induced signal transductions between cyclin D and cyclin E. This is the first report about an interaction among the growth factors on signal transductions and G1 cyclins in primary cultured rat hepatocytes. However, the transcription factors, which occur in response to MAPK activation and regulation of the cyclin D1 gene, are still not known. The complex interplay of transcription factors induced by growth factors in the transcriptional regulation of the cyclin D1 gene is currently under investigation.

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