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Continuous HGF Supply from HGF-Expressing Fibroblasts Transplanted into Spleen Prevents CCl_4-Induced Acute Liver Injury in Rats

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Hepatocyte growth factor (HGF), first identified as a potent mitogen for mature hepatocytes, has been reported to have various activities. We investigated protective effect of continuous HGF supply on carbon tetrachloride (CCl_4)-induced acute liver injury in rats. We transfected immortalized but not tumorigenic rat fibroblasts (Rat-1) with an expression plasmid containing the human HGF cDNA and established several cell lines expressing HGF. The biological activity of HGF produced by these cell lines was confirmed by its mitogenic effect on rat hepatocytes *in vitro.* Either one of the high-HGF-producer cell lines or parental Rat-1 cell line was transplanted into a syngenic rat spleen. Twelve days after transplantation, each rat was intraperitoneally injected with CCl_4 and sacrificed 48 h after CCl_4 injection. In rats with continuous HGF supply significantly lower serum glutamic-pyruvic transaminase (GPT) level was observed compared to its marked elevation in control rats and the degree of hepatocyte damage was slight on histological analysis. These results indicate that continuous HGF supply effectively inhibits CCl_4-induced acute liver injury and may suggest the possibility that this system would be useful on various liver diseases.

Hepatocyte growth factor (HGF), first identified as a potent mitogen for mature hepatocytes (1,2), has been reported to have various activities (3,4,5). A recent report has demonstrated that intermittently injected recombinant HGF markedly stimulates the growth of hepatocytes in mouse liver and protects the integrity of hepatocytes in vivo against hepatitis caused by hepatotoxin (6). In the case of clinical p for liver disease, transportal route would be more desirable than peripheral intravenous injection and continuous infusion may be more efficient than intermittent supply. In this study, we at first established rat fibroblast cell lines overexpressing human HGF and transplanted them into syngenic rat spleen to supply HGF for liver continuously. Then we investigated its protective effect on carbon tetrachloride (CCl_4)-induced acute liver injury in rats. In this study, we report that continuous HGF supply markedly suppressed acute hepatitis in rats caused by CCl_4.

MATERIALS AND METHODS

Animals. Male F344 rats weighing 200 to 250 g (SRL, Hamamatsu, Japan) were used. All animals received humane care. A nutritionally balanced rodent diet and water were provided *ad libitum.*

Plasmids. The recombinant expression plasmid, pUC-SR α/HGF, which expresses human HGF under the control of SR α promoter was provided by T. Nakamura. pSV2bsr is a recombinant plasmid (7) used for selection which allows expression of the Blasticidin S (BS) resistance gene (Funakoshi, Tokyo, Japan).

Cells and transfection. One day before transfection, immortalized but not tumorigenic F344 rat fibroblast cell line Rat-1 cells (8) (approximately 2×10^5 cells) were plated into a 6.0 cm dish with Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) containing 10% fetal calf serum (GIBCO, BRL, USA), penicillin (50 units/ml) and streptomycin (50 μg/ml) (standard medium). Rat-1 cells were transfected with 7.2 μg of plasmid pUC-SR α/HGF and 0.8 μg pSV2bsr by the calcium phosphate method (9). Forty eight hours after transfection, transfectants were selected with BS (5 μg/ml, Funakoshi, Tokyo, Japan) and resultant BS-resistant colonies were harvested and propagated, colony by colony, using a stainless-steel cylinder.

ELISA assay. Conditioned medium derived from parental Rat-1 cells or each established cell lines was collected from

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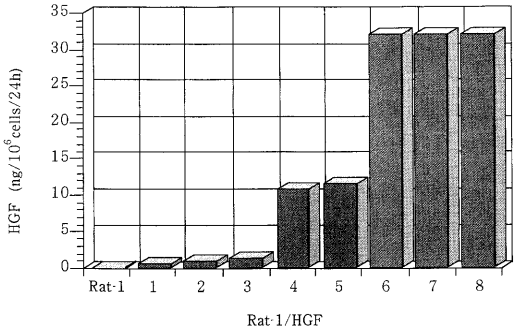

FIG. 1. HGF production of various Rat-1-derived cell lines.

subconfluent 24-well dish cultures in standard medium. HGF concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Institute of Immunology, Tokyo, Japan).

Bioassay for HGF activity. Biological activity of HGF produced by transfected cells was determined using adult rat hepatocytes in primary culture. Hepatocytes were isolated from adult male F344 rat as originally described by Seglen (10), and the cells were plated onto the lower compartments of the 6 well Transwell (Costar, MA, USA) at a density of 10^5 cells/well. The culture medium was the standard medium supplemented with 10^{-9}M insulin, 10^{-9}M dexamethasone and 0.5 μg/ml aprotinin. Twenty four hours after plating, each upper compartment on which 2×10^5 Rat-1 cells or HGF-producing Rat-1 cells were plated 2 days before was added and the every culture medium was replaced with the medium containing 5-bromo-2′-deoxyuridine (BrdU) (400 μ M, Sigma Chemical Co., St. Louis, USA). As a control, primary hepatocytes alone were cultured in the lower compartment. Seventy two hours after plating the lower compartments were fixed with acid ethanol for anti-BrdU staining.

Immunohistochemical staining of wells with anti-BrdU antibody. Anti-BrdU staining was performed using HISTOFINE SAB-PO(M) kit (Nichirei, Tokyo, Japan). Briefly, endogenous peroxidase was inactivated in 0.3% H_2O_2 in absolute methanol. The wells were washed with phosphate buffered saline (PBS), and DNA was denatured in 2 N HCl for 1 h. After neutralization in 0.1 M borate buffer (pH 8.5), the wells were incubated with monoclonal antibody against BrdU (Becton Dickinson, San Jose, CA) diluted 1:100 with 1% bovine serum albumin in PBS and incubated for 1 hr at room temperature. After washing with PBS, the wells were incubated with biotin-conjugated rabbit antimouse IgG for 10 min at room temperature followed by incubation with peroxidase-conjugated streptoavidin for 5 min. After washing with PBS, the enzyme reaction was initiated by adding substrate solution containing diaminobenzidine. The labeling index of hepatocytes was determined by counting more than 2,000 nuclei.

Transplantation. Parental Rat-1 cells or HGF-producing Rat-1 cells (approximately 3×10^6 cells) suspended in 0.5 ml DMEM were injected directly into the spleen of F344 rats ($n = 5, 8$ respectively) through 22G needle. At once the injection sites were ligated with 2-0 silk to minimize leakage of cells. As a cell-free control experiment, DMEM alone was injected ($n = 6$).

Assessment of liver injury. Twelve days after transplantation, CCl_4 (Wako, Osaka, Japan) dissolved in equal volume of olive oil were intraperitoneally injected at a dose of 1.5 ml/g body weight. Forty eight hours after CCl_4 injection, the animals were sacrificed and blood was collected from portal vein. Immediately after blood collection, each liver was removed for histochemical study (hematoxylin and eosin staining; HE staining) to observe histological change. To evaluate the degree

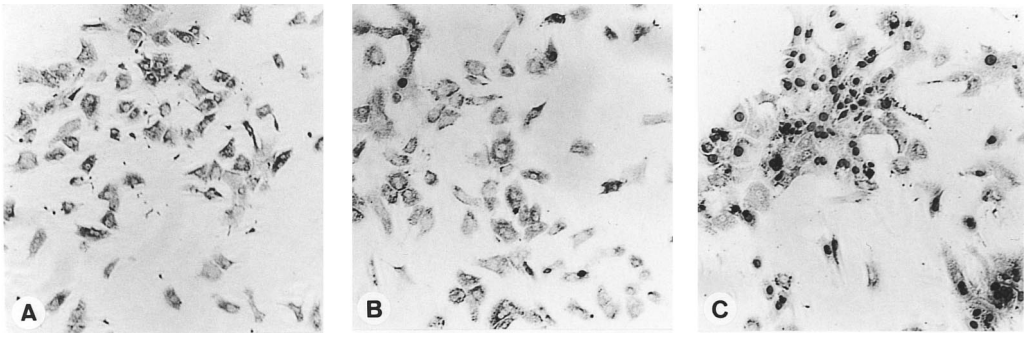

FIG. 2. Anti-BrdU staining of rat primary hepatocytes alone (A) and hepatocytes cocultured with Rat-1 cells (B) or with Rat-1/HGF 6 cells. (C) (original magnification × 700).

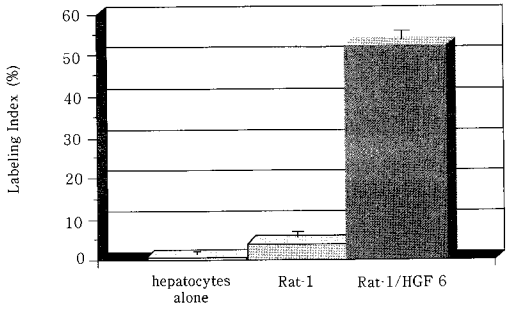

FIG. 3. Labeling index of rat primary hepatocytes alone and hepatocytes cocultured with Rat-1 cells or with Rat-1/HGF 6 cells.

of liver injury after CCl_4 administration, the amount of GPT, a representative cytosolic enzyme of hepatocyte, were assayed with an autoanalyzer.

Statistical evaluation. Student's *t* test was used to compare the mean values of groups.

RESULTS

Establishment of Cell Lines Stably Expressing HGF

Eight BS-resistant cell lines designated as Rat-1/HGF 1∼8 were established. Rat-1/HGF 1∼8 cells were morphologically spindle-shaped cells apparently different from parental Rat-1 cells. Data for HGF production of these lines are listed in Fig. 1. HGF was not detectable in the conditioned medium from parental Rat-1 cells. On the contrary, human HGF cDNA-transfected cells showed various amounts of human HGF production. Among 8 cell lines, 3 cell lines of Rat-1/HGF 6∼8 were found to produce high levels of HGF (32.0 ng/10^6 cells/24 h), therefore we used Rat-1/HGF 6 in the following experiments.

Biological Activity of Human Recombinant HGF Produced by Rat-1/HGF 6 Cells on Rat Hepatocytes in Primary Culture

The labeling index of primary cultured hepatocytes alone, hepatocytes co-cultured with Rat-1 or Rat-1/HGF 6 were 0.6%, 3.9%, 52.3% respectively (Fig. 2, 3). This finding evidently shows HGF produced by the Rat-1 transfectants serves as a potent mitogen for primary rat hepatocytes.

Effect of HGF from Transplanted Rat-1/HGF 6 Cells on CCl_4-Induced Acute Liver Injury

Histological findings showed that hepatocytes around the central veins were necrotic in both control rats and rats transplanted with Rat-1 cells (Fig. 4A, B). However, the degree of liver injury

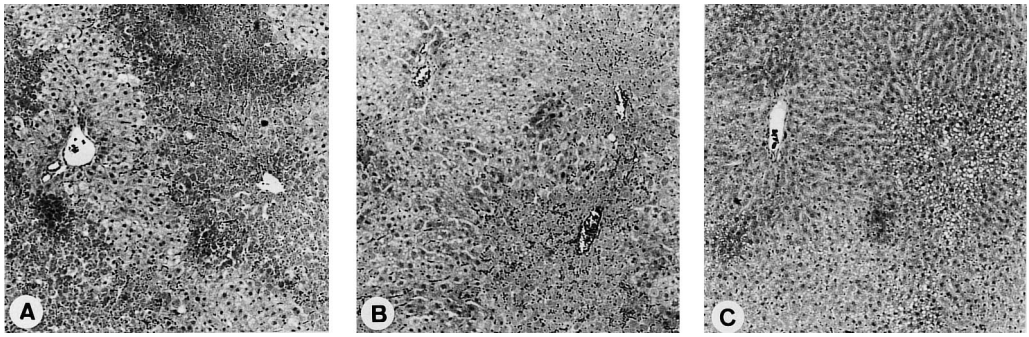

FIG. 4. Histology of rat liver 48 h after CCl_4 injection from a control rat (A) and rats transplanted with Rat-1 cells (B) or with Rat-1/HGF 6 cells (C) (original magnification × 359).

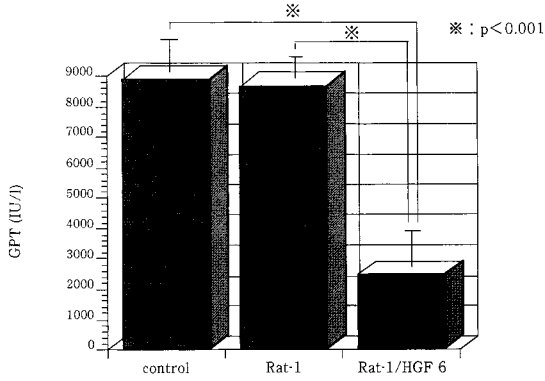

FIG. 5. Serum GPT level of various rats with or without transplantation of Rat-1/HGF 6 cells into spleen 48 h after CCl_4 injection.

was remarkably reduced in rats transplanted with Rat-1/HGF 6 cells (Fig. 4C). Namely only steatosis was found in hapatocytes around the central veins. Accordingly, the increase of GPT caused by CCl_4 was markedly suppressed by continuous HGF supply from Rat-1/HGF 6 cells in spleen (Fig. 5).

DISCUSSION

We established cell lines producing much higher amounts of HGF than the cell lines previously reported (11) and demonstrated the usefulness of a system continuously supplying *de novo* synthesized HGF for liver on CCl_4-induced acute liver injury model. As far as we know, there have been no report which describes continuous HGF supply *via* the portal vein. There may be two methods to supply HGF continuously to liver. One is recombinant HGF injection through a catheter placed in the portal system such as the superior mesenteric vein using a syringe pump. The other is the implantation of HGF-producing cells into upstream organs of liver as we did. In the former case, it is thought difficult to preserve the biological activity of unstable cytokines such as HGF for a prolonged time. But in the latter case, *de novo* synthesized HGF is immediately supplied to the liver continuously. Besides, once the cells are transplanted they would produce HGF for a long time as they are originated from immortalized rat fibloblasts.

Recently Moullier et al intraperitoneally implanted collagen lattices containing genetically modified autologous fibroblasts secreting human β-glucuronidase and observed a sustained secretion of the enzyme in dogs (12). For clinical application, autologous human skin fibroblasts could be available to transfect with cDNA. HGF is a hepatotrophic factor for liver regeneration and has a potent antihepatitis effect in vivo (6). Furthermore HGF has anti-proliferative effects on several tumor cell lines, including hepatocellular carcinoma cells (5,13), and inhibits growth of experimental rat hepatocellular carcinomas induced by diethylnitrosamine (14). Therefore if sufficient dose of HGF could be supplied to liver continuously, the progression of hepatitis and tumor growth would be considerably suppressed. Thus our system would be greatly expected to be used for various liver diseases in the future.

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