of Medicine, Kyoto University, approved all experimental protocols. Animal care was under the supervision of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. All experimental procedures were performed in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*.

Cochlear Explant Culture

On postnatal day 3 (P3), ICR mice were deeply anesthetized with sevoflurane and decapitated. The temporal bones were dissected, and the cochleae were freed from surrounding tissues in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. After removal of the cochlear lateral wall, auditory epithelia were dissected from the cochlear modiolus. The tissue pieces were then attached to glass mesh inserts (Falcon, Billerica, MA) and cultured initially in serum-free minimum essential medium (Invitrogen, Eugene, OR) supplemented with 3 g/L glucose and penicillin G for 24 hours. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Because hair cells in the apex are resistant to aminoglycosides,[11,12] basal turns of the cochlea were used in this study.

Neomycin Application, Cell Survival, and HGF Protection Assay

The explants were then transferred to the medium containing neomycin sulfate (Sigma-Aldrich, St. Louis, MO) at concentrations of 0, 0.1, 0.3, 1, or 3 mM, with six to 10 cochleae incubated at each concentration. For HGF protection assay, we examined effects of 20 ng/mL recombinant human HGF (rhHGF, Sigma-Aldrich) with the same neomycin concentrations. We also used 1 mM neomycin media supplemented with recombinant human HGF at concentrations of 0, 4, 20, or 100 ng/mL to estimate optimal HGF concentration.

Cultures were maintained for 24 hours and at the end of the culture period, samples were fixed for 15 minutes in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Specimens were then rinsed with PBS, incubated in 1% bovine serum albumin (BSA) with 0.2% Triton X-100 for 30 minutes and immersed in Alexa 488-labeled phalloidin (1:200; Invitrogen) for 30 minutes. Samples were examined with a Leica TCS-SP2 confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). To quantify hair cell loss in the cochlea after various treatments, inner hair cells (IHCs) and outer hair cells (OHCs) in the auditory epithelia were counted over a 100-μm longitudinal distance from two separate regions in the basal turn of each culture (totaling 200 μm).

c-Met Expression in the Cochlea

Temporal bones of P3 ICR mice (n = 4) were collected under deep anesthesia. Cochleae were perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer and immersed in the same fixative at room temperature for 2 hours. After ethylenediaminetetraacetic acid decalcification, the specimens were embedded in optimum cutting temperature compound (Tissue Tek, Miles Inc., Elkhart, IN) and sectioned at 15 μm using a cryostat. Midmodiolar sections were provided for immunohistochemistry. Sections were briefly refixed with 4% paraformaldehyde and blocked with 1% BSA with 0.2% Triton X-100 for 30 minutes. Primary antibodies were rabbit polyclonal anti-c-Met (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-myosin VIIa (1:500, Proteus Bioscience Inc., Ramona, CA). Alexa-Fluor 488-conjugated goat anti-rabbit IgG (1:200; Invitrogen) were used as the secondary antibody, and Zenon Alexa Fluor 555 rabbit IgG labeling kit (Invitrogen) was used for anti-myosin VIIa staining. Specimens were then incubated in PBS containing 2 mg/mL DAPI (Invitrogen) for nuclear staining.

Alteration of c-Met Levels in Cochlear Explants

We employed Western blotting to estimate alterations of c-Met expression in cochlea explants following neomycin application. Cochlear explants were cultured without glass mesh, using medium containing neomycin sulfate at concentrations of 0, 0.3, 1, or 3 mM for 24 hours. Ten cochleae were used for each condition. Explants were then quickly collected and homogenated in ice-cold radio immunoprecipitation assay buffer with protease inhibitor cocktail (Nakalai Tesque, Kyoto, Japan). Five micrograms of each extract was separated on a 4% to 15% Tris-HCL gradient polyacrylamide gel (Bio-Rad, Hercules, CA) and then transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). Membranes were blocked for 40 minutes with 5% BSA in Tween-TBS (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) and stained overnight with anti-c-Met antibody or antiactin antibody (Sigma) at 4°C. After three washes the membranes were incubated in a 1:20000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 hour and developed using the ECL plus Western blotting detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). Experiments were repeated three times and pictures were processed with Adobe Photoshop CS (Adobe Systems, San Jose, CA), and band intensity was measured using ImageJ software (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, MD).

Lipid Peroxidation Assay

Lipid peroxidation was assessed in cultures treated with neomycin, in the presence or absence of HGF, by measuring expression of 4-hydroxynonenal (HNE) immunohistochemically.[13] Explants were labeled with mouse anti-HNE monoclonal antibody (1:8; Oxis Research, Portland, OR) and Alexa-Fluor 568 goat anti-mouse IgG (1:200; Invitrogen) as the primary and secondary antibodies. Specimens were then counterstained with Alexa 488 phalloidin and examined with a fluorescence microscope. All images were taken with the same exposure and shutter speed. Red fluorescence intensity was measured using ImageJ software.

Statistical Analysis

Overall effects of HGF on hair cell numbers were analyzed by 2-way factorial analysis of variance (ANOVA) using the Statcel2 application (OMS Publishing, Saitama, Japan), with *P* values below .05 considered statistically significant. For interactions that were significant, multiple paired comparisons were analyzed using the Tukey-Kramer test. Measurements of HNE staining intensity were analyzed with 1-way ANOVA with the Tukey-Kramer test.

RESULTS

Dose-Dependent Hair Cell Loss by Neomycin

First, we tested a dose-response relationship between neomycin concentrations and hair cell counts. The addition of 0-3 mM neomycin for 24 hours significantly reduced hair cell numbers in both IHC and OHC regions (Fig. 1A, C, E, G). The addition of 1 mM of neomycin destroyed approximately 73% of the IHCs and 64% of the OHCs (Fig. 1E, I). The hair cell density

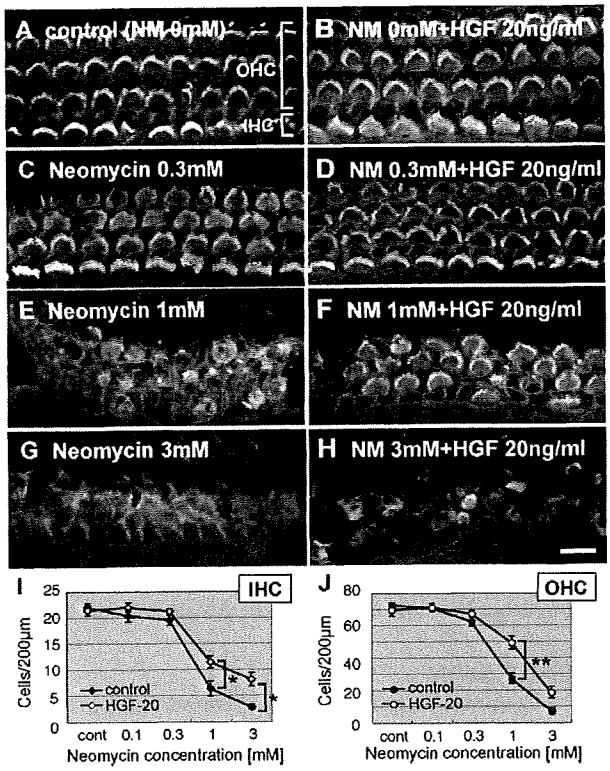

Fig. 1. Dose-dependent auditory hair cell loss by neomycin and its protection by hepatocyte growth factor (HGF). Cochleae were cultured for 24 hours in various concentrations of neomycin with or without HGF. (A–H). Photomicrographs of phalloidin-labeled cochlear cultures. Outer hair cell (OHC) consists of three rows of triangular-shaped cells that have phalloidin-positive, inverted v-shaped stereocilia bundles on top of them. Inner hair cells (IHC) are oval-shaped cells that have flat, u-shaped stereocilia bundles. Bar = 10 μm. (A). A control showing normal arrangement of IHCs and OHCs. (C, E, G). Examples of cochlear cultures treated with neomycin at a concentration of 0.3 mM (C), 1 mM (E), and 3 mM (G). (B, D, F, H). Examples of cochlear cultures treated with 20 ng/mL HGF and neomycin at a concentration of 0 mM (B), 0.3 mM (D), 1 mM (F), and 3 mM (H). (I, J). Hair cell count following neomycin and HGF-supplemented cultures. Filled symbols represent counts from control cultures without HGF and open symbols from HGF- supplemented cultures. In HGF-free cultures, the hair cell density decreased systematically as the neomycin concentration increased. HGF significantly attenuated the loss of IHC ($P < .05$) and OHC ($P < .001$) in neomycin-damaged cochleae (2-way analysis of variance). Post hoc analyses with Tukey-Kramer tests for multiple pairwise comparisons showed that IHC loss for HGF plus neomycin was significantly less ($P < .05$) than for neomycin alone at neomycin concentrations of 1 and 3 mM, and also enhanced the survival of OHCs at 1 mM neomycin ($P < .01$). *$P < .05$. **$P < .01$. Bars in (I) and (J) represent standard errors.

decreased as the neomycin dose increased until virtually all hair cells were absent from the auditory epithelium cultured in 3 mM neomycin.

Dose-Response Effects of HGF Against Neomycin

To assess whether HGF has protective effects against neomycin damage, we examined the power of HGF on a quantitative assessment of dose-response relationship of the hair cells to various concentrations of neomycin. We administered 20 ng/mL HGF to 0, 0.1, 0.3, 1, or 3 mM neomycin cultures (Fig. 1B, D, F, H). The addition of 20 ng/mL HGF markedly enhanced IHC and OHC survival. HGF even promoted the survival of a substantial number of hair cells at the highest neomycin dose, 3 mM (Fig. 1G vs. H). Two-way ANOVA analyses showed that HGF has a significant effect on both remaining IHC and OHC numbers ($P = .000097$ and $P = .0000011$, respectively). Tukey-Kramer tests for multiple pairwise comparisons showed that IHC losses for HGF at 1 or 3 mM neomycin was significantly less ($P < .05$) than those for neomycin alone conditions (Fig. 1I). Twenty ng/mL HGF also significantly ($P < .01$) enhanced the survival of OHCs at 1 mM neomycin condition (Fig. 1J). These data demonstrated that HGF exerts significant protective effects against neomycin-induced hair cell damage.

Optimal Concentration of HGF for Protection

We then administered 4 to 100 ng/mL HGF to these cultures (Fig. 2). Based on the above dose-response experiment, 1 mM neomycin was used. When HGF was administered alone to control cultures at 4 to 100 ng/mL, the number of hair cells present in the samples was comparable to untreated controls indicating that HGF alone had no negative or mitogenic effects at these concentrations (Fig. 1B). In neomycin-

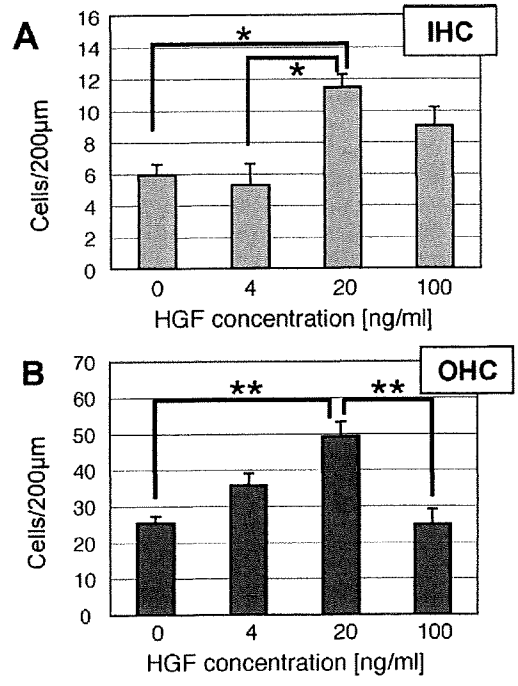

Fig. 2. Effects of different concentrations of hepatocyte growth factor (HGF) on the survival of cochlear hair cells. Cochleae were incubated with different concentrations of HGF in the presence of 1 mM neomycin. (A) Inner hair cell (IHC) and (B) outer hair cell (OHC) counts in HGF-treated cultures. HGF significantly increased hair cell survival at concentrations up to 20 ng/mL. *$P < .05$. **$P < .01$. Bars represent standard errors.

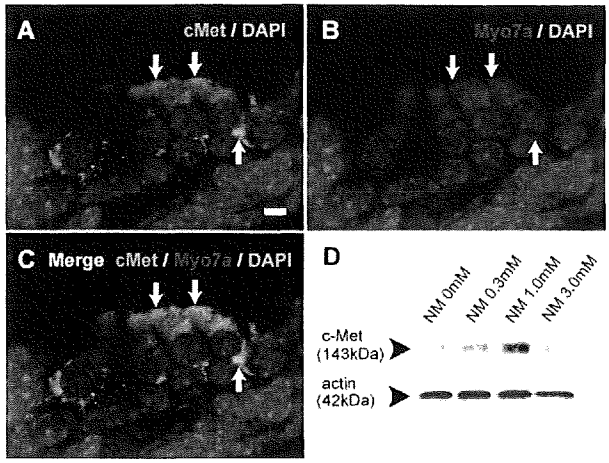

Fig. 3. c-Met expression in the organ of Corti increases after neomycin insult. (A, B, C). c-Met localization (green) in a normal postnatal day 3 cochlea. Specimen was counterstained with anti-myosin VIIa (red) and DAPI (blue). Arrows indicate punctuate c-Met staining in the hair cells. Bar = 10 μm. (D). Induction of c-Met expression in the neomycin-injured cochlea. Western blotting analyses showed a remarkable increase of c-Met expression, peaking when neomycin was applied at 1.0 mM. Relative band intensities were 1.00, 1.12 ± 0.26, 1.94 ± 0.48, and 1.17 ± 0.35 for 0, 0.3, 1.0, and 3.0 mM neomycin (n = 10 in each condition).

supplemented cultures, adding 4 ng/mL of HGF had little effect on IHC survival. In comparison, HGF treatment at 20 ng/mL elicited 64% increase of remaining cell counts. However, at concentration of 100 ng/mL HGF, no further increases in the number of IHCs were seen, indicating that HGF effects saturated at 20 ng/mL concentration. A 1-way ANOVA revealed significant treatment effects for HGF ($P = .018$). Tukey-Kramer tests for multiple pairwise comparisons showed significant differences between the 0 and 20 ng/mL neomycin ($P < .05$) and 4 and 20 ng/mL neomycin groups ($P < .05$). HGF had similar effects on OHC survival (Fig. 2B). HGF treatment at 20 ng/mL elicited almost a two-fold increase of remaining cell counts. A 1-way ANOVA revealed significant effects for HGF ($P = .000049$). Tukey-Kramer tests for multiple pairwise comparisons showed significant differences between the 0 and 20 ng/mL neomycin ($P < .01$) and 20 and 100 ng/mL neomycin groups ($P < .01$). Based on these findings, the optimal concentration of HGF to 1 mM neomycin damage was estimated as 20 ng/mL for both IHCs and OHCs.

c-Met Localization in the Auditory Epithelia

To reveal whether cochlear protection mechanism of HGF is mediated by HGF/c-Met paracrine coupling, we first examined c-Met expression in the cochlea using immunohistochemistry (Fig. 3A, B, C). The expression of c-Met was detected punctate in both IHCs and OHCs, most densely at the apical region of the OHCs and the nerve ending (basal) area of IHCs and OHCs. In contrast, surrounding supporting cells expressed minimal amount of c-Met.

c-Met Expression Increases After Neomycin Insult

We then tested whether neomycin damage could increase c-Met expression in the hair cells. We examined c-Met expression in cochlear explant cultures after 24 hours of 0 to 3 mM neomycin application. Western blotting revealed that c-Met expression peaked when neomycin was applied at 1.0 mM (Fig. 3D). Relative band intensity was 1.94 ± 0.48 compared to that of 0 mM neomycin. At 3.0 mM neomycin, band intensity decreases, corresponding to fewer hair cells remaining in the auditory epithelia at this neomycin concentration (Fig. 1G).

Antioxidant Effects of HGF

We also investigated the antioxidant effect of HGF, as another mechanism by which its protective effect might function. HNE expression in explant cultures increased in the presence of neomycin (Fig. 4). Adding HGF to the cultures resulted in a significant reduction in HNE expression ($P = .00049$). HGF showed its greatest effect at a concentration of 4 ng/mL, at which HNE production was attenuated to 59.0% of the level in the absence of HGF.

DISCUSSION

The present study has demonstrated that the application of HGF attenuates neomycin-induced hair cell death in cochlear explants. In our experimental setting, quantitative assessments of hair cell numbers following neomycin application exhibits that both IHC and OHC numbers decrease as the neomycin dose increases. In this setting we examined the effects of 20 ng/mL HGF and the results demonstrated significant effects of HGF on dose-dependent hair cell loss by neomycin, which strongly supports our hypothesis that HGF is a potent protectant for the cochlear hair cells. Also, in using a concentration of 1 mM neomycin, which provides a significant lesion to the hair cells, we showed that the optimal concentration of HGF is 20 ng/mL for hair cell protection in explant cultures.

HGF protection of neuronal cells is believed to involve various mechanisms including HGF/c-Met

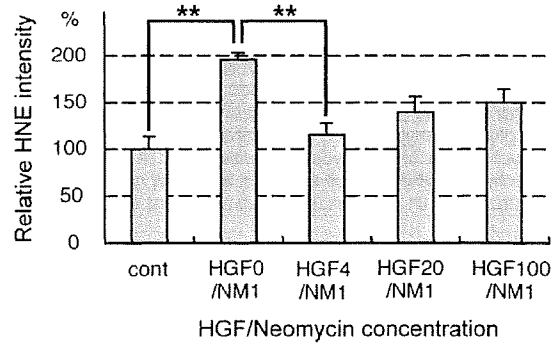

Fig. 4. Hepatocyte growth factor (HGF) treatment reduced lipid oxygenation. Lipid oxygenation was measured in cultures treated with neomycin and HGF as the intensity of immunohistochemical labeling for 4-hydroxynonenal (HNE). Relative HNE staining intensity in the organ of Corti was significantly increased in the presence of 1 mM neomycin, but significantly reduced with the addition of 4 ng/mL HGF. **$P < .01$. Bars represent standard errors.

signaling.[5,6] We thus investigated involvement of HGF/c-Met signaling in hair cell protection by HGF against neomycin toxicity. Immunohistochemistry in normal P3 mouse cochleae revealed the presence of c-Met in cochlear hair cells. In addition, Western blotting demonstrated an increase of c-Met expression after neomycin-induced damage. These findings indicate involvement of paracrine coupling between HGF and c-Met in the auditory epithelium and the central nervous system.[5] Also, the increase of c-Met expression after neomycin-induced damage justifies exogenous HGF application for hair cell protection.

HGF could also protect hair cells from damage by reducing the oxidative stress generated by neomycin. Several researchers have reported that HGF exerts antioxidant activity by enhancing reactive oxygen species (ROS) scavenging and suppressing ROS production.[14,15] We have previously shown that lipid peroxidation caused by hydroxyl radicals increases in the auditory epithelium during cisplatin-induced hearing trauma.[13] In this study, our results indicated that lipid peroxidation also occurred in the neomycin-damaged cochlea, and HGF successfully attenuated HNE expression at a concentration of 4 ng/mL. We therefore consider that the antioxidant activity of HGF may in part play a role in the mechanisms by which it protects hair cells.

CONCLUSION

We have provided evidence for the direct survival-promoting effect of HGF on cochlear hair cells. Our data indicate that damaged auditory epithelia express c-Met receptors and HGF is a candidate as a delivering drug to the cochlea for treatment of sensorineural hearing loss.

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Increased Rate of Death Related to Presence of Viremia Among Hepatitis C Virus Antibody–Positive Subjects in a Community-Based Cohort Study

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The overall mortality of patients infected with hepatitis C virus (HCV) has not been fully elucidated. This study analyzed mortality in subjects positive for antibody to HCV (anti-HCV) in a community-based, prospective cohort study conducted in an HCV hyperendemic area of Japan. During a 10-year period beginning in 1995, 1125 anti–HCV-seropositive residents of Town C were enrolled into the study and were followed for mortality through 2005. Cause of death was assessed by death certificates. Subjects with detectable HCV core antigen (HCVcAg) or HCV RNA were considered as having hepatitis C viremia and were classified as HCV carriers; subjects who were negative for both HCVcAg and HCV RNA (i.e., viremia-negative) were considered as having had a prior HCV infection and were classified as HCV noncarriers. Among the anti–HCV-positive subjects included in the analysis, 758 (67.4%) were HCV carriers, and 367 were noncarriers. A total of 231 deaths occurred in these subjects over a mean follow-up of 8.2 years: 176 deaths in the HCV carrier group and 55 in the noncarrier group. The overall mortality rate was higher in HCV carriers than in noncarriers, adjusted for age and sex (hazard ratio, 1.53; 95% confidence interval, 1.13-2.07). Although liver-related deaths occurred more frequently among the HCV carriers (hazard ratio, 5.94; 95% confidence interval, 2.58-13.7), the rates of other causes of death did not differ between HCV carriers and noncarriers. Among HCV carriers, a higher level of HCVcAg (≥100 pg/mL) and persistently elevated alanine aminotransferase levels were important predictors of liver-related mortality. *Conclusion:* The presence of viremia increases the rate of mortality, primarily due to liver-related death, among anti–HCV-seropositive persons in Japan. (HEPATOLOGY 2009;50:393-399.)

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Hepatitis C virus (HCV) was identified 20 years ago. It is now known that between 50% and 85% of acute HCV infections become chronic[1-3]; after developing chronic infection, spontaneous HCV clearance is very rare. Approximately 170 million people worldwide are infected with HCV, and chronic HCV infection is a major health problem. HCV is a common cause of fatal liver disease, including liver cirrhosis and hepatocellular carcinoma (HCC). However, the liver-related mortality rate associated with chronic HCV infection is highly variable across different populations. In

Abbreviations: ALT, alanine aminotransferase; anti-HCV, antibody to HCV; CI, confidence interval; GGT, gamma-glutamyltransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVcAg, hepatitis C virus core antigen; HR, hazard ratio; IFN, interferon.

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patients that have been infected for more than 20 years, the occurrence of liver cirrhosis, HCC, and liver disease–related mortality are reported to be 10%-50%, 1%-23%, and 4%-15%, respectively.[4-6]

The range in published HCV-related mortality rates is due in part to the variability in the natural history of HCV infection as well as in the subjects studied. Some HCV-positive individuals have persistently normal alanine aminotransferase (ALT) levels and exhibit no clinical symptoms. Persons with this phenotype were often not included in previous hospital-based studies that focused on liver-related mortality in patients with HCV-associated liver disease/cirrhosis.[4,5] A few studies have systematically examined the risk of causes of death after HCV infection in a community-based setting[7-9]; however, the status of HCV viremia was not clear in these studies. In addition, the age range of HCV-infected subjects followed for mortality can vary considerably, with some cohort studies conducted in subjects whose average age was younger than 45 years[1,10-13] and others among older individuals.[9,14,15] To overcome some of these limitations, we analyzed mortality in 1125 subjects positive for antibody to HCV (anti-HCV) with data on viremia status, who were enrolled in a population-based cohort study in an HCV hyperendemic area of Japan between 1995 and 2005. These subjects were followed prospectively until death or until the end of the study in December 2005.

Patients and Methods

Study Population. Since 1993, we have been following anti–HCV-seropositive residents in a hyperendemic area (Town C) of Japan. The overall prevalence of anti-HCV positivity is higher (20.6%) in this region than in the surrounding area.[16] Town C is a small town in midwestern Miyazaki Prefecture, Japan, and the Town C HCV Study is a cohort study examining the natural course of HCV infection.[17-20] A general health examination program, begun in 1993, has been conducted annually for residents over 20 years of age. An ultrasonography-based liver disease screening program was initiated in 1994 to detect HCC in Town C residents who were identified as positive for anti-HCV. A total of 1321 anti–HCV-positive residents were enrolled into the cohort from 1994 through the last liver disease screening in 2006. Informed consent was obtained from subjects at the time of enrollment. The study was approved by the human subjects committees of the Harvard School of Public Health, the University of Miyazaki Faculty of Medicine, the Boston University School of Public Health, and the Kagoshima University Graduate School of Medical and Dental Sciences.

Our analysis focuses on the 1125 subjects with hepatitis C viremia data between 1995 and February of 2005, who were followed for mortality from the beginning of 1996 through the end of 2005. Anti–HCV-seropositive subjects with detectable HCV core antigen (HCVcAg) or HCV RNA were considered to be persistently infected with HCV and were classified as HCV carriers. Anti–HCV-positive subjects who were negative for HCVcAg and HCV RNA were assumed to have had a prior HCV infection and were classified as noncarriers. Subjects who underwent oral or intravenous administration of medical herbs or other palliative therapies or who had received interferon therapy were not excluded from the analyses. A subgroup analysis was conducted on HCV carrier subjects with at least three independent ALT measurements obtained at an annual general health examination or liver disease screening; ALT levels ≥ 35 were considered abnormal.

Follow-Up. For this analysis, follow-up started at the date of first HCV viremia measurement (baseline) and ended at date of death or December 31, 2005. During the course of the study, 12 residents moved to other areas, and their follow-up time was censored at that point; no other subjects were lost to follow-up. Cause of death was based on the information from the death certificate and was classified into one of seven categories: HCC, liver disease excluding HCC, neoplasms excluding HCC (i.e., other neoplasms), stroke, heart disease, pulmonary disease excluding lung cancer, and other/unknown causes.

Laboratory Methods. Serum anti-HCV antibodies were detected using second-generation enzyme immunoassay testing (Immunocheck F-HCV antibody; International Reagents Co., Kobe, Japan) or third-generation chemiluminescence enzyme immunoassays (Lumipulse Ortho II; Ortho-Clinical Diagnostics, Tokyo, Japan). In the anti–HCV-positive residents, serum levels of HCVcAg were tested with a fluorescence enzyme immunoassay (Immunocheck F-HCV Ag Core; International Reagents Co., Kobe, Japan),[21] with a detection threshold of 8 pg/mL. The presence of HCV RNA was determined by reverse transcription polymerase chain reaction (Amplicor HCV Monitor, version 1.0 [Nippon Roche, Tokyo, Japan] or version 2.0 [Nippon Roche or Roche Diagnostics K.K., Tokyo, Japan]) in study subjects whose HCVcAg levels were below the detection threshold.

Serologically defined HCV genotype (HCV serotype) was determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping; International Reagents Co., Tokyo, Japan). If the HCV serotype could not be determined, the HCV genotype was examined (HCV Core Genotype; SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b

Table 1. Baseline Characteristics of Anti-HCV Antibody-Positive Subjects in Town C HCV Study

Characteristics	All Patients (n = 1125)	HCV Carriers (n = 758)	HCV Noncarriers (n = 367)	*P* Value
Age (years)				
Mean (±SD)	64.2 (±11.1)	64.9 (±10.6)	62.6 (±11.9)	0.007
Range	28-97	32-97	28-90	
Sex				
Male	456 (40.5%)	313 (41.3%)	143 (39%)	0.46
Female	669 (59.5%)	445 (58.7%)	224 (61%)	
ALT (IU/L)	40 ± 42.8 (1062)	47 ± 47.5 (719)	25.3 ± 25 (343)	<0.001
GGT (IU/L)	35.8 ± 46 (912)	39.1 ± 50.7 (612)	29.2 ± 33.6 (300)	<0.001
HCV core antigen level (pg/mL)				
Mean (±SD)		207.5 (±208.4)	-	
Median		140	-	
Range		20-1445	-	
HCV serotype				
I		463 (64.5%)	-	
II		220 (30.6%)	-	
Indeterminate		35 (4.9%)	-	
HBs antigen				
Positive	6 (0.6%)	4 (0.6%)	2 (0.6%)	0.99
Negative	948 (99.4%)	638 (99.4%)	310 (99.4%)	
History of alcohol intake				
Daily	365 (34.3%)	236 (32.9%)	129 (37.2%)	
Occasionally	206 (19.4%)	140 (19.5%)	66 (19.0%)	0.37
None	493 (46.3%)	341 (47.6%)	152 (43.8%)	
History of blood transfusion				
Yes	165 (15.7%)	101 (14.3%)	64 (18.6%)	0.07
No	885 (84.3%)	605 (85.7%)	280 (81.4%)	

Abbreviations: ALT, alanine aminotransferase; GGT, gamma-glutamyltranspeptidase; HBs antigen, hepatitis B surface antigen; HCV, hepatitis C virus.

with serotype II. No other HCV genotype was detected in this study population.

Statistical Analysis. One-factor analysis of variance, χ^2 tests, Fisher's exact tests, and the Mann-Whitney U tests were used, when appropriate, for statistical comparisons of the baseline characteristics of the HCV carrier and noncarrier groups of subjects. Cox proportional hazards regression was used to obtain hazard ratios (HRs) and 95% confidence intervals (CIs) that were adjusted for age and sex; for the analyses of cause-specific mortality, subjects who died from a different cause were censored at the time of death. The cumulative incidence of death was analyzed by the Kaplan-Meier method, and differences in the survival curves were evaluated by the log-rank test. Statistical analyses were performed using Statistical Analysis System (SAS, version 9.1; SAS Institute, Cary, NC), STATVIEW (version 5.0; Abacus Concepts, Berkeley, CA), or SPSS (SPSS Inc., Chicago, IL) software programs. A *P* value less than 0.05 was considered to be statistically significant.

Results

Demographic Characteristics of Study Subjects. As shown in Table 1, 758 (67.4%) of the anti–HCV-positive subjects were HCV carriers (i.e., positive for HCVcAg or HCV RNA), with a mean age at enrollment of 64.9 years. The HCV noncarrier group, who were considered to have had a prior HCV infection, included 367 subjects whose mean age at enrollment was 62.6 years. On average, the HCV carriers were older and had higher levels of ALT and gamma-glutamyltransferase (GGT) than the noncarriers, at baseline. In contrast, there were no significant differences between the two groups with respect to sex, alcohol intake, or history of blood transfusions. The number of subjects positive for hepatitis B surface antigen was small and not significantly different between the two groups. Sixty-seven subjects reported that they had previously received interferon (IFN) therapy, all of whom were categorized as HCV carriers when they entered the study. Fifteen of these subjects were treated prior to entering the study, five were treated during the study, and one was treated both prior to and during the study; for the other 46 subjects, the timing of IFN treatment was unknown. Although the results of IFN therapy could not be fully determined for these 67 subjects, 41 of 44 with available data in 2005 were positive for HCV RNA at that time and only three (7%) were negative for HCV RNA.

Overall and Cause-Specific Mortality. Over an average of 8.2 years of follow-up, 231 deaths occurred among the 1125 subjects (Table 2). The overall mortality

Table 2. Cause of Death in Subjects Positive for Anti-HCV Antibody

Cause of Death	All Patients	HCV Carriers	HCV Noncarriers
All causes	231	176	55
1. All liver-related deaths	76	70	6
a. HCC	45	41	4
b. Non-HCC	31	29	2
2. Neoplasms excluding HCC	41	28	13
3. Stroke	30	20	10
4. Heart disease	22	13	9
5. Pulmonary disease excluding lung cancer	32	22	10
6. Other/unknown	30	23	7

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

rate was 25.0 per 1000 person-years in this study population. Most deaths were liver-related, with 45 due to HCC and 31 to other liver diseases including cirrhosis, hepatic failure, and ruptured esophageal varix. The next most frequent cause of death was other neoplasms (n = 41), followed by pulmonary disease excluding lung cancer (n = 32), stroke (n = 30), other/unknown causes (n = 30), and heart disease (n = 22).

Of the 231 deaths, 176 were in the HCV carrier group and 55 were in the noncarrier group (Table 2). After adjusting for age and sex, HCV carriers had a significantly higher overall mortality rate (HR, 1.53; 95% CI, 1.13-2.07), compared to HCV noncarriers (Table 3). The elevated mortality rate among the subjects with evidence of HCV viremia was due to a much higher occurrence of liver-related deaths (HR, 5.94; 95% CI, 2.58-13.7). In contrast, HCV viremia was not significantly associated with death from other malignancies, stroke, heart disease, or pulmonary disease. The cumulative risk of death, based on Kaplan-Meier estimates, was 28.0% for the HCV carrier group and 17.9% for the HCV noncarrier group over 10.3 years (Fig. 1), a statistically significant difference ($P < 0.001$).

Table 3. The Association of HCV Viremia with Causes of Mortality Among Anti-HCV Antibody-Positive Subjects in Town C HCV Study

Cause of Death	HR	95% CI
All causes	1.53	(1.13, 2.07)
1. All liver-related deaths	5.94	(2.58, 13.7)
a. HCC	4.85	(1.73, 13.5)
b. Non-HCC	8.11	(1.94, 33.8)
2. Neoplasms excluding HCC	1.04	(0.54, 2.02)
3. Stroke	0.89	(0.41, 1.90)
4. Heart disease	0.68	(0.29, 1.60)
5. Pulmonary disease excluding lung cancer	1.05	(0.50, 2.22)
6. Other/unknown	1.59	(0.68, 3.71)

Abbreviations: CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio.

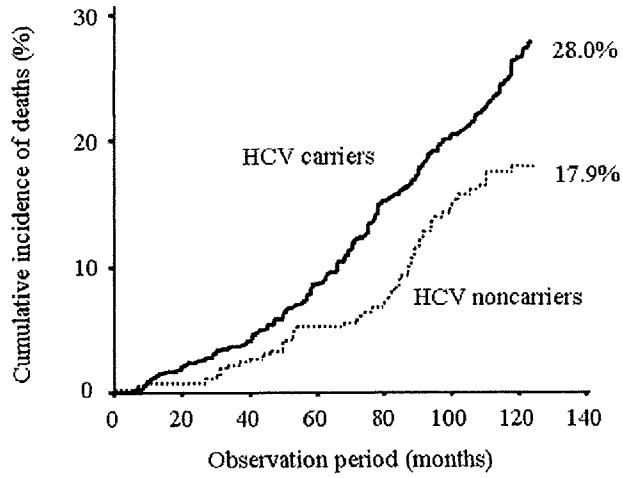

Fig. 1. Cumulative incidence of all-cause deaths in HCV carriers and noncarriers.

Predictors of Mortality Among HCV Carriers. The age-adjusted and sex-adjusted association between HCV serotype and HCVcAg level and mortality was examined among the subjects with HCV viremia. Compared to HCV serotype II, those with serotype I infection did not have a higher rate of overall (HR, 1.04) or liver-related mortality (HR, 1.12); however, having an indeterminate HCV serotype was related to both overall (HR, 3.59; 95% CI, 2.1-6.1) and liver-related death (HR, 2.12; 95% CI, 0.78-5.75). Of note, both serotype I infection (HR, 2.21; 95% CI, 0.91-5.33) and indeterminate HCV infection (HR, 3.89; 95% CI, 0.97-15.7) appeared to increase HCC mortality. In addition, a significantly increased rate of liver-related death was associated with a higher level (≥100 pg/mL) of HCVcAg (HR, 1.81; 95% CI, 1.08-3.06); the effect of higher HCVcAg level was stronger with respect to other liver-related death (HR, 2.58; 95% CI, 1.04-6.41) than to HCC death (HR, 1.48; 95% CI, 0.77-2.82). HCVcAg level had no effect on overall mortality among the HCV carriers (HR, 1.06).

In a subgroup analysis of 719 HCV carrier subjects who had data for at least three separate ALT measurements, 173 had persistently normal ALT levels while 141 had persistently abnormal levels. Subjects whose ALT levels fluctuated were not included in the analysis. Adjusting for age and sex, overall mortality (HR, 2.23; 95% CI, 1.37-3.61) and liver-related death (HR, 11.0; 95% CI, 4.35-27.9) were significantly higher for HCV carriers with persistently elevated ALT than for those with persistently normal ALT. The strongly elevated rate of liver-related mortality was evident for death due to both HCC (HR, 11.1) as well as other liver-related disease (HR, 14.5).

Discussion

Our study indicated that liver-related mortality is strongly associated with the presence of HCV viremia among persons who are seropositive for anti-HCV antibodies and that HCVcAg and ALT levels were predictors of liver-related mortality in HCV carriers. In this study population, the age distribution of anti–HCV-positive subjects, the prevalence of viremia, and the frequency of HCV serotype I were similar to previously reported data in Japan.[22-25] Japan has the highest incidence rate of HCC attributed to HCV infection among developed countries. Tanaka et al. estimated that HCV infection was spread in Japan during the 1920s, whereas HCV was widely disseminated in the United States in the 1960s.[26] The authors suggested that the HCC burden in the United States will likely increase in the next two or three decades, possibly to a level equal to that currently experienced in Japan.

Several studies have examined mortality in patients with HCV. Seeff et al. provided mortality data for 222 transfusion-associated hepatitis C cases and 377 control patients after approximately 25 years of follow-up.[27] Kamitsukasa et al. also reported mortality data for 302 HCV-infected patients with tuberculosis sequelae who had received a blood transfusion.[15] Although both studies showed that liver-related mortality was significantly higher in the disease groups than in the control groups, liver-related mortality was not the main cause of death. Kamitsukasa et al. reported that the main cause of death for approximately 45% of the patients in their study was tuberculosis sequelae.[15] Similar results were obtained in patients with inherited bleeding disorders and hepatitis C, where the main cause of death was human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS).[28] Moreover, there was no significant difference between patients with and without hepatitis C in the overall mortality rates in the study by Seeff et al. In contrast, our study showed that all-cause mortality and liver-related mortality with or without HCC were significantly higher in the HCV carrier group than in the noncarrier group. The incidence of HCC in Caucasian patients with HCV-related cirrhosis has been reported to be 1.2% in the United States,[29] whereas the incidence in Japanese patients is reportedly between 6% and 7%.[30] Furthermore, HCV-related cases in some studies included subjects with previous HCV infections.[15,27] Ethnic-dependent and racial-dependent variation in the rates of HCC, the composition of the comparisons groups, and/or complications unrelated to liver disease, such as tuberculosis sequelae or HIV/AIDS, may have resulted in differences in the patient prognoses between our study and previous studies.

It has been reported that HCC was the main cause of liver-related death in patients with compensated cirrhosis due to HCV infection.[31,32] Kasahara et al. found that 74% of liver-related death in patients with chronic hepatitis C who had not received IFN therapy was due to HCC.[33] Although HCC was more frequently observed than other liver-related deaths in our study, the proportion of HCC among all liver disease deaths (59% in the HCV carrier group) was relatively low compared to that study.[33] This occurrence may have been because the causes of death were obtained from death certificates in our study and cases of severe hepatic failure due to HCC may have been classified as liver disease excluding HCC.

A large community-based linkage study that included 78,438 individuals with hepatitis C indicated that the risk of dying from drug-related causes was significantly greater than from liver-related causes; however, the incidence of liver-related deaths was greater than that of drug-related deaths in patients older than 45 years.[7] In addition, other studies have shown that age appears to be an important risk factor that affects HCC development[14] and that the risk of cirrhosis is related to the patient's age at the time of infection and to disease activity.[34,35] These reports, which focused on patients with transfusion-associated chronic hepatitis C, suggest that the younger the patients are at the time of infection, the lower the rate of progression. Although the exact dates of infection and HCC diagnosis were not clear in our study population, the median age at enrollment was older than 60 years. Thus, the incidence of liver-related deaths might be expected to be greater than deaths from other causes.

In our study, HCV serotype I, which included HCV genotypes 1a and 1b, was found in 64.5% of the HCV carrier subjects in whom serotype was measured, whereas serotype 2, which included genotypes 2a and 2b, was detected in 30.6% of patients. These results agree with the overall distributions of HCV genotypes and serotypes in the entire Japanese population, which show that genotype 1b is the most prevalent genotype at 70%.[36] Several studies have demonstrated that genotype 1b is associated with severe liver disease, including cirrhosis and HCC.[37,38] In this study, there was an apparent association between HCV serotype I infection and mortality due to HCC. Other studies, however, have not found an effect of HCV genotype on liver disease development.[39,40] In addition, although an association of indeterminate serotype with mortality was observed (HR = 3.6), the reason for this finding is not clear. A larger study is needed to elucidate the role of genotype in the prognosis of HCV infection.

HCV RNA levels have also been reported to be associated with the progression of chronic hepatitis C.[41,42] Although the level of HCV RNA was not quantified in this study, HCVcAg levels, which are known to correlate with HCV RNA levels,[21] were assessed by fluorescence enzyme immunoassay. We observed that high HCVcAg levels were predictive of liver-related mortality, including death due to HCC, in the HCV carriers. The precise mechanism underlying HCV infection–dependent hepatocarcinogenesis is not clear. However, a study of transgenic mice that express the HCV core protein demonstrated that this protein was important in HCC development.[43] Of interest, Moucari et al. reported that insulin resistance is a specific feature of chronic hepatitis C and associated with high serum HCV RNA levels.[44] A significant increase in the incidence of diabetes has also been seen in subjects with high titer of HCV core protein compared to subjects who were negative for anti-HCV.[45] Moreover, significant fibrosis is associated with insulin resistance,[44] and diabetes mellitus is known to increase the risk of primary liver cancer in the presence of other risk factors such as hepatitis C.[46] Thus, HCVcAg levels might be associated with liver-related mortality through the development of HCV-induced insulin resistance or diabetes mellitus.

We have previously shown that elevated ALT levels are an important predictor of HCC among HCV carriers in this study population.[19] In the current analysis, ALT, aspartate aminotransferase, and GGT levels at enrollment were significantly higher in subjects who died due to a liver-related disease compared with subjects who died from other causes (data not shown). In addition, after adjusting for age and sex, overall mortality (HR, 2.23) and liver-related death (HR, 11.0) were significantly higher for HCV carriers with persistently elevated ALT than for those with persistently normal ALT.

Our study had several limitations. First, data regarding liver histology were lacking. It is likely that HCV carriers had more cirrhosis than did HCV noncarriers, given that more HCV carriers died of HCC and non-HCC liver deaths (Table 2). However, we were unable to examine this possibility directly. Information on platelet counts, which are generally inversely correlated with hepatic fibrosis, was available for a subset of subjects. Based on data obtained in 1996, mean platelet counts were significantly lower in HCV carriers (n = 539; $18.4 \times 10^4/\mu L \pm 5.6 \times 10^4/\mu L$) than in HCV noncarriers (n = 277; 21.3 ± 6.0). In addition, data from the last examination attended after 2001 showed that the persistently elevated ALT group had lower mean platelet counts (n = 94; $14.5 \times 10^4/\mu L \pm 5.5 \times 10^4/\mu L$) than did the persistently normal ALT group (n = 123; 21.8 ± 7.3). These findings suggest that the presence of viremia may increase the rate of hepatic fibrosis, especially in HCV carriers with high ALT levels.

Second, although the effect of IFN therapy may have implications with respect to the overall death rates in the study population, information on treatment was limited. However, the proportion of treated subjects with an observed sustained viral response to IFN was small (7%). Data on socioeconomic factors, which are strongly related to mortality outcomes,[47] also were not available in this study. We would not expect much variation in socioeconomic status in the study population, because the cohort included only Japanese subjects who resided in a small rural community where farming is the principal occupation. In addition, all subjects in the study population had health insurance. Thus, we believe that socioeconomic factors and IFN therapy likely did not greatly affect the rate of mortality in our study population.

In conclusion, the results of this prospective 10-year follow-up study showed a strong effect of HCV carrier status on liver-related mortality among anti-HCV–seropositive individuals. Moreover, high HCVcAg and ALT levels were important predictors of liver-related death in this population. Monitoring HCV load and ALT level in HCV carriers may be important for identifying those individuals at increased risk for HCC or other liver disease, particularly among older carriers who are less likely to respond to HCV treatment.

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Review Article

Translational research to identify clinical applications of hepatocyte growth factor

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Hepatocyte growth factor (HGF), originally purified from the plasma of patients with fulminant hepatic failure, has been shown to carry out various physiological functions. HGF not only stimulates liver regeneration, but also acts as an antiapoptotic factor in *in vivo* experimental models. Therefore, HGF is a promising therapeutic agent for the treatment of fatal liver diseases, including fulminant hepatic failure. After performing a number of preclinical tests, our group began an investigator-initiated registered phase I/II clinical trial of patients with fulminant hepatic failure to examine the safety and clinical efficacy of recombinant human HGF. In this article, we will discuss the basic research results as well as the translational research that underpins current attempts to use HGF in various clinical settings.

Key words: fulminant hepatic failure, HGF, liver regeneration, translational research

INTRODUCTION

SINCE HEPATOCYTE GROWTH factor (HGF) was purified for the first time in 1986, a number of studies have examined this paracrine factor, resulting in the elucidation of its various physiological functions. The molecular mechanisms underlying these functions of HGF are currently being examined in detail. For example, *in vivo* experimental systems have been used to clarify the functions of HGF. Through these studies, HGF has shown potential as a therapeutic agent for the treatment of a number of intractable diseases. Our group has worked for years to make recombinant human HGF (rh-HGF) available as a drug. In September 2005, we began an investigator-initiated ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use)-GCP (Good Clinical Practice)-registered phase I/II clinical trial for the treatment of fulminant hepatic failure. In this article, we will discuss the published experimental results, starting with the basic research and continuing through to the translational research that has sought to uncover the clinical applications of HGF.

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PURIFICATION AND STRUCTURAL ANALYSIS OF HGF

WE INITIALLY IDENTIFIED a factor in the plasma from patients with fulminant hepatic failure that strongly stimulated DNA synthesis in primary cultured rat hepatocytes.[1] We were then able to successfully purify this humoral factor, and named it HGF.[2] Approximately 40 μg of HGF was obtained from 1 L of plasma from patients with fulminant hepatic failure. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis indicated that the molecular weight of HGF is approximately 90 kDa, and its native form consists of a heterodimer with an approximately 60-kD heavy chain (α chain) and an approximately 30-kDa light chain (β chain), which are connected through a disulfide bond (Fig. 1). Next, we cloned human HGF cDNA from a human placental cDNA library and examined the primary structure of the protein.[3,4] Human HGF is synthesized as a single chain precursor (prepro HGF), which consists of 728 amino-acid residues. After a 31-amino-acid N-terminal signal peptide is removed, it is secreted from cells as pro-HGF. Then, the molecule is

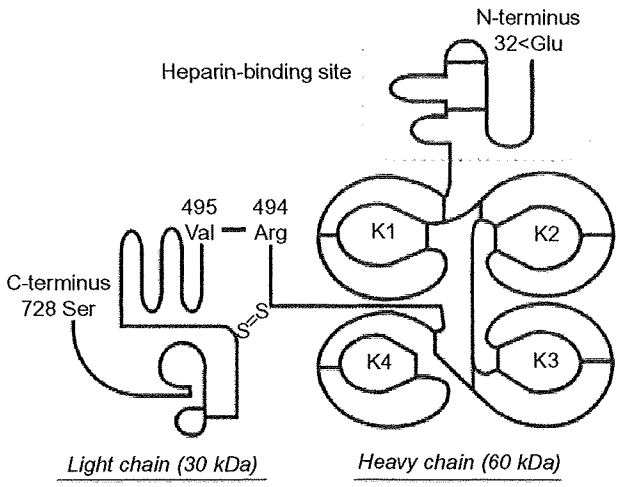

Figure 1 Primary structure of human hepatocyte growth factor (HGF). After human HGF is synthesized as a single chain precursor (prepro HGF) with 728 amino-acid residues, the signal peptide containing 31 N-terminal amino-acid residues is removed, and human HGF is secreted from the cell as pro-HGF. Then, this molecule is cleaved between the Arg494 and Val495, converting it to the mature heterodimer form of HGF.

cleaved between Arg494 and Val495, resulting in the mature HGF heterodimer. Variants of HGF carrying amino-acid substitutions at the cleavage site are not cleaved and have no biological activity, even though they are able to bind to HGF receptors. Therefore, the conversion to the heterodimer is essential for the physiological activities of HGF.

EXPRESSION OF THE MATURE FORM OF HGF

PRO-HGF, WHICH does not show any significant biological activity, interacts with the extracellular matrix, especially types I, III, V, and VI collagen.[5] The extracellular matrix not only stores pro-HGF but also facilitates the binding of mature HGF to c-Met and the subsequent signal transduction.[6] The conversion from pro-HGF to the mature form of HGF is primarily catalyzed by the serine protease HGF activator (HGFA),[7] whereas factor XII has a weak conversion activity.[8] In addition, urokinase-type plasminogen activator, tissue plasminogen activator, and matriptase can also catalyze the conversion to the mature form of HGF.[9] HGFA, which shows a high degree of homology with factor XII, a protein from the coagulation fibrinolytic system,[7] is also synthesized as a precursor protein, and is converted to an active form by the serine proteases thrombin or coagulation factor Xa.[8] Thus, HGF is activated by a serine protease cascade as is observed in the coagulation fibrinolytic system.

HGFA is produced by hepatic parenchymal cells, exists primarily as an inactive form in the blood, and is activated under hemorrhagic conditions. Although active HGF is not detected in hepatic tissues after partial hepatectomy,[10] HGFA was activated in liver tissues in a rat model of carbon tetrachloride (CCl_4)-induced liver injury; HGFA expression increased 1–6 h after CCl_4 administration, and decreased after 24 hours.[11,12] Additionally, HGFA activity was induced in the renal tissues of rats following renal injuries.[11] Therefore, HGF is specifically activated in injured tissues, and the active form of HGF is involved in the repair of injured tissues. Additionally, Kitamura *et al.*[13,14] have purified the HGFA inhibitors HAI-1 and HAI-2, two Kunitz-type protease inhibitors, from the culture supernatant of human MKN45 gastric cancer cells. Although HAI-1 is strongly expressed in human placenta, kidney, pancreas, prostate, and small intestine, it is only weakly expressed in liver tissues. Recent studies have demonstrated that the expression of HAI-1 is up-regulated in injured tissues, and that HAI-1 acts to suppress HGFA activity. Thus, HAI-1 plays an important role in HGF activation.[15,16]

PHYSIOLOGICAL EFFECTS OF HGF

HGF AT CONCENTRATIONS of 1 ng/mL or greater strongly enhances the proliferation of primary cultured rat hepatocytes.[2] Although HGF was initially considered to be a specific growth factor for hepatocytes, it has been shown that HGF also stimulates the proliferation of various epithelial, endothelial, and mesenchymal cells.[17] Recent investigations have shown that HGF is produced by IMR-90 human embryonic lung fibroblasts as tumor cytotoxic factor, which inhibits proliferation and induces apoptosis in certain types of cancer cells.[18] Moreover, scatter factor (SF), which was isolated as a factor that results in the scattering of epithelial cell colonies by stimulating the motility and migration of individual cells, was actually found to be HGF.[19] When Madin-Darby canine kidney cells were cultured with HGF in a collagen gel, tubular structures formed (tubulogenesis).[20] Induction of morphogenesis, such as lumen formation, follows cell scattering and cellular proliferation, and these effects are related to each other. Additionally, HGF suppresses apoptosis and stimulates angiogenesis.[21] More recently, an inhibitory effect of HGF on dendritic cell functions was reported.[22]

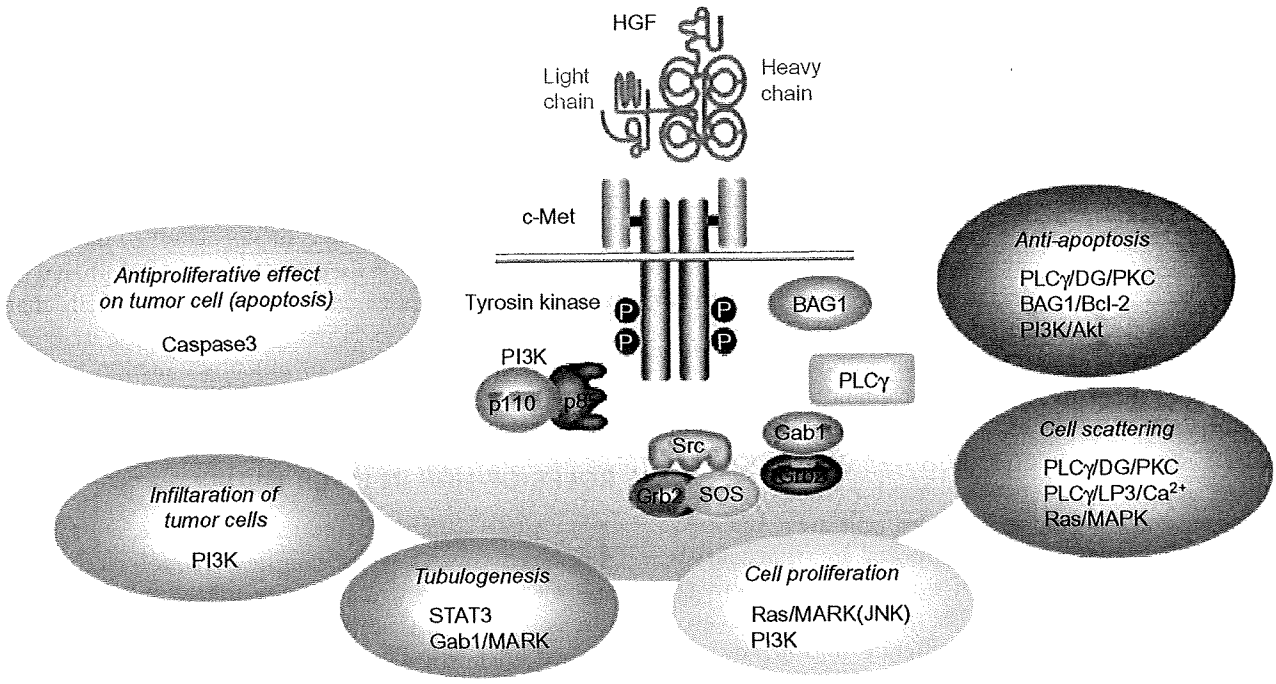

Figure 2 Hepatocyte growth factor (HGF) receptor (c-Met) and its signalling. Although the various physiological effects of HGF are expressed through c-Met, the signalling is thought to depend on which adaptor protein binds to the multifunctional docking site (MDS) in the C-terminal region of the β chain to recruit the components of the signal transduction cascade.

c-Met, the specific receptor for HGF, consists of a 50-kDa α chain and a 145-kDa β chain. These chains are connected through a disulfide bond. The HGF binding site and tyrosine kinase domain are found in the β chain.[23,24] Upon HGF binding, c-Met autophosphorylates two critical tyrosine residues to create a unique multisubstrate docking site. This site, which is located in the C-terminal cytoplasmic domain, serves to recruit downstream signalling molecules and adaptor proteins, thereby amplifying the cellular responses to HGF via multiple signalling pathways (Fig. 2).[25–27] These pathways have been shown to be essential for a variety of HGF-induced cellular changes.[28,29] On the other hand, recent studies have reported that, although HGF induces a transient activation of c-Met in rat primary cultured hepatocytes at a high cell density, this activation is quickly ameliorated by LAR tyrosine phosphatase.[30] Moreover, phosphorylation of Ser985 in the juxtamembrane domain of c-Met also suppresses HGF-induced c-Met activation.[31,32] Elucidating the mechanisms that negatively regulate c-Met activation will contribute to our understanding of controlled responses to HGF in normal tissues as well as processes mediating intercellular adhesion and tissue regeneration.

A number of investigations have shown that HGF and c-Met play critical roles in the invasive growth of tumor cells.[33] Through its pleiotropic effects on cell scattering, proteinase expression, antiapoptotic signaling, cell proliferation, and angiogenesis, HGF-induced signaling may contribute to multiple steps during malignant tumor progression, including loss of cell-cell adhesion, cell migration, and invasiveness.[34,35] Moreover, high levels of HGF and/or overexpression of c-Met are frequently observed in many types of cancer, and often correlate with poor prognoses.[29]

ROLES OF HGF IN LIVER REGENERATION

LIVER REGENERATION IS induced by a reduction of hepatic volume, such as hepatic resection or loss of hepatocytes due to viruses or drugs; following hepatic resection or injury, mature hepatocytes, which are normally in the 0 phase, shift to the 1 phase upon stimulation with interleukin-6 and tumor necrosis factor (TNF)-α. This reversible effect is essential for growth stimulation. Various growth factors can stimulate hepatocytes at the 1 phase to proliferate beyond the 1/S checkpoint.[36,37] HGF promotes the proliferation of

mature hepatocytes,[2,38] and stimulates hepatic regeneration together with multiple growth factors, such as transforming growth factor (TGF)-α.[39] HGF-mediated signals in hepatocytes are transmitted into the nucleus following c-Met tyrosine phosphorylation primarily through the Ras/MAPK system, resulting in increased cyclin 1 expression.[40] The stimulatory effect of HGF on liver regeneration has also been observed *in vivo* using normal and partially hepatectomized rats, in which treatment with HGF increases the liver weight and the serum albumin concentration.[41]

Additionally, when liver regeneration is induced under conditions in which the proliferation of mature hepatocytes is impaired – for example, by treatment with 2-acetylaminofluorene (2-AAF) followed by partial hepatectomy (PH), hepatic progenitor (stem) cells can be observed.[36,37] These oval-shaped cells are a heterogeneous cell population in the periportal area that consists of cells at different stages of differentiation with egg-shaped nuclei. When 2-AAF/PH rat models were treated with HGF, these oval cells proliferate, and hepatic differentiation was accelerated.[42] It has also been reported that the proliferation of these oval cells is enhanced by adenoviral gene transduction of the HGF gene.[43] Thus, HGF stimulates the proliferation of not only mature hepatocytes but also hepatic progenitor cells, which appear following hepatic injury. On the other hand, hepatoblasts can differentiate into hepatocytes or cholangiocytes. Dexamethasone, oncostatin M, HGF, and an additional signal from the extracellular matrix are required for the induction of hepatic differentiation of hepatoblasts isolated from 14 to 14.5-day-old mouse embryonic livers.[44] These findings suggest that HGF plays an important role in hepatic differentiation as well as the proliferation of hepatoblasts/hepatic progenitor (stem) cells during hepatic development and regeneration. The detailed molecular mechanisms of these functions, however, are unknown.

Recently, several investigations have demonstrated that stem cells, which can differentiate into a variety of cells in addition to blood cells, exist in bone marrow, and that bone marrow stem cells contribute to liver regeneration.[45–48] HGF has been shown to induce the hepatic differentiation of c-Met-bearing, pluripotent progenitor cells from *in vitro* bone marrow experiments.[49,50] Moreover, when bone marrow cells or purified hematopoietic stem cells were transplanted to a murine model of fatal hereditary tyrosinemia, the parenchyma in the recipient liver was reconstituted with hepatocytes derived from the transplanted bone marrow cells, resulting in the rescue of recipient animals.[51,52] Other studies, however, have clearly demonstrated that this phenomenon was not due to hepatic differentiation of the transplanted bone marrow cells but was instead a result of cell fusion between the transplanted bone marrow-derived cells and the native hepatocytes from the recipient.[53–55] Additionally, a controversy about whether bone marrow cells transplanted to a model of liver injury differentiate into hepatocytes[56] or into non-parenchymal cells,[57–59] such as sinusoidal endothelial or stellate cells, has not been settled. Although cells that can differentiate into hepatocytes may exist in bone marrow, the contribution of this phenomenon to liver regeneration should be clarified in future investigations.

ROLES OF HGF IN LIVER INJURY

IN VARIOUS ANIMAL models of acute hepatic failure induced by D-galactosamine, lipopolysaccharide, and CCl_4, HGF attenuates liver injury and improves survival.[60–64] For example, a single dose of rh-HGF rescued mice with fatal hepatic failure induced by agonistic antiFas antibodies (Jo2 antibodies) (Fig. 3). This result was mediated by the antiapoptotic effect of HGF, which is exerted through Bcl-xL. Moreover, it was recently reported that the antiapoptotic effect of HGF involves the PI-3K/Akt signal transduction system and the formation of a complex of c-Met and Fas.[65,66]

Hepatic regeneration is impaired in liver cirrhosis, and studies using animal models of liver cirrhosis have shown that decreased HGF and c-Met expression in cirrhotic liver tissues, which is caused by impaired activa-

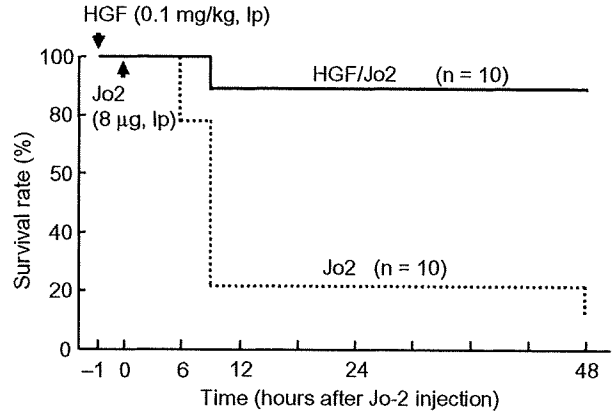

Figure 3 Hepatocyte growth factor (HGF) improves the survival rate of mice with Jo2-induced lethal hepatic failure. When mice with fatal hepatic failure induced by Jo2 antibodies were pretreated with injections of HGF into the peritoneal cavity, the survival rate markedly improved.

tion of HGF and hypoxia, contribute to impaired liver regeneration.[67,68] Moreover, TGF-β expression is induced during the early stages of CCl_4-induced liver cirrhosis following partial hepatectomy, which also results in a suppression of DNA synthesis in hepatocytes.[69] On the other hand, when rh-HGF (0.3 mg/kg) is intravenously administered to rats with dimethylnitrosamine (DMN)-induced liver cirrhosis, the development of hepatic fibrosis is suppressed.[70] This antifibrogenic effect of HGF is not only observed with cirrhosis induced by DMN but also in models of CCl_4- or thioacetamide (TAA)-induced cirrhosis, in which reduced type I collagen and TGF-β mRNA expression levels as well as enhanced expression of albumin mRNA have been observed.[71–77] Additionally, treatment with rh-HGF increased the number of thrombocytes in cirrhotic rats with thrombocytopenia.[78] When partial hepatectomy was performed on cirrhotic livers induced by TAA or CCl_4, followed by HGF treatment, postoperative hepatic functions and the enhancement of liver regeneration were ameliorated.[79,80] Based on these effects of HGF, rh-HGF is an attractive candidate as an antifibrogenic agent for liver cirrhosis.

CLINICAL APPLICATIONS OF HGF

WE HAVE ESTABLISHED an enzyme-linked immunosorbent assay to specifically measure serum levels of the active form of human HGF, and have examined serum HGF levels in patients with various liver diseases.[81–83] Serum HGF concentrations transiently increased in patients with acute hepatitis, reaching a maximum value that was less than 1 ng/mL. In patients with fulminant hepatic failure, however, serum levels of HGF were greater than 1 ng/mL even prior to hepatic encephalopathy, and serum concentrations of HGF were highest in cases that resulted in death. Therefore, measuring serum HGF levels, which is commonly performed in Japan, can be useful for predicting fulminant progression of acute liver diseases and patient prognoses. We are currently working on a semiquantitative HGF measurement kit that allows healthcare providers to quickly and easily ascertain serum HGF levels.[84]

Because HGF inhibits apoptosis and the development of hepatic fibrosis as well as promoting liver regeneration, rh-HGF is promising as a new therapeutic modality for intractable liver diseases, including fulminant hepatic failure and liver cirrhosis. Therefore, we have been collaborating with a pharmaceutical company to make rh-HGF available as a drug.

A pharmacokinetic study of rh-HGF intravenously administered as a bolus indicated that the half-life of rh-HGF is short. c-Met tyrosine phosphorylation in liver tissues, however, was induced even following a single intravenous injection of rh-HGF.[85] Additionally, intravenously administered rh-HGF was primarily targeted to the liver. In addition to liver tissues, an increase in human HGF levels was observed in the adrenal gland, spleen, and kidney.

We did not observe any problematic findings in the single-dose toxicity test. An increase in the urinary excretion of protein and albumin, however, was observed in a repeated-dose toxicity study in rats. Proliferative changes in the glomeruli were also observed in renal tissues. These renal findings, however, were reversible, and an increase in the serum creatinine concentration was not observed.[70] Further, intravenous injections of HGF have been reported to produce dose-related reductions in arterial pressure through venodilatation, which may be mediated by nitric oxide.[86] Indeed, a single bolus injection of rh-HGF moderately decreased the systolic blood pressure of miniature swine in a general pharmacological test (unpubl. data). It should be noted that cardiac toxicities, including left ventricular dysfunction or arrhythmia, were not observed.

Because HGF is a growth factor that stimulates the proliferation of various types of cells including hepatocytes, potential HGF-induced carcinogenesis should be carefully considered. Regarding these effects, for which enhanced and sustained HGF expression induces or suppresses hepatocarcinogenesis in transgenic mice, conflicting results have been reported.[87–89] When rats with liver cirrhosis induced by a choline-deficient, amino acid-defined diet over a period of nine months followed by hepatocarcinogenesis were repeatedly administered rh-HGF, the development of precancerous nodules and hepatocellular carcinomas was not affected by the treatment[90] Even if additional experiments fail to uncover the effects of HGF on carcinogenesis, however, the possibility of HGF-induced carcinogenesis cannot be completely excluded. Therefore, clinical studies of rh-HGF should include a frank assessment of risks vs. benefits.

Fulminant hepatic failure is an acute, progressive, and fatal liver disease. Based on a national survey in Japan, the only effective method to rescue patients with fulminant hepatic failure is liver transplantation, which contributes to an increase in the survival rate (70–80%). Only approximately 25% of all patients, however, are able to undergo liver transplantation, and the survival rate of the patients who do not receive a new liver is still extremely low (20–30%). Therefore, novel therapeutic modalities that have been verified in clinical trials are urgently needed. Thus, we began a clinical trial of

rh-HGF for patients with fulminant hepatic failure. Although rh-HGF has never been administrated to human subjects and the potential for oncogenesis has not been completely excluded, the validity of a clinical trial for patients with fulminant hepatic failure is thought to be ethically sound. Additionally, because HGF and c-Met are known to play a critical role in invasive growth and metastasis of malignant tumor cells, subjects who are suffering from cancer or have recently recovered from cancer should be excluded from these clinical trials.

In September 2005, we started a phase I/II clinical trial of rh-HGF for patients with fulminant hepatic failure as an investigator-sponsored ICH-GCP registered trial. This clinical trial is designed for subjects with fulminant hepatic failure and late-onset hepatic failure, who require a liver transplantation, but cannot undergo the procedure for various reasons, such as an appropriate donor is not available. The survival rate for the subjects that qualified for this clinical trial was approximately 18%. This trial protocol begins with a low dose of rh-HGF, for which the efficacy and safety have been confirmed in preclinical tests. After the safety of a repeated dose of rh-HGF in a group of four subjects is confirmed by an independent data monitoring committee, the dose is increased in a stepwise fashion (cohort ascending-unit-dose design). As of June 2008, four patients were enrolled in this clinical trial. Although intravenous injections of rh-HGF moderately decreased patient blood pressure, the urinary excretion of albumin did not increase following repeated rh-HGF doses. In two patients, hepatic failure gradually progressed, and they died 68 and 28 days after encephalopathy occurred, respectively. The other two patients were rescued. An independent data monitoring committee reviewed the data for the four subjects (cohort 1), and submitted an advisory report that found that a repeated dose of rh-HGF did not induce severe side-effects. Consequently, the transition to the next four subjects (cohort 2), in which the dose of rh-HGF will be doubled, was approved.

CONCLUSIONS

HGF ACTS AS a stimulator of liver regeneration as well as an antiapoptotic factor. We have been developing rh-HGF as a potential therapeutic agent since its discovery, and have recently initiated an ICH-GCP-registered phase I/ II study for the treatment of fulminant hepatic failure. On the other hand, HGF has various biological effects not only on hepatocytes but also on other epithelial and endothelial cells, and is considered to be an important agent for the regeneration/repair of injured tissues. Therefore, if the safety of rh-HGF can be established through this clinical trial, rh-HGF may become a new treatment strategy for various intractable diseases. Thus, HGF, which was originally identified in the plasma of patients with fulminant hepatic failure (the bedside), has been extensively examined in the laboratory (the bench), and is now returning as a therapeutic agent for various intractable diseases (the bedside).

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