

Anti-inflammatory effects of hepatocyte growth factor: induction of interleukin-1 receptor antagonist

Clemens Molnar¹, Elena R. Garcia-Trevijano², Othmar Ludwiczek¹, Dominique Talabot³, Arthur Kaser¹, Jose M. Mato², Gernot Fritsche¹, Günter Weiss¹, Cem Gabay³, Matias A. Avila², Herbert Tilg¹

¹Clinical Division of General Internal Medicine, Clinical Department of Internal Medicine, Innsbruck Medical University, ANICHSTRASSE 35, 6020 INNSBRUCK, Austria

²Division of Hepatology and Gene Therapy, School of Medicine, University of Navarra, Pamplona, Spain

³Division of Rheumatology, University Hospital of Geneva, Geneva, Switzerland

Correspondence: H. Tilg
<herbert.tilg@uibk.ac.at>

ABSTRACT. Hepatocyte growth factor (HGF) prevents liver failure in various animal models including endotoxin-induced acute liver failure. We were interested to find out whether human HGF exerts anti-inflammatory effects by modulation of cytokine synthesis. Therefore, human HepG2 cells were cultured with increasing concentrations of HGF. HGF dose-dependently upregulated the production of interleukin-1 receptor antagonist (IL-1Ra). Incubation of HepG2 cells with interleukin-1 β (IL-1 β) caused an increase in IL-1Ra levels, while interleukin-6 (IL-6) had no effect on IL-1Ra synthesis. Co-stimulation of HepG2 cells with HGF + IL-1 β resulted in a synergistic effect on IL-1Ra mRNA and protein expression. Stimulation of freshly isolated mouse hepatocytes from male C57 BL/6 mice with HGF increased IL-1Ra mRNA and protein synthesis dose-dependently. A co-stimulation with HGF and IL-1 β had a synergistic effect on IL-1Ra mRNA expression but only a partially additive effect on IL-1Ra protein synthesis. HGF-induced IL-1Ra production was significantly decreased by the mitogen-activated protein kinase (MAPK) inhibitor PD98059. Accordingly, HGF stimulation specifically increased MAPK-dependent signalling pathway (p42/44). In contrast, in preactivated PBMC mRNA expression and protein synthesis of IL-1Ra, interleukin-10 (IL-10) and tumor necrosis factor- α (TNF- α) were unaffected after stimulation with HGF. In conclusion, our data suggest that HGF exerts anti-inflammatory effects by modulating the signal transduction cascade leading to increased expression of IL-1Ra, which might explain the protective and regenerative properties of this cytokine in animal models of liver failure.

Keywords: HGF, hepatocytes, HepG2, monocytes, IL-1Ra

INTRODUCTION

Hepatocyte growth factor (HGF) is the most potent mitogen for mature parenchymal hepatocytes in primary culture [1], and seems to be a hepatotrophic factor that acts as a trigger for liver regeneration after partial hepatectomy and liver injury [2, 3]. Several reports have shown that HGF is a multifunctional and pleiotropic cytokine affecting various target cells [4-7]. HGF acts as a mitogen, morphogen and motogen [4-7]. HGF has been shown to prevent liver failure in various models, acting as an anti-hepatitis cytokine [8, 9]. Rats injected intravenously with HGF showed reduced hepatotoxic responses to α -naphthylisothiocyanate administration [8]. These results were confirmed by Ishiki *et al.*, who demonstrated protective effects of HGF in mice injected with α -naphthylisothiocyanate or CCl₄ [9]. Some of the possible mechanisms for these protective properties of HGF, such as increased hepatic prostaglandin (PG) E2 production following D-galactosamine administration, and suppressed activation of the Fas and Fas ligand system, have

been elucidated [10, 11]. Interaction with the cytokine cascade could be another mode of action.

The liver is thought to play a central role in limiting local and systemic inflammation. Indeed, a hepatocyte-specific inhibitor of RNA and protein synthesis, D-galactosamine, sensitizes animals to the lethal effects of lipopolysaccharide (LPS) and tumor necrosis factor (TNF), suggesting that proteins synthesized in the liver in response to LPS and inflammatory cytokines somehow attenuate the biological effects of these mediators [12, 13]. TNF is a prototypic, pro-inflammatory cytokine, which has been demonstrated to be of importance in animal models of acute liver failure [14]. Gene transfer with human TNF receptor p55, a natural inhibitor of TNF, has been shown to reduce mortality in lethal endotoxemia and thereby to attenuate liver inflammation [15]. On the other hand it has been demonstrated that the prototype anti-inflammatory cytokine, namely interleukin-10 (IL-10), is also able to prevent experimental liver failure [15, 16]. Recently, it has been shown in a mouse model of turpentine-induced inflammation, that the magnitude of the nonspecific inflammatory

response and its successful outcome results from the balance between interleukin-1 (IL-1) and interleukin-1 receptor antagonist (IL-1Ra) [17]. The physiological function of IL-1Ra has been demonstrated using IL-1Ra-knockout mice, which were more susceptible to lethal endotoxemia than wild-type mice [18]. In an animal model of experimental hepatitis, the protective role of IL-1Ra was demonstrated using blocking monoclonal antibodies against IL-1Ra [19]. The elevated ratio of IL-1Ra to IL-1 β in patients with fulminant hepatic failure who survived compared with those who died also suggests a protective role for IL-1Ra in liver failure [20]. Therefore, besides HGF, a whole panel of pro- and anti-inflammatory cytokines seems to be involved in the pathophysiology of acute liver injury.

It is well known that pro-inflammatory cytokines such as IL-1 α and IL-1 β enhance HGF gene expression *in vitro* [21, 22]. In contrast however, it is not known whether HGF is able to modulate cytokine synthesis. In the present study, we examined the production of the acute phase protein IL-1Ra by HepG2 cells and by mouse hepatocytes, in response to HGF treatment. Beilmann *et al.* demonstrated a marked induction of *c-met* HGF receptor expression in activated monocytes, in particular under conditions resembling inflammation, suggesting that HGF might activate monocytes during inflammation and modulate specific monocyte/macrophage functions at the site of the inflammatory reaction [23]. Therefore, we additionally examined whether HGF affects production of the pro-inflammatory cytokine TNF- α or the anti-inflammatory cytokines IL-10 and IL-1Ra in human mononuclear cells.

MATERIALS AND METHODS

Reagents

Recombinant human HGF was provided by Genentech Inc. (South San Francisco, CA, USA). Phorbol 12-myristate 13-acetate (PMA), phytohemagglutinin (PHA) and endotoxin (LPS; *E.coli* 055:B5) as well as collagenase and polymyxin B sulfate were from Sigma Chemical Co. (Vienna, Austria). RPMI 1640 and phosphate-buffered saline (PBS) were from Seromed (Biochrom KG, Berlin, Germany). Minimal essential medium (MEM), fetal bovine serum (FBS), non-essential amino acids, glutamine and antibiotics (penicillin and streptomycin) were from Invitrogen (Karlsruhe, Germany). IL-1 β was from R&D Systems (Minneapolis, USA). Mitogen-activated protein kinase (MAPK)/extracellular regulated kinase kinase-1 (MEK-1) inhibitor PD98059 and p38-MAPK inhibitor SB203580 were from Calbiochem (San Diego, CA, USA).

HepG2 cell culture

The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (Bethesda, MD, USA) at passage number 76. The cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), streptomycin (100 mg/mL) and penicillin (100 units/mL). Cells were grown at 37 °C in 75 cm² plastic flasks in a humidified atmosphere containing 5% CO₂. The medium was replaced twice a week. Every seven days the cells were trypsinized and subcultured at a ratio of 1:6. For experi-

ments, cells were seeded at a density of 0.7x10⁶/mL in six-well polystyrene plates containing 2 mL of medium. All experiments were performed with addition of 5 μ g/mL polymyxin B to rule out the possibility that LPS-contamination might stimulate IL-1Ra. HepG2 cells were incubated for 48 h until they reached confluence, subsequently medium was changed and respective cytokines were added in RPMI 1640. Supernatants were collected after 2 h, 4 h, 8 h, 12 h and 24 h and cytokine detection was carried out using ELISA.

Mouse hepatocyte isolation and treatments

Hepatocytes were isolated from male C57 BL/6 mice (three months old) by the two step EGTA-collagenase perfusion method [24]. Cell viability was measured using the Trypan blue-exclusion test: it was always above 90% in all experiments. Cells were seeded at a density of 3x10⁶ cells per dish onto collagen-coated 60 mm dishes in MEM supplemented with 10% FBS plus non-essential amino acids, glutamine and antibiotics (penicillin 100 units/mL and streptomycin 100 mg/mL). After 2 h of culture, medium was replaced by fresh medium without serum, and then cells were treated with increasing concentrations of HGF (0.1, 10, 100 ng/mL) and/or IL-1 β (1 ng/mL) for the indicated time periods (6 h for RNA isolation and 24 h for ELISA). Supernatants were collected and cytokine detection was carried out using an ELISA.

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from the heparinized blood from healthy volunteers as previously described [25]. In all experiments, except those with LPS, 5 μ g/mL of polymyxin B were included in the culture medium. PMA and PHA were used at a final concentration of 1 and 10 ng/mL. As stimulation with PHA and LPS has been demonstrated to cause an upregulation in HGF-receptor messenger RNA (mRNA) [23], in some experiments PBMC were preincubated for 15 h with PMA, PHA or LPS before addition of HGF. The total incubation period in these experiments was 48 h. After incubation, PBMC cultures were frozen and thawed three times to allow for the release of intracellular cytokines. After centrifugation, cytokine detection was carried out using an ELISA. The amount of IL-1Ra, IL-10 and TNF- α reported in these experiments represents the total amount generated (secreted and cell-associated).

Elisa

Commercially available „sandwich“ ELISA kits were used to determine cytokine levels in the supernatants and cell lysates of HepG2 cells and PBMC. Human TNF- α and IL-1Ra were analysed using an ELISA obtained from R&D Systems, and IL-10 was measured using an ELISA obtained from CLB (Amsterdam, The Netherlands) according to the manufacturer's instructions. The lower detection limit for these assays was 5 pg/mL, and they did not show any cross-reactivity with various other cytokines. Levels of murine IL-1Ra were measured in supernatants from mouse primary hepatocytes using a modification of a specific ELISA [26], as previously described [27].

Northern blot analysis

HepG2 cells were seeded at a concentration of 0.7×10^6 /mL in a six-well plate for 48 h and then stimulated with HGF, IL-6 \pm HGF and IL-1 β \pm HGF for 4 h. The supernatants were discarded and the cell monolayer was washed twice with PBS. Then total RNA was then isolated by means of the guanidinium-isothiocyanate phenol extraction method using the RNA-Clean system (AGS GmbH, Heidelberg, Germany) following the manufacturer's instructions. 1×10^7 PBMC were suspended in RPMI 1640 and 5% FCS and cultured for 4, 8, 12 and 20 h, respectively, after stimulation with HGF, PMA \pm HGF, PHA \pm HGF or LPS \pm HGF. In parallel experiments, cells were preincubated with PMA, PHA or LPS for 15 h and then stimulated with HGF for an additional 5 h. PBMC were washed once with PBS. Total cellular RNA was prepared as described above. Northern blot analysis was performed as previously described [25]. The TNF probe used was a 505-bp fragment of the human TNF complementary DNA (cDNA) subcloned in pUC18 (R&D Systems). The IL-1Ra probe was a 480-bp fragment of human IL-1Ra cDNA subcloned in pUC18 (R&D Systems). The IL-10 probe was a fragment of 1.6 kb of human IL-10 cDNA, subcloned in the plasmid vector pcDSRalpha (ATCC, Bethesda, MD, USA). Control-hybridizations were performed with the rat cDNA of the housekeeping gene glyceraldehyde-3-phosphatedehydrogenase (GAPDH).

Total RNA isolation in mouse hepatocytes

After stimulation, hepatocyte cultures were washed with cold PBS, subsequently lysed in acid guanidinium-isothiocyanate and total RNA was purified as described [28]. The reverse transcriptase reaction was performed with 1 μ g of total RNA, random hexamers (Roche, Mannheim, Germany), and MMLV reverse transcriptase (GIBCO, Gaithersburg, MD, USA) according to the manufacturer's directions.

TaqMan real-time polymerase chain reaction (PCR)

Primers and probes. TaqMan real-time PCR [29, 30] primers and the TaqMan probe were designed using the Primer Express Software (PE Applied Biosystems, Vienna, Austria). Primers were purchased from MWG Biotech (Ebersberg, Germany), probes were from Microsynth (Balgach, Switzerland). For quantification of IL-1Ra mRNA, the following primers were used: 5'-TCT CCT TCT CAT CCT TCT GTT TCA T-3', 5'-CTT GCA GGG TCT TTT CCC AG-3'; the TaqMan probe 5'-CAG AGG CAG CCT GCC GCC C-3' was labeled with the reporter fluorescent dye 6-carboxyfluorescein (FAM) at the 5' end and with the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3' end. For quantification of IL-1Ra mRNA levels in mouse hepatocytes, the TaqMan real-time PCR (Perkin Elmer, Vienna, Austria) method was used. For the standard curve, serially diluted mRNA prepared from mouse spleen was subjected to reverse transcription as described above. Generation of the standard curve and the PCR reaction were performed as described elsewhere [31]. To minimize intra-assay and interassay variability caused by differences in reverse transcriptase efficiency, IL-1Ra quantities were normalized to the amount of 18S-rRNA in specimens. For

18S-rRNA quantification, the 18S-rRNA reaction mix (Perkin Elmer) was used.

Immunoblot analysis

Cells were lysed by adding 1X SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). Then, cell extracts were sonicated for 15 seconds, heated to 95 °C for 5 min and microcentrifuged for 5 min, before 20 μ L of each sample were loaded onto a 12.5% SDS-PAGE gel. After the transfer blot, nitrocellulose membranes (Amersham, UK) were washed with 25 mL TBS for 5 min at room temperature (RT). Membranes were subsequently incubated in 25 mL of blocking buffer (1X TBS, 0.1% Tween-20 with 5% w/v nonfat dried milk) for 1h at RT. Then, blots were washed three times with 1X TBS, 0.1% Tween-20, before incubation with the primary antibody (rabbit polyclonal IgG, affinity purified phospho-p44/42 MAPK Thr202/Tyr204, Cell Signaling Technology, USA). After another three washing steps, membranes were incubated with an HRP-conjugated secondary antibody (HRP goat anti rabbit, DAKO, Denmark), washed three times and subsequently incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA). Finally, membranes were exposed to X-ray film (Amersham, UK).

Statistics

Data are presented as mean \pm SEM. Statistical analysis was performed using the nonparametric Wilcoxon test. *p* values less than 0.05 were considered to be significant.

RESULTS

Effects of HGF on IL-1Ra synthesis in HepG2 cells

HepG2 cells were cultured with HGF alone or in combination with IL-6 or IL-1 β for 24 h. Stimulation with HGF showed a dose-dependent pattern of IL-1Ra induction. This effect was observed after stimulation with 1 ng/mL HGF, and reached a maximum with 100 ng/mL HGF (3.4 ng/mL IL-1Ra *versus* 0.45 ng/mL in control supernatants) (*figure 1*). Time course experiments with HGF alone (1 ng/mL; 10 ng/mL) showed an elevation of IL-1Ra levels already after 2 h, reaching a maximum after 8 h (1 ng/mL) and 12 h (10 ng/mL), respectively and then a slight decline within 24 h (*figure 2*). Whereas IL-1Ra levels were unaffected after stimulation with IL-6 alone, IL-6 slightly potentiated the HGF-induced IL-1Ra production (data not shown). HGF exhibited a strong synergistic effect on IL-1 β -induced IL-1Ra production (*figure 3*). IL-1 β is known to be a potent inducer of IL-1Ra production in HepG2 cells and hepatocytes [32]. IL-1 β at a concentration of 1 ng/mL had a stronger stimulatory effect than HGF at any concentration tested (0.1 to 100 ng/mL) (data not shown).

Effects of HGF on IL-1Ra mRNA expression in HepG2 cells

HepG2 cells were incubated with increasing concentrations of HGF (0.1 to 10 ng/mL) alone or in combination with IL-6 or IL-1 β for 4 h. RNA was isolated as described

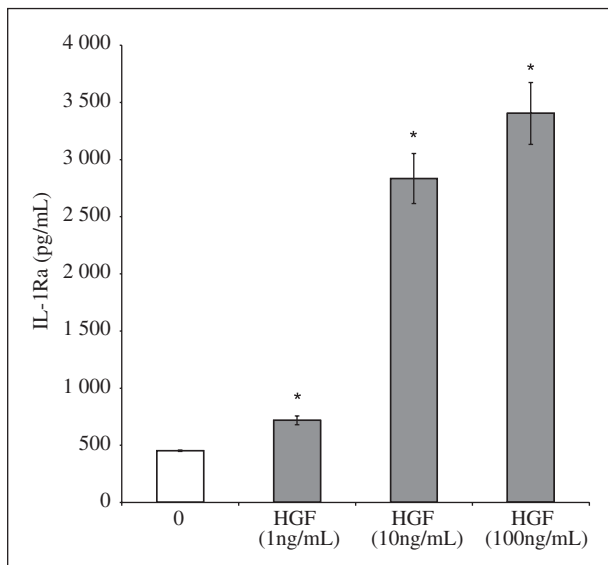


Figure 1

HGF induces IL-1Ra in HepG2 cells. HepG2 cells were cultured in medium alone (open bar) or stimulated with HGF (grey bars) for 24 h. Values are expressed as mean \pm SEM (n = 6); (*P < 0.05 compared to medium alone).

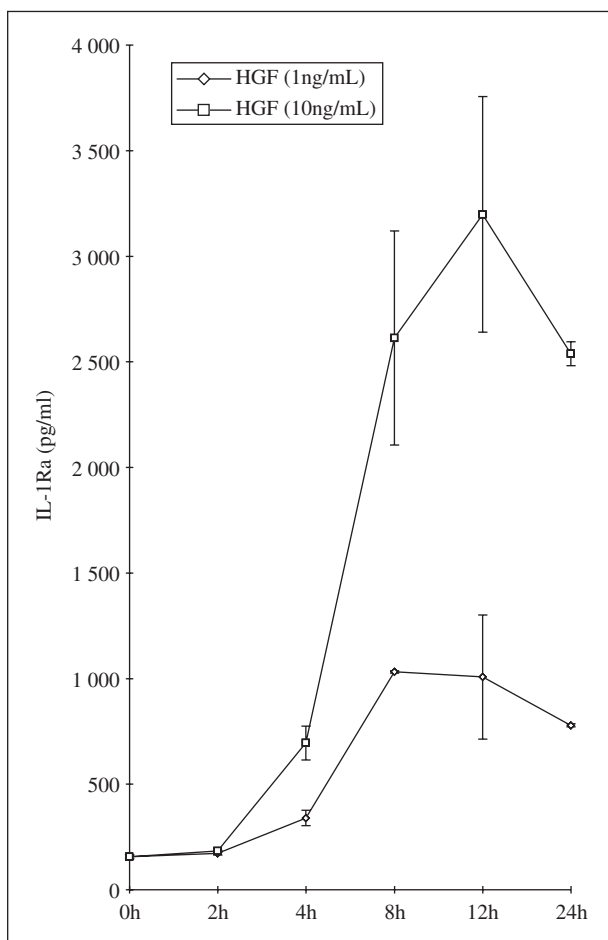


Figure 2

Time course of IL-1Ra production. HepG2 cells were cultured with HGF for 2, 4, 8, 12 and 24 h. Data represent mean \pm SEM (n = 3).

in Materials and methods. Culture medium alone caused a weak expression of IL-1Ra, and HGF dose-dependently

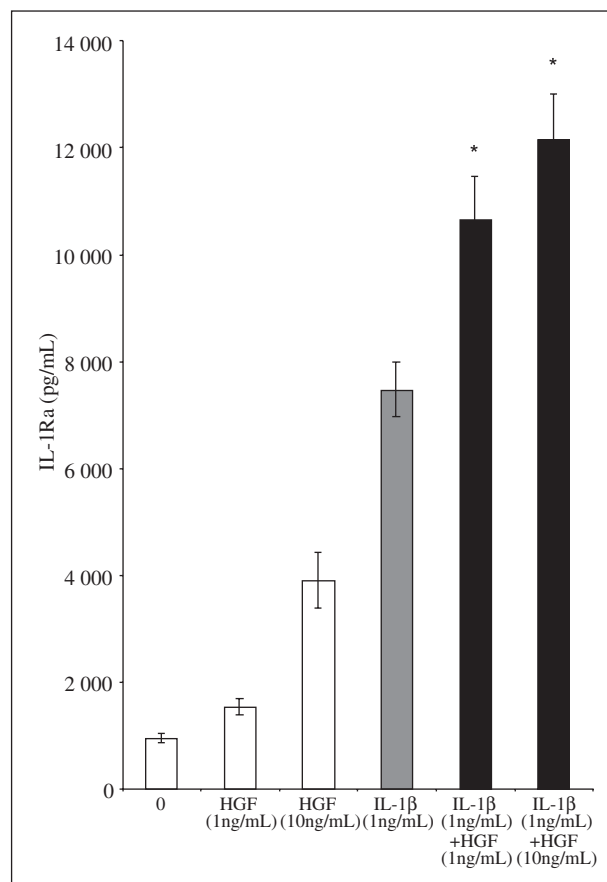


Figure 3

Synergistic effect of HGF on IL-1 β -induced IL-1Ra production in HepG2 cells. HepG2 cells were cultured in the absence or presence of HGF (open bars), IL-1 β (grey bar) or with a combination of HGF and IL-1 β (solid bars) for 24 h. Values are expressed as mean \pm SEM (n = 6); (*P < 0.05 compared to IL-1 β alone).

upregulated IL-1Ra mRNA expression (figure 4A). IL-1 β was a potent inducer of IL-1Ra mRNA expression (figure 4B). In accordance with the protein data, costimulation with HGF enhanced IL-1 β -induced IL-1Ra mRNA levels (figure 4B). IL-1Ra mRNA expression was unaffected after incubation with IL-6 alone (data not shown), but co-stimulation with HGF dose-dependently upregulated IL-1Ra mRNA expression (data not shown).

Effects of HGF on IL-1Ra synthesis in mouse hepatocytes

To see if the effects described above for a human hepatocellular carcinoma cell line could also be seen for primary hepatocytes, we treated freshly isolated hepatocytes with increasing concentrations of HGF (0.1, 10, 100 ng/mL) \pm IL-1 β (1 ng/mL) for 24 h. HGF dose-dependently upregulated the synthesis of IL-1Ra, an effect, which was found even with 0.1 ng/mL HGF and reached a maximum with 100 ng/mL HGF (10.7 ng/mL IL-1Ra versus 7.9 ng/mL in control supernatants) (figure 5). The high basal levels of IL-1Ra, especially after stimulation with IL-1 β (1 ng/mL), masked the synergism between HGF and IL-1 β on protein accumulation in conditioned media, and a significant effect was only observed at the highest concentration tested (15.4 \pm 1.4 ng/mL IL-1Ra with IL-1 β versus 16.6 \pm 1.6 ng/mL IL-1Ra with IL-1 β + 100 ng/mL HGF, P < 0.05)

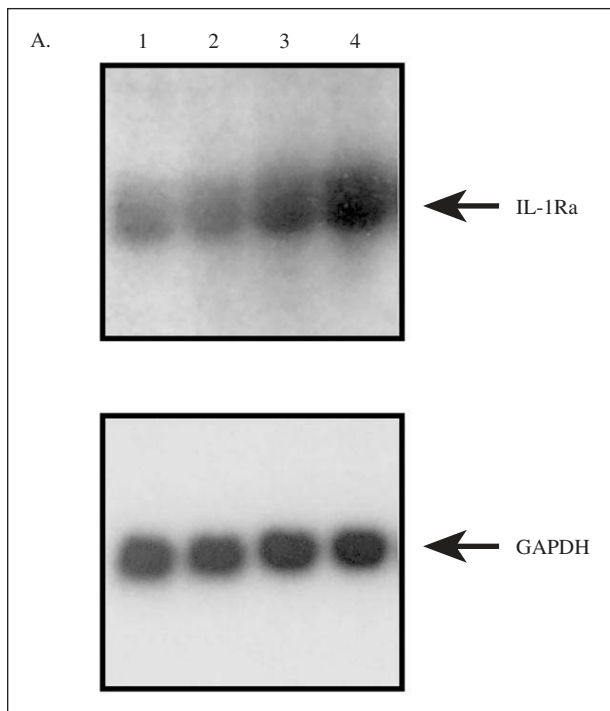


Figure 4A

Induction of IL-1Ra mRNA expression by HGF in HepG2 cells. HepG2 cells were cultured in medium alone (lane 1) or stimulated with 0.1, 1 and 10 ng/mL HGF (lane 2, 3, 4, respectively). After 4 h, total RNA was extracted, gel-electrophoresed, blotted onto nylon membranes and hybridized with IL-1Ra and GAPDH probes.

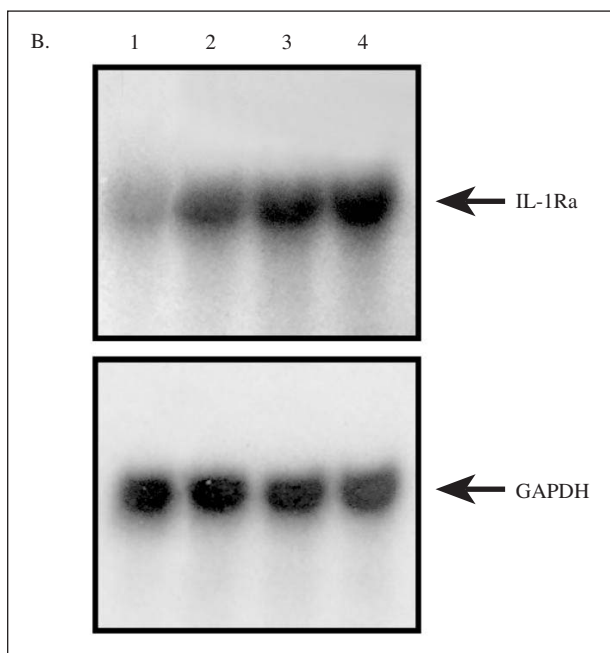


Figure 4B

Costimulatory effect of HGF on IL-1β-induced IL-1Ra mRNA expression in HepG2 cells. HepG2 cells were cultured in medium alone (lane 1), or stimulated with 1 ng/mL IL-1β (lanes 2-4) in the absence (lane 2) or presence of 1 and 10 ng/mL HGF (lane 3, 4, respectively). After 4 h, total RNA was extracted, gel-electrophoresed, blotted onto nylon membranes and hybridized with IL-1Ra and GAPDH probes.

(data not shown). As shown for HepG2 cells, IL-1β at a concentration of 1 ng/mL had a stronger stimulatory effect than HGF at any concentration tested (0.1 to 100 ng/mL).

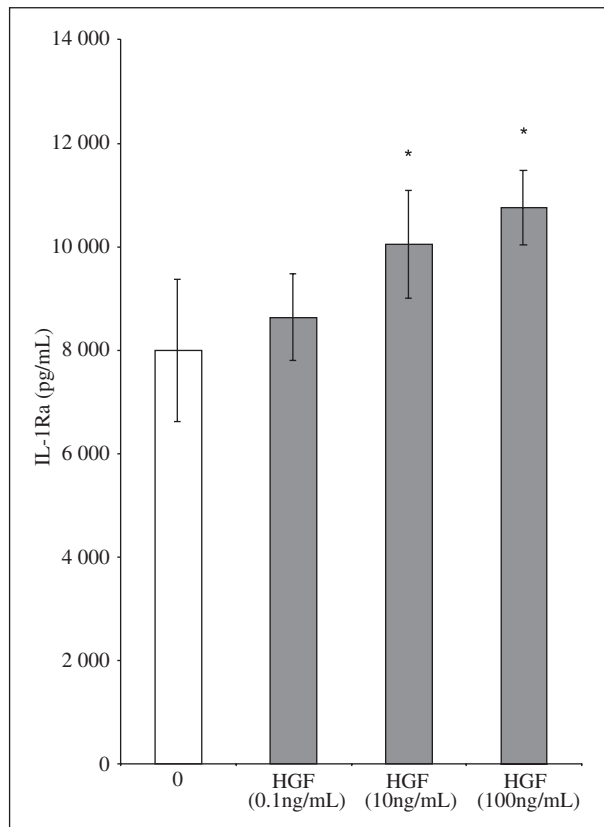


Figure 5

HGF induces IL-1Ra in mouse hepatocytes. Hepatocytes were cultured in medium alone (open bar) or stimulated with HGF (grey bars) for 24 h. Values are expressed as mean ± SEM (n = 6); (*P < 0.05 compared to medium alone).

Effects of HGF on IL-1Ra mRNA expression in mouse hepatocytes

Mouse hepatocytes were incubated with HGF alone, IL-1β alone or with a combination of these two cytokines for 6 h. HGF alone dose-dependently upregulated IL-1Ra mRNA expression and significantly increased IL-1β-induced IL-1Ra mRNA expression (figure 6). This clear synergistic effect, in contrast to the only slight synergistic effect of HGF on IL-1β-induced IL-1Ra protein synthesis, might be explained by the sometimes high spontaneous and IL-1β-induced IL-1Ra production at the protein level in murine hepatocytes, which could have concealed the synergism between HGF and IL-1β.

Effects of HGF on cytokine synthesis and mRNA expression in human PBMC

PBMC were stimulated with LPS, PMA or PHA in the absence or presence of increasing concentrations of HGF (1 to 100 ng/mL). In addition, PBMC were preactivated with PMA, PHA or LPS and subsequently stimulated with HGF. After a 48 h incubation period, cell lysates were analysed for IL-1Ra, IL-10 and TNF-α concentrations. In all experiments performed HGF was not able to modulate cytokine synthesis itself or to affect LPS-, PMA- or PHA-induced cytokine synthesis (data not shown).

HGF alone was not able to upregulate the expression of IL-1Ra mRNA or to affect the marked upregulation of IL-1Ra mRNA expression observed with PMA and LPS

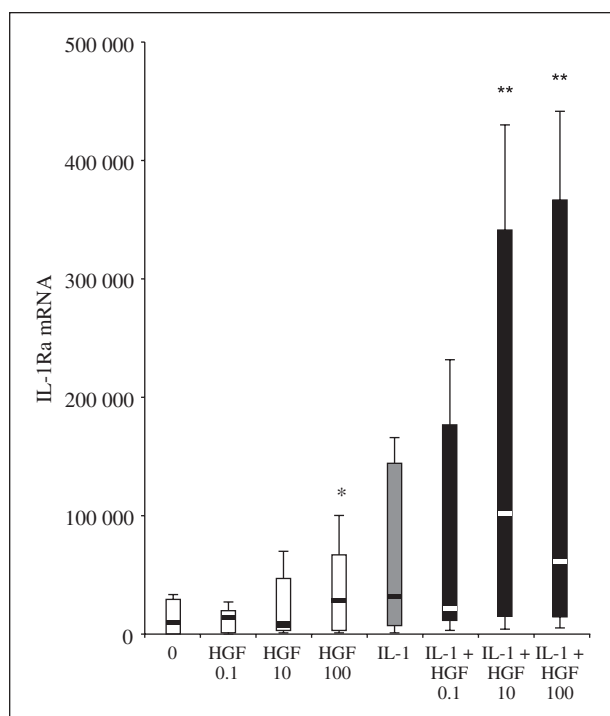


Figure 6

HGF induces IL-1Ra mRNA expression in mouse hepatocytes and potentiates IL-1 β -induced IL-1Ra mRNA expression. Hepatocytes were cultured in the absence or presence of HGF (ng/mL) (open bars), IL-1 β (1 ng/mL) (grey bar) or with a combination of HGF and IL-1 β (solid bars). After 6 h, total RNA was isolated and PCR performed as described in Material and methods. Values are expressed as mean \pm SEM (n = 6); (*P < 0.05 compared to medium alone and **P < 0.05 compared to IL-1 β alone).

(data not shown). Moreover, HGF failed to modulate PMA/PHA-induced TNF- α mRNA and IL-10 mRNA expression (data not shown). Also, after preactivation with PMA, PHA or LPS, HGF had no effect on IL-1Ra, IL-10 and TNF- α mRNA expression (data not shown).

Modulation of signal transduction pathways by HGF

To study the mechanism underlying IL-1Ra induction by HGF, we investigated the modulatory potential of this cytokine on signal transduction pathways underlying IL-1Ra expression. To this end, we first studied the effects of pharmacological inhibition of signal transduction processes by analysing the influence of the MAPK inhibitors, MEK-1 inhibitor PD98059 and p38-MAPK inhibitor SB203580 on HGF-mediated IL-1Ra induction in HepG2 cells. By treatment with PD98059 (1 to 35 μ M), HGF-induced IL-1Ra synthesis was significantly decreased. This effect was already seen with 1 μ M and reached a maximum with 35 μ M (figure 7). However, treatment with the p38-MAPK inhibitor SB203580 (35 μ M) had no significant effect on HGF-induced IL-1Ra synthesis (figure 7).

In order to further elucidate the regulatory network between HGF and signal transduction processes, we performed Western blot experiments using an antibody directed against phosphorylated p42/44 MAPK. As can be seen in figure 8, HGF specifically and rapidly stimulated the phosphorylation of p42/44 in HepG2 but not in THP-1 human monocytic cells (not shown).

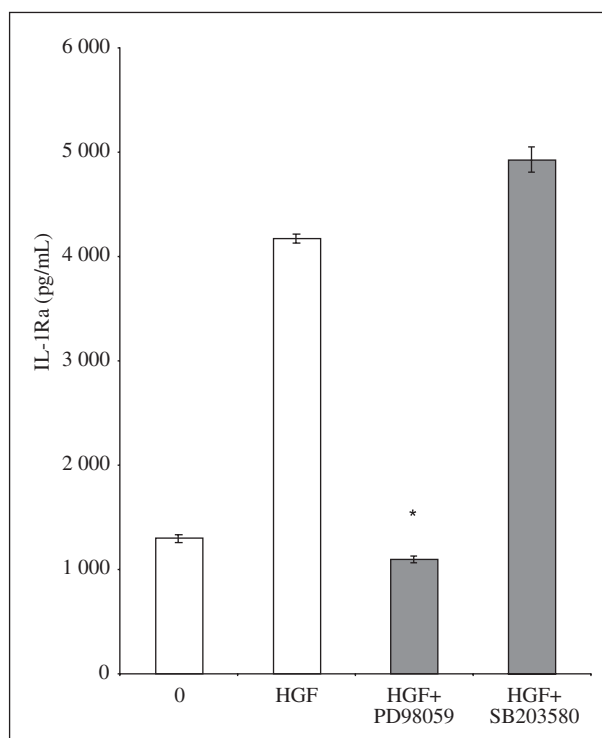


Figure 7

Suppression of HGF-mediated IL-1Ra induction by MEK-1 inhibitor PD98059 in HepG2 cells. HepG2 cells were cultured in the absence or presence of HGF (10 ng/mL) (open bars) or treated with HGF (10 ng/mL) + PD98059 (35 μ M) or HGF (10 ng/mL) + SB203580 (35 μ M) (grey bars) for 24 h. Values are expressed as mean \pm SEM (n = 4); (*P < 0.05 compared to HGF alone).

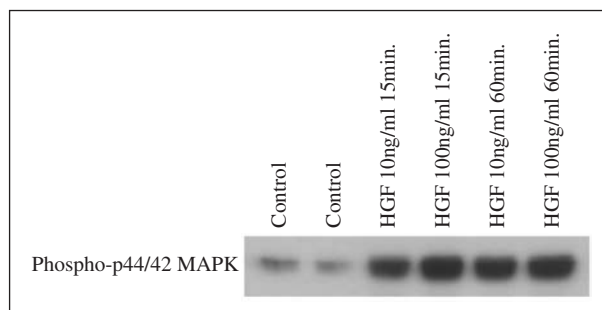


Figure 8

HGF induces the phosphorylation of stress-induced MAPK. HepG2 cells were stimulated with the indicated dosages of HGF for 15 to 60 min or left untreated. Total protein lysates were then prepared, and Western blotting for the stress-induced MAPK p42/44 (MEK1/2) was carried out as detailed in Materials and methods. One of three representative experiments is shown.

DISCUSSION

HGF, originally described as a potent mitogen for hepatocytes, has, in recent years, been identified as a multifunctional cytokine. HGF exerts protective actions in several acute phase models and is able to prevent acute liver injury in rats. Nevertheless, the regenerative effect of HGF is not liver-specific as it has also been detected in the gastrointestinal tract [33] and in the kidney [34]. HGF has also been shown to possess cytoprotective effects on hepatocytes during hepatitis caused by hepatotoxins such as α -naphthylisothiocyanate or CCL₄ in various animal models [8, 9]. Consistent with this anti-hepatitis effect of

HGF *in vivo*, elevated HGF mRNA expression could be detected in liver biopsies from patients with acute alcoholic hepatitis, and a positive correlation between elevated HGF serum levels and hepatocyte proliferation in the biopsies was demonstrated [35, 36]. Hemodialysis is associated with elevated HGF serum levels [37]. In hepatitis C virus positive patients, subjected to hemodialysis, liver damage caused by the hepatitis C virus was reduced compared to hepatitis C virus-positive patients without renal disease and lower HGF serum levels, suggesting that HGF may be responsible for this anti-hepatitis effect *in vivo* [37]. It has been reported that HGF has a protective effect on interferon gamma (IFN- γ)-induced cytotoxicity in mouse hepatocytes [38]. HGF inhibits DNA fragmentation induced by IFN- γ in primary cultures of hepatocytes, suggesting that HGF inhibits apoptosis caused by IFN- γ [38]. Some of the potential mechanisms involved in the anti-hepatitis properties of HGF have been identified. Okano *et al.* provided evidence that this protective effect might be mediated via increased hepatic PG E2 production [10]. Kosai *et al.* showed that HGF inhibits excessive activation of Fas and Fas ligand by blocking the Fas-mediated signal pathway, which plays a key role in the development of fulminant hepatic failure caused by hepatitis virus infection or endotoxin [11]. Additionally, HGF induces Bcl-xL mRNA expression and protein synthesis in the liver, a protein belonging to the family of the proto-oncogene Bcl-2, which has an inhibitory effect on Fas-mediated cell death [11].

As cytokines are involved in various models of acute liver failure [14, 15], we thought that modulation of cytokine synthesis by HGF in HepG2 cells and hepatocytes could be a mode of action for its protective effects. HepG2 cells, a hepatoma cell line widely used for the study of acute phase proteins such as IL-1Ra, retain many functions of parenchymal hepatocytes and have been shown to express the high affinity *c-met* HGF receptor [39]. Systemic inflammation is associated with elevated IL-1 and IL-6 levels resulting in a systemic acute phase response. IL-1 plays a key role in the pathogenesis of chronic inflammation and septic shock [40]. IL-6 regulates a broad spectrum of acute phase proteins in the liver and is an important component of signaling pathways leading to liver regeneration [41]. Primary hepatocytes as well as HepG2 cells, have been previously shown to express mRNA encoding for the secreted form of IL-1Ra, but not for intracellular IL-1Ra [42, 43]. We show here that HepG2 cells and mouse hepatocytes synthesized IL-1Ra when stimulated with HGF, and that this effect was potentiated especially after co-culture with IL-1 β . Based on the findings that HGF serum levels and HGF mRNA expression in the liver increase markedly following acute hepatitis, one may speculate that HGF exerts, at least partially, its regenerative ability by induction of anti-inflammatory cytokines such as IL-1Ra [44, 45]. Our data provide further evidence for a role of HGF in the interaction between the stromal environment and mononuclear cells, as polymorphonuclear cells have been identified as a source of HGF in patients with alcoholic hepatitis [46] and HGF has been shown to be able to control the immunomodulatory functions of monocytes by induction of IL-6 [47].

Several signalling events have been reported after HGF receptor activation [48-51]. MAP kinases are thought to be implicated in a variety of cellular functions such as arachi-

donic acid release and cell growth [52, 53]. It has been shown that HGF activated MAP kinases and arachidonic acid release in primary cultured rat hepatocytes [54, 55]. We show here that MEK-1 inhibitor PD98059 decreases the HGF-induced IL-1Ra synthesis in HepG2 cells. Accordingly, HGF specifically increased the phosphorylation of MAPK p42/44 (MEK1/2) in HepG2 cells indicating that HGF modulates IL-1Ra expression by stimulating stress-induced MAPK signalling cascades.

Another mode of action for the anti-inflammatory effects of HGF might be the regulation of pro- and anti-inflammatory cytokines in monocytes. Galimi *et al.* showed that endotoxin- and IL-1 β -activated monocytes clearly expressed the HGF receptor, while the receptor transcript was barely detectable in nonactivated monocytes. Secretion of biologically active HGF by activated monocytes implies an autocrine stimulation [47]. In our experiments we used an approach similar to that used by Beilmann *et al.* [23], preactivating monocytes with PHA, PMA and LPS and then stimulating with HGF. We found however, no effects of HGF on IL-1Ra, TNF- α and IL-10 mRNA expression or protein synthesis. In contrast to the data obtained for HepG2 cells, no phosphorylation of MAPK p42/44 (MEK1/2) was observed in the human monocytic cell line THP-1. These results might explain the different effects of HGF on IL-1Ra production in hepatocytes versus monocytes.

Besides the anti-hepatitis effect, HGF is thought to play an important role in suppressing the onset of hepatic fibrosis induced by different hepatotoxins [56]. As shown in a rat model, HGF gene therapy caused a complete resolution of fibrosis induced by dimethylnitrosamine [57]. In this study, repeated administration of HGF suppressed the expression of transforming growth factor (TGF)- β 1 mRNA induced by dimethylnitrosamine. TGF- β 1 is known to play a major role in the progression of liver cirrhosis [57]. Also IL-1Ra has been demonstrated to limit dimethylnitrosamine-induced fibrosis *in vivo* by competitively blocking the binding of IL-1 to its receptor [58]. Our data suggest that the protective and regenerative properties of HGF observed in several animal models might be partially mediated by its ability to regulate synthesis of IL-1Ra, a natural by occurring inhibitor of inflammation.

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REFERENCES

1. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem Biophys Res Commun* 1984; 120: 1450.

2. Thaler FJ, Michalopoulos GK. Hepatopietin A: partial characterization and trypsin activation of a hepatocyte growth factor. *Cancer Res* 1985; 45: 2545.
3. Kinoshita T, Tashiro K, Nakamura T. Marked increase of HGF mRNA in non-parenchymal liver cells of rats treated with hepatotoxins. *Biochem Biophys Res Commun* 1989; 165: 1229.
4. Boros P, Miller CM. Hepatocyte growth factor: a multifunctional cytokine. *Lancet* 1995; 345: 293.
5. Michalopoulos GK, Zarnegar R. Hepatocyte growth factor. *Hepatology* 1992; 15: 149.
6. Matsumoto K, Nakamura T. Hepatocyte growth factor: molecular structure, roles in liver regeneration, and other biological functions. *Crit Rev Oncog* 1992; 3: 27.
7. Matsumoto K, Nakamura T. Emerging multipotent aspects of hepatocyte growth factor. *J Biochem (Tokyo)* 1996; 119: 591.
8. Roos F, Terrell TG, Godowski PJ, Chamow SM, Schwall RH. Reduction of alpha-naphthylisothiocyanate-induced hepatotoxicity by recombinant human hepatocyte growth factor. *Endocrinology* 1992; 131: 2540.
9. Ishiki Y, Ohnishi H, Muto Y, Matsumoto K, Nakamura T. Direct evidence that hepatocyte growth factor is a hepatotrophic factor for liver regeneration and has a potent antihepatitis effect in vivo. *Hepatology* 1992; 16: 1227.
10. Okano J, Shiota G, Kawasaki H. Protective action of hepatocyte growth factor for acute liver injury by D-Galactosamine in transgenic mice. *Hepatology* 1997; 26: 1241.
11. Kosai K, Matsumoto K, Nagata S, Tsujimoto Y, Nakamura T. Abrogation of fas-induced fulminant hepatic failure in mice by hepatocyte growth factor. *Biochem Biophys Res Commun* 1998; 244: 683.
12. Galanos C, Freudenberg MA, Reutter W. Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc Natl Acad Sci USA* 1979; 76: 5939.
13. Lehmann V, Freudenberg MA, Galanos C. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. *J Exp Med* 1987; 165: 657.
14. Kusters S, Tiegs G, Alexopoulou L, et al. In vivo evidence for a functional role of both tumor necrosis factor (TNF) receptors and transmembrane TNF in experimental hepatitis. *Eur J Immunol* 1997; 27: 2870.
15. Rogy MA, Auffenberg T, Espat NJ, et al. Human tumor necrosis factor receptor (p55) and interleukin 10 gene transfer in the mouse reduces mortality to lethal endotoxemia and also attenuates local inflammatory responses. *J Exp Med* 1995; 181: 2289.
16. Santucci L, Fiorucci S, Chiorean M, et al. Interleukin 10 reduces lethality and hepatic injury induced by lipopolysaccharide in galactosamine-sensitized mice. *Gastroenterology* 1996; 111: 736.
17. Josephs MD, Solorzano CC, Taylor M, et al. Modulation of the acute phase response by altered expression of the IL-1 type 1 receptor or IL-1ra. *Am J Physiol Regul Integr Comp Physiol* 2000; 278: R824.
18. Hirsch E, Irikura VM, Paul SM, Hirsh A. Functions of interleukin 1 receptor antagonist in gene knockout and overproducing mice. *Proc Natl Acad Sci USA* 1996; 93: 11008.
19. Fujioka N, Mukaida N, Harada A, et al. Preparation of specific antibodies against murine IL-1ra and the establishment of IL-1ra as an endogenous regulator of bacteria-induced fulminant hepatitis in mice. *J Leukoc Biol* 1995; 58: 90.
20. Sekiyama KD, Yoshida M, Thomson AW. Circulating proinflammatory cytokines (IL-1 beta, TNF-alpha, and IL-6) and IL-1 receptor antagonist (IL-1Ra) in fulminant hepatic failure and acute hepatitis. *Clin Exp Immunol* 1994; 98: 71.
21. Matsumoto K, Okazaki H, Nakamura T. Up-regulation of hepatocyte growth factor gene expression by interleukin-1 in human skin fibroblasts. *Biochem Biophys Res Commun* 1992; 188: 235.
22. Tamura M, Arakaki N, Tsubouchi H, Takada H, Daikuhara Y. Enhancement of human hepatocyte growth factor production by interleukin-1 alpha and -1 beta and tumor necrosis factor-alpha by fibroblasts in culture. *J Biol Chem* 1993; 268: 8140.
23. Beilmann M, Odenthal M, Jung W, Van de Woude GF, Dienes HP, Schirmacher P. Neoeexpression of the c-met/hepatocyte growth factor-scatter factor receptor gene in activated monocytes. *Blood* 1997; 90: 4450.
24. Douglas DN, Dolinsky VW, Lehner R, Vance DE. A role for SP 1 in the transcriptional regulation of hepatic triacylglycerol hydrolase in the mouse. *J Biol Chem* 2001; 276: 25621.
25. Kaser A, Molnar C, Tilg H. Differential regulation of interleukin 4 and interleukin 13 production by interferon alpha. *Cytokine* 1998; 10: 75.
26. Ruth JH, Bienkowski M, Warmington KS, Lincoln PM, Kunkel SL, Chensue SW. IL-1 receptor antagonist (IL-1ra) expression, function and cytokine-mediated regulation during mycobacterial and schistosomal antigen-elicited granuloma formation. *J Immunol* 1996; 156: 2503.
27. Gabay C, Porter B, Fantuzzi G, Arend WP. Mouse IL-1 receptor antagonist isoforms: complementary DNA cloning and protein expression of intracellular isoform and tissue distribution of secreted and intracellular IL-1 receptor antagonist in vivo. *J Immunol* 1997; 159: 5905.
28. Garcia-Trevijano ER, Latasa MU, Carretero MV, Mato JM, Avila MA. S-Adenosylmethionine regulates MAT1A and MAT2A expression in cultured rat hepatocytes: a new role for S-adenosylmethionine in the maintenance of the differentiated status of the liver. *FASEB J* 2000; 14: 2511.
29. Oldach D. „Real-time“ polymerase chain reaction. *Gastroenterology* 1999; 116: 763.
30. Freeman WM, Walker SJ, Vrana KE. Quantitative RT-PCR: pitfalls and potential. *Biotechniques* 1999; 26: 112.
31. Zoller H, Koch RO, Theurl I, et al. Expression of the duodenal iron transporters divalent-metal transporter 1 and ferroportin 1 in iron deficiency and iron overload. *Gastroenterology* 2001; 120: 1412.
32. Gabay C, Smith Jr. MF, Eidlén D, Arend WP. Interleukin 1 receptor antagonist (IL-1Ra) is an acute-phase protein. *J Clin Invest* 1997; 99: 2930.
33. Fukamachi H, Ichinose M, Tsukada S, et al. Hepatocyte growth factor region specifically stimulates gastro-intestinal epithelial growth in primary culture. *Biochem Biophys Res Commun* 1994; 205: 1445.
34. Kawaida K, Matsumoto K, Shimazu H, Nakamura T. Hepatocyte growth factor prevents acute renal failure and accelerates renal regeneration in mice. *Proc Natl Acad Sci USA* 1994; 91: 4357.
35. Fang JWS, Bird GLA, Nakamura T, Davis GL, Lau JYN. Hepatocyte proliferation as an indicator of outcome in acute alcoholic hepatitis. *Lancet* 1994; 343: 820.
36. Hillan KJ, Logan MC, Ferrier RK, et al. Hepatocyte proliferation and serum hepatocyte growth factor levels in patients with alcoholic hepatitis. *J Hepatol* 1996; 24: 385.
37. Rampino T, Arbustini E, Gregorini M, et al. Hemodialysis prevents liver disease caused by hepatitis C virus: role of hepatocyte growth factor. *Kidney Int* 1999; 56: 2286.
38. Morita M, Watanabe Y, Akaike T. Protective effect of hepatocyte growth factor on interferon-gamma-induced cytotoxicity in mouse hepatocytes. *Hepatology* 1995; 21: 1585.
39. Tajima H, Matsumoto K, Nakamura T. Regulation of cell growth and motility by hepatocyte growth factor and receptor expression in various cell species. *Exp Cell Res* 1992; 202: 423.
40. Dinarello CA. Interleukin-1 and Interleukin-1 Antagonism. *Blood* 1991; 77: 1627.

41. Maeda A, Nagino M, Takeuchi E, Sano T, Kurumiya Y, Nimura Y. Interleukin 6 in bile as an indicator of liver function after hepatectomy in patients with biliary tract carcinoma. *Br J Surg* 1999; 86: 458.
42. Gabay C, Porter B, Guenette D, Billir B, Arend WP. Interleukin-4 (IL-4) and IL-13 enhance the effect of IL-1 on production of IL-1 receptor antagonist by human primary hepatocytes and hepatoma HepG2 cells: differential effect on C-reactive protein production. *Blood* 1999; 93: 1299.
43. Gabay C, Gigley J, Sipe J, Arend WP, Fantuzzi G. Production of IL-1 receptor antagonist by hepatocytes is regulated as an acute-phase protein in vivo. *Eur J Immunol* 2001; 31: 490.
44. Shoitto G, Okana J, Kawasaki H, Kawamoto T, Nakamura T. Serum hepatocyte growth factor levels in liver diseases: clinical implications. *Hepatology* 1995; 21: 106.
45. Masuhara M, Yasunaga M, Tanigawa K, et al. Expression of hepatocyte growth factor, transforming growth factor alpha and transforming growth factor beta 1 messenger RNA in various human liver diseases and correlation with hepatocyte proliferation. *Hepatology* 1996; 24: 323.
46. Taieb JC, Delarche V, Paradis P, et al. Polymorphonuclear neutrophils are a source of hepatocyte growth factor in patients with severe alcoholic hepatitis. *J Hepatol* 2002; 36: 342.
47. Galimi F, Cottone E, Vigna E, et al. Hepatocyte growth factor is a regulator of monocyte-macrophage function. *J Immunol* 2001; 166: 1241.
48. Okano Y, Mizuno K, Osada S, Nakamura T, Nozawa Y. Tyrosine phosphorylation of phospholipase C in c-met/HGF receptor-stimulated hepatocytes: comparison with HepG2 hepatocarcinoma cells. *Biochem Biophys Res Commun* 1993; 190: 842.
49. Mine T, Kojima I, Ogata E, Nakamura T. Comparison of effects of HGF and EGF on cellular calcium in rat hepatocytes. *Biochem Biophys Res Commun* 1991; 181: 1173.
50. Osada S, Nakashima S, Saji S, Nakamura T, Nozawa Y. Hepatocyte growth factor (HGF) mediates the sustained formation of 1,2-diacylglycerol via phosphatidylcholine-phospholipase C in cultured rat hepatocytes. *FEBS Lett* 1992; 297: 271.
51. Graziani A, Gramaglia D, Cantley LC, Comoglio PM. The tyrosine-phosphorylated hepatocyte growth factor receptor associates with phosphatidylinositol 3-kinase. *J Biol Chem* 1991; 266: 22087.
52. Nishida E, Gotoh Y. The MAP kinase cascade is essential for diverse signal transduction pathway. *Trends Biochem Sci* 1993; 18: 128.
53. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem* 1993; 268: 14553.
54. Adachi T, Nakashima S, Saji S, Nakamura T, Nozawa Y. Roles of prostaglandin production and mitogen-activated protein kinase activation in hepatocyte growth factor-mediated rat hepatocyte proliferation. *Hepatology* 1995; 21: 1668.
55. Stolz DB, Michalopoulos GK. Comparative effects of hepatocyte growth factor and epidermal growth factor on motility, morphology, mitogenesis, and signal transduction of primary rat hepatocytes. *J Cell Biochem* 1994; 55: 445.
56. Matsuda Y, Matsumoto K, Yamada A, et al. Preventive and therapeutic effects in rats of hepatocyte growth factor infusion on liver fibrosis/cirrhosis. *Hepatology* 1997; 26: 81.
57. Ueki T, Kaneda Y, Tsutsui H, et al. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nat Med* 1999; 5: 226.
58. Mancini R, Benedetti A, Jezequel AM. An interleukin-1 receptor antagonist decreases fibrosis induced by dimethylnitrosamine in rat liver. *Virchows Arch* 1994; 424: 25.