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BRIEF ARTICLE

Hepatotropic growth factors protect hepatocytes during inflammation by upregulation of antioxidative systems

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Abstract

AIM: To investigate effects of hepatotropic growth factors on radical production in rat hepatocytes during sepsis.

METHODS: Rat hepatocytes, isolated by collagenase perfusion, were incubated with a lipopolysaccharide (LPS)-containing cytokine mixture of interleukin-1β, tumor necrosis factor-α and interferon-γ to simulate sepsis and either co-incubated or pre-incubated with hepatotropic growth factors, e.g. hepatocyte growth factor, epidermal growth factor and/or transforming growth factor-α. Cells were analyzed for glutathione levels. Culture supernatants were assayed for production of reactive oxygen intermediates (ROIs) as well as NO_2^-, NO_3^- and S-nitrosothiols. To determine cellular damage, release of aspartate aminotransferase (AST) into the culture medium was analyzed. Activation of nuclear factor (NF)-κB was measured by electrophoretic mobility shift assay.

RESULTS: Rat hepatocytes treated with the LPS-containing cytokine mixture showed a significant increase in ROI and nitrogen oxide intermediate formation. AST leakage was not significantly increased in cells treated with the LPS-containing cytokine mixture, independent of growth-factor co-stimulation. However, pretreatment with growth factors significantly reduced AST leakage and ROI formation while increasing cellular glutathione. Application of growth factors did not result in increased NF-κB activation. Pretreatment with growth factors further increased formation of NO_2^-, NO_3^- and S-nitrosothiols in hepatocytes stimulated with LPS-containing cytokine mixture. Thus, we propose that, together with an increase in glutathione increased NO_2^-, NO_3^- formation might shift their metabolism towards non-toxic products.

CONCLUSION: Our data suggest that hepatotropic growth factors positively influence sepsis-induced hepatocellular injury by reducing cytotoxic ROI formation *via* induction of the cellular protective antioxidative systems.

Key words: Primary human hepatocytes; Hepatocyte proliferation; Cytokines; Hepatotropic growth factors; Nitric oxide; Glutathione

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INTRODUCTION

After partial hepatectomy, the remaining liver tissue undergoes rapid regeneration of its lost mass. Although it has been studied for many years, the exact mechanisms and interactions of this regenerative process are still the focus of many investigations[1-4]. Despite advances in surgical techniques and perioperative management, liver failure occasionally occurs after extended hepatectomy often being associated with postoperative infections that lead to multiple organ failure and death[5,6].

Although a two-thirds resection of the liver is not fatal, there is increased sensitivity to endotoxin, caused by up-regulation of the toll-like receptor 4, in the period following experimental hepatectomy[7]. Thus, intravenous injection of a sub-lethal dose of lipopolysaccharide (LPS) 48 h after surgery results in a high mortality in rats[8]. LPS directly activates Kupffer cells (the hepatic macrophages) to produce the tumor necrosis factor (TNF)-α and other inflammatory cytokines[9] through activation of the transcription factor, nuclear factor (NF)-κB. During liver regeneration, however, cytokines as well as hepatotropic growth factors have been well demonstrated to be involved in the process of tissue regeneration[10].

Numerous publications suggest a direct link between nitric oxide (NO) production, cellular loss of glutathione (GSH) and reduction of glutathione reductase activity. Thus, depletion of GSH reduces cellular NO levels while increasing superoxide formation, because GSH is an important cofactor for NO synthase[11-16]. Togo *et al*[17] suggest that NF-κB is the major transcription factor regulating the initial steps of liver regeneration. Growth factors, by different mechanisms, play an essential role in cell growth, proliferation, differentiation and DNA synthesis[18-21]. Certain interplays between cytokines and growth factors indeed seem to exist. Inflammatory cytokines increase the intracellular radical formation if not being blocked by intracellular antioxidative systems, e.g. GSH[22]. Therefore, it might be possible that adequate proliferation and regeneration occurs after partial hepatectomy, and the interplay of growth factors and cytokines could be shifted towards protective proliferation rather than hepatocellular injury.

Using an experimental model of sepsis/inflammation, we investigated the effects of hepatotropic growth factors, hepatocyte growth factor (HGF), epidermal growth factor (EGF) and/or transforming growth factor (TGF)-α on radical production and glutathione content in rat hepatocytes that were exposed to an inflammatory cytokine mixture of interferon (IFN)-γ, TNF-α and interleukin (IL)-1β, including LPS.

MATERIALS AND METHODS

Isolation, culture and treatment of primary rat hepatocytes

Rat hepatocytes were isolated from healthy Sprague-Dawley rats with a body weight between 250 and 300 g (Fa. Harlan-Winkelmann, Borchen, Germany) in accordance with the institutional guidelines of the Charité (Berlin, Germany) by collagenase P (Boehringer, Mannheim, Germany) digestion as described previously[23]. Hepatocytes were separated from non-parenchymal cells by differential centrifugation at 50 *g*. Cells were further purified by density gradient centrifugation using 30% Percoll (Pharmacia, Piscataway, NJ, USA). Hepatocyte purity, assessed by microscopy, was > 95% and viability, examined by trypan blue exclusion method, was consistently > 90%. Immediately after isolation, hepatocytes were plated onto gelatin-coated culture dishes (5×10^4 cells/cm^2) in Williams medium E (0.5 mmol/L L-arginine, 1 μmol/L insulin, 15 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum). The next day, experiments were performed in serum-free medium. To imitate inflammation, cells were stimulated with a cytokine mixture (CM) consisting of 100 U/mL IFN-γ, 500 U/mL TNF-α, 10 U/mL IL-1β and 10 μg/mL LPS (*Escherichia coli* 111:B4) for 24 h. To investigate the effect of growth factors on inflammation, cells were either co-stimulated or pretreated (12 h) with 20 ng/mL HGF, 30 ng/mL EGF and/or 20 ng/mL TGF-α.

Measurement of NO_2^-, NO_3^- and S-nitrosothiols

Culture supernatants were assayed for the stable end products of NO oxidation (NO_2^- and NO_3^-) and S-nitrosothiols using modified procedures based on the Griess reaction as described previously[24].

Aspartate aminotransferase measurement

In order to evaluate cellular damage, culture supernatants were measured for aspartate aminotransferase (AST) leakage using commercially available reaction kits (Roche Diagnostics, Mannheim, Germany).

Determination of cellular GSH levels

To evaluate total cellular GSH levels [GSH + oxidized glutathione (GSSG)] cells were suspended in 1 mL metaphosphoric acid (3%) and centrifuged at 1000 *g* for 5 min. Supernatants were adjusted to pH 7.5-8.0 with K_2CO_3. Total cellular GSH was assayed, using an enzymatic recycling procedure, as described previously[22]. Reduced GSH was sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic-acid) (DTNB) to GSSG. The rate of DTNB formation was monitored at 412 nm and glutathione content was determined from a standard curve. To determine GSSG, GSH was masked with 2-vinylpyridine. Then, GSSG was reduced by NADPH to GSH in the presence of glutathione reductase to react again with DTNB. Oxidized and

reduced GSH that may be released in the supernatant were measured in the same way. All data were normalized to total protein content determined from cell pellets.

Determination of superoxide (O_2^-)

Release of O_2^- into culture supernatant was measured by monitoring the superoxide dismutase-dependent reduction of 160 μmol/L ferricytochrome c at 550 nm and 37℃, where 1 mol O_2^- reduces 1 mol ferricytochrome c[14].

Measurement of NF-κB activation

The NF-κB binding activity was analyzed by electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from cells that were homogenized in sucrose buffer (2.1 mol/L sucrose, 10 mmol/L HEPES, 1 mmol/L $MgCl_2$, 5 mmol/L NaF, 0.5 mmol/L Leupeptin, 0.5 mmol/L pepstatin, 5 mmol/L aprotinin, 1 mmol/L DTT, and 0.1 mmol/L PMSF). Nuclei were separated by centrifugation (35 000 *g*, 3 h, 4℃) and washed twice in sucrose washing buffer. Nuclei were resuspended in high-salt buffer (20 mmol/L HEPES at pH 7.9, 1.5 mmol/L $MgCl_2$, 440 mmol/L NaCl, 0.2 mmol/L EDTA, 25% glycerin, 5 mmol/L NaF, 0.1 mmol/L PMSF, 0.5 mmol/L leupeptin, 0.5 mmol/L pepstatin, 5 mmol/L aprotinin, and 1 mmol/L DTT). After incubation on ice for 50 min, nuclei were spun down (14 000 *g*, 15 min, 4℃). Following quantification, protein extracts were stored at -70℃. NF-κB binding activity was performed as described previously[25]. The DNA probe used for EMSA corresponded to the high-affinity kB sequence found in the mouse κ light chain enhancer. Two oligonucleotides (sense 5'-AGCTTGGGGACTTTCCACTAGTACG-3', antisense 5'-AATTCGTACTAGTGGAAAGTCCCCA-3') were annealed to generate a double-stranded probe. Labeling was accomplished by the Klenow fragment of DNA polymerase I in the presence of dGTP, dCTP, dTTP and α[^{32}P] dATP. After labeling, the probe was added to 5 μg nuclear protein and 5 μg poly-dI-dC (Pharmacia Biotech Enzyme GmbH, Freiburg, Germany). Binding reactions were carried out in 10 mmol/L Tris-HCl (100 mmol/L NaCl, and 4% glycerol, pH 7.5) for 30 min on ice. DNA-protein complexes were resolved by electrophoresis in a 4% non-denaturing polyacrylamide gel. Monoclonal antibodies raised against various NF-κB subunits (p50, p52, rel A/p65, C rel, and rel B; Santa Cruz Biotechnology, Heidelberg, Germany) were used to confirm the nature of the DNA-protein complex. Competition assay was performed using unlabeled κB probe in 10- 50- and 100-fold concentrations[25].

Statistical analysis

Results are expressed as mean ± SE of at least five independent experiments ($N = 5$) measured in triplicates ($n = 3$). Data sets were compared by Kruskal-Wallis followed by Dunn's multiple comparison test (GraphPad Prism software; El Camino Real, Sunnyvale, CA USA). $P < 0.05$ was taken as minimum level of significance.

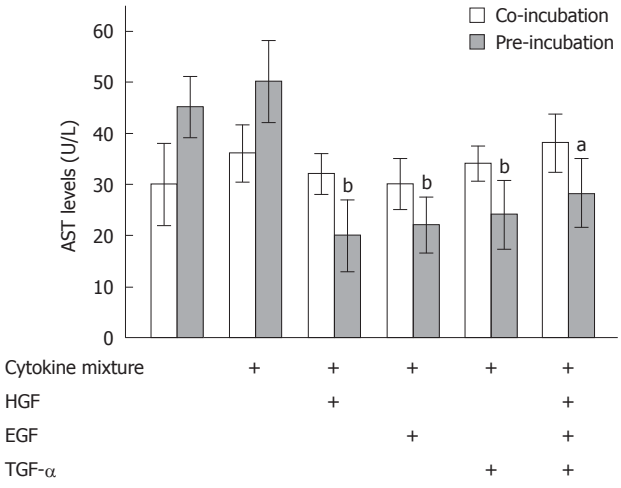

Figure 1 Pre-stimulation with growth factors significantly reduces cellular damage induced by lipopolysaccharide-containing cytokine mixture. Primary rat hepatocytes ($N = 5$, $n = 3$) treated with lipopolysaccharide (LPS)-containing cytokine mixture (CM) for 24 h showed a slight increase in aspartate aminotransferase (AST) leakage into the culture supernatant. Co-incubation with the hepatocyte growth factor (HGF), epidermal growth factor (EGF) and transforming growth factor (TGF)-α, individually or in combination, did not reduce AST leakage significantly (empty bars). However, preincubation with these hepatotropic growth factors resulted in a significant reduction in AST leakage (grey bars). $^aP < 0.05$, $^bP < 0.005$ *vs* corresponding rat hepatocytes treated with LPS-containing CM alone.

RESULTS

Measurement of AST leakage in rat hepatocytes pre- and co-treated with growth factors

Incubation of primary rat hepatocytes ($N = 5$, $n = 3$) with LPS-containing CM led to a slight increase in AST levels in the culture supernatant (30.0 ± 8.0 to 36.0 ± 5.6 U/L and 45.0 ± 6.0 to 50.0 ± 8.0 U/L). Co-incubation with growth factors (20 ng/mL HGF, 30 ng/mL EGF and/or 20 ng/mL TGF-α) individually or in combination did not reduce AST leakage (Figure 1, empty bars). However, preincubation with growth factors significantly reduced AST leakage in cells treated with LPS-containing CM (Figure 1, grey bars).

Determination of reactive oxygen intermediate levels in rat hepatocytes co-treated with growth factors

Incubation of primary rat hepatocytes ($N = 5$, $n = 3$) with the LPS-containing CM slightly increased reactive oxygen intermediate (ROI) production (63.0 ± 6.7 to 80.0 ± 9.0 pmol O_2^-/min every 10^5 cells), while the intracellular glutathione levels were not notably affected as compared to untreated controls. Co-incubation with all growth factors combined slightly reduced ROI levels (Figure 2A and B, empty bars).

Determination of cellular GSH from rat hepatocytes pretreated with growth factors

Rat hepatocytes ($N = 5$, $n = 3$) were pretreated with growth factors 12 h prior to incubation with LPS-containing CM. As observed before, LPS-containing CM

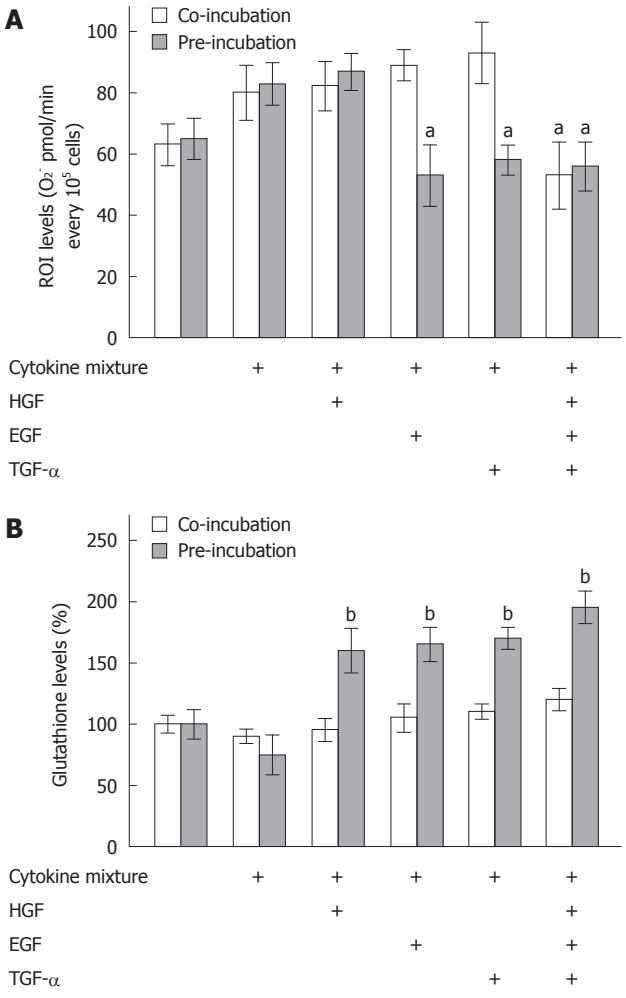

Figure 2 Increased oxidative stress in rat hepatocytes treated with lipopolysaccharide-containing cytokine mixture. A: Treatment of primary rat hepatocytes (N = 5, n = 3) with lipopolysaccharide (LPS)-containing cytokine mixture (CM) for 24 h caused a slight increase in O_2^- production. B: Cellular glutathione levels were not significantly affected by this treatment. Co-incubation with single hepatocyte growth factor (HGF), epidermal growth factor (EGF) or transforming growth factor (TGF)-α did not reduce reactive oxygen intermediate (ROI) production significantly. Co-incubation with the hepatotropic growth factor mixture alone was able to reduce ROI production significantly. Furthermore, co-incubation with the growth factors did not alter cellular glutathione levels (empty bars). On the other hand, preincubation with these growth factors, individually or in combination, significantly reduced ROI production (except for pretreatment with HGF alone). Preincubation with the different growth factors increased cellular glutathione significantly (grey bars). $^aP < 0.05$, $^bP < 0.001$ *vs* corresponding rat hepatocytes treated with LPS-containing CM alone.

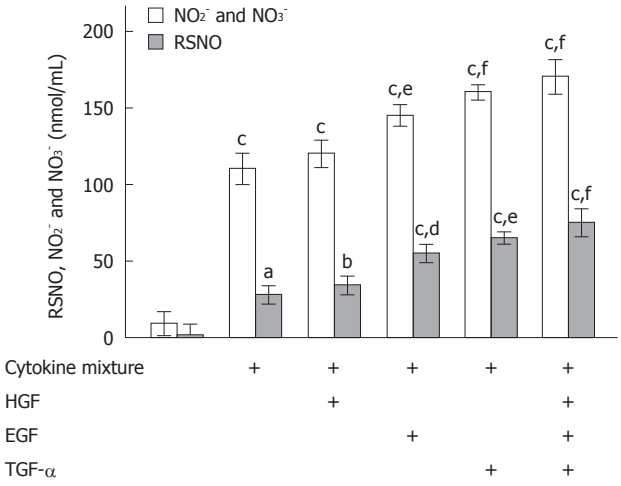

Figure 3 Hepatotropic growth factors increase nitric oxide formation in primary rat hepatocytes. NO_2^- plus NO_3^- (empty bars) and RSNO (grey bars) levels demonstrated a significant increase after hepatocyte (N = 5, n = 3) pretreatment with growth factors and subsequent stimulation with lipopolysaccharide (LPS)-containing cytokine mixture (CM), if compared to treatment with LPS-containing CM alone [except for pretreatment with hepatocyte growth factor (HGF) alone]. $^aP < 0.05$, $^bP < 0.005$, $^cP < 0.001$ *vs* corresponding untreated rat hepatocytes; $^dP < 0.05$, $^eP < 0.005$, $^fP < 0.001$ *vs* corresponding rat hepatocytes treated with LPS-containing CM alone. NO: Nitric oxide; EGF: Epidermal growth factor; TGF: Transforming growth factor.

slightly increased ROI production (65.0 ± 6.7 to 70.0 ± 7.0 pmol O_2^-/min every 10^5 cells) without a notable effect on intracellular glutathione levels (Figure 2A and B, grey bars). However, pretreatment with growth factors, both individually or in combination, significantly reduced ROI production by subsequent stimulation with LPS-containing CM. At the same time, intracellular glutathione levels were significantly increased. This goes along with the reduction in AST leakage observed with growth-factor-pretreated cells (Figure 1, grey bars). Combination of all three growth factors did not further decrease ROI production or increase intracellular glutathione compared to treatment with single growth factors. Pretreatment with HGF alone was not able to reduce ROI production by subsequent stimulation with LPS-containing CM.

Determination of NO_2^-, NO_3^- and S-nitrosothiol formation in rat hepatocytes pretreated with growth factors

Incubation of hepatocytes (N = 5, n = 3) with the LPS-containing CM led to a significant increase in NO production as compared to untreated controls. Formation of stable end products of NO oxidation (NO_2^- and NO_3^-) and S-nitrosothiols was even more increased in hepatocytes pretreated with growth factors when subsequently stimulated with LPS-containing CM. Pretreatment with HGF alone did not further increase NO_2^-, NO_3^- and S-nitrosothiols compared to stimulated cells without pretreatment (Figure 3). This was in accordance with the lack of reduction of ROIs under the same conditions.

Determination of NF-κB activation in rat hepatocytes stimulated with LPS-containing CM pretreated with or without growth factors

Rat hepatocytes, with and without pretreatment with growth factors, were stimulated with LPS-containing CM. NF-κB activation was measured at 0.5, 1, 2, 3 and 6 h after stimulation by EMSA. NF-κB was markedly increased 6 h after stimulation with LPS-containing CM (Figure 4A). Pretreatment with the combined or individual growth factors did not further increase NF-κB activation (Figure 4B). Moreover, growth factors alone (without LPS-containing CM) were not able to cause NF-κB expression (Figure 4B). The competition assay

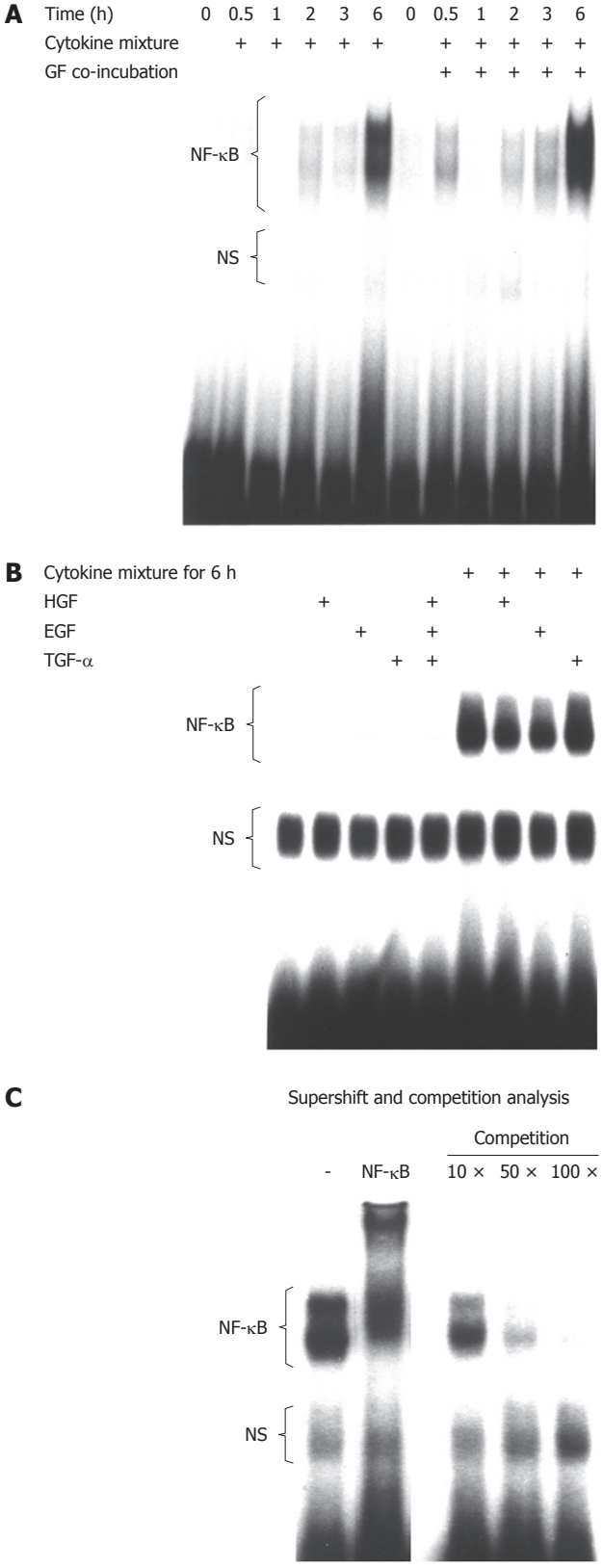

Figure 4 No additional nuclear factor-κB activation by hepatotropic growth factors. Electrophoretic mobility shift assay for nuclear factor (NF)-κB after hepatocyte pretreatment with hepatotropic growth factors and subsequent stimulation with lipopolysaccharide (LPS)-containing cytokine mixture (CM) demonstrated that stimulation with growth factors combined (A) or individually (B) did not further increase NF-κB expression when compared to stimulation with LPS-containing CM alone. Super shift and competition analysis (C) of NF-κB. HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; TGF: Transforming growth factor.

using an excess of unlabeled κB probes demonstrated the specificity of the signal (Figure 4C).

DISCUSSION

Recovery after partial hepatectomy requires an adequate interplay between hepatotropic growth factors and cytokines, as both factors are markedly involved and obviously well-balanced in the process of residual liver tissue proliferation and regeneration[26,27]. In this context, it has been reported that IL-6 plays a crucial role for regeneration, because it is supposed to prime remnant hepatocytes, in a way that they can fully respond to growth factors and enter a pre-replicative phase (G1)[26-28]. However, in our earlier studies, we have found that addition of IL-6 to hepatocyte cultures does not alter ROI or nitrogen oxide intermediate production in the presence of other inflammatory cytokines. When using the mentioned growth factors, there was also a lack of significant alterations in ROIs, and intracellular glutathione was seen. This suggests that growth factors have no direct impact on radical formation, cellular injury and/or cellular antioxidative protection systems.

Under septic or inflammatory conditions, as in the case of any infectious post-operative complication, when both plasma HGF and inflammatory cytokine levels are increased[29-31], cytokine and growth factor compositions might be different. Indeed, increased cytokine levels and protein--protein interactions may have positive and negative effects on liver regeneration[32,33]. Thus, IL-1β is markedly expressed during inflammation, and acts as a very potent inhibitor of hepatocyte proliferation[34]. Clinically observed, severe infections may seriously affect the post-operative course after liver resection, which results in an increased incidence of liver insufficiency and patient loss[6,35,36].

Obviously, cytokines and growth factors act in a well-balanced process under normal regenerative conditions. To gain a better understanding of the avoidance of the deleterious effects of postoperative infectious complications following liver resection, the interplay of growth factors and cytokines was a focus or our attention.

As cytokine reduction is hard to achieve if inflammation has already occurred, we focused our analysis on the effects of hepatotropic growth factor (pre)treatment in hepatocytes exposed to an inflammatory LPS-containing CM.

In the present study, we could demonstrate that growth factors, namely HGF, EGF and/or TGF-α may positively influence influenced cytokine-induced hepatocellular injury. In pre-treated hepatocytes, we found increased NO levels, while the expression of NF-κB was comparable to untreated controls. Our results confirm the study of Kaido *et al*[37] who have reported on successful prevention of post-operative liver failure in cirrhotic rats by continuous HGF supply. They have shown that rats with HGF-secreting fibroblasts (genetically modified to secret rat HGF and implanted in syngeneic rat spleen 7 d prior to exposition exposure of to hepatotoxins)

showed a dramatic resistance to carbon tetrachloride- and LPS-induced liver injury, which resulted in a significantly improved survival rate (80% *vs* 20%). In the same line of evidence, Kosai *et al*[38] have shown that HGF treatment 6 h and 30 min before and 3 h after intra-peritoneal LPS administration resulted in a significant increase of survival in mice (75% *vs* 0%). Although not focusing on pathophysiological interactions of HGF and cytokines, they clearly described HGF-related hepatic protection in case of severe endotoxemia[37,38].

Although several mechanisms may lead to hepatocyte injury, oxidative stress with increased radical formation as a consequence of inflammation, sepsis or ischemia-reperfusion, plays an important role. Intracellular antioxidative systems, e.g. p38-mitogen activated protein kinase or p21 may protect the cells, but they also decrease the hepatocyte proliferation rate by inhibiting hepatic DNA synthesis during the late G1 phase[39,40]. Other intracellular antioxidative systems include upregulation of enzymes e.g. heme oxygenase-1 by NF-κB[41]. We hypothesize that increased glutathione synthesis reduces the amount of cytotoxic radical formation. As further mechanisms improve oxygen supply, subsequent NO-dependent vasodilatation may contribute to the growth-factor-related protection of rat hepatocytes during sepsis. This could explain the results of Seto *et al*[42] who have observed that HGF pretreatment attenuates LPS-induced sinusoidal endothelial cell injury and intra-sinusoidal fibrin deposition.

However, further studies are required because this kind of cell protection was present only in hepatocyte pretreatment. Indeed, direct stimulation of rat hepatocytes with growth factors had no impact on intracellular ROI levels, glutathione content or AST levels under septic conditions.

Nevertheless, this aspect could provide new therapeutic options in case of partial hepatectomy. Pretreatment with hepatotropic growth factors may potentially decrease the incidence of postoperative liver insufficiency in patients undergoing extended liver resection, and subsequent infectious complications by shifting the postoperative course towards growth-factor-related liver tissue proliferation rather than cytokine-related cellular injury.

COMMENTS

Background

The exact mechanisms and interactions of the regenerative process in the liver after partial hepatectomy remain unclear. The well-balanced interplay of liver growth factors and cytokines is strongly interfered when any infectious postoperative complications occur. This effect leads to higher mortality *via* radical formation.

Research frontiers

The deleterious effects of postoperative infectious complications following liver resection have not been examined adequately. In particular, the interplay of pretreated growth factors and cytokines was studied.

Innovations and breakthroughs

The main reason for increased survival of growth-factor-pre-treated hepatocytes is the intracellular antioxidative system that prevents cell-damaging radical formation. Nitric oxide production during sepsis especially increases cell survival.

Applications

Pretreatment with hepatotropic growth factors can be a new therapeutic option in case of patients undergoing extended liver resection and may potentially decrease the incidence of postoperative liver insufficiency.

Terminology

Partial hepatectomy describes the process by which tumors are surgically removed from the liver. Cytokines are regulatory proteins that are released by cells of the immune system and act as mediators in the generation of an immune response.

Peer review

This paper is interesting and reports a large number of experiments. The methodology is well described and the results are clearly shown.

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