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

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Supported by The Federal Ministry of Research (BMBF - 01 GN0984)

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Received: July 12, 2010 Revised: August 16, 2010
Accepted: August 23, 2010
Published online: May 7, 2011

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 NO₂⁻, NO₃⁻ 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 NO₂⁻, NO₃⁻ and S-nitrosothiols in hepatocytes stimulated with LPS-containing cytokine mixture. Thus, we propose that, together with an increase in glutathione increased NO₂⁻, NO₃⁻ 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.

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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. 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.

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. Thus, intravenous injection of a sub-lethal dose of lipopolysaccharide (LPS) 48 h after surgery results in a high mortality in rats. LPS directly activates Kupffer cells (the hepatic macrophages) to produce the tumor necrosis factor (TNF-α) and other inflammatory cytokines 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.

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. Togo et al. 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. 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. 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, Borechen, Germany) in accordance with the institutional guidelines of the Charité (Berlin, Germany) by collagenase P (Boehringer, Mannheim, Germany) digestion as described previously. 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 × 10⁶ cells/cm²) 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 NOx, NO· and S-nitrosothiols

Culture supernatants were assayed for the stable end products of NO oxidation (NOx and NO·) and S-nitrosothiols using modified procedures based on the Griess reaction as described previously.

Aspartate aminotransferase measurement

In order to evaluate cellular damage, culture supernatants were measured for aspartate aminotransf erase (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 K2CO3. Total cellular GSH was assayed, using an enzymatic recycling procedure, as described previously.

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
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.
slightly increased ROI production (65.0 ± 6.7 to 70.0 ± 7.0 pmol O₂⁻/min every 10⁵ 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 pretreatment 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⁻, NO₃⁻ 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⁻ and NO₃⁻) 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⁻, NO₃⁻ 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
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. 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). 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, 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. Thus, IL-1β is markedly expressed during inflammation, and acts as a very potent inhibitor of hepatocyte proliferation. 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.

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 of 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 pretreatment 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 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. 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.

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. Other intracellular antioxidative systems include upregulation of enzymes e.g. heme oxygenase-1 by NF-κB [31]. 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. [32] 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 successively reduce the incidence of postoperative liver insufficiency in case of patients undergoing extended liver resection and may potentially decrease the incidence of postoperative liver insufficiency.

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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
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S-Editor Wang YR L-Editor Kerr C E-Editor Zheng XM