Hepatoprotection by Human Epidermal Growth Factor (hEGF) against Experimental Hepatitis Induced by d-Galactosamine (d-GalN) or d-GalN/Lipopolysaccharide

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The hepatoprotective effects of recombinant human epidermal growth factor (hEGF) on chemically and immunologically induced experimental liver injury models were examined. The hEGF clearly decreased serum transaminase levels in d-galactosamine (d-GalN) and d-GalN/lipopolysaccharide (LPS)-induced liver injury models under sub-lethal conditions. However, it has not significantly changed either serum or in vitro tumor necrosis factor (TNF)-α production or in vitro nitric oxide (NO) production, suggesting that the hepatoprotection by EGF is not mediated by inhibiting these pathological mediators produced in d-GalN and d-GalN/LPS-induced liver injury.

Key words human epidermal growth factor (hEGF); hepatoprotective effect; liver injury cytokotoxic molecules

N-Galactosamine (d-GalN) or d-GalN/lipopolysaccharide (LPS)-induced liver injury in mice has been reported to be among the immunopathologically damaged diseases in liver.1–3 Rapidly released tumor necrosis factor (TNF)-α or superoxide, which act as cytotoxics against hepatocytes from macrophage/Kupffer cells, are known to be the main pathological mediators in these models.4,5 These were also clearly demonstrated from experiments with some compounds, such as rolipram, a phosphodiesterase type IV inhibitor, and dinutyl cAMP (dbcAMP), or monoclonal antibody, which could inhibit these molecules.4–6 Some naturally-occurring products have been reported to possess a hepatoprotective effect in these injury models in a similar manner.6,7

Epidermal growth factor (EGF), known to be produced by platelets, macrophages, epidermal and dermal,8,9 plays a central role in epidermal cell growth, wound healing with inhibition of scar formation,9,10 and has an anti-apoptotic effect through the mitogen-activated protein kinase pathway.11 In addition, it has been reported that EGF stimulates the proliferation of some cells such as hepatocytes and fibroblasts.12,13

In the course of our toxicity test, we found that EGF significantly decreased normal serum transaminase levels (data not shown), although the mechanism has not yet been elucidated. In addition, several in vitro experiments demonstrated the possible hepatoprotective effect of EGF,14,15 and a hepatotomy experiment showed EGF production.12 These led us to hypothesize that EGF may have an in vivo hepatoprotective effect against experimental liver disease models. Moreover, recent biotechnology allowed us to raise the production yield of recombinant human EGF, resulting in it being economically beneficial to develop to medicine.

On the basis of the background described above, we decided to examine the in vivo hepatoprotective effect of human recombinant EGF (hEGF) in these experimental hepatitis models, as well as the effect on the production of cytoxic molecules such as TNF-α and nitric oxide (NO).

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats 7—8 weeks old, weighing 200—220 g were used for the d-GalN-induced liver injury model. Male BALB/c mice, 7 weeks, weighing 23—26 g were used for the d-GalN/LPS-induced liver injury model. All animals were purchased from B & K Laboratory Animal Center (Fremont, CA, U.S.A.) and maintained under a 12 h light/dark cycle in a temperature and humidity controlled room. The animals were fed with a laboratory pelleted chow and given water ad libitum.

Materials Recombinant human epidermal growth factor (hEGF, DWP401), which is identical with human EGF, with 99.9% purity in HPLC analysis, was provided by the Biotechnology Department of Daewoong R & D Center (Sungnam, Korea). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assay kits were purchased from Wako Pure Chemical (Osaka, Japan). Dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-dicarboxylate (DDB) was provided by the Chemistry Department of Daewoong R&D Center (Sungnam, Korea). Pentoxifylline, nitro-L-arginine methyl ester (L-NAME) and lipopolysaccharide (LPS, E. coli 0111:B4) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rolipram was purchased from Calbiochem (La Jolla, CA, U.S.A.). Fetal bovine serum (FBS), penicillin, streptomycin and RPMI 1640 were obtained from Gibco (Grand Island, NY, U.S.A.). RAW264.7 cells, murine macrophages, were from ATCC (Rockville, MD, U.S.A.). Murine TNF-α ELSA kit was purchased from Amersham Life Science Co. (Little Chalfont, Buckinghamshire, U.K.). All other chemicals were of the highest grade from Sigma.

TNF-α Production For in vitro TNF-α production,16 RAW 264.7 cells maintained in RPMI 1640 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5% FBS and grown at 37 °C, with 5% CO2 in humidified air, were seeded. After incubation for 18 h in 24-well plates, stimul and the various concentrations (0.1, 1, and 10 μg/ml) of hEGF solubilized with PBS and diluted with RPMI 1640

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were then added to the wells for another 6 or 24 h incubation in the presence of stimuli. For \textit{in vivo} TNF-α determination, serum was collected from mice 1.5 h after \( \alpha \text{-GalN/LPS} \) injection. TNF-α content was determined in the culture supernatant and serum using a murine TNF-α ELISA kit.

Nitric Oxide (NO) \textit{Production in Vitro} After preincubation of RAW 264.7 cells for 18 h (1 x 10^5 cells/ml), the various concentrations of hEGF in the presence of LPS (1 μg/ml) were incubated for 24 h. Nitrates in culture supernatants were measured, as described previously, by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl samples of medium, respectively, for 10 min at room temperature. The nitrite concentration (μM) was calculated from a sodium nitrite standard curve. The detection limit of the assay is 0.5 μM. As a standard drug, L-NAME, a generally well-known inducible nitric oxide synthase inhibitor, was used.

\( \alpha \text{-GalN or \( \alpha \text{-GalN/LPS} \)-Induced Liver Injury} \) Liver injury was induced by \( \alpha \text{-GalN/LPS} \) according to the method reported previously. In each group 11 rats or mice were used. After 12 h fasting, mice were injected i.p. with 700 mg/kg of \( \alpha \text{-GalN} \) only or with 10 μg/kg of LPS. EGF was administered two times at doses of 0.01, 0.1 or 1 mg/kg, s.c., 3 and 1 h before the \( \alpha \text{-GalN/LPS} \) challenge. The blood ALT and AST levels were examined at 8 and 24 h after \( \alpha \text{-GalN/LPS} \) challenge to measure the extent of liver damage. Blood was collected and serum was stored at -70°C until used. As standard drugs, DDB, a clinically well-known hepatoprotective agent, and rolipram, a phosphodiesterase IV inhibitor, were used in each experiment.

\textbf{Measurement of Serum Transaminases} At 8 and 24 h after \( \alpha \text{-GalN} \) or \( \alpha \text{-GalN/LPS} \) injection, blood samples were collected from the abdominal aorta with heparinized syringes under ether anesthesia. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured spectrophotometrically using commercial test reagents (Wako Pure Chemical, Osaka, Japan). The absorbance change at 340 nm that was associated with the oxidation of NADH was monitored, and the activities were expressed in IU/L, according to the manufacturer's manual.

\textbf{Statistical Analysis} All values expressed as mean±S.E.M., were obtained from \( n \) number of experiments. Student's \( t \)-test for unpaired observation between control and experimental samples was carried out for statistical evaluation of differences; \( p \) values of 0.05 or less were considered statistically significant.

\textbf{RESULTS AND DISCUSSION}

Hepatocytes primed with cytokines are known to proliferate in response to several growth factors such as hepatocyte growth factor, transforming growth factor-α, and EGF, as demonstrated from partial hepatectomy. Of them, EGF is reported to play a central role in epidermal cell growth, the maturation of intestinal organs and wound healing, in addition to stimulation of the proliferation of some cells such as hepatocytes and fibroblasts. In the course of our toxicity test, we also found that EGF decreases normal serum transaminase levels (data not shown). These led us to hypothesize that EGF may have a hepatoprotective effect against \textit{in vivo} hepatitis models, although \textit{in vitro} activities were already suggested.

Indeed, considering that hEGF decreased serum transaminase levels increased by \( \alpha \text{-GalN} \) or \( \alpha \text{-GalN/LPS} \) (Tables 1 and 2), hEGF appears to be cytoprotective toward damaged liver cells, as shown with positive control drugs such as DDB and rolipram, similar to previous reports. These effects were strikingly exhibited at both 8 and 24 h after inducing experimental hepatitis under sub-lethal conditions; no lethality was evident in 5 d (data not shown). However, at these doses EGF did not change the lethality under lethal conditions which were caused by very early stage excessive cytotoxic molecules such as TNF-α, in which 80% of mice were killed within 1 d (data not shown). Thus, we assume that EGF may not be protective in extremely severe liver damage induced mainly by the cytotoxics. Both \textit{in vivo} and \textit{in vitro} TNF-α release and NO production level also seem to suggest that these cytotoxic molecules may not be target molecules of EGF pharmacology in experimental hepatitis models (Fig. 1). However, pentoxifylline (IC\textsubscript{50}=249 μM) and rolipram (IC\textsubscript{50}=86.3 nm) strongly inhibited TNF-α release, and L-
Fig. 1. Effect of hEGF on TNF Release in α-GalN/LPS-Primed Mice and LPS-Stimulated RAW264.7 Cells

(A) Serum TNF-α was assayed 1.5 h after α-GalN/LPS challenge, as described in Materials and Methods. (B) In vitro TNF-α production was evaluated from culture supernatants of lipopolysaccharide (1 μg/ml)-stimulated RAW264.7 (1×10⁶ cells/ml) in the presence of various concentrations of hEGF, rolipram and pentoxifylline, as described in Materials and Methods. Data represent mean±S.E.M. of 4 separate observations. * Significantly different from the α-GalN/LPS-treated group (p<0.05). ** Significantly different from the α-GalN/LPS-treated group (p<0.01).

NAME (IC₅₀ = 196.3 μM) suppressed NO production, indicating that these conditions are reproducible, as reported previously.¹²,²²

In our long-term treatment experiment with hEGF, we found that EGF decreased normal serum transaminase levels and increased liver weights (data not shown), indicating that EGF may increase hepatocyte viability and the number of the cells through stimulating DNA synthesis.¹²,¹³ These seem to be possible mechanisms by which hEGF displays its hepatoprotective effect. In particular, however, the hepatoprotective effect of hEGF at 8 h after treatment of α-GalN/LPS appears to be not enough to explain the mechanism, suggesting that in addition to enhancing DNA synthesis, EGF may directly activate either an in vivo hepatoprotective mechanism or prevent cell membrane damage, rather than block cytoxic molecule production elicited by α-GalN/LPS at either the early or late stage of pathogenesis under the sub-lethal conditions. Indeed, several in vitro models suggested a possible inhibitory mechanism by which EGF protects acute hepatocyte damage induced by CCl₄ and acetaminophen, including maintaining the stability of the cellular lipid membrane and affecting the glutathione metabolism of hepatocytes,¹⁴,¹⁵ although the exact in vivo mechanism remains to be resolved.

In acute hepatitis induced by chemicals and bacterial products such as endotoxin, the cytotoxic molecules are known to critically participate in liver damage, resulting in the malfunction of detoxification and the metabolism of chemically toxic compounds or endotoxin.¹⁻³,²² These have led to developing antagonists against the cytotoxic molecules, although it has been demonstrated that some clinically available hepatoprotective agents such as silymarin and bile acids inhibit or increase TNF-α production.²⁴ Several cAMP-enhancing compounds such as dibutyryl cAMP, rolipram and pentoxifylline are among the examples. They are reported to possess inhibitory effects on TNF-α production and hepatoprotective effects in hepatitis models.²⁵,²⁶ Under our conditions, these compounds also significantly attenuated in vitro and in vivo TNF-α production (Fig. 1). This seems to provide us with an idea, which will be tested, that the combination treatment with EGF and these compounds as an injectable formulation which could be rapidly delivered would synergistically raise the hepatoprotective effect at both early and late stages through blocking TNF-α production, thereby increasing either the direct cytoprotective effect or hepatocyte proliferation. In particular, several acute and emergency hepatic diseases such as endotoxemia and septic shock would be expected to be a suitable target of these formulations. To clarify this, however, detailed study will be followed in future experiments.

In this study, we have first demonstrated the in vivo efficacy of EGF on both α-GalN and α-GalN/LPS-induced hepatitis models. An effective injection with EGF would be advantageous against acute liver diseases, but further investigation of the mechanism and efficacy of combination treatment with soluble TNF-α antagonist is required.

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