Therapeutic Potential of Cardiotrophin 1 in Fulminant Hepatic Failure

Dual Roles in Antiapoptosis and Cell Repair

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Hypothesis: Administration of cardiotrophin 1 (CT-1) can treat experimental fulminant hepatic failure (FHF).

Design: Rat model with FHF induced by D-galactosamine (D-gal).

Setting: Fulminant hepatic failure is a rapidly progressive disease that lacks effective nonsurgical treatment. Cardiotrophin 1 is a member of the interleukin 6 family that can protect cells from damage in some animal disease models.

Animals: A rat model of FHF was induced by an intraperitoneal injection of D-gal (1.4 g/kg of body weight). Cardiotrophin 1 was administered at different time points after D-gal injection.

Results: Administration of CT-1 at 12 and 18 hours had a survival rate of 80% (12/15) and 70% (7/10), respectively, which was significantly higher than that of non-treatment (28% [5/18]). In addition, improvement of liver histologic findings, shortening of activated clotting time, and decrease in serum levels of total bilirubin and alanine aminotransferase were detected with CT-1 treatment. Administration of CT-1 decreased apoptotic cells and increased Ki-67 cells in the liver tissues. In vitro, CT-1 administration significantly decreased apoptotic cells and sequentially down-regulated the expression of proapoptotic molecules and up-regulated the expression of antiapoptotic molecules at different culture periods. D-galactosamine culture induced morphologic damage in a hepatocyte cell line, which was greatly improved by CT-1 administration. In addition, CT-1–treated cells demonstrated increased expression of glycoprotein 130 and up-regulation of cyclin D1 and heat shock protein 90.

Conclusion: Cardiotrophin 1 may improve the outcome of D-gal–induced FHF through its effects on antiapoptosis and cell repair.

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Fulminant hepatic failure (FHF) is a rapidly progressive disease that leads to a high mortality rate, despite extensive supportive treatment.1,2 Liver transplantation is the only treatment that may cure FHF, but this approach is limited by organ shortage and by lifelong immunosuppression that is required after the operation. In addition, patients die while awaiting liver transplantation.3 Although some liver support systems were reported to be effective in prolonging animal survival and in improving the neurologic and biologic conditions of patients with FHF, these systems alone had no significant effect on patient survival.4-5 As a result, they were only regarded as a useful approach to bridge patients with FHF to liver transplantation. Therefore, identifying a more effective therapeutic strategy is crucial for improving the outcome of patients with FHF.

Cardiotrophin 1 (CT-1) is a member of the interleukin (IL) 6 family of cytokines, which can induce hypertrophy of cardiac myocytes.6,7 The effects of CT-1 are documented in several disease models, such as myocardial infarction and ischemia-reperfusion injury.8,9 The receptor of CT-1 (glycoprotein 130 [gp130]) is expressed in different organs and tissues (such as the heart, lung, and muscle), and the therapeutic potentials of CT-1 are demonstrated in these organs.10-12 Recent studies13,14 revealed that CT-1 could protect liver cells from concanavalin A–induced damage. However, in these studies, CT-1 was administered before injection of the cytotoxic agent; therefore, the effects of CT-1 were investigated in the aspect of preven-
tion but not of treatment. This is inapplicable in the clinical setting, as patients with FHF already have severe liver cell injury at the time of diagnosis.

In the present study, the therapeutic role of CT-1 was investigated in a rat model with FHF. The dual roles of CT-1 in antiapoptosis and in cell repair were also studied.

### METHODS

#### ANIMALS AND EXPERIMENTAL GROUPS

Adult male Dark Agouti rats, weighing 200 to 250 g, were obtained from the Animal Laboratory Unit, The University of Hong Kong. All animals received humane care according to the Guide for the Care and Use of Laboratory Animals prepared by The University of Hong Kong. The study was approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong. Fulminant hepatic failure in rats was induced by an intraperitoneal injection of D-galactosamine (D-gal) (Sigma-Aldrich Inc, St Louis, Mo) at a dose of 1.4 g/kg of body weight. At different time points after D-gal injection, the rats were divided into the following 5 experimental groups: (1) no treatment (n=18), (2) CT-1 (2 µg per rat through the penile vein; Calbiochem, San Diego, Calif) administered at 6 hours (n=5), (3) CT-1 administered at 12 hours (n=15), (4) CT-1 administered at 18 hours (n=10), and (5) CT-1 administered at 24 hours (n=5). According to power estimation,5 5 rats in each group were sufficient to achieve our experimental objective. However, we added more rats in the 12-hour and 18-hour groups to confirm survival significance of the findings. The animals in all the treatment groups received a second intraperitoneal injection of CT-1 (1 µg per rat) at 24 hours after the first dose of CT-1. Survival time was recorded. Tissue and blood samples were collected at 0, 18, 24, 36, and 48 hours after D-gal injection (≥3 animals at each time in each group).

After the animals were humanely killed, half of the liver tissues were fixed in 10% buffered formalin and embedded in paraffin. The paraffin-embedded tissue was cut into 5-µm-thick sections for histologic studies using hematoxylin-eosin staining. The sections were also subjected to immunohistochemical staining for TUNEL (terminal deoxynucleotidyl transferase–mediated biotin–deoxyuridine 5-triphosphate nick-end labeling) (a marker for cell apoptosis; Roche, Basel, Switzerland) and for Ki-67 (Ki-67 staining displayed a nuclear antigen expressed in all phases of the cell cycle, except G0 and early G1, and was used as a marker for cell proliferation50; BD Biosciences Pharmingen, San Diego). Five fields were randomly chosen on each section, and positive-staining cells were counted using Meta-...

### Statistical Analysis

Comparisons of serum levels of total bilirubin and alanine aminotransferase, activated clotting times, and apoptotic cells and Ki-67 cells in the liver tissues with or without CT-1 treatment were performed using t test (GraphPad Software Inc, San Diego). Comparisons of apoptotic cells and gp130 cells between different groups in the hepatocyte cell line were performed using 1-way analysis of variance (GraphPad Software Inc). P<.05 was considered statistically significant.

**Table. Survival Rates at 48 Hours After D-Galactosamine (D-gal) Injection**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. in Group</th>
<th>Survival Rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>18</td>
<td>27.7</td>
</tr>
<tr>
<td>Cardiotrophin 1 administration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h after D-gal injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>20.0</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>80.0*</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
<td>70.0†</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>20.0</td>
</tr>
</tbody>
</table>

*P<.05 compared with no treatment group.
†P<.05 compared with no treatment group.

Normal rat hepatocyte cell line CRL-1439 was purchased from American Type Culture Collection (Manassas, Va). Cells were maintained in Ham's F12K medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, Carlsbad, Calif) at 37°C in a humidified atmosphere of 5% carbon dioxide.

### Treatment Regimens

After starvation in serum-free medium for 12 hours, the cells received the following 2 combinations of D-gal and CT-1: (1) Ham's F12K medium containing 10% FBS, 40mM D-gal, and CT-1 at the concentrations of 0.5µM, 1µM, and 2µM, with culture for 6, 12, and 24 hours, respectively, and (2) the cells were first treated with Ham's F12K medium containing 10% FBS and 40mM D-gal for 12 hours, and then the culture condition was changed to Ham's F12K medium containing 1% FBS, with or without different CT-1 concentrations, for another 12 hours. The cells were then harvested and subjected to Western blot and flow cytometry analyses.

### Western Blot

Total cell lysate was extracted using ristocetin-induced platelet agglutination buffer. Thirty micrograms of total protein was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel for electrophoresis. Antibodies were purchased from Upstate Biotechnology (polyclonal rabbit anti-rat apoptosis protease activator protein 1 [Apaf-1] antibody; Waltham, Mass); Cell Signaling Technology, Inc (monoclonal mouse anti-rat caspase 9 antibody, polyclonal rabbit anti-rat Bax antibody, and monoclonal mouse anti–rAT cyclin D1 antibody; Beverly, Mass); eBioscience (polycyclonal rabbit anti–rat Bcl-2 antibody; San Diego); and Stressgen Biotechnologies (monoclonal mouse anti–rat heat shock protein 90 [Hsp90] antibody; Palo Alto, Calif).

### Flow Cytometry

After the D-gal and CT-1 treatment, the cells were harvested and labeled with annexin V antibody (BD Biosciences Pharmingen) or with phycoerythrin-conjugated polyclonal goat anti–rat gp130 antibody (R&D Systems Inc, Minneapolis, Minn) and detected using an FACSCalibur flow cytometer (Becton Dickinson Immuno cytometry Systems, San Jose, Calif). Appropriate isotypes of irrelevant antibodies were used as control specimens.
RESULTS

CT-1 PROLONGED SURVIVAL OF ANIMALS WITH FHF

Our preliminary pilot study demonstrated that more than 8 (80%) of 10 rats died within 48 hours after D-gal injection. Using 48 hours as a cutoff time point, we defined survivors and nonsurvivors and compared 48-hour survival rates among different groups. When no treatment was given, 72.3% of rats died within 48 hours after D-gal injection (Table). Significantly improved survival was observed in the groups receiving CT-1 administration at 12 hours (12/15 [80%] \( P < .005 \)) and at 18 hours (7/10 [70%] \( P < .05 \)) after D-gal injection. There was no improvement in animal survival when CT-1 was administered at 6 hours or at 24 hours after D-gal injection.

At the parallel time points (36 and 48 hours), the nontreated livers were pale with scattered spots of hemorrhage on the surface, whereas CT-1–treated livers had a normal liver appearance. The histologic findings of D-gal–treated livers demonstrated extensive areas of necrosis and hemorrhage; CT-1–treated livers had a normal liver morphologic structure (Figure 1A). Microscopically, there was no obvious difference in

Figure 1. Nontreated livers vs cardiotrophin 1 (CT-1)– and D-galactosamine (D-gal)–treated livers. A, D-galactosamine–treated livers displayed pallor and extensive hemorrhagic spots on the surface, whereas CT-1–treated livers had a normal liver appearance. B, The histologic findings of D-gal–treated livers demonstrated extensive areas of necrosis and hemorrhage; CT-1–treated livers had a normal liver morphologic structure. NT indicates no treatment.
Histologic findings in cells with and without CT-1 treatment at 18 and 24 hours after D-gal injection. However, at 36 and 48 hours after D-gal injection, the nontreated livers displayed extensive areas of necrosis and hemorrhage and damage of the liver architecture, whereas the CT-1–treated livers demonstrated normal liver structure with scattered areas of cell infiltration (Figure 1B).

Beginning at 24 hours after D-gal injection, significantly elevated serum levels of total bilirubin (A) and alanine aminotransferase (ALT) (B) and shortend the D-galactosamine (D-gal)–induced prolongation of activated clotting time (ACT) (C). NT indicates no treatment; asterisk, *P<.05 compared with the levels before D-gal injection; and dagger, †P<.05 compared with NT at the parallel time points. To convert bilirubin to micromoles per liter, multiply by 17.1.

In contrast to apoptosis, a slight increase in Ki-67 cells was detected in livers after D-gal injection. Following CT-1 treatment, a dramatic increase in Ki-67 cells was found, with a peak at 24 hours (Figure 4).

**CT-1 DECREASED APOPTOTIC CELLS INDUCED BY D-GAL**

In vitro, D-gal cultures at 12 and 24 hours induced an increase in apoptotic cells, with higher numbers at 24 hours. However, when the D-gal–containing medium was replaced by the normal culture condition, no further increase in apoptotic cells was observed (data not shown). Cardiotrophin 1 coculture with D-gal resulted in a decrease in apoptotic cells, but the effect was only observed at 24 hours (Figure 5).

**CT-1 AFFECTED DIFFERENT MOLECULES IN THE APOPTOTIC PATHWAYS AT DIFFERENT TIME POINTS**

In vitro, an increased caspase 9 level was observed at 6 hours after D-gal injection, whereas there was no change in the expression of Apaf-1, Bax, or Bcl-2. Cardiotrophin 1 coculture with D-gal resulted in a decrease in apoptotic cells, but the effect was only observed at 24 hours (Figure 5).
regulation of Apaf-1 was detected and was possibly inhibited by the CT-1 coculture with D-gal. At the same time point, an increased expression of Bcl-2 was observed with the CT-1 coculture. However, at the 24-hour time point, there was no observable change in the caspase 9, Apaf-1, or Bcl-2 levels, but significant down-regulation of Bax was detected when D-gal was cocultured with CT-1 (Figure 6).

CT-1 FACILITATED THE REPAIR OF DAMAGED HEPATOCYTES

At 12 hours after D-gal injection, the cells lost their oblong jagged shape and became oval, with some of the cells floating in the culture medium. After culture with Ham’s F12K and 1% FBS alone for another 12 hours, the attached cells still demonstrated the unhealthy shape (Figure 7A). However, when CT-1 was added to the culture medium, the cells gradually displayed a normal epithelial shape, especially with CT-1 at 1µM (Figure 7B and C).

CT-1 INCREASED THE EXPRESSION OF gp130 AND CELL CYCLE–RELATED MOLECULES

When the cells received D-gal treatment for 12 hours and then Ham’s F12K and 1% FBS treatment for another 12 hours, an increase in gp130 cells was detected. However, when the culture medium during the second 12 hours was replaced with Ham’s F12K and 1% FBS con-
When the cells received D-gal treatment for 12 hours and then Ham's F12K and 1% FBS treatment for another 12 hours, down-regulation of cyclin D1 and Hsp90 was detected. However, when the cells were cultured with Ham's F12K and 1% FBS containing CT-1 during the second 12 hours, the expression of cyclin D1 and Hsp90 was up-regulated, prominently at 1µM (Figure 9).

COMMENT

Unlike the study by Bustos et al., the present study focused on the therapeutic role of CT-1 in FHF. We used different regimens of CT-1 to treat FHF and found that CT-1 administration significantly prolonged animal survival when it was given at 12 hours and at 18 hours after D-gal injection. Cardiotrophin 1 improved neither animal survival nor liver histologic findings when it was administered at 6 hours or 24 hours after D-gal injection. Two explanations are possible, one being that the toxicity of D-gal did not reach its peak level at 6 hours and the other being that CT-1 could not overcome the extremely severe liver cell damage at 24 hours after D-gal injection. Therefore, the therapeutic role of CT-1 in D-gal-induced FHF was limited to a narrow window.

The IL-6 family plays an important role in liver mass restoration and repair after injury by actions on apoptosis and cell proliferation. Researchers demonstrated that IL-6 has an antiapoptotic effect in hepatocytes by blocking transforming growth factor β–induced apoptosis. In addition, tumor necrosis factor α and IL-6 seem to prime hepatocytes to regenerate, probably by triggering the G0 to G1 phases of liver cell proliferation. A member of the IL-6 family, CT-1 demonstrated antiapoptotic effects in the present study. Beginning at 18 hours after D-gal injection, a decrease in apoptotic cells in the liver tissues was observed after CT-1 treatment. However, these effects seemed to be more prominent at the 36-hour and 48-hour time points. In vitro, CT-1 also significantly decreased the number of apoptotic cells induced by D-gal. Because the mechanism of apoptosis demonstrated a stepwise manner, involving various molecules in different periods during its progression, it is of interest to determine how CT-1 affects the molecules in the apoptotic pathway. The present study revealed that CT-1 affected different molecules in the apoptotic pathways during different time points and that the effects of CT-1 were not prominent during the late phase of apoptosis. This might represent molecular evidence why CT-1 did not improve animal survival when it was administered at 24 hours after D-gal injection, when the proapoptotic pathway had already proceeded to the downstream molecules and cell death had already been extensive.

However, an inhibitory effect on hepatocyte proliferation was observed when IL-6 was administered to soluble IL-6 receptor transgenic mice at an early stage following hepatectomy. This contradictory effect was suggested by the elevated induction of suppressors of cytokine signaling that eventually inhibited gp130 stimulation–enhanced liver regeneration. This probably explains why CT-1 did not prolong survival when it was administered to rats at 6 hours after D-gal injection. Therefore, the timing of CT-1 administration might be an important factor in the outcome with respect to liver injury and liver regeneration.
Although the anti-apoptotic effects of CT-1 are well documented in different disease models, and some similar phenomena were observed in the present study, we considered that the therapeutic role of CT-1 in a D-gal-induced FHF model relied more on its cell repair effects, because it seemed that the increase in proliferating cells with CT-1 treatment was more predominant than the decrease in apoptotic cells. The in vitro data also demonstrated that administration of CT-1 to the D-gal-damaged cells could facilitate morphologic improvement of these cells. Moreover, administration of CT-1 to the damaged cells could reverse the down-regulation of the proliferation-related molecules cyclin D1 and Hsp90 that were induced by D-gal. All of these data suggest the importance of CT-1 in mediating cell repair after D-gal-induced damage.

The interaction of CT-1 and its receptor gp130 is crucial for signal transduction. In some studies, gp130 expression paralleled CT-1 expression in disease models, but there was no evidence of a direct relationship between CT-1 and gp130 expression. In the present study, during cell repair when the injured cells were stimulated by CT-1, an increase in gp130 cells was observed. However, this stimulatory effect of CT-1 on gp130 seemed to be inconspicuous in normal cells, suggesting that CT-1 might enhance receptor expression only in a disease state. The possible mechanism remains to be elucidated.

When we replaced the D-gal-treated medium with normal culture medium containing 1% FBS, there was no further increase in apoptotic cells, suggesting that removal of the causative source was crucial for improving the outcome of the disease. This is supported by a study demonstrating that the recirculating system of the molecular adsorbents could decrease drug toxicity and improve patient outcome. In addition, we postulated that toxic substance removal might facilitate CT-1 in repairing the damaged cells. Because CT-1 treatment alone did not achieve 100% animal survival even when it was administered at 12 hours after D-gal injection, we propose that a combination of liver support and CT-1 treatment might achieve a better outcome among patients with FHF.

In conclusion, CT-1 is a possible treatment for FHF but has a limited therapeutic window of administration. The effects of CT-1 on antiapoptosis and cell repair contribute to the improved outcome of FHF.

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REFERENCES


Invited Critique

As demonstrated by Ho et al, CT-1 treatment in a D-gal rat model of hepatic failure had profound antiapoptotic effects and promoted cell repair and proliferation that improved survival. The beneficial effects were observed when animals were treated at 12 and 18 hours after induction of FHF but not at 6 and 24 hours. The authors deduce that the lack of improvement in survival at 6 hours may be due to early generation of suppressors of cytokine signaling and, hence, an inhibition of the beneficial CT-1 actions. If this were the case, one would expect that the suppression could be overcome by increasing the concentrations of administered CT-1, and this can and should be experimentally tested. Equally important would be the identification of such inhibitors. In addition to dose-response curves, given the few numbers of animals in the 6-hour group, it would behoove the authors to perform additional experiments at this time to account for known variability in biological responses even among inbred animals.

Further investigation of the mechanisms and critical mediators of CT-1–induced hepatoprotection might include the use of anti-Fas monoclonal antibody–induced FHF (which is known to mediate massive cellular apoptosis and liver failure) and the use of knockout strains. In addition, future studies should include FHF induced by other causes to define the conditions that favor a therapeutic effect for exogenous cytokine therapy. Finally, cytokine treatment may have implications for strategies to enhance liver regeneration after major resections and, perhaps, graft survival in liver transplantation.

Based on the merit of animal studies, we believe that the therapeutic potential of the interleukin 6 family of cytokines warrants further experimentation in anticipation of preclinical trials.

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