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Inhibition of gap junctions relieves the hepatotoxicity of TNF-α

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ABSTRACT. The aim of this study was to observe the influence of gap junction (GJ) functional changes on the hepatotoxicity of TNF-α. Three different methods were employed to study functional effects of the GJ inhibition: 1) pretreatment with a GJ inhibitor; 2) inoculation of cells at high and low densities; and 3) inhibition of the expression of connexin 32 (Cx32) by small inhibitory RNA transfection. We then observed the influence of these treatments on hepatotoxicity following treatment with different concentrations of TNF-α for various duration. The hepatotoxicity of TNF-α was observed to occur in a dose- and time-dependent manner; after pretreatment inhibition, the hepatotoxicity of TNF-α was significantly reduced ($P < 0.01$). The hepatotoxicity of TNF-α was also found to be remarkably lower in cells that had been inoculated at low density (as measured by the amount of GJ formation among cells) than in those inoculated at density ($P < 0.01$). In addition, following Cx32 inhibition, the hepatotoxicity of TNF-α was significantly decreased ($P < 0.01$) as well. Together, these results suggest that inhibition of GJ function or of its component Cx32 significantly

decreases the hepatotoxicity of TNF-α, and that the expression of Cx32 plays an important role in the hepatotoxicity of TNF-α.

Key words: Gap junction; TNF-α; BRL-3A cells; Connexin 32; siRNA; Oleamide

INTRODUCTION

Liver ischemia/reperfusion (I/R) injury is a common clinical pathophysiological process, and severely affects patient recovery. It has been well documented that TNF-α is an important mediator of inflammation, mediating the spread and development of the immune reaction from a central site, which is a key factor in hepatic I/R injury (Scales et al., 1994; Suzuki and Toledo-Pereyra, 1994; Imanishi et al., 1997). The pathological processes that mediate liver I/R injury include center granulocyte aggregation, protein enzyme leakage, and hepatocyte necrosis (Imanishi et al., 1997); however, the role of TNF-α in hepatic I/R injury is still not completely determined. The identification of a means for reducing the sensitivity of liver cells to TNF-α, as well as for inhibiting the TNF-α-mediated inflammation and its spread, might alleviate the overall TNF-α-mediated liver I/R injury and systemic inflammatory response and provide significant prognostic value.

The gap junction protein connexin (Cx) is a kind of channel protein wherein six Cx proteins are bound together into one half-channel, termed a hemi-channel; two hemi-channels on adjacent cell membranes form an intact channel, called a gap junction (GJ). The GJ allows material less than 1 kDa in molecular weight to pass directly between the two adjacent cells. It has been demonstrated that GJs mediate GJ intercellular communication (GJIC), which plays important roles in the processes of cell growth and development (Li et al., 2010), the regulation of cell proliferation and apoptosis, and in the synchronization of cell activity, which is necessary to maintain a stable intracellular environment (Huang et al., 2001; Kalvelyte et al., 2003). However, whether GJ functional changes can influence the hepatotoxicity of TNF-α has not been reported in the literature. Therefore, this study focused on the influence of GJ functional changes on the hepatotoxicity of TNF-α.

MATERIAL AND METHODS

Material

TNF-α was obtained from PeproTech (Rocky Hill, NJ, USA), and siRNA was a product of Guangzhou Ruibo Bio Co., Ltd. (Guangzhou, China). siRNA sequences were designed and manufactured by the company as a pre-determined product and the sequences were as follows: Cx32-siRNA1: 5-GCC UCU CAC CUG AAU ACA AdTdT-3; Cx32-siRNA2: 5-GCA UCU GCA UUA UCC UCA AdTdT-3; Cx32-siRNA3: 5-CAC CAA CAA CAC AUA GAA AdTdT-3). Lipofectamine 2000 was sourced from Invitrogen (Carlsbad, CA, USA); CCK-8 was purchased from Dojindo (Shanghai, China); Cx32 monoclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA), as were 0.25% pancreatin, dimethylsulfoxide (DMSO), and oleamide. Dulbecco's modified Eagle's medium (DMEM; low-glucose) was a product of Hyclone (Logan, UT, USA); newborn calf serum and the fluorochrome calcein-AM were obtained from Gibco (Gaithersburg, MD, USA); other common reagents were all domestic

analytical reagents.

Methods

Cell culture

Immortalized rat liver cell line BRL-3A was purchased from the Chinese Academy of Sciences (Shanghai, China). BRL-3A cells were grown using conventional subculture techniques in DMEM culture containing 10% newborn calf serum at 37°C and 5% CO_2 and under saturated humidity.

Detection of the cytotoxic effects of TNF-α on BRL-3A cells and of the respective influence of GJ functional inhibition

To determine the cytotoxic effects of TNF-α on BRL-3A cells, we inoculated 100 µL (5 x 103) cells into each well of a 96-well plate. Following culture for 24 h, the cells were incubated with TNF-α at 10, 20, 40, or 60 ng/mL, with six repeated wells for each group. The surviving fractions of each treated cell group were detected at 24, 48, and 72 h. We also assessed negative (non-TNF-α treated) and blank (medium only, without cells) controls. Cultures were terminated after TNF-α treatment for 24-72 h. For detection, 10% volume CCK-8 buffer was added to each well 2 h prior to culture termination; the plate was placed in an incubator for 2 h, and then the optical density value at 450 nm was measured with a Multiscan Spectrum instrument (model ELx800; Bio-Tek); The different testing conditions consisted of inoculation of cells to high or low density, or treatment with 25 µM oleamide for 1 h to impair GJ function, followed by determination of the fraction of surviving cells after treatment with 40 ng/mL TNF-α for 72 h.

Western blot detection of Cx32 expression levels in siRNA-transfected cells

Prior to transfection, cells were inoculated on 6-well plates, and antibiotic-free medium was added to every well. The cell density was approximately 30-50% at the time of transfection. The siRNA-lipo2000 mixture was added to culture plates containing cells and culture solution, to a final siRNA concentration of 50 nM according to manufacturer's protocol. After culture for 5 h, the medium was replaced and cells were cultured in an incubator for approximately an additional 72 h. The cells transfected with Cx32-targeting siRNAwere collected and lysed for 30 min in cell lysis buffer with protease inhibitors on ice. All proteins were extracted from the cells. The protein concentration of each group was detected using a BCA quantitative assay. The protein concentrations were equilibrated across groups through dilution with cell lysis buffer, and aliquots were then mixed with loading buffer and boiled. The samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (the proteins were run through the stacking gel at 80 V and through the separation gel at 100 V and 12% of acrylamide was used), transferred to nitrocellulose membranes at 4°C, blocked with 5% nonfat milk in Tris-buffered saline-Tween 20 (TBSTM) at room temperature for 30 min, and incubated with the primary antibody at 1:2000 in TBSTM overnight at 4°C. Following incubation, membranes were washed with TBST three times for 5 min each and then incubated

with the appropriate secondary antibodies in TBSTM at room temperature for 0.5 h. Following the second incubation, the membranes were washed with TBST three times for 10 min each, and bound antibodies were visualized with ECL substrate (Forevergen, Guangzhou, China) and then detected and quantified using the ChemDoc XRS system (Bio-Rad Laboratories, Berkeley, CA, USA).

Detection of intercellular GJ function in BRL-3A cells by the parachute assay

The parachute assay was used to determine intercellular GJ function. BRL-3A cells were preloaded with the fluorescent indicator 25 μM calcine-AM allowing the calcine-AM to enter the cells (donor cells) at 37°C for 30 min. Afterward, the calcein-AM solution was aspirated from the well, and cells were rinsed three times with PBS for 5 min. Preloaded cells were trypsinized, and the cell suspension was 500 cells/mL to generate the "donor cells". To initiate the assay, the cell culture medium was first aspirated from the unloaded "acceptor cells", and each well was rinsed one time with PBS. The freshly prepared "donor cells" were added to the monolayer of "acceptor cells". The assay was incubated at 37°C for 4 h to allow the formation of gap junction channels. After the formation of stable GJs, the small molecule calcine-AM (which exhibits green fluorescence) from the "donor cells" will enter the adjacent cell (receiver cells) through the GJ; this can be observed using a fluorescent inverted microscope. Recording the number of receiver cells around a donor cell serves as an index of GJ function (Koreen et al., 2004; Wentlandt et al., 2005). To observe the effects of GJ inhibition on calcine-AM transfer, oleamide was dissolved with DMSO to a final concentration of 25 μM and added to the culture. After 1-h induction, the changes in green fluorescence transmission among cells were observed. Similarly, the changes in green fluorescence transmission among cells were observed 24 h following siRNA transfection.

Statistical analysis

Data were analyzed using the SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). The *t*-test was performed for comparison between two groups. Analysis of variance (ANOVA) was performed for comparisons among multiple groups. According to the least significant difference law, ($P < 0.05$) was considered to be statistically significant.

RESULTS

Concentration- and time-dependent effects of TNF-α cytotoxicity on BRL-3A cells

We initially determined whether BRL-3A cells were sensitive to TNF-α-mediated cytotoxicity by examining the surviving fraction of cells after application of different concentrations of TNF-α onto BRL-3A cells for various duration using the CCK-8 assay. Results showed that as the TNF-α concentration and treatment time increased, the surviving fraction of BRL-3A cells decreased remarkably (Figure 1). It followed that the cytotoxicity of TNF-α toward BRL-3A cells showed concentration- and time-dependent effects. Therefore, we chose the treatment parameters of 40 ng/mL and 72 h for further studies, as these led to the most obvious degree of cytotoxicity; we then observed the

effects of GJIC function on TNF-α hepatotoxicity, as described below.

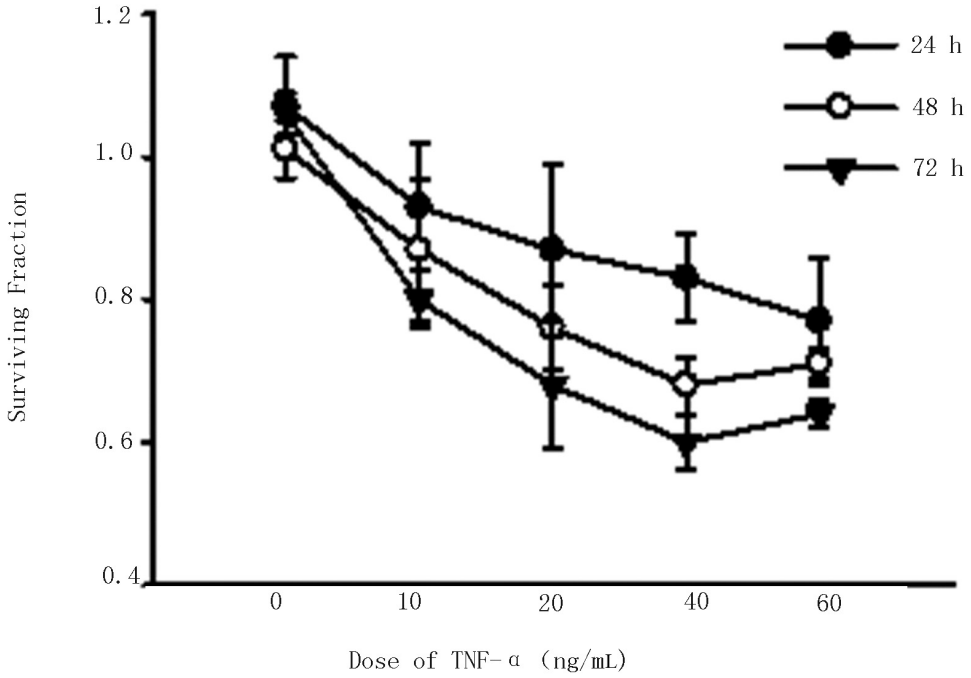

Figure 1. Surviving fraction of BRL-3A cells treated with TNF-α for 24, 48, and 72 h (mean ± standard deviation, N = 4).

Functional inhibition of GJIC can relieve the hepatotoxicity of TNF-α

According to the literature, functional changes in GJIC can affect the cytotoxicity of chemotherapy drugs; therefore, we altered the function of GJIC in the BRL-3A cells using various methods designed to inhibit Cx32, the main component of GJs, and observed the influence of GJIC function on TNF-α hepatotoxicity.

The results shown in Figure 2 demonstrate that TNF-α had marked effect of the survival of BRL-3A cells when these had been inoculated at high density; this condition permitted direct contact between cells and the formation of GJIC. At high density, the surviving cell fraction was 41.7%; this increased substantially to 78.4% when cells were inoculated at low cell density; i.e., no direct contact was possible between cells, and GJIC was not formed. Subsequently, we treated the BRL-3A cells with the Cx32 inhibitor oleamide, and found that the fraction of surviving cells remarkably increased in the high-density cell inoculation condition; however, no obvious influence was seen on cells incubated at low density. The results therefore appeared to be GJIC-dependent and suggested that GJIC had an obvious influence on hepatotoxicity, and that functional inhibition of Cx32-containing GJIC structures can relieve the hepatotoxicity of TNF-α.

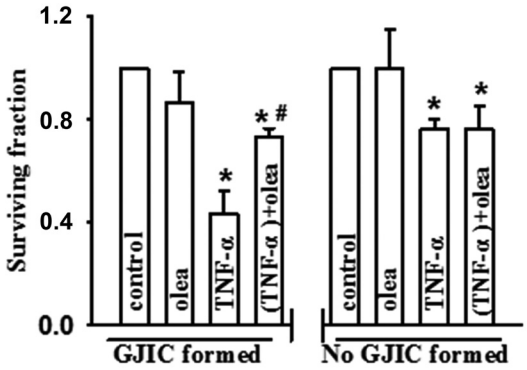

Figure 2. Effect of high- and low-density vaccination cells and oleamide pretreatment on the cytotoxicity of TNF-α in BRL-3A cells (means ± standard deviation, N = 3). *P < 0.01 *vs* control group; #P < 0.01 *vs* TNF-α group; GJIC = gap junctional intercellular communication, olea = oleamide.

Functional disruption of GJIC through knockdown of its Cx32 components can relieve the hepatotoxicity of TNF-α

In order to further confirm the results described above, we successfully inhibited the expression of Cx32 in BRL-3A cells using Cx32-specific siRNAs. Three different siRNAs targeting the Cx32 gene were evaluated for their gene-silencing efficiency. Protein expression was measured, since changes in transcript levels do not necessary reflect changes in protein levels. Cx32-siRNA3 was by far the most effective at downregulating Cx32 expression. The nonspecific siRNA had no effects on protein expression (Figure 3A and B). In addition, the parachute assay showed that the function of GJIC was remarkably inhibited as well following Cx32-specific knockdown (Figures 4 and 5). Furthermore, the parachute assay demonstrated that following the inhibition of Cx32 expression, the hepatotoxicity of TNF-α to BRL-3A cells dramatically declined.

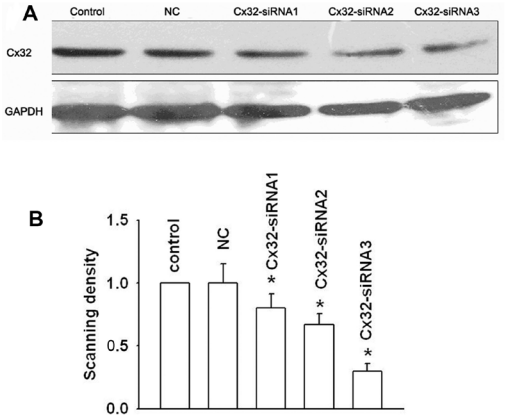

Figure 3. Effect of siRNA transfection on the expression of Cx32 in BRL-3A cells. **A.** Western blot analysis of Cx32 showing decreased protein expression following treatment with Cx32-specific siRNA. Nonspecific siRNA had no effect on Cx32 protein expression. **B.** Immunoblots quantified by densitometry to determine the effectiveness of gene expression knockdown by each siRNA. Quantified results were normalized to the untransfected control sample (means ± standard deviation, N = 3). *P < 0.01 *vs* control group; NC = nonspecific siRNA.

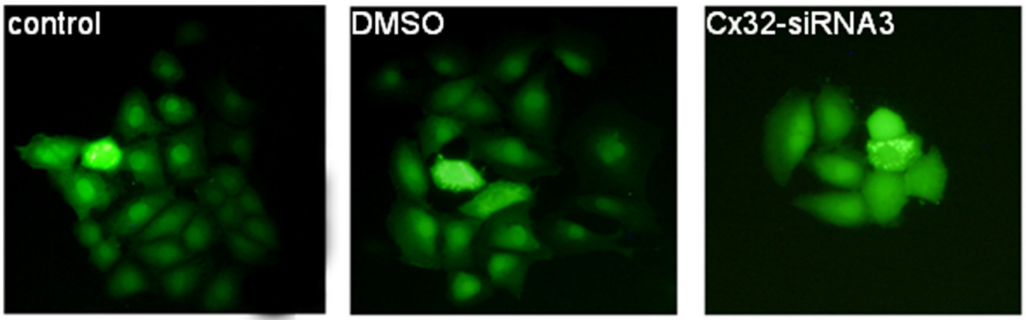

Figure 4. Dye coupling through the GJs between BRL-3A cells 24 h after transfection with Cx32-siRNA3, as shown by a parachute dye-coupling assay. Control: the dye coupling through the GJs in the control group, 100X; DMSO: the dye coupling through the GJs in the DMSO group, 100X; Cx32-siRNA3: the dye coupling through the GJs in the siRNA group, 100X. GJ = gap junction.

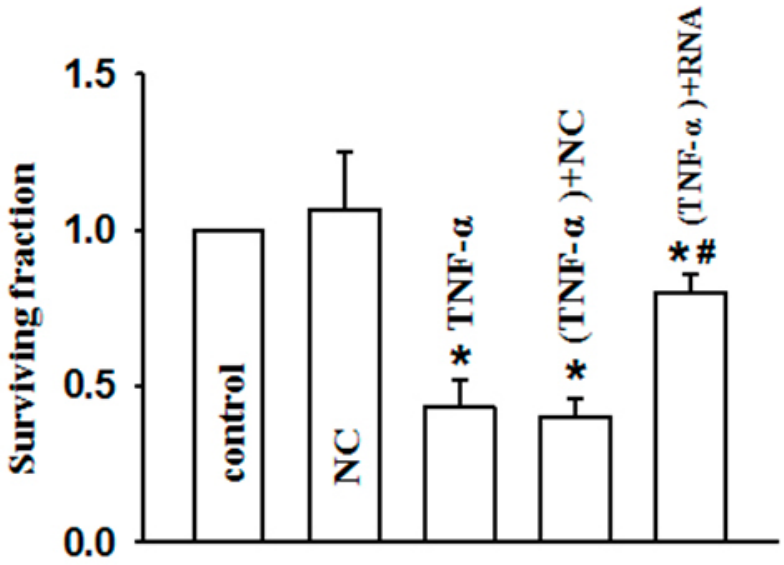

Figure 5. Effect of transfection of siRNA targeting Cx32 on the cytotoxicity of TNF-α in BRL-3A cells (means ± standard deviation, N = 3). *P < 0.01 *vs* control group; #P < 0.05 *vs* TNF-α group; NC = nonspecific siRNA, RNA = Cx32-siRNA3.

DISCUSSION

The results from this study showed that as the TNF-α concentration and treatment time increased, the surviving fraction of treated BRL-3A cell decreased, which indicated that the cytotoxicity of TNF-α to BRL-3A cells was concentration- and time-dependent. It has previously been confirmed that the direct toxicity, oxidative damage, and inflammatory effects of TNF-α are involved in the pathological physiological processes of liver injury (Klebanoff

et al., 1986; Leist et al., 1994; Polyak et al., 1997). Subsequent research has, however, suggested that the mechanisms underlying the hepatotoxicity caused by TNF-α are complicated and need further study.

Large numbers of GJs exist between the liver cells of healthy individuals; GJs constitute 3% of the hepatic membrane. Three kinds of Cx, which make up the GJs, have been described: Cx32, Cx43, and Cx26. The cells used in this study primarily expressed Cx32, with lesser amounts of Cx26 and Cx43. All three methods utilized in this study to functionally inhibit GJ function: high- and low-density cell culture, oleamide treatment, and siRNA transfection-mediated inhibition of Cx32 expression demonstrated that inhibiting the function of GJs led to a decrease in the hepatotoxicity of TNF-α. As a corollary, we also attempted to utilize retinoic acid (Wu et al., 2013) to enhance GJ function; however, after retinoic acid treatment, mass cell mortality occurred for unknown reasons. Therefore, the question of whether enhancement of the function of GJs can enhance the cytotoxicity of TNF-α to BRL-3A cells remains uncertain.

Previous studies have shown that enhancement of the function of tumor GJIC can reduce the effective concentrations of chemotherapy drugs (Tanaka and Grossman, 2004; He et al., 2009). Usually, two methods are utilized to enhance the function of GJIC. First, the expression of Cx is induced by drug treatment, which stimulates the formation of GJs among cells, facilitating direct communication by and the spread of molecular signals through GJ channels. Second, by changing the voltage, pH value, phosphorylation, or other physiological parameters (Wentlandt et al., 2006), it is possible to ensure that the GJ channels remain open. A growing number of studies have shown that GJ protein-mediated GJIC is closely related to the "bystander effect" (Jensen and Glazer, 2004), and it is thought that GJ enhancement of TNF-α hepatotoxicity might also be related to this effect. GJs are known to be required for the bystander effect seen with low-dose I/R. In this phenomenon, I/R-damaged cells pass a signal to unirradiated cells, triggering certain signaling pathways and producing genomic instability and cytotoxicity in the untreated cells. Then in this study, TNF-α not only caused cell death directly but also transmitted "cell death" signals through GJs. These signals could be comprised of IP3, Ca^{2+}, or other small molecular substances (Evans et al., 2006); in addition, the signal molecules responsible for apoptosis/necrosis could also spread to adjacent cells through GJ channels, causing cell death and the expansion of cell death to adjacent cells. By inhibiting the function of GJs, these death signal substances would not be spread through the GJs, which would decrease the level of cell death, and reduce the hepatotoxicity of TNF-α.

The finding from this study that the hepatotoxicity of TNF-α was altered coordinate with GJ functional change demonstrated that when GJ function is downregulated or lost, the toxicity of TNF-α is relieved; furthermore, these findings indicate that Cx32 plays an important role in the hepatotoxicity of TNF-α. These results suggested that a likely strategy for limiting TNF-α-mediated liver I/R injury and systemic inflammation might be the inhibition and downregulation of GJ function. GJs are therefore proposed as target for the development of new and effective anti-inflammatory drugs.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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