Original Article

Different Effects of Plasma from Patients with Acute on Chronic Liver Failure on the Proliferation and Biotransformation Function of C3A Cells

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Objective To investigate the effects of plasma from patients with acute on chronic liver failure on the proliferation and biotransformation function of C3A cells in vitro, and provide experimental data for C3A cells to be efficiently used in the bioartificial liver system.

Methods C3A cells were incubated in 100% normal human plasma (NHP) and 100% abnormal plasma (AP) from patients with acute on chronic liver failure. Growth morphology of the two groups were observed under inverted microscope and scanning electron microscope. The method of methyl thiazolyl tetrazolium (MTT) was conducted for the proliferation activities of C3A cells. The cellular apoptosis rates were assessed by the flow cytometer. The biotransformation function of cells was evaluated through diazepam metabolic amount assay. The concentrations of epithelial growth factor (EGF), transforming growth factor- α (TGF- α) and interleukin-1 (IL-1) were detected in plasma of the two groups.

Results A: The proliferation activities of C3A cells incubated in 100% AP for 24, 48, 72, 96 and 120 hours were significantly higher than that in 100% NHP ($P < 0.01$). B: Observation under the inverted microscope indicated that the cells in 100% AP were growing faster than those in 100% NHP after cells attached to the plastic at 24 and 48 hours. The same phenomena was observed under the scanning electronic microscope. C: The C3A cells cultured in both groups of plasma showed the same apoptosis rate at 48 hours and there was no statistical difference. D: The diazepam metabolic value of C3A cells incubated in 100% AP for 24, 72 and 120 hours were lower than that in 100% NHP and were statistically different ($P < 0.01$). E: The concentrations of TGF- α , EGF and IL-1 in AP were significantly higher than that in NHP ($P < 0.01$).

Conclusions Compared with normal human plasma, the plasma from patients with acute on chronic liver failure has more obvious effect to facilitate the proliferation of C3A cells, but also decreases partial biotransformation function of C3A cells.

Key words: Liver failure; Plasma; Cell culture; Proliferation; Bioartificial liver

The potential for liver regeneration is considerable and patients with liver failure might be expected to survive if a temporary detoxification device were available for liver support during the acute phase of the illness. The key component of the bioartificial liver is the cultured hepatocytes. Plasma from the patients flows across the membrane and comes in to contact with cells in the bioreactor and exchange substance through the semi-permeable membrane. As the detoxification of plasma by hepatocytes is essential to all¹ bioartificial liver designs, it would be important to investigate the direct interactions between liver failure plasma and the cells. Some researchers concentrated

on the effects of the plasma from patients with acute liver failure or fulminant hepatic failure on hepatocytes in bioreactor and proved that the patient plasma could inhibit DNA, RNA and protein synthesis and was cytotoxic to cells in the bioreactor.¹⁻³ In Asia, chronic liver disease such as viral or alcoholic hepatitis is very common. Most of the patients who received artificial liver treatment suffered from acute on chronic liver failure. However, there have been very few studies concerned about the effect of plasma from those patients on the cultured cells in the bioreactor. Here we characterized the functional capacity of hepatocytes cultured in those patients' plasma. As the cellular source of extracorporeal liver-assist device (ELAD[®]), the C3A cell line has almost normal liver function and is easily cultured in vitro, therefore we used C3A cells to demonstrate the effect of the plasma from patients

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with acute on chronic liver failure on the proliferation and biotransformation function of C3A cells.

MATERIALS AND METHODS

Plasma

Normal human plasma (NHP) anticoagulated by heparin were taken as normal control which were obtained from 12 healthy persons aged 25-50 years old with normal liver function. Abnormal plasma (AP) were acquired from 12 patients with acute on chronic liver failure, 6 male and 6 female, aged 26-60 years old, hospitalized in the Artificial Liver Treatment and Training Center, Beijing You'an Hospital, Capital Medical University, from May 2004 to December 2004. All the patients had a history of chronic hepatitis B before the onset of liver failure. The average value of total biliary acid in plasma of these 12 patients was $312.2 \pm 65.7 \mu\text{mol/L}$, with total bilirubin as $347.3 \pm 68.2 \mu\text{mol/L}$, and HBV DNA all negative in the plasma.

Major reagents

Major reagents were produced by the following companies: MEM tissue culture media (Invitrogen Corporation; Grand Island, NY, USA), superior grade fetal calf serum (HyClone; Logan, UT, USA), methyl thiazolyl tetrazolium (MTT, Sigma; St. Louis, MO, USA), propidium iodide (PI, Bender Medsystems Inc; Burlingame, CA, USA), diazepam metabolic testing kit (Testing Components Corporation; Ithaca, NY, USA), transforming growth factor- α (TGF- α), epidermal growth factor (EGF), interleukin-1 (IL-1) testing kit (R&D Systems Inc; Minneapolis, MN, USA).

The proliferation activities of C3A cells tested by methyl thiazolyl tetrazolium method

C3A cells were inoculated into 96 well plates, with 2.0×10^3 /well. The supernatants were discarded at 24, 28, 72, 96 and 120 hours after cells attaching to the plastic, and then the culture media were replaced by 100% NHP and 100% AP, respectively. At 144 hours, 5 mg/ml MTT was added to each well. The supernatants were discarded after 4 hours incubation and then DMSO was added. Samples were checked by Model 550 Microplate Reader (Bio-Rad Laboratories, Inc; Hercules, CA, USA) at 570 nm wave length absorbance.

The morphology observation

C3A cells were inoculated onto the cover slips in the culture well, with 1.0×10^5 /well. The supernatants were discarded after cells attaching to the cover slips and then 100% NHP and 100% AP were added separately. The supernatants were discarded at 24 hours and 48 hours and then cover slips were washed with PBS and

fixed with methanol. After that the cover slips were observed under the inverted microscope.

The cover slips were placed in the 6-well plates and 100% NHP and 100% AP were replaced separately after cells attaching to the cover slips and then cells were incubated with the plasma for 48 hours. After that, the cells were fixed with 2% formaldehyde polymer-2.5% glutaraldehyde in 4°C for 2 hours. After washed with PBS, the cells were coated with gold and observed under the scanning electronic microscope (SEM) for the morphology.

The apoptosis of cells assessed by flow cytometer

C3A cells were inoculated into the 6 well plates, with 2.0×10^5 /well. The supernatants were discarded after cells attaching to the plastic and then 100% NHP and 100% AP were added separately. After 48 hours, the suspended cells were collected into the test tubes for centrifugation. Total of 250 μl RNase and 250 μl propidium iodide were added and stored at 4°C and kept in the dark for 30 minutes. The FACS Calibur flow cytometer was applied for the examination.

Test for diazepam metabolic function

C3A cells were inoculated into the 24 well plates, with 5×10^4 /well. The supernatants were discarded after cells attaching to the plastic and then 100% NHP and 100% AP were added separately as well as 10 μg pure diazepam (0.5 $\mu\text{g}/\mu\text{l}$, 20 μl). The metabolic values were assessed at 24, 72 and 120 hours following the test kit instructions.

Cytokine detection

The concentrations of TGF- α , EGF, IL-1 in the plasma of the two groups were detected through the same lot of cytokine test kit. ELISA assay was conducted and the testing procedure in test kit instructions was followed.

Statistical analysis *t* test for two independent samples and modified Bonferroni test were applied in data analysis by SPSS 11.0 software.

RESULTS

MTT analysis of C3A cells in NHP and AP culture conditions

The optical density (*A*) values of C3A cells cultured in the plasma increased with time going. It was observed that C3A cells incubated in 100% AP had a faster growth rate than those in 100% NHP. With regards to *A* values from the MTT test at 24, 48, 72, 96 and 120 hours, the *A* values in 100% AP group were higher than those in 100% NHP group. There was a significant statistical difference ($P < 0.01$, by modified Bonferroni test) (Figure 1).

Figure 1. MTT analysis of C3A cells in NHP and AP culture conditions.

Notes: Mean MTT results of 6 experiments of C3A cells cultured in 100% NHP and 100% AP, when seeded at 96 well plates, with 2.0×10^3 /well ($P < 0.01$, by modified Bonferroni test).

Figure 2. C3A cells were cultured for 48 hours.

Notes: A: Inverted microscope (200 ×, 100%NHP); B: The cell amount was much more in the same area, compared with 100% NHP. (Inverted microscope 200 ×, 100%AP); C: SEM 500 ×, 100% NHP; D: SEM 500 ×, 100% AP

The morphology observation

The cells incubated in both groups of plasma were observed under inverted microscope. Cells in both groups were with normal morphology when attached to the plastic. The cell amount in 100% AP at 24 hours was much more than that in 100% NHP and was

more obvious at 48 hours (Figure 2A and 2B). When observed under the scanning electronic microscope (SEM), the morphology of cells in both groups of plasma at 48 hours was normal and filar-like pseudopod and cellular salience could be seen. Cells in the divided phase in 100% AP were more than that in 100% NHP

Figure 3. The comparison of diazepam metabolic value of cells.

Notes: The cells were cultured in 100% and 100% AP at different time when seeded at 24 well plates, with 5×10^4 /well. $P < 0.01$, by modified Bonferroni test. Results are the mean $\pm$ SEM of 6 experiments.

Table 1. The 48 hours apoptosis rate of C3A cells cultured in two different groups of the plasma ($\bar{x} \pm s$)

Groups	n	apoptosis rate (%)	G ₂ /G ₁	%, CV
100% AP	12	2.992 $\pm$ 0.6271	1.96 $\pm$ 0.103	6.72 $\pm$ 0.502
100% NHP	12	2.545 $\pm$ 0.6258 ^a	1.89 $\pm$ 0.021	7.85 $\pm$ 0.447

Notes: ^a $P > 0.05$ for 100% AP vs. 100% NHP; G₂/G₁ indicates the ratio between tetraploid and diploid. The normal value is 2; % CV is variable coefficient and normal value is less than 8.

Table 2. The examination of cytokines in two different groups of plasma

Groups	n	TGF- α (pg/ml)	EGF (pg/ml)	IL-1 (pg/ml)
100% NHP	12	9.37 $\pm$ 0.21	0.33 $\pm$ 0.012	554 $\pm$ 7.6
100% AP	12	17.21 $\pm$ 0.95 ^a	2.76 $\pm$ 0.06 ^a	1782 $\pm$ 20.5 ^a

Note: ^a $P < 0.01$ for 100% AP vs. 100% NHP

(Figure 2C and 2D).

Flow cytometer applied for the analysis of apoptosis

The rates of apoptosis of both groups at 48 hours were similar, with a lower level and the statistical difference was not significant (Table 1).

Diazepam metabolic assay for the C3A cells incubated in different plasma

The diazepam metabolic value of C3A cells slightly increased as the culture time prolonged. Compared in the same culture time, the diazepam metabolic value of the cells incubated in 100% AP was lower than that in 100% NHP at 24, 72 and 120 hours which showed significant difference ($P < 0.01$, by modified Bonferroni test) (Figure 3).

Examination of cytokines in both groups of plasma

The concentrations of TGF- α , EGF and IL-1 in 100% AP were much higher than that in 100% NHP. There was a significant difference between the two groups (Table 2, by t test, two-tailed).

DISCUSSION

C3A cell line was subcloned from the hepatoblastoma HepG2 cell line. These two cell lines are similar but

C3A is well differentiated with improved liver specific functions, such as production of albumin and alpha fetal protein, ureagenesis and glycogenesis, etc. The C3A cell line can grow in the hollow fiber cartridge which has been used in the bioartificial liver research for a long time.⁴ The results from some researchers showed the concentrations of bilirubin, biliary acid, endotoxin and tumor necrosis factor α in plasma of patients with liver failure were much higher than that in the normal plasma. Hepatocytes incubated with the plasma from patients with acute liver failure or fulminant hepatic failure showed severe damage of the hepatocyte membrane and inhibition of cell proliferation, protein synthesis and biological transformation function.¹⁻³

However, there were few reports on the effects of the plasma from patients with acute on chronic liver failure on hepatocyte function. Under such a background, we studied the effects of the plasma from the patients with acute on chronic liver failure on C3A cells proliferation, apoptosis and biological transformation. All patients included in the study had a history of chronic hepatitis B. Before liver failure occurred, inflammation, fibrosis and regeneration had already existed in their liver tissues. Difference from the results of previous studies of acute liver failure was the speed of cell proliferation, which was obviously higher in AP than that in NHP from patients with

acute on chronic liver failure under the observation of morphology and by MTT assay. Regarding the apoptosis, there was no remarkable difference between AP and NHP groups, both were on a lower level.

Polypeptide growth factors are cellular factors related to cell growth and development. EGF is a growth stimulating factor with relatively small molecule. EGF concentration in patient plasma and expression of liver EGF receptors were remarkably increased while massive hepatocyte necrosis and partial hepatectomy occurred. Cyclin A is a key element in regulation of cell cycle and DNA synthesis. When cells are exposed to certain concentrations of EGF in vitro, the cyclin A in cells are distinctively ascending.⁵ EGF is a strong stimulating factor which can stimulate mitosis and proliferation of many types of tissues and cells.⁶ Hirata et al showed that 2 µg/L EGF in the media could strongly stimulate the proliferation of the cells in vitro.⁷ The mean EGF concentration we detected in AP was 2.76 µg/L, which is 100 times higher than that in healthy person. As a result, the C3A cells grew faster in AP, which provided evidence of close relationship between the growth speed and the high concentration of EGF. TGF-α is a cellular factor which plays a role in the adjustment of normal and tumor cell proliferation and differentiation. The enhanced TGF-α expression could result in a rise in DNA synthesis in hepatocytes and make a stronger function for cell mitosis promoting.^{8,9} Some researchers in recent studies considered that EGF took part in hepatocyte proliferation during the early stage while TGF-α played a role in the late stage. Beside the two factors, heparin-binding growth factor (HBGF), insulin-like growth factor (IGF), interleukin-6 (IL-6), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), etc., also play functions to promote cell mitosis, but their role in promoting cellular proliferation are not as important as EGF and TGF-α.¹⁰ Patients with acute on chronic liver failure had a background of chronic liver inflammation and fibrosis, and the signals of hepatocyte necrosis in these patients could cause more polypeptide growth factor synthesis and stimulate the hepatocyte mitosis and proliferation, which rarely happens in patients with acute liver failure. The phenomenon is one reason for different effects of plasma from the patients with acute or acute on chronic liver failure on C3A proliferation. There was no remarkable effect of the plasma from patients with acute on chronic liver failure on C3A cells apoptosis. The cytotoxic T lymphocyte (CTL) mostly caused hepatocyte apoptosis through the mediation of FasL/Fas system in these patients.¹¹ But culture system in vitro could not exert effect due to lack of cellular components. It could be speculated that the effect of factors for promoting

cell proliferation in plasma of acute on chronic liver failure patients was greater than that for inhibiting cell proliferation.

The diazepam metabolic value of C3A cells slightly increased as the culture time prolonged because the cell amount were significant more than before. The diazepam metabolic value of C3A cells incubated in AP from patients with acute on chronic liver failure was lower than that in NHP, which were the same as previous studies on acute liver failure. Cytochrome P450 (CYP) which relate to the detoxification and metabolism of the derivative compound is mainly expressed in endoplasmic reticulum of the hepatocytes. Among the cytochrome P450 family, 3A4 plays a major role on the diazepam metabolism.¹² Elmar et al believed that interleukin-1 was one of the most important factors leading to the down-regulation of CYP function. CYP function was down-regulated by interleukin-1 in the process of lipid-amylose induced inflammation.¹³ Studies in vivo and in vitro made it clear that interleukin-1 down-regulated CYP function by suppressing the transcription of cytochrome gene.^{14,15} Bertini et al found that factors such as IL-1, TNF-α, interferon-γ could inhibit the activities of cytochrome P450 in vivo experiment with primary rat hepatocytes, while only interleukin-1 could play the same role in vitro experiments.¹⁶

We detected the concentration of interleukin-1 in AP and found it was much higher than that in NHP. Patients suffered from acute on chronic liver failure usually present much lower leukocyte chemotaxis and increased permeability of the intestine. The intestinal microbial flora is apt to shift. The microbial overgrowth and invasion to the portal vein are enhanced while the clearance capability of the liver decrease. This results in higher incidence of bacteremia which could increase cytokines such as IL-1, IL-6, etc., in serum. In this experiment, reasons on decreased diazepam metabolic function of cells in AP was partly attributed to the increased concentration of IL-1 in plasma. Another possible reason is related to biliary acid. Amie et al reported that if the media contained 0.5% biliary acid, it could reduce P450 activities in the primary cultures of rat hepatocytes.¹⁷ High concentration of biliary acid in AP probably had some impact on the CYP function. Besides the negative effect of IL-1, biliary acid probably correlated with decreased function of CYP and corresponding depressed diazepam metabolic value.

In order to fully ensure the functional activities of cells in the bioreactor, plasma of the patient should be more meticulously dealt with prior to the treatment of bioartificial liver. For example, biliary acid need to be filtered out in order to eliminate their negative effect on metabolic function of the hepatocytes. The useful

substance such as positive growth factor need to be selectively preserved because they are useful for cell proliferation in the bioreactor.

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