Effect of Flow Load on Hepatic Function in Co-Culture of Hepatocytes with Hepatic Stellate Cells and Endothelial Cells: Relationship between Hepatic Function and Nitric Oxide Concentration in vitro*

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Abstract
To date, no study has described the effects of media flow load on a co-culture model of hepatocytes (HC), hepatic stellate cells (HSC) and endothelial cells (EC). Furthermore, no research has been reported regarding the influence of nitric oxide (NO) concentration in such a co-culture model. Therefore, we developed co-culture models that include two or three of these cell types, and assayed their hepatic functions both in static culture and under flow load. We also measured the NO concentration in each models and inhibited NO production of cells. In static culture, the HC+HSC and HC+HSC+EC models demonstrated higher hepatic function than in the model containing HC alone. Under flow load, all models exhibited higher hepatic function than in static culture. The HC+HSC and HC+HSC+EC models under flow demonstrated the highest hepatic function observed under any condition. In almost all models, NO concentration exhibited the same tendency to increase along with hepatic function, and NO improved hepatic function in HC+HSC model under flow load. Inhibition of NO production decreased small levels of hepatic function in HC+HSC and HC+HSC+EC models under flow load. We conclude that co-culture and flow load positively impact hepatic function, and that HSC and NO are related to improvements in hepatic function. Furthermore, we consider that the presence of HSC is responsible for other aspects of improvement in hepatic function.

Key words: Hepatocytes, Hepatic Stellate Cells, Endothelial Cells, Co-Culture, Flow Load, Nitric Oxide

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1. Introduction

In recent years, bioartificial liver (BAL) has been developed in order to treat patients with severe liver failure; several types of BAL have succeeded in bridging liver transplantation (1)(2)(3). BAL converts ammonia into urea and produces important materials, such as albumin (4)(5). However, maintaining the metabolic performance of BAL is difficult, and hepatic capacity is therefore limited, because hepatocytes in BAL lose their metabolic functions in vitro (6)(7). To overcome these difficulties, researchers have discovered several methods for improving hepatic function in vitro; these methods involve co-culture hepatocytes with nonparenchymal cells and imposing flow on the growth medium.

In co-culture models, hepatocytes are cultured with nonparenchymal cells. Nonparenchymal cells are liver cells other than hepatocytes, e.g. stellate cells, Kupffer cells and endothelial cells; these cell types are all important for hepatic function (8)(9)(10). Co-culture with Kupffer cells increases secretion of albumin by hepatocytes, an important hepatic function (11)(12); co-culture with stellate cells improves hepatocyte detoxification functions (13)(14). Many studies have described co-culture of hepatocytes with a single other type of cell; however, no researcher has yet carefully studied the consequences of co-culturing hepatocytes with more than two types of cells.

On the other hand, flow load is another important element for improvement of hepatic performance. The term ‘flow load’ refers to the concept that the flow of the growth medium affects hepatocytes in vitro, and that this flow influences hepatic function (15)(16). When hepatocytes were cultured in rotating radial flow-type bioreactor, albumin secretion was increased 3-fold (15); other hepatic functions, such as urea synthesis and Cy P450 secretion, were also improved (17)(18). These results suggest that flow influences hepatocytes and increased albumin secretion, but these studies did not examine the effects of co-culture under flow load. In other words, the two conditions have only been examined separately, and the relationship between the effects of flow load and co-culture in vitro remains unclear.

Two methods, co-culture and flow load, have improved hepatic performance in vitro, but the causes of hepatic improvement were not clear. According to Adawai et al., one important component was nitric oxide (NO) (19), which influences many types of organs; specifically, an increase in the NO concentration allows recovery of liver cirrhosis in vivo (20)(21). NO is able to ameliorate diseases of livers in vivo, but the effect of NO in vitro has not yet been carefully studied, in part because it is difficult to control the NO concentration in vitro. Under standard culture conditions, molecules of NO oxidize in 5~7 seconds; researchers have not yet determined the best ways to control NO concentration in vitro. Endothelial cells mainly produce NO under shear stress, which improves the ability of endothelial cells to produce NO in vitro (22)(23).

This study had three main objectives: to establish a system for static co-culture of three hepatic cell types; to determine the influence of flow on the co-culture model; and to elucidate the relationship between NO and hepatic function. Initial investigations of the influence of co-culture of these three cell types were performed by culturing hepatocytes with stellate cells and/or endothelial cells and measuring improvement in one hepatic function, namely ammonia decomposition. Next, we applied flow load to co-culture models containing two or three cell types, and observed the influence of flow on these models. Finally, to investigate the relationship between hepatic functions in vitro and NO concentration, we measured the NO concentration of each model both in static culture and under flow load, and compared trends in ammonia decomposition with trends in NO concentration.
2. Materials and Methods

2.1 Cell culture

In this study, we used three types of cells: immortal hepatocyte cells (RHT33, RIKEN, Japan), immortal hepatic stellate cells (RI-T, HSRRB, Japan), and Lung Microvascular Endothelial Cells (rLMVEC, Vec Technologies, USA). All cell types were derived from rat. RHT33 exhibits normal hepatic functions in vitro. These cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, USA) containing 10% Fetal Bovine Serum (FBS, Biological Industries, Israel) and 1% penicillin/streptomycin. All cultures were maintained in a humidified atmosphere of air + 5% CO₂ at 37°C. Throughout the manuscript, we abbreviate hepatocytes as “HC”, hepatic stellate cells as “HSC”, and endothelial cells as “EC”.

![Fig. 1 Schematic diagram of the co-culture model, composed of hepatocytes, a collagen gel layer containing hepatic stellate cells, a spacer ring, and endothelial cells.](image)

2.2 Co-culture model

On φ60-mm dishes (BD Falcon, USA), 1.5×10⁷ cells/dish HC were seeded and grown to confluence. Next, we overlaid collagen gel (BD Falcon, USA) mixed with 2.7×10⁶ cells/dish HSC. To make 0.1-mm collagen gel layers, spacer rings (Microseiko, Japan) were used. One day later, 6.5×10⁸ cells/dish EC were seeded on top of the collagen gel layer. After 7 days, we verified that ECs covered the surface, and then used successfully established cultures for subsequent experiments. This model is termed ‘HC+HSC+EC model’. In the absence of HSC in collagen gel, the model is termed ‘HC+EC model’; in the absence of EC seeded on the collagen gel layer, the model is termed ‘HC+HSC model’.

2.3 Flow device

In order to apply controlled levels of flow to cultured cells, we used the same parallel plate-type flow chamber described previously (24). The two flat surfaces of the chamber and the top of the collagen gel layer were held ~200 µm apart using a silicone rubber gasket. The flow medium was DMEM with serum and antibiotics containing 1.0 mmol/L ammonium chloride (KOSO Chemical Company, Japan). We chose a flow volume that resulted in shear stress of 0.6 Pa, which is the same shear stress that occurs in a liver blood vessel. In this study, volumes of medium were 20 mL in static culture and under flow load.

2.4 Counting of numbers of cells

Before experiments, cells were imaged and counted using the Image Processing utility from Image J (25). Each cell was in its own layer: HC were in the bottom layer, HSC were in the middle layer, and EC were on the top surface (Fig. 1). The layers were imaged, allowing us to count only a specific type of cell. Figure 2 shows phase-contrast images. Next, we calculated the number of cells in a 600-mm² area. Four images were shot from different points, and cell counts obtained for each image; the cell count for a given experiment is defined as the average of these four counts (Table 1).
Fig. 2  Phase-contrast images of each cell layer. After 7 days, EC covered the surface of collagen gel layer. (a) bottom layer, HC; (b) middle layer, HSC; (c) top layer, EC.

Data are presented as the means ± SD (N = 6).

<table>
<thead>
<tr>
<th>Culture Model</th>
<th>Hepatocyte (10^7 / dish)</th>
<th>Hepatic Sulfate Cell</th>
<th>Endothelial Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>7.90 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HC+HSC</td>
<td>7.94 ± 0.86</td>
<td>1.44 ± 0.34</td>
<td>0</td>
</tr>
<tr>
<td>HC+EC</td>
<td>8.12 ± 0.54</td>
<td>0</td>
<td>2.74 ± 0.57</td>
</tr>
<tr>
<td>HC+HSC+EC</td>
<td>7.92 ± 0.87</td>
<td>1.32 ± 0.87</td>
<td>3.83 ± 0.31</td>
</tr>
</tbody>
</table>

2.5 Assay of hepatic function

Ammonia decomposition was chosen as the marker of hepatic function in this study, because HC convert harmful ammonia into relatively harmless urea. After 1 day of static culture and 1 day of flow, we calibrated the ammonia absorbance using the Ammonia-Test Wako kit (WAKO, Japan). HC mainly detoxify ammonia, but HC, HSC and EC produce ammonia. Because of this, in this study the initial ammonia concentration was defined as the total of 1.0 mmol/L which medium contained and the ammonia concentration which cells produced. The time-course exchanges in ammonia concentration are fitted by the least squares method on the basis of the reaction kinetic equation:

\[-\frac{dC}{dt} = K_m \cdot C\]

where \( C \) is the concentration of ammonia, \( K_m \) is the ammonia decomposition rate constant and \( t \) is culture time \((26)\). We calculated \( K_m \) from ammonia concentrations before and after. Furthermore the amount of ammonia decomposition is defined as the difference between 1.0 mmol/L ammonia concentration and the concentration calculated by that equation and the \( K_m \) when the before ammonia concentration is 1.0 mmol/L. The loss of ammonia due to sources, e.g. adsorption by the chamber materials; evaporation, other than HC activity is negligible.
2.6 NO concentration assay

To assay NO concentration in the flow medium, NO fluorescence was calibrated using a NO2/NO3 Assay Kit-FX (DOJINDO, Japan). In the medium, both NO2 and NO3 were present. NO3 was reduced to NO2, and NO2 concentration was assayed. For purposes of NO determinations, we employed DMEM without phenol red (Invitrogen, USA), because phenol red interrupts fluorescence assay.

2.7 Inhibition of NO production

Under flow load, we added 10 mmol/L L-NAME (L-NG-Nitroarginine methylester, Sigma-Aldrich, Japan) in culture medium. L-NAME inhibits the activation of NOS (Nitric Oxide Synthase), which is involved in NO production. Subsequently, we calculated ammonia decomposition as described in section 2.5.

2.8 Statistical analysis

Data are presented as means ± standard deviation. The statistical significance of experimental observations was determined by one-way analysis of variance (ANOVA) followed by Scheffe test, with the level of significance set at P < 0.05. Descriptive statistical analyses were performed using Microsoft Office Excel 2003.

3. Results

3.1 Changes of hepatic function in static culture

Levels of ammonia decomposition in static culture are shown in Fig. 3. The unit of ammonia decomposition is moles of ammonia decomposed per liter per hepatocyte cells per 1 day. Amounts of ammonia which HC, HC+HSC, HC+EC and HC+HSC+EC produced were 5.3±0.1 mmol/L, 10.3±0.1 mmol/L, 8.6±0.1 mmol/L and 14.2±0.1 mmol/L, respectively.

HC+HSC and HC+HSC+EC models exhibited significantly higher decomposition levels than the HC model (*p<0.05). The HC+HSC+EC model also exhibited a significantly higher decomposition level than the HC+EC model (†p<0.05). This result demonstrates that the HC+HSC and HC+HSC+EC models decomposed more ammonia than the HC model.

Fig. 3  Amount of ammonia decomposition in static culture when hepatocytes (HC) were co-cultured with hepatic stellate cells (HSC) and/or endothelial cells (EC). Ammonia decomposition is defined as the difference in ammonia concentration before experiment and after 24 h. An increase in ammonia decomposition was observed in the HC+HSC and HC+HSC+EC models. Data are presented as the means ± SD. *p<0.05 versus HC model; †p < 0.05 versus HC+EC model.

3.2 Influence of flow on the co-culture models

Levels of ammonia decomposition in static culture and under flow load are shown in Fig. 4. The unit of ammonia decomposition is moles of ammonia decomposed per liter per hepatocyte cells per 1 day. Amount of ammonia which HC, HC+HSC, HC+EC and HC+HSC+EC produced were 7.8±0.4 mmol/L, 15.6±0.4 mmol/L, 13.7±0.2 mmol/L and
21.7±0.5 mmol/L, respectively.

All models decomposed more ammonia under flow load than under static culture; these differences were all significant (*p<0.05). All co-culture models (HC+HSC, HC+EC and HC+HSC+EC) showed significant higher decomposition levels than the HC model (†p<0.05). This result demonstrates that hepatic function improves under flow when hepatocytes are co-cultures with one or two types of nonparenchymal cells. Specifically, there is a significant improvement in function when hepatocytes are co-cultured with hepatic stellate cells. HC+HSC+EC model demonstrated the highest ability to decompose ammonia.

Fig. 4  Amount of ammonia decomposition under flow load. Ammonia decomposition was measured as in Figure 3. White bars, static culture; gray bars, flow load. All models showed significant differences under flow load relative to static culture. Nonparenchymal cells, HSC and EC, influenced ammonia decomposition. The HC+HSC+EC model under flow load showed the highest capacity for ammonia decomposition, relative to all other models both in static culture and under flow load. Data are presented as the means ± SD. *p<0.05 versus HC model in static culture; †p < 0.05 versus HC model under flow load.

3.3 NO concentration in static culture

The NO concentration of each model in static culture is shown in Fig. 5. NO concentration increased in the following order: HC<HC+EC<HC+HSC+EC<HC+HSC. There was no significant difference between the HC+HSC model and HC+HSC+EC model. HC+HSC and HC+HSC+EC models exhibited significantly higher concentrations of NO than the HC model (*p<0.05). The HC+HSC+EC model also exhibited a significantly higher concentration of NO than the HC+EC model (†p<0.05).

Fig. 5  Nitric oxide (NO) concentration in static culture. NO concentrations in the HC+HSC and HC+HSC+EC models were higher than in the HC model; the concentrations in the HC+EC and HC models were similar to one another. Data are presented as the means ± SD. *p<0.05 versus HC model; †p < 0.05 versus HC+EC model.
3.4 NO concentration under flow load

NO concentrations in the HC model under static culture, and all models under flow load, are shown in Fig. 6. NO concentration of each model increased in the following order: HC<HC+HSC<HC+EC<HC+HSC+EC. HC+EC and HC+HSC+EC models showed significantly higher concentrations of NO than the HC model (†p<0.05).

Fig. 6  NO concentration under flow load. NO concentration was measured as in Figure 5. Under flow load, the HC+HSC+EC and HC+EC models exhibited higher NO concentrations than other models containing no EC. Data are presented as the means ± SD. *p<0.05 versus HC model in static culture; †p < 0.05 versus HC model under flow load.

3.5 Effects of inhibiting NO synthesis in ammonia decomposition

Levels of ammonia decomposition under flow load in the presence or absence of L-NAME are shown in Fig. 7. With the exception of the HC model, all models exhibited significantly decreased ammonia decomposition (*p<0.05). In particular, the HC+EC model exhibited the same level of ammonia decomposition as the HC model upon addition of L-NAME. Functional declines in the HC+HSC and HC+HSC+EC models were smaller than in the HC+EC model.

Fig. 7  Effects of L-NAME on ammonia decomposition under flow load. L-NAME decreased ammonia decomposition in the HC+HSC, HC+EC and HC+HSC+EC models. Data are presented as the means ± SD. White columns, no L-NAME; gray columns, +L-NAME. *p<0.05 versus models containing no L-NAME; †p < 0.05 versus HC model of L-NAME.

4. Discussion

In this study, we constructed co-culture models containing three cell types, and measured improvements of hepatic function and NO concentrations, both in static culture and under flow load. In static culture, models containing HSC increased hepatic function and exhibited high NO concentrations. Under flow load, all models increased hepatic function. Upgrades of hepatic function have a relationship with HSC and NO concentration under flow load.
Figure 3 illustrates changes of hepatic function in the static co-culture models. Hepatic function in HC+HSC model was 1.2 times higher than in the HC model. Abu-Absi et al. demonstrated that when HC isolated from rat livers were cultured with immortalized HSC, albumin secretion was 2.3 times higher than when HC were cultured alone (13). Those authors used albumin secretion as a marker for hepatic function, whereas we measured ammonia decomposition. Granted, albumin secretion and ammonia decomposition are controlled by distinct metabolic pathways, but both studies are consistent in their finding that co-culture of HC with HSC improves hepatic function. On the other hand, another past study suggested that hepatic function does not improve when HC are co-cultured with EC (27). In this paper, Fig. 3 shows that co-culture of HC with EC does not improve ammonia decomposition, but co-culture HC and HSC does so. We did not observe that co-culture of HC with EC improves hepatic function in static culture.

In the HC model, ammonia decomposition under flow load is 2-fold higher than in static culture (Fig. 4). Miyazawa et al. demonstrated that secretion of albumin under 1 Pa flow increases 3-fold relative to static culture (13). On the other hand, no past study has described hepatic functions in either the HC+HSC or HC+EC model under flow load. Our measured values for the HC+HSC and HC+EC models under flow load therefore represent novel findings. Past studies also did not describe hepatic function of the HC+HSC+EC model under flow load; however, some earlier research bears on similar issues. Torii demonstrated that when isolated liver tissue was placed under flow load, albumin secretion increased 4-fold relative to the HC model (28). The earlier work measured different endpoints than those used in this study, e.g., albumin and cell number, but it is still apparent that flow improves hepatic function in a range of models. Based on these findings, we conclude that flow influences all models and improves hepatic function.

Our studies demonstrate that co-culture and flow load improved hepatic function. We hypothesized that this improvement of hepatic functions might be mediated by HSC and nitric oxide (NO). The molecular mechanism by which HSC and NO influence hepatic function remains unknown, but Adawi described that an NOS inactivator exacerbated cirrhosis of the liver in vivo (19)(20). A few in vivo studies have also shown that NO improves hepatic function, but to date there has been no reported research performed in vitro. In this study, we measured NO concentration in each condition, and asked whether HSC and NO improve hepatic function in vitro.

The trend in NO concentration was similar to the trend in ammonia decomposition and models containing HSC showed high levels of NO concentration (Figs. 3, 5). Thus, we consider that NO levels and HSC were correlated with improved hepatic function in static culture. On the other hand, under flow load, except the HC+HSC model, this trend in NO concentration was similar to the trend in ammonia decomposition under flow load (Figs. 4, 6). In the HC+HSC model, the rise in NO concentration was smaller than in the HC+HSC+EC model, but hepatic function in the HC+HSC model was the same as in the HC+HSC+EC model. From these results, we hypothesize that HSC and NO are related to improvement of hepatic function, both in static culture and under flow load. However, under flow load, effects of HSC were larger than effects of NO with regard to improvement of hepatic function. Because of this, in the next experiment, we inhibited cellular NO production under flow load, and investigated how HSC and NO are involved in improvement of hepatic function.

Figure 7 shows that inhibition of NO production decreased hepatic function in all models, except for the HC model. It is known that HC and EC produce NO, but it is unclear whether HSC produce NO (29). The reason why hepatic function did not decline in the HC model is that the level of NO production is small in the HC model; therefore, no effect of NO inhibition could be detected under this experiment condition. In the HC+EC model, inhibition of NO production decreased hepatic function by about 34%. On the other hand, in
the HC+HSC and HC+HSC+EC models, hepatic functions declined by about 17%. Amounts of ammonia decomposition increased by about 52% in HC+HSC and HC+HSC+EC models which did not contain L-NAME. We considered that HSC raised hepatic function by about 35%. In models that contain HSC, we have not relevant factors other than NO, but we speculate that other important factors include extracellular matrix synthesis and secretion of hepatocyte growth factors \(^{(13)}\).

Our study demonstrates that the HC+HSC+EC model under flow load is the best model for recreating improved hepatic function. We observed that three cell types, especially HSC, increase hepatic function both in static culture and under flow load. In static culture, a rise in NO concentration invokes an increase in ammonia decomposition; in contrast, inhibition of NO production decreases hepatic function in co-culture models under flow load. These results suggest NO influences improvement of hepatic function. Additionally, we conclude that HSC make a greater impact on the improvement of hepatic function than NO, at least under conditions of under flow load.

Improvement of hepatic functions \textit{in vitro} is clinically important. Our study suggests three methods for improvement of these functions: co-culturing HC with HSC+EC, imposing flow load, and increasing NO concentration. Future studies are needed to construct a more sophisticated liver model \textit{in vitro} using these two methods, and to explain the observations made regarding the behavior of the HC+HSC model under flow load.

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References

(23) Noris, M., Morigli, M., Donadelli, R., Aiello, S., Foppolo, M., Todeschini, M., Orisio, S,


