Development of a Leukocyte Removal Filter for Hepatitis C Therapy and the Possibility of Multipurpose Treatment

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Abstract: Hepatitis C virus infects and causes inflammation in extrahepatic organs and the liver. Patients with chronic hepatitis C disease receive interferon-based treatment, and both interferon and interferon-free therapies are expensive. We attempted to eliminate hepatitis C virus-infected cells using extracorporeal circulation with a unique filter. We created two types of the filters with syndiotactic polystyrene (SPS) resin sheets in a solution that dissolved hydroxypropyl cellulose (HPC) and cellulose acetate (CA) in oleyl alcohol, respectively. First, hepatitis C virus-positive cells were confirmed in the blood and mononuclear cells of hepatitis C virus-infected volunteers, showing that mononuclear cells phagocytosed the hepatitis C virus. Next, we extracted blood from three healthy volunteers and examined the blood cell removal rate using various filters. The HPC-SPS (fiber diameter, 3.5 µm) showed the highest mononuclear cell removal rate and the lowest platelet removal rate. HPC-SPS reduced hepatitis C virus-positive cells in patients with the hepatitis C virus, and the removal rate of monocytes containing the hepatitis C virus was 77.2%. Thus, HPC-SPS may serve as filter to remove mononuclear cells and granulocytes from blood. Although a technique using extracorporeal circulation is required to apply HPC-SPS, the technical cost is lower than IFN-free drug therapy. We believe that HPC-SPS can be utilized for the treatment of HCV- and leukocyte-related diseases.

Keywords: Hepatitis C virus, Apheresis, Leukocyte, Syndiotactic Polystyrene, Hydroxypropyl Cellulose

1. Introduction

In 2015, approximately 70 million people worldwide were infected with the hepatitis C virus (HCV). Today, roughly 1.7 million people are estimated to be infected with HCV in Japan. Although the number of newly infected HCV patients has decreased over time due to improvements in screening methods for blood transfusions, HCV has not been fully eradicated because many years can elapse between the acquisition of HCV infection and the development of noticeable symptoms, such as hepatitis. Between 60%-80% of HCV-infected patients become HCV carriers, most of whom progress from acute to chronic hepatitis, and the probability of spontaneous remission is extremely low (0.2%). Between 15% and 30% of chronic HCV infections have the potential to cause cirrhosis within 20 years [1]. HCV infects monocytes and lymphocytes, inducing inflammation in the liver as well as other organs. Yamabe et al. demonstrated that HCV-infected lymphocytes induce cryoglobulinemia, which is associated with the risk of developing membranoproliferative glomerulonephritis [2]. Furthermore, Matsumori et al. demonstrated that HCV-infected mononuclear cells were associated with myocarditis and cardiomyopathies [3, 4], suggesting that HCV-infected blood cells are transported in the bloodstream to many other organs besides the liver. Thus, the development of novel treatments to remove infected blood cells could be used to treat HCV.

In the current study, we focused on leukocyte reduction filters used for blood transfusion [5]. We confirmed HCV infection in mononuclear cells and used blood from healthy volunteers to investigate filtration materials for effective mononuclear cell removal.
2. Materials and Methods

2.1. Immunohistochemistry

We confirmed HCV phagocytosis by mononuclear cells using immunohistochemistry, according to Matsumori et al. [3]. Briefly, we obtained 20-mL blood samples from three HCV-infected volunteers and isolated the mononuclear cells from heparinized venous blood by density gradient centrifugation (800 × g, 20 min, without braking) using Lymphoprep™ (Axis-Shield Diagnostics Ltd., Dundee, Scotland). Then, the mononuclear cells were homogenized in 1 mL of phosphate-buffered saline (PBS) using an ultrasonic homogenizer, centrifuged at 1500 × g for 10 min, and washed in 1 mL of PBS three times. We prepared slides of the cells and extract solutions. After drying and fixing the slides with 100% ethanol, we applied mouse monoclonal antibodies against the HCV-core antigen (1:100 dilution) using Lumipulse Presto Ortho HCV (Fujirebio Inc, Tokyo, Japan) and incubated overnight at 4°C. Then, the slides were incubated with a secondary antibody using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. This was followed by incubation with a TSA Biotin System Kit (NEN Life Science Products, Boston, MA, USA) for signal amplification.

2.2. Filter Material

Solutions were created by dissolving hydroxypropyl cellulose (HPC) and cellulose acetate (CA) in oleyl alcohol, respectively. Then, two types of syndiotactic polystyrene (SPS) resin sheets, with diameters of 1.8 and 3.5 µm, respectively, were added to each solution and incubated at 25°C for 24 h. After incubation, the sheets were air-dried and surface coated with each material by heat treatment at 70°C for 30 min. The filter is shown in Figure 1. We performed perfusion tests using 6 mL of blood from each of the healthy volunteers with SPS, HPC-SPS, and CA-SPS filters, respectively, at a speed of 30 mL/min through the column. The blood cells that passed through the column were destroyed using a supersonic wave homogenizer, and the HCV antigen density was measured. Additionally, the cells obtained using density gradient centrifugation with Lymphoprep™ were also destroyed using supersonic waves, and the HCV antigen density was measured. Whole blood smears and slides of the isolated leucocytes obtained using Lymphoprep™ were prepared before and after the perfusion experiment and were immunostained using mouse monoclonal antibody for HCV-core antigen. Hematoxylin and eosin staining was then performed.

2.3. Perfusion Experiment with HCV-Positive Blood

A cylindrical column was manufactured using a da Vinci All-in-One (AiO) 3D printer (XYZ Printing, San Diego, CA, USA) (Figure 2). The best material among the SPS, HPC-SPS, and CA-SPS filters was chosen as the filter material in the cylindrical column. The cylindrical column was perfused with 500 mL of heparinized saline, and all solutions were drained to prevent dilution of the blood sample.

Figure 2. Cylindrical column image. We manufactured a cylindrical column using 3D printer (XYZ Printing, San Diego, CA, USA).

We obtained 30-mL blood samples from 14 people who were HCV antigen-positive (genotype Ib). The perfusion experiments were performed using 25 mL of blood at a speed of 30 mL/min through the column. The blood cells that passed through the column were destroyed using a supersonic wave homogenizer, and the HCV antigen density was measured. Additionally, the cells obtained using density gradient centrifugation with Lymphoprep™ were also destroyed using supersonic waves, and the HCV antigen density was measured. Whole blood smears and slides of the isolated leucocytes obtained using Lymphoprep™ were prepared before and after the perfusion experiment and were immunostained using mouse monoclonal antibody for HCV-core antigen. Hematoxylin and eosin staining was then performed.

2.4. Ethical Approval

All subjects enrolled in this research have given their informed consent, which has been approved by the relevant institutional committees on human research, and this protocol has been found acceptable by them. The study protocol was reviewed and approved by the Himeji Dokkyo University Ethics Committee (number: 14-17) and the Ethics Committees of Sugita Genpaku Memorial Obama Municipal Hospital, Nabeya Clinic, Public Obama Hospital, Tango municipal institution Yasaka Hospital, and Otsuka clinic.

3. Result

HCV-positive cells were confirmed in the blood and mononuclear cells of HCV-infected volunteers (Figure 3). The
platelet removal rates using HPC-SPS were 32.5% and 31.5% for fiber diameters of 1.8 and 3.5 µm, respectively, which were lower than for the other materials. However, the removal rate for granulocytes using 3.5-µm fibers was the highest with HPC-SPS (87.0%) compared with CA-SPS (57.4%) and SPS (38.6%). The removal rate for monocytes using 3.5-µm fibers was also the highest with HPC-SPS (91.2%) compared with CA-SPS (79.7%) and SPS (41.1%) (Table 1). The adsorption of monocytes and granulocytes to the fibers was confirmed by Giemsa staining. The number of cells adsorbed to the HPC-SPS fiber was higher than that for CA-SPS and SPS fibers (not shown).

A cylindrical column was prepared using HPC-SPS with 3.5 µm fibers. The removal rates of HCV from whole blood and plasma were 59.2±20.0% (P=0.0251) and 57.0±25.8% (P=0.0463), respectively. Only monocytes were separated from the whole blood, and the perfusion product was destroyed using a supersonic wave homogenizer. The removal rates of HCV in the perfusion for monocytes was 77.2±20.5% (P=0.0492), which was significantly reduced from the HCV levels prior to the perfusion (Figure 4). Additionally, immunostaining revealed that HCV levels were reduced after perfusion compared with before perfusion (Figure 5).

### Table 1. Blood cell removal rates (%).

| Fiber diameter (µm) | 1.8  | 3.5  |
|---------------------|------|------|
|                     | SPS  | CA-SPS | HPC-SPS | SPS  | CA-SPS | HPC-SPS |
| Blood component     |      |       |        |      |       |        |
| Platelets           | 98.2 | 40.9  | 32.5   | 98.9 | 42.1  | 31.5   |
| Granulocytes        | 98.5 | 97.8  | 83.7   | 38.6 | 57.4  | 87     |
| Monocytes           | 97.7 | 95.3  | 79     | 41.1 | 79.7  | 91.2   |

4. Discussion

Treatment for chronic hepatitis C generally includes liver-supporting therapy and antiviral therapy. Liver-supporting therapy slows the progression of cirrhosis, while antiviral therapy is a fundamental treatment to eliminate HCV from the body. Interferon (IFN) therapy for the treatment of hepatitis C began in 1986 [6]. Subsequently, combination therapy with IFN and ribavirin (RBV) in 1998 and pegylated interferon (PEG-IFN) and RBV in 2001 improved therapeutic efficiency [7-10]. Protease inhibitors with PEG-IFN and RBV were first approved as direct-acting antiviral agents in 2011 [11]. The newest combination therapy, featuring nonstructural protein 5A inhibitors (ledipasvir) and nonstructural protein 5B polymerase inhibitors (sofosbuvir), was approved as IFN-free drug therapy in 2015 [12]. IFN-free drug therapies have long been sought due to their expected efficacy in many patients, including those experiencing IFN side effects, those with high HCV viral loads, those of Black ethnicity, elderly patients, and those with HIV infection. However, because ledipasvir–sofosbuvir is extremely expensive and must be taken daily for 12 weeks, IFN-free therapy is impossible in countries without comprehensive medical insurance systems. The highest HCV
prevalence rates occur in poor developing countries in Africa and Asia, whereas the developed, industrialized nations in Europe and North America have low prevalence rates [13]. The World Health Organization has asked countries to take advantage of the recent reductions in the cost of diagnosis and viral hepatitis treatment and to scale-up investments for disease elimination [1]. The Government of India has announced free testing and treatment for hepatitis B and C as part of its universal health coverage plan, which could decrease the annual treatment costs for hepatitis B and C. Additionally, the Government of Pakistan procured hepatitis C curative treatment at similarly low prices, and the provision of curative treatment to all those presently diagnosed with hepatitis C could reduce healthcare costs in Pakistan within 3 years [1].

In this study, we confirmed HCV infection in mononuclear cells and also detected HCV antigen in the homogenized cell extract. Therefore, it is clear that HCV is capable of infecting mononuclear cells. HCV-infected patients experience not only liver damage but also inflammation of extrahepatic organs [2-4]. We considered that HCV-infected mononuclear cells could be transported to other organs through the bloodstream, contributing to elevated HCV-related inflammation. Therefore, monocyte removal could suppress the inflammation induced by HCV-infected blood cells. In this study, we eliminated mononuclear cells using extracorporeal circulation, which is an established and useful technique in hemodialysis, artificial livers, and cardiopulmonary bypass [14-16]. A dialyzer and adhesion filter have been applied during extracorporeal circulation to eliminate waste and specific substances [16, 17]. When using dialyzers and adhesion filters, the device materials are determined according to the substances to be removed. In this study, we applied materials used as leukocyte reduction filters during blood transfusions to remove HCV-infected mononuclear cells. Our results showed that the use of SPS resin as the filter material decreased the amount of circulating platelets and mononuclear cells. One concern when using SPS resin is the possible attachment and coagulation of platelets in extracorporeal circulation. This problem has also been observed in hemodialysis research [18, 19]. Thus, we aimed to include materials with very few hydroxyl groups to maintain hydrophilicity for suppressing the activation of coagulation factors and complement while removing mononuclear cells; we also attempted to coat the surface of the SPS resin with HPC and CA, which are both hydrophilic polymers. Both HPC-SPS and CA-SPS showed reduced platelet removal rates compared with SPS resin alone. Furthermore, HPC-SPS demonstrated a higher leucocyte removal rate but a lower platelet removal rate than CA-SPS. Therefore, we believe that HPC-SPS may serve as a suitable filter to remove mononuclear cells. HPC-SPS removed HCV-infected blood cells. HPC is a derivative of cellulose with both water solubility and organic solubility. Since cellulose derivatives are currently used for absorptive filtration of viruses, HPC will also be effective for removing floating HCV [20]. However, there were individual differences in the levels of HCV among the patients. In patients with a high level of HCV, the removal rate is limited, so improvements, such as an increased filter and column size, are necessary. Kotera et al. showed that polysulfone membranes had strong HCV antigen adsorption in HCV dialysis patients using in vitro experiments and mathematical models, and treatment every 2 days reduced the amount of HCV by approximately 30%, with 60% of the 30% rebounding in the next hemodialysis treatment. However, if this procedure is performed daily, the rebound amount will be suppressed, which should lead to a decreased HCV level. Compared with this result, our HCV removal rates using HPC-SPS were very high. Using the mathematical model of Kotera et al., it can be expected that the HCV level before the treatment will be significantly reduced by approximately 5-fold [21, 22]. Alternatively, increasing the number of perfusions may decrease the amount of HCV.

Granulocyte–monocyte apheresis has previously been proposed for the treatment of inflammatory bowel disease, particularly ulcerative colitis [23]. Therefore, HPC-SPS could also be applied to the treatment of ulcerative colitis due to its high granulocyte removal rate. We believe that HPC-SPS can be utilized not only for HCV-related diseases but also for leukocyte-related diseases.

5. Conclusion

We confirmed HCV infection in mononuclear cells and examined the most effective removal filter for mononuclear cells using healthy blood. HPC-SPS demonstrated a high leukocyte removal rate alongside low levels of platelet activation. HPC-SPS have a synergistic effect on the removal of both HCV-infected blood cells and floating HCV because HPC is a cellulose derivative and is also effective in removing free HCV. Although a technique using extracorporeal circulation is required to apply HPC-SPS, the technical cost is lower than IFN-free drug therapy. This therapy in addition to extracorporeal circulation can be used for HCV patients in developing countries. We believe that HPC-SPS can be utilized for the treatment of HCV- and leukocyte-related diseases.

Future Perspective

In the future, it is necessary to increase the number of HCV patients. Since expensive drug treatment is difficult in developing countries, we aim to build a system that can use extracorporeal circulation therapy for HCV patients in developing countries. Regarding the treatment method, the treatment time and the number of treatments required will be examined in detail.

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