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Cytokines in adipose-derived mesenchymal stem cells promote the healing of liver disease

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Abstract

Adipose-derived mesenchymal stem cells (ADSCs) are a treatment cell source for patients with chronic liver injury. ADSCs are characterized by being harvested from the patient's own subcutaneous adipose tissue, a high cell yield (*i.e.*, reduced immune rejection response), accumulation at a disease nidus, suppression of excessive immune response, production of various

growth factors and cytokines, angiogenic effects, anti-apoptotic effects, and control of immune cells via cell-cell interaction. We previously showed that conditioned medium of ADSCs promoted hepatocyte proliferation and improved the liver function in a mouse model of acute liver failure. Furthermore, as found by many other groups, the administration of ADSCs improved liver tissue fibrosis in a mouse model of liver cirrhosis. A comprehensive protein expression analysis by liquid chromatography with tandem mass spectrometry showed that the various cytokines and chemokines produced by ADSCs promote the healing of liver disease. In this review, we examine the ability of expressed protein components of ADSCs to promote healing in cell therapy for liver disease. Previous studies demonstrated that ADSCs are a treatment cell source for patients with chronic liver injury. This review describes the various cytokines and chemokines produced by ADSCs that promote the healing of liver disease.

Key words: Cell transplantation therapy; Cytokine; Hepatocytes; Liquid chromatography with tandem mass spectrometry; Liver cirrhosis; Adipose-derived mesenchymal stem cells

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Core tip: We previously showed that conditioned medium of adipose-derived mesenchymal stem cells (ADSCs) promoted hepatocyte proliferation and improved the liver function in a mouse model of acute liver failure. Furthermore, as reported by many other groups, the administration of ADSCs improved liver tissue fibrosis in a mouse model of liver cirrhosis. A comprehensive protein expression analysis by liquid chromatography with tandem mass spectrometry showed that the various cytokines and chemokines produced by ADSCs have the ability to promote the healing of liver disease. In this review, we examine the ability of the expressed protein components of ADSCs to promote healing in cell therapy for liver disease.

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INTRODUCTION

We and others have conducted clinical studies in patients with symptoms of autoimmune liver disease^[1], hepatitis C^[2,3], bacterial infection^[4], acute liver failure liver^[5],

nonalcoholic fatty liver disease^[6] and cirrhosis. We have also conducted translational research that bridges basic research using hematopoietic cells^[7-14], hepatic stellate cells (HSCs)^[15-18], embryonic stem cell-derived hepatocytes^[19-22], bioartificial livers^[23-26], animals^[27-33] and clinical research in humans.

Thanks to their expected therapeutic efficacy, mesenchymal stem cells^[34] are currently under clinical evaluation as a cell source for cell therapy in trials of regenerative medicine for a broad spectrum of diseases. Since adipose-derived mesenchymal stem cells (ADSCs)^[35,36] are obtained from the patient's abdomen by liposuction, cell procurement is relatively easy (large numbers of cells can be obtained by minimally invasive treatment). ADSCs can avoid immune rejection if they are autografted^[37-39]; however, similarly to other cell sources, they are subject to immune rejection if allogeneic or xeno cell transplantation is performed. Mesenchymal stem cells are used for medical treatment worldwide. Since ADSCs are not a mainstream therapeutic cell, we have been performing clinical studies of treatments using ADSCs. For this reason, cell therapy using ADSCs is now performed in many public and private hospitals worldwide. In this review, we examine the effects of ADSCs in cell therapy for liver diseases, focusing on the proteins secreted by ADSCs. We previously reported that the proteins expressed by human ADSCs cultured using Dulbecco's Modified Eagle's medium [10% fetal bovine serum (FBS)] and clinical-grade chemically-defined medium showed 98% similarity, demonstrating that the proteins expressed by ADSCs cultured in media for research and clinical use largely coincide^[40,41]. However, using animal-derived components in the process of culturing cells for treatment is associated with a risk of transmitting pathogenic infections derived from animals (e.g., bovine spongiform encephalopathy or swine fever). Furthermore, when animal-derived ingredients are used the risks and quality may vary in individual lots. Thus, the use of chemically defined media with recombinant protein is recommended for large-scale culture conditions, such as the manufacturing of therapeutic cells for industrial use. In this background, we use the medium containing FBS as the control medium for the cultivation of research cells, while chemically-defined media is the first choice for culturing therapeutic cells. In addition, we do not deny the option of using a supplement that uses infectious and highly safe virus-tested human serum as a raw material for culturing therapeutic cells.

Gene ontology (GO) facilitates^[42,43] the development of a common vocabulary to describe biological concepts. Terms defined in GO are called GO terms (GO is a classification of biological phenomena that associates genes with their known [reported in the existing literature] biological role structured based on given criteria), which are divided into three categories: Biological processes, cellular components, and molecular functions. The Gene Ontology Consortium (<http://www>.

geneontology.org/) is a database of functional information that aims to describe biological phenomena in standardized terms. In recent years, liquid chromatography with tandem mass spectrometry (LC-MS/MS) has been used to perform a GO classification of comprehensive expression data using protein analysis software programs. Both a comprehensive expression analysis of proteins using LC-MS/MS and a protein GO analysis were performed according to methods that we reported previously^[40,41,44].

In normal liver tissue, blood flows from the portal vein through the central veins, each of which supplies sufficient blood to the hepatocytes of a single hepatic lobule (Figure 1). The blood supply to hepatocytes remains sufficient in carbon tetrachloride (CCL4: 0.5 mL/kg)-administered acute liver failure model in mice^[40,41]. Liver failure is defined as a group of diseases associated with the development of symptoms such as jaundice, ascites, hepatic encephalopathy, bleeding tendency, or the like, due to a decrease in the number of hepatocytes or a decrease in their function. Acute liver failure is defined by the presence of necrosis and inflammation in normal liver tissue, with the period until the onset of symptoms of hepatic insufficiency being within 8 wk. Cases in which the onset of symptoms is 8–24 wk or > 24 wk are classified as delayed liver failure (late onset hepatic failure; LOHF) and chronic liver failure, respectively. The American Association for the Study of Liver Diseases published a position paper on acute liver failure in 2005 to unify the terms and disease concepts^[45].

In contrast to this acute model, the preparation of the CCL4 (2.0 mL/kg)-administered cirrhosis model mouse requires more than 6 wk. The liver tissue in this mouse model of liver cirrhosis shows a marked increase in the fiber component of the fibrous septa. This results in the separation of some lobules by fiber components, and the creation of pseudo-lobules. At the same time, the blood supply to the lobules decreases and liver cell necrosis occurs (Figure 2). Chronic liver injury also results in the differentiation of astrocytes into myofibroblastoid cells, in turn causing the pathogenesis of fibrotic liver injury^[46]. Given this background, the factor most necessary for the improvement of acute liver failure symptoms is hepatocyte growth factor (HGF). In contrast, the most important factors for the improvement of the symptoms of chronic liver failure (*i.e.*, liver cirrhosis) are: (1) growth factors; (2) inhibition of the inflammation of hepatic stellate cells; and (3) angiogenic factors.

When the disordered repair process is delayed or inhibited after liver damage from drugs, trauma, inflammation, or other insults, liver regeneration is insufficient and hepatic failure develops. In hepatic tissue repair, in addition to growth factors that promote hepatocyte proliferation, angiogenic factors that promote hepatic microvascular remodeling are important. In addition, the extracellular matrix in the liver is mainly

produced in hepatic stellate cells. The ability of hepatic stellate cells to produce extracellular matrix is low in the normal liver. However, in the fibrous liver, it is known that hepatic stellate cells are activated to differentiate into myofibroblasts and their ability to produce extracellular matrix markedly increases. Interleukin-6 (IL-6), tumor necrosis factor- α , HGF, and other factors secreted from HSCs, hepatic sinusoidal endothelial cells and Kupffer cells are thought to have the greatest influence on hepatocyte proliferation^[47]. Angiogenesis is a physiological phenomenon in which a new blood vessel branch is branched from an existing blood vessel to construct a vascular network. The various factors involved in angiogenesis include fibroblast growth factor, vascular endothelial cell growth factor (VEGF), angiopoietin, and platelet derived growth factor (PDGF).

GROWTH FACTORS IMPROVING THE SYMPTOMS OF LIVER CIRRHOSIS

HGF acts as a HGF and metabolic regulator and promotes hepatocyte proliferation^[48]. The hepatic development of the liver is the origin of the gut tube, which is formed by the accumulation of hematopoietic cells^[49]. Thus, the idea that HGF expressed by hematopoietic cells promotes the regeneration of hepatocytes is plausible. Liver tissue regeneration using HGF may accordingly be considered a treatment method that reproduces the original development of the liver. HGF is expressed by both HSCs and ADSCs.

Our group previously investigated the clinical application of organ preservation solution^[50–56]. We found that the expression level of HGF mRNA did not decrease in ADSCs, even when they were stored in preservation solution for 16 h after separation from adipose tissue. In addition, we found no difference between the expression levels of HGF using glucose-free University of Wisconsin and glucose-containing (5.6 mmol/L) Hank's Balanced Salt Solution. This result shows that the expression of HGF by ADSCs does not decrease after separation from adipose tissue. Moreover, the expression is not affected by the glucose concentration. In addition, the expression of VEGF showed a similar tendency. In short, ADSCs constantly express HGF and VEGF both *in vivo* and *in vitro*^[57]. A recent theory suggests that the biliary tree functions as a source of liver and pancreatic stem cells and progenitor cells. VEGF is secreted by the biliary tree as a response to stress^[58]. From these developmental perspectives, HGF and VEGF secreted by ADSCs appear to have a marked promoting effect on hepatocyte proliferation.

Our experiments showed that the administration of ADSC conditioned medium (CM) from a single vein rapidly promotes the cellular proliferation of mouse hepatocytes (Figure 3). The proteins associated with a growth function (GO analysis), identified by the presen-

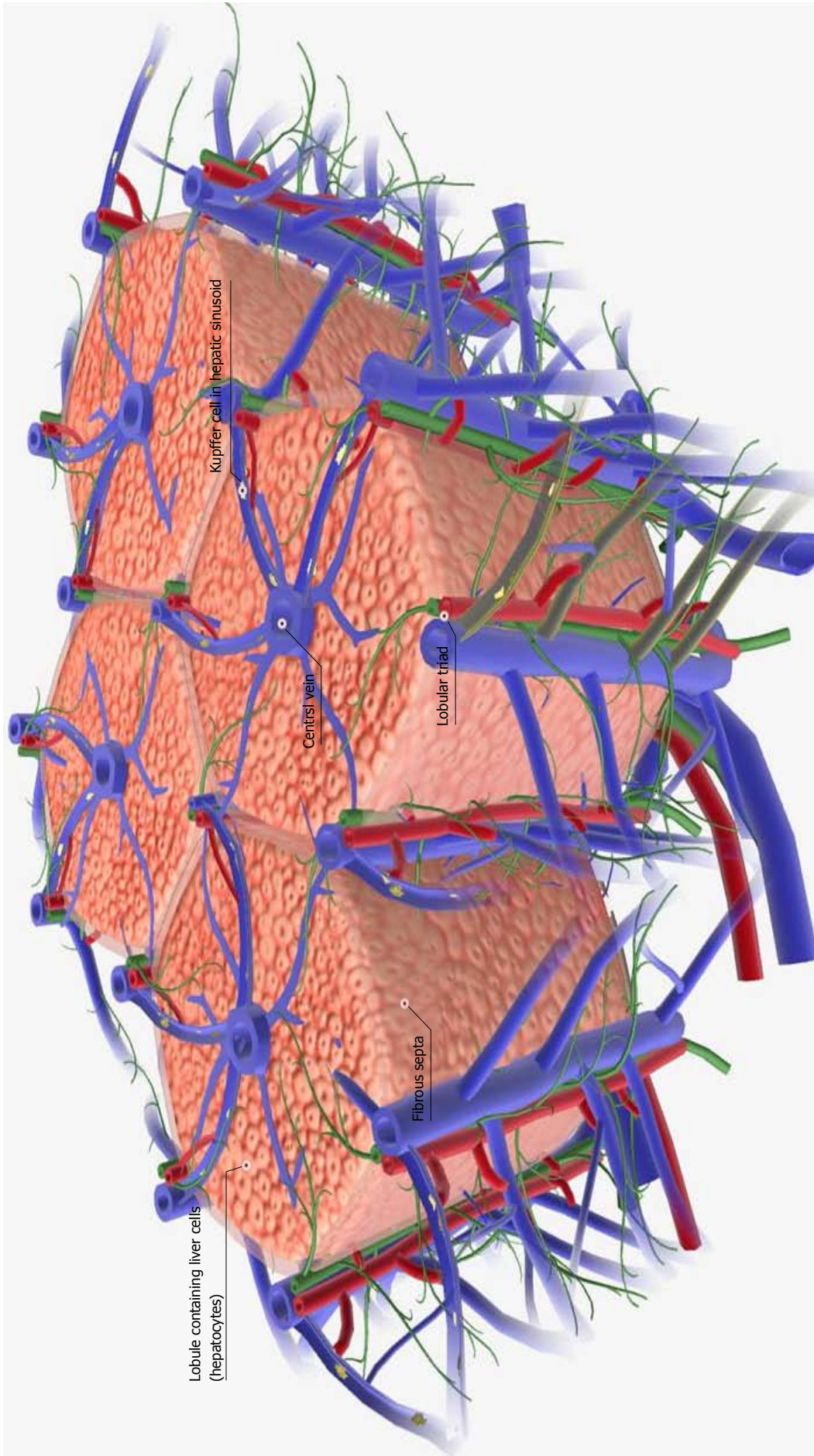


Figure 1 Schematic diagram of a normal liver tissue model. The liver is a digestive organ that filters and detoxifies blood from the digestive tract. It also produces proteins, such as albumin, and synthesizes cholesterol and bile. The functional portion of the liver tissue is organized into hexagonal columns called liver lobules. Each liver lobule contains hundreds of individual liver cells (hepatocytes) and a large central vein. Lobular portal triads, which contain branches from the hepatic portal vein, hepatic artery and bile duct, are located at the points of the hexagonal lobule. Blood from the branches of the hepatic artery joins the blood of the hepatic portal vein branches, forming hepatic sinusoids. Hepatic sinusoids are lined with specialized cells called Kupfer cells, which help collect debris and detoxify the blood. All hepatic sinusoids in the liver lobule drain into the central vein. Adjacent lobules are separated by a thin fibrous septa. Images were obtained from BIODIGITAL HUMAN 3.0 (<https://human.biodigital.com/index.html>) (BioDigital, Broadway, NY, United States).

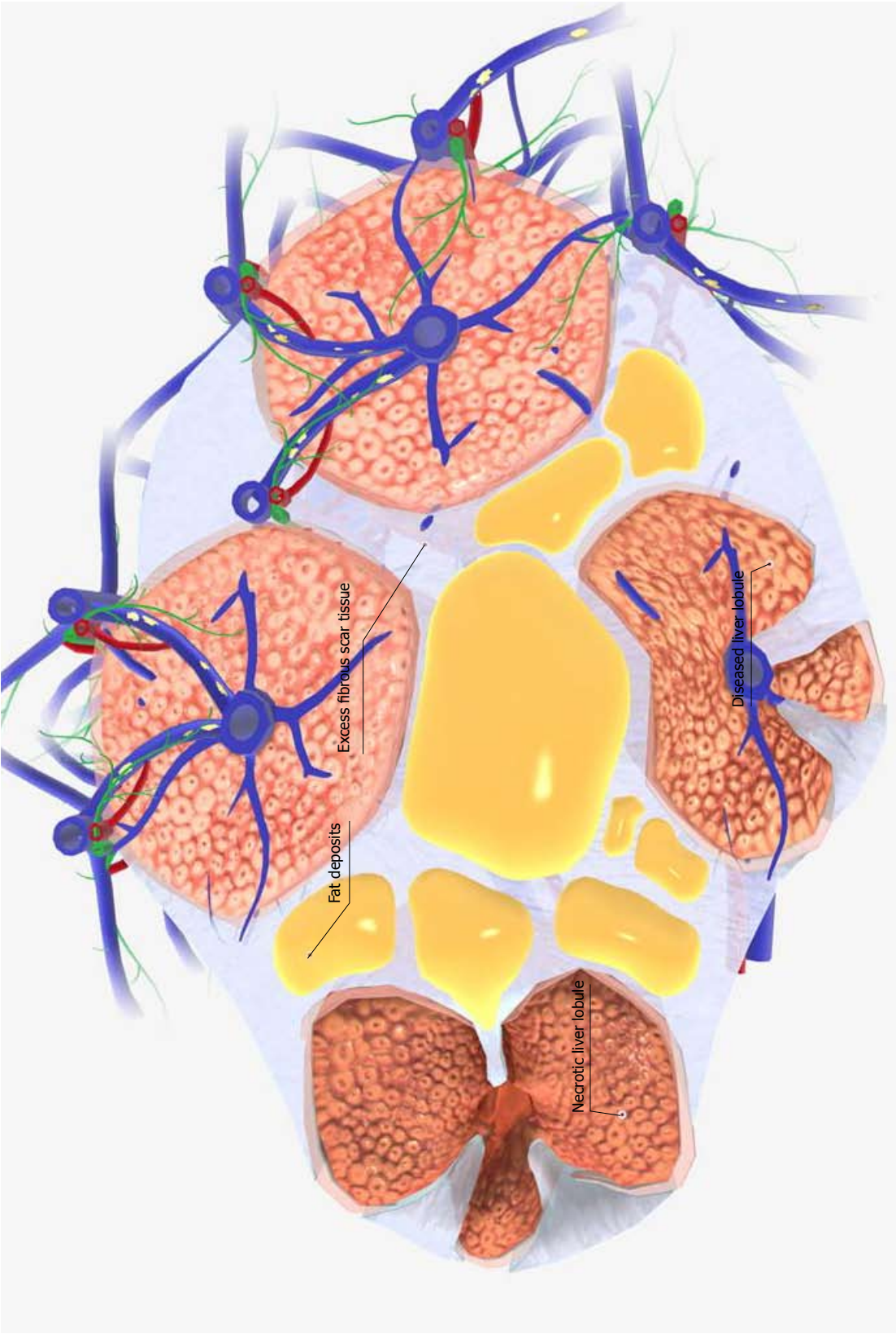


Figure 2 Schematic diagram of a liver cirrhosis tissue model. The functional portion of the liver tissue is organized into hexagonal columns called liver lobules. Each liver lobule contains hundreds of individual liver cells (hepatocytes). In healthy liver tissue, adjacent lobules are separated by thin fibrous septa. However, liver cirrhosis involves thickening of the fibrous septa that separate lobules, and the deposition of fat. As a result, the blood flow in the lobules is disturbed and hepatocyte necrosis occurs. Also, the fibrous septa that separate the lobules transform the lobules and produce pseudolobules. Although it has a wide variety of causes, liver cirrhosis is most commonly caused by chronic alcohol abuse, chronic hepatitis, and nonalcoholic fatty liver disease. Images were obtained from BIODIGITAL HUMAN 3.0 (<https://human.biodigital.com/index.html>) (BioDigital, Broadway, NY, United States).

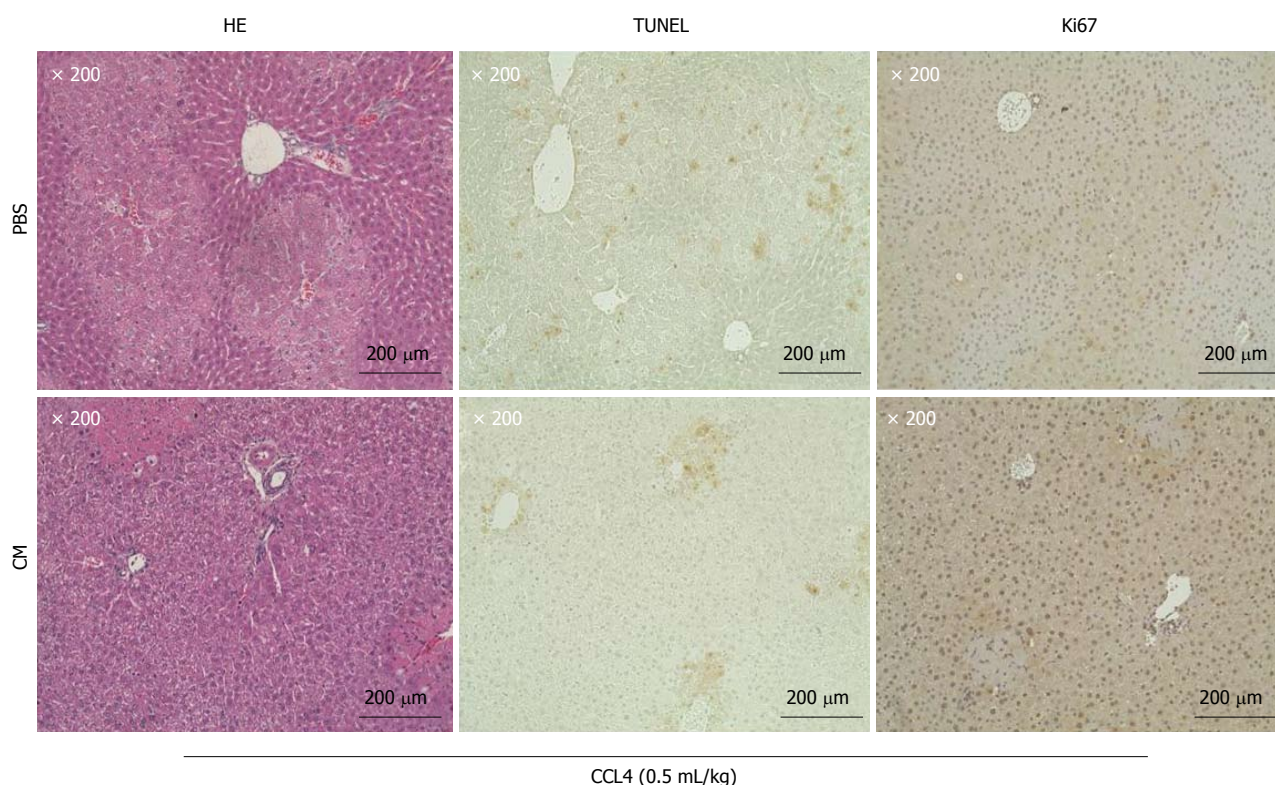


Figure 3 Culture supernatant concentrate significantly improved the symptoms of acute liver failure caused by the administration of CCL4. Micrographic images of Hematoxylin and Eosin (HE) staining (left panels), TUNEL assay (middle panels) and tissue immunostaining of Ki67 (right panels) of liver specimens. Microscopic images of liver specimens 20 h after the administration of PBS (upper panels) and CM (lower panels) via the mouse tail vein. Fragmented DNA generated in the process of apoptosis can be detected by the TUNEL (TdT-mediated UTP nick end labeling) method. Ki67 protein present in the nucleus of cells in G1, S, G2 and M cycles (cell growth phase) was detected using immunostaining to identify cells in the growth phase in liver tissue. It was also used to count the number of positively stained cells in images of TUNEL-stained sections ($\times 200$). The numbers of positively stained cells in the PBS and CM groups were 14.00 ± 4.54 and 8.25 ± 5.57 , respectively ($n = 4$; $P = 0.19$). The numbers of cells with positively stained nuclei on images of Ki67-stained sections ($\times 200$) were also counted. The numbers of cells with positively stained nuclei in the PBS and CM groups were 9.25 ± 7.61 and 116.25 ± 3.06 , respectively ($n = 4$; $P < 0.01$).

ce of ADSC-CM, were Periostin (POSTN), P component (SAP), semaphorin 7A (SEM7A), and Inactive tyrosine-protein kinase $7^{[40,41]}$. Periostin, which is encoded by the POSTN gene, has been reported to be an extracellular factor that promotes hepatosteatosis^[59,60]. Nevertheless, much remains unknown about proteins with the ability to promote the cellular proliferation of hepatocytes. For example, SAP, a protein that is expressed in hepatocytes and secreted into serum, is known to be involved in processes associated with immune regulation, such as the action of opsonins^[61], but whether SAP is involved in the cellular proliferation of hepatocytes is unknown. Further, SEM7A is known to contribute to transforming growth factor (TGF)- β -mediated hepatic fibrosis^[62], but whether it promotes hepatocyte cell proliferation is unknown. Future studies should therefore investigate whether the growth-associated proteins that are newly identified by GO analyses promote the cellular proliferation of hepatocytes in CCL4-induced liver impairment. What is certain is that HGF and VEGF secreted by ADSCs are among the key factors promoting the proliferation of hepatocytes.

INFLAMMATION INHIBITOR OF HEPATIC STELLATE CELLS

The Jun amino-terminal kinases (JNK) signaling pathway is involved in the activation of HSCs^[63,64]. JNK1 plays a major role in the upregulation of the α -SMA expression in HSCs under the stress conditions induced by TGF- β during liver fibrosis^[65]. We previously reported the clinical application of organ preservation solution with a JNK inhibitory peptide (11R-JNKI)^[66-68] and 8R-sJNKI(-9)^[69]. The design of these cell-permeable inhibitory peptides is not only significant for *in vivo* studies, but also for future attempts to design inhibitors of liver fibrosis for the clinical treatment of liver cirrhosis. In addition, we previously reported that Arg-Gly-Asp (RGD) peptide^[70] and Rho-kinase inhibitor^[71] suppresses liver fibrosis. Our experiments show that the administration of ADSCs (1×10^6 cells) from a total of three veins at a twice weekly interval rapidly improves the fibrosis of excessive mesenchyme around mouse hepatocytes (Figure 4). When ADSCs are administered via the mouse tail vein, they cause pulmonary embolism, which has a high pro-

Table 1 Relationship between immunomodulatory protein secreted by adipose-derived mesenchymal stem cells and liver cirrhosis

UniProt/SWISS-PROT ID	Description	Reference
FINC	Fibronectin	[74]
CO1A2	Collagen alpha-2(I) chain	
CO1A1	Collagen alpha-1(I) chain	
CATB	Cathepsin B	[73]
TSP1	Thrombospondin-1	
CFAH	Complement factor H	[75]
GAS6	Growth arrest-specific protein 6	
LEG1	Galectin-1	[77]
PTX3	Pentraxin-related protein PTX3	
C1S	Complement C1s subcomponent	[78]
SEM7A	Semaphorin-7A	
CLUS	Clusterin	[79]
G3P	Glyceraldehyde-3-phosphate dehydrogenase	
PXDN	Peroxidase homolog	[80]
SRCL	Soluble scavenger receptor cysteine-rich domain-containing protein	
SSC5D		[81,82]
CD248	Endosialin	
SPON2	Spondin-2	[80]
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	
CD109	CD109 antigen	[81]
CFAB	Complement factor B	
CATL1	Cathepsin L1	[82]
MFAP5	Microfibrillar-associated protein 5	
MIF	Macrophage migration inhibitory factor	[83]
CXCL5	C-X-C motif chemokine 5	
ADAM9	Disintegrin and metalloproteinase domain-containing protein 9	[84]
CATK	Cathepsin K	

ability of causing the death of the mouse. Our group developed a method to safely administer ADSCs using heparin^[72]. The proteins associated with the immune system process (GO analysis) identified by the presence of ADSC-CM were FINC, CO1A2, CO1A1, CATB, TSP1, CFAH, GAS6, LEG1, PTX3, C1S, SEM7A, CLUS, G3P, PXDN, SRCL, CD248, SPON2, ENPP2, CD109, CFAB, CATL1, MFAP5, MIF, CXCL5, ADA M9, and CATK (Table 1)^[40,41]. Among these ADSC-secreted proteins, we found no studies reporting a relationship in the field of liver cirrhosis and hepatic stellate cells for FINC, CO1A2, CATB, CFAH, LEG1, C1S, SEM7A, CLUS, G3P, PXDN, SRCL, SPON2, ENPP2, CD109, CFAB, CATL1, MFAP5, ADAM9, or CATK. It is expected that these proteins will be investigated in future studies.

Type I collagen and fibronectin are also reported to be components of hepatic fibrosis^[73,74]. It is therefore unlikely that CO1A1 and CO1A2, which are secreted by ADSCs, suppress the excess activity of HSCs. Thrombospondin-1, a matricellular glycoprotein that is secreted by many cell types, modulates a variety of cellular functions by binding to extracellular proteins and/or cell surface receptors. Thrombospondin-1 might contribute to liver fibrosis not only as an activator of TGF- β , but also as a modulator of angiogenesis^[75]. In the normal liver, growth arrest-specific gene 6 (Gas6) is mainly expressed in Kupffer cells. The expression of Gas6 increases in activated HSCs and

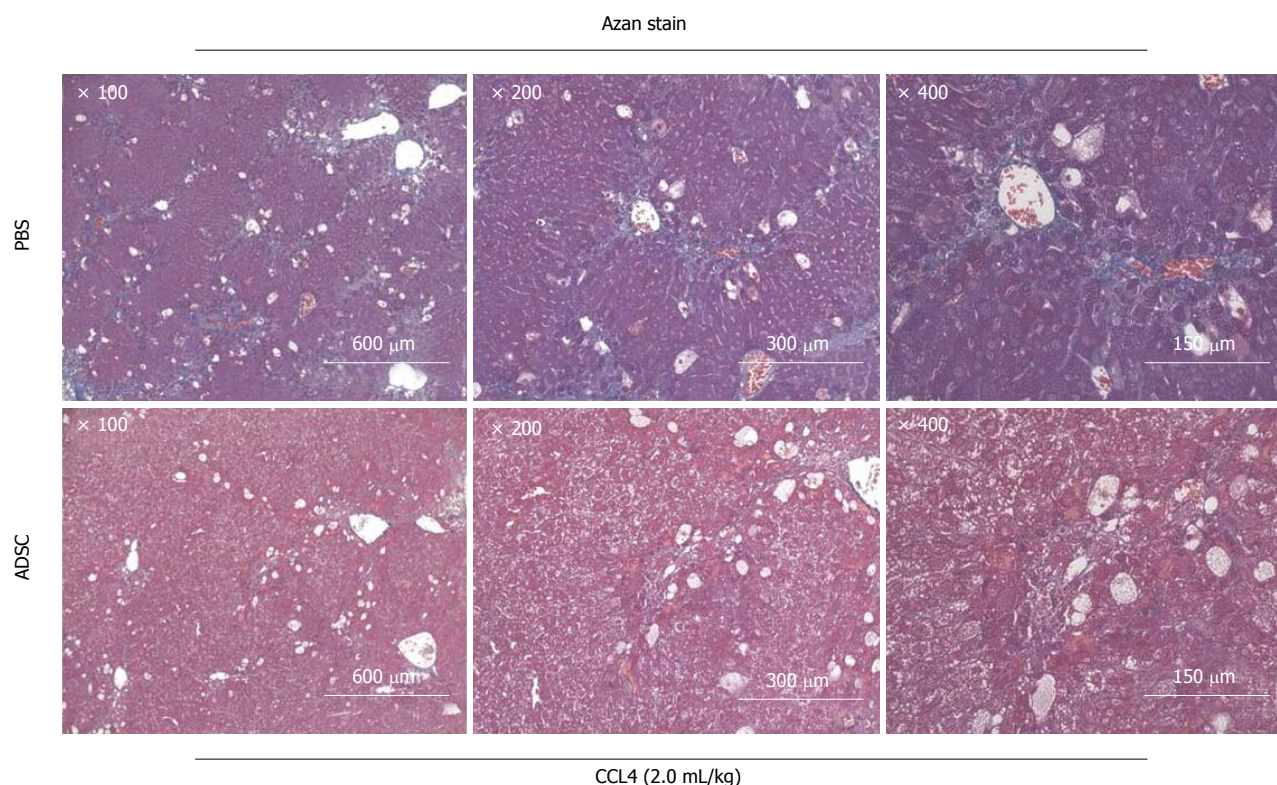


Figure 4 Adipose-derived mesenchymal stem cells improved symptoms of tissue fibrosis in cirrhosis caused by the administration of CCL4. Micrographic image of Azan staining of liver specimens. Azan staining is a fibrous connective tissue staining method that differentiates collagen fibers and muscle fibers. The fibrous connective tissue in the tissue section was stained blue. Microscopic images of the liver specimens 20 h after the administration of PBS (upper panels) and adipose-derived mesenchymal stem cells (ADSC) (lower panels) via the mouse tail vein. In the ADSC administration group, fibrosis and pseudolobule formation were ameliorated. Microscopic images ($\times 100$ - 400) of the same tissue section.

macrophages after acute CCL4 administration^[76]. Given that Gas6 and Axl are reported to be necessary for HSC activation^[77], Gas6 secreted by ADSCs seems to have no effect in inhibiting the activity of HSCs. Pentraxin 3 (PTX3) is expressed and released by hematopoietic cells and stromal cells and is an essential component of innate immunity. IL-1 induces the production of PTX3 by Kupffer cells, endothelial cells and biliary duct epithelial cells. PTX3 is reported to be a biomarker of liver fibrosis in response to hepatic injury^[78]. These reports indicate that PTX3 secreted by ADSCs is unlikely to suppress the activation of HSCs. CD248 (endosialin) is a stromal cell marker expressed on fibroblasts and pericytes. During liver injury, myofibroblasts are the main source of fibrotic matrix. Liver fibrosis was reported to be suppressed in CD248 knockout mice^[79], suggesting that it is unlikely that CD248 secreted by ADSCs inhibits hepatic fibrosis. Macrophage migration inhibitory factor (MIF) is a pleiotropic inflammatory cytokine that has been implicated in various inflammatory diseases. MIF knockout mice were reported to have strongly increased fibrosis in a mouse model of chronic liver injury model. This phenomenon was accompanied by no change in the infiltration of intrahepatic immune cells. MIF has an anti-fibrotic effect on the liver via the MIF receptor (CD74). In addition, recombinant MIF protein has a similar anti-

fibrotic effect^[80]. These results indicate that MIF secreted by ADSCs is a major component in the suppression of liver fibrosis. CXCL5 is the best known for its function as a neutrophil chemotactic factor and activator, with a molecular structure similar to that of IL-8, 4, 5. CXCL5 is released from monocytes, neutrophils, epithelial cells, fibroblasts and smooth muscle during inflammation. Interestingly, CXCL5 has a proliferative effect on rat hepatocytes. The use of a neutralizing antibody of CXCL5 slowed the liver regeneration rate after partial hepatectomy^[81]. The plasma CXCL5 levels are low in patients with chronic liver disease, and CXCL5 may be involved in the pathogenesis of chronic liver disease^[82]. These results strongly indicate the possibility that CXCL5 secreted by ADSCs also promotes hepatocyte proliferation. These findings indicated that MIF is one of the components that suppress liver fibrosis among the ADSC-secreted proteins that we identified. In addition, CXCL5 was identified as a component that promotes hepatocyte proliferation. Of course, the function of these proteins has been previously reported. Table 1 shows 26 different proteins classified as immunomodulatory (GO analysis). The 18 proteins indicated by N/A have not been reported in the liver field, and further research into them is anticipated. Note that proteins that were not classified as immunomodulatory (by a GO analysis) may

Table 2 Relationship between angiogenesis protein secreted by adipose-derived mesenchymal stem cells and liver cirrhosis

UniProt/SWISS-PROT ID	Description	Reference
PAI1	Plasminogen activator inhibitor 1	PAI-1 regulates angiogenesis via effects on extracellular matrix proteolysis and cell adhesion [87]
FSTL1	Follistatin-related protein 1	Knockdown of Fstl1 attenuates hepatic stellate cell activation through the TGF- β 1/Smad3 signaling pathway [88]
POSTN	Periostin	POSTN, a ligand of α v β 3/5 integrins, as an effector protein in SULF2-induced angiogenesis [89]
MMP2	72 kDa type IV collagenase	MMP2 has an important role in the preservation of liver vascular homeostasis [91]
TSP1	Thrombospondin-1	TSP1 was reported to be increased in HSCs isolated from the liver of CCl4-induced cirrhosis model mice [92]
TIMP1	Metalloproteinase inhibitor 1	Inhibition of TIMP1 was reported to promote angiogenesis by increasing cell motility during fibrovascular invasion [93]
FBLN3	EGF-containing fibulin-like extracellular matrix protein 1	FBLN3 has been reported as an angiogenesis antagonist regulating cell morphology, growth, adhesion and motility [95]
MFGM	Lactadherin	MFGM promote angiogenesis via enhanced PDGF-PDGFR β signaling mediated by cross-talk of the integrin growth factor receptor [97]

also have effects on the liver.

ANGIOGENIC FACTORS

EGF, VEGF and HGF, which are expressed by ADSCs, have a strong angiogenic effect on liver tissue^[83-85]. Table 2 lists eight types of proteins [Plasminogen activator inhibitor 1 (PAI1), follistatin-related protein 1 (FSTL1), periostin (POSTN), matrix metalloproteinases 2 (MMP2), TSP1, metalloproteinase inhibitor 1 (TIMP1), Fibulins 3 (FBLN3) and Lactadherin (MFGM)] affecting angiogenesis from among 101 types of proteins secreted by ADSCs. PAI1 is a member of a family of proteins that inhibit plasminogen activators^[86]. Although the binding of VEGF to vitronectin induces strong angiogenic signaling, this is inhibited by competitive binding to PAI1^[87]. PAI1 secreted from ADSCs is therefore thought to inhibit angiogenesis by VEGF in liver tissue. FSTL1 is a secretory glycoprotein belonging to the follistatin and SPARC family. FSTL1 was reported to be highly expressed in fibrotic human liver tissue and activated HSCs^[88]. FSTL1 has the effect of promoting the activity of HSCs. It is therefore unlikely that FSTL1 secreted by ADSCs affects angiogenesis. POSTN, an extracellular matrix (ECM) molecule of the fasciclin family, has roles in vascular cell differentiation and migration^[89]. We therefore hypothesize that POSTN secreted by ADSCs may promote angiogenesis of the liver. MMPs are a family of over 24 zinc-dependent endopeptidases capable of degrading virtually any component of the ECM^[90]. MMP2 plays an important role in the preservation of liver vascular homeostasis *via* its participation in the TGF- β activation process^[91]. MMP2 secreted by ADSCs-and many other cell types-is therefore considered to be one of the main factors. TSP1 was reported to be increased in HSCs isolated from the liver in a mouse model of CCL4-induced cirrhosis. In

liver samples of patients with alcohol cirrhosis and non-alcoholic steatohepatitis-related cirrhosis, TSP1 levels were reported to be increased^[92]. It is thought that TSP1 expressed in the liver has the effect of promoting liver fibrosis. If so, it would be unlikely that TSP1 secreted by ADSCs promotes liver angiogenesis. TIMP1 is a widely expressed inhibitor of MMPs. Given that the inhibition of TIMP1 promotes angiogenesis by increasing cell motility during fibrovascular invasion^[93], TIMP1 secreted by ADSCs may inhibit liver angiogenesis. FBLNs, a versatile family of extracellular matrix proteins, comprise a small family of widely expressed ECM proteins^[94]. FBLN3 has been reported to be an angiogenesis antagonist that regulates cell morphology, growth, adhesion and motility^[95]. It is therefore unlikely that FBLN3 secreted by ADSCs promotes liver angiogenesis. MFGM interacts with α v β 3 and α v β 5 integrins and alters both VEGF-dependent Akt phosphorylation and neovascularization^[96]. MFGM was reported to promote angiogenesis *via* enhanced PDGF-PDGFR β signaling mediated by cross-talk of the integrin growth factor receptor^[97]. Thus, MFGM secreted by ADSCs is considered to be one of the main components promoting liver angiogenesis. We recently reported that ADSCs strongly express MFGM and that human MFGM protected dopamine neurons in a rat model of Parkinson's disease model^[98]. At the present stage, there are no reports on a therapeutic method for the disease-specific selection of therapeutic cells that reference to the list of protein components expressed by therapeutic stem cells.

Regarding cell therapy using ADSCs, we believe that excellent effects on immune response control by cell adhesion can be expected based on reports in the literature. For treatment with ADSC-CM, we used a method to concentrate ADSC-CM 20 times using a 10k filter. ADSC-CM is a liquid and has the advantage of

being able to pass through both a 0.22- μ m sterilizing filter and a 0.10- μ m virus removal filter. We think that lowering the hurdles to cell therapy by taking advantage of the simple adjustment of the solution will contribute to a wide range of medical needs.

These results suggest that VEGF, HGF, EGF, MMP2, POSTN, and MFGM secreted by ADSCs promote hepatic angiogenesis. Among the 101 proteins expressed by ADSCs, we identified three proteins that promote angiogenesis from among eight proteins reported to be involved in angiogenesis. The further development of research into the 93 other proteins is expected.

A study of one-shot stem cell therapy reported the effects of bone marrow-derived mesenchymal stem cell treatment in 53 cirrhosis patients^[99]. Although study period was sufficient to observe short-term symptomatic improvement over a period of days to weeks, it has been reported that there is no improvement in symptoms over the longer term (more than a few months). Although this study did not involve the use of ADSCs, it showed that stem cell treatment improves the symptoms of cirrhosis over the short term. However, it shows that one-shot stem cell therapy does not reset the pathological state of cirrhosis through natural healing power leading to recovery. In this paper, we reported that ADSC has three angiogenesis-inducing effects, which promoted the proliferation of hepatocytes, which suppressed the fibrosis of the liver tissue. We believe that medical stakeholders and patients will be more likely to challenge clinical studies of ADSCs in the treatment of cirrhosis. However, it is unlikely that ADSCs play a direct role in controlling all of the pathological conditions of cirrhosis in the body. Thus, we are of the opinion that cell therapy using ADSCs and treatment using ADSC-CM will be useful as supplementary treatments.

CONCLUSION

The factors necessary for improvement of the symptoms of chronic liver failure (*i.e.*, liver cirrhosis) are: (1) growth factors; (2) inhibitors of hepatic stellate cell inflammation; and (3) angiogenic factors. (1) It is certain that ADSC-secreted HGF and VEGF are among the factors that promote the proliferation of hepatocytes. In addition, CXCL5 was identified as a component that promotes hepatocyte proliferation; (2) MIF, which was one of the ADSC-secreted proteins that we identified that suppressed liver fibrosis; and finally (3) ADSC-secreted VEGF, HGF, EGF, MMP2, POSTN and MFGM are factors that promote hepatic angiogenesis. It seems that the therapeutic effect on the symptoms of cirrhosis is based on ADSC-secreted growth factors, anti-inflammatory effects on stellate cells, and the anti-fibrotic and angiogenic effects of ADSC-secreted proteins.

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