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| Original Paper |

Reduction of Liver Fibrosis by Xenogeneic Human Umbilical Cord Blood and Adipose Tissue-derived Multipotent Stem Cells without Treatment of an Immunosuppressant

Yeong-Geon Lee[1,2,3,*], Jae-Woong Hwang[1,2,3,*], Sang-Bum Park[1,2,3], Il-Seob Shin[5], Soo-Kyung Kang[4], Kwang-Won Seo[1,2,3], Yong-Soon Lee[1,2] and Kyung-Sun Kang[1,2,3,**]

[1]Lab of Stem Cell and Tumor Biology, Dept of Veterinary Public Health, [2]BK21 Program for Veterinary Science, [3]Adult Stem Cell Res Center, College of Veterinary Medicine, [4]Lab of Veterinary Biotech, College of Veterinary Medicine and BK21 Program for Veterinary Science, Seoul Nat'l Univ, Seoul 151-742, Korea, [5]Stem Cell Res Institute, RNL BIO CO, Ltd, Seoul 153-768, South Korea

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Abstract : Therapy using stem cells for the liver fibrosis is a prospective alternative to overcome the insufficiency of transplantable liver donor. Here, we demonstrated xenogeneic human cell therapy for the treatment of rat liver fibrosis without the use of an immunosuppressant. Liver fibrosis was induced by dimethylnitrosamine for 5 weeks in six-week-old male Sprague-Dawley rats. Human umbilical cord blood- and adipose tissue-derived multipotent stem cells were injected intravenously by the tail vein after one week. Blood samples were collected and liver samples were stained with Masson's trichrome in order to evaluate the amount of fibrosis. After the cell injection, the level of total protein, albumin, alanine transaminase and aspartic acid transaminase was recovered to the similar level of the normal rats. The liver weight per body weight increased after the cell injection. Collagen fiber, near the portal triad and marginal region, was reduced, significantly. Taken together, it is suggested that xenotransplantation of multipotent stem cells might be a candidate for the treatment of liver fibrosis without the use of an immunosuppresant.

Key words: *Liver fibrosis, multipotent stem cells, xenegeneic transplantation umbilical cord blood, adipose tissue*

1. Introduction

Liver disease is one of the major causes of death in many countries.[1] Liver cirrhosis are typically the primary causes of mortality.[2] In the course of progression to cirrhosis, liver fibrosis is a pivotal and necessary stage.[3] Liver fibrosis is the wound healing process associated with various liver injuries and it is characterized by continuous collagen deposition in the extra-cellular matrix(ECM).[4] Therefore, it is very important to prevent the development of liver fibrosis and to determine the mechanism involved in development of liver fibrosis. To reduce the fibrosis of the liver, many clinical trials using multipotent stem cells(MSCs) have been demonstrated as followings; MSCs derived from bone marrow were administered intravenously to the liver-injured rats or mice and they were homed to the injured liver, reducing the fibrosis of the liver.[5-7] Recently, it was reported that the BM-derived MSCs could functionally contribute to liver fibrosis.[8]

MSCs are known to be derived from many adult tissues, such as bone marrow(BM),[9-11] adipose tissue(AD)[12] and umbilical cord blood(UCB).[13-17] Multipotent adult progenitor cells(MAPCs) from human BM could differentiate into hepatocyte-like cells *in vitro* which acquired functional characteristics of hepatocytes.[18] And also *in vivo*, human MSCs xenografted directly to rat liver could differentiate into human hepatocytes without fusion.[19] MAPCs introduced into an early blastocyst could contribute to most somatic cell types including endodermal cells.[20] Recently, it was demonstrated that MSCs from human AD could differentiate into hepatic lineage *in vitro*.[21] Hepatic differentiation of MSCs from human UCB was also reported *in vitro*[17,22,23] and *in vivo* without fusion.[24]

Site specific differentiation after systemic cell infusion and the immunomodulatory effect of MSCs was demonstrated in various literatures.[25-27] Thus, not only autograft but also allograft or xenograft of MSCs could be applied by systemic

*Tel: +82-2-880-1246; Fax: +82-2-876-7610
e-mail: kangpub@snu.ac.kr (Kyung-Sun Kang)

infusion to various animal models of diseases without any immunosuppressant.

A dimethylnitrosamine(DMN)-induced liver fibrosis model has many advantages. The high level of fibrosis in the liver, low mortality rate and stable duration make it a good potential screening model.[28,29]

In the present studies, xenogeneic UCB- or AD-derived MSCs were used to determine if they could be a good therapeutics for the DMN-induced liver fibrosis of rats. Here, we firstly report that human MSCs can reduce rat liver fibrosis without any immunosuppressant.

2. Materials and Methods

2.1 Cell Isolation and Expansion

UCB sampleswere donated from full-termed delivery women with informed consent in the Seoul City Borame Hospital. This experiment was approved by the Borame Hospital Institutional Review Board(IRB) and the Seoul National University Institutional Review Board(SNUIRB number 0603/001-002, Seoul, Korea).

As described in our previously published papers on UCB-MSCs,[30] after thawing at 37°C, cord blood cells were separated into a low-density mononuclear fraction(<1.077 g/mL) by Ficoll-Paque Plus(GE Healthcare AB, Uppsala, Sweden, http://www.amersham.com), and the mononuclear cells were washed and suspended in a culture medium(Dulbecco's modified Eagle's medium low glucose, Gibco, Grand Island, NY, USA), containing 20% fetal bovine serum (Gibco) and were then seeded in T-25 flasks at a concentration of 4×10^6 to 5×10^6 cells per cm^2. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The suspended cells were transferred into new flasks after three days. The medium was changed every 7 days thereafter. The cells were passaged by trypsinization(0.005% trypsin/ethylenediaminetetraacetic acid; Gibco) upon reaching 80-90% confluence and replated at 5×10^4 cells per cm^2. The cells were cultured and expanded for 4-6 weeks from the primary culture and were prepared under one or two passages before being used for clinical trials.

Human AD-MSCs were isolated from subcutaneous adipose tissue. AD was obtained from tissue removed after elective cosmetic liposuction. Liposuction waste tissue was digested with 1 mg collagenase type I(Gibco) per mL of Dulbecco's Modified Eagle Medium(DMEM; Gibco) for 1 hr at 37 °C with intermittent shaking. The floating adipocytes were separated from the precipitating stromal fraction by centrifugation. The stromal cells were then plated in tissue culture flasks in stromal media(DMEM, Gibco) with 10% fetal bovine serum(Gibco). After 12 hr, the culture media was replaced with Defined Keratinocyte-SFM(Gibco) with 5% fetal bovine serum(Gibco). Media was changed every other day and the cells were allowed to grow until near confluence. After 1 week, sufficient cells were generated for trypsinization and storage in liquid nitrogen or for subculturing before being used for clinical trials.

2.2 Animals and Experimental Design

One hundred and five Sprague-Dawley(SD) rats aged 5 weeks old(SLC INC, Shizuoka-ken, Japan) were purchased and acclimated for 5 days. All rats were bred and maintained under constant conditions at a temperature of 22±3 °C, 50±20% humidity with 12 h light and 12 h dark cycles. Water and feed were accessible to the rats ad libitum. The animals and experimental procedure were approved by the Seoul National University Institute of Laboratory Animal Resources(Approve number SNU-051227-2, Seoul, Korea).

After 5-day acclimation, rats were divided into 2 groups. Group I(n=15) served as the negative control(NC). Group II(n=54) was the DMN-induced liver fibrosis group. DMN was intra-peritoneally injected at a concentration of 1 μL/g, diluted to 1% using saline on the first three consecutive days for 8 weeks.[3] Blood was taken from the ophthalmic venous plexus in Group I and II, by three rats each group on the 2nd week after DMN induction in order to monitor whether or not the liver was in state of fibrosis. Serological and histopathological examination was performed in Group I and II on the 5th and 6th week. DMN induction was terminated on the 8th week when all rats were dead(Fig 1A).

Liverfibrosis was induced to 36 rats by the same method above for 5 weeks in order to evaluate the effect of MSCs. The rats were divided into 3 groups. Group III(n=12) for the only cell-vehicle control(VC), Group IV(n=12) and V(n=12) for the UCB- and AD-MSCs-administered groups each. UCB- and AD-MSCs were injected by the tail vein at a concentration of 1×10^6 cells/100 μL PBS per rat after one week, respectively. And the same volume of vehicle(100 μL PBS) was injected by the same route(Fig 1B). Three rats were exsanquinated under ether anesthesia and sacrificed at each groups on the 1st and 4th week after MSCs administration in order to monitor the liver condition.

Blood samples were collected from abdominal vein and examined for the clinical biochemistry. Blood was taken from the ophthalmic venous plexus on the 2nd and 3rd week

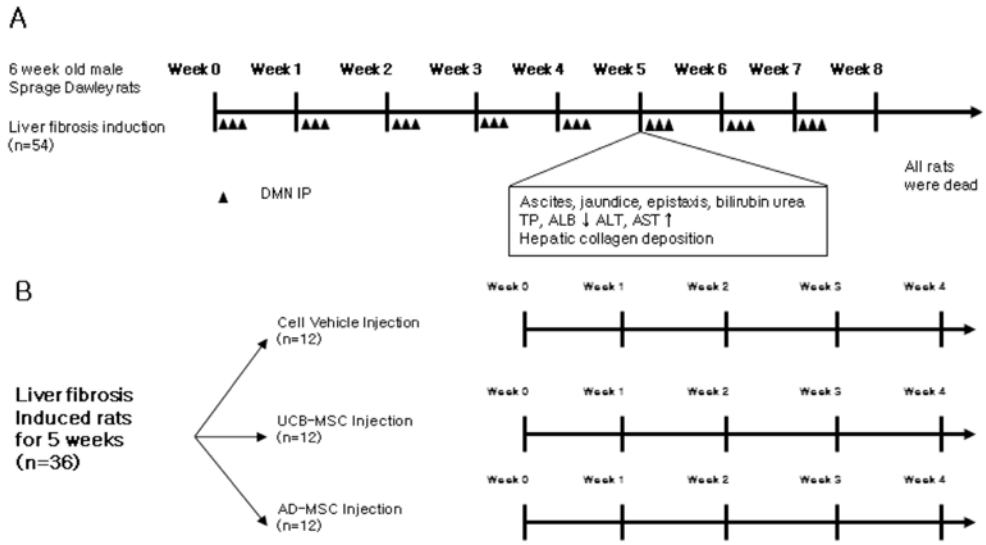

Figure 1. Liver fibrosis induced by DMN(A). 69 rats were divided into 2 groups. Negative control(n=15), DMN-induced liver fibrosis group(n=54). For 8 weeks, liver fibrosis was induced by DMN i.p. at three consecutive days each week. General examination including ascites, jaundice, epistaxis and bilirubin urea was performed. Serological and histopathological examination was performed on the 2nd, 5th and 6th week after the induction (A). The evaluation of MSCs effect on the liver fibrosis induced rats (B). Liver fibrosis was induced for 5 weeks in 36 rats. After one week, administration was performed to the following 3 groups each: Cell vehicle control(n=12), UCB-MSC group(n=12) and AD-MSC group(n=12). General examination and serological analysis was performed every week after the cell injection. Histopathological examination was performed on the 1st and 4th week after the cell injection.

after the cell injection. Liver samples were fixed in the 10% neutral buffered formalin to be used for the histopathological analysis.

2.3 General Examination

All of the animals were observed every other day for clinical signs such as ascites, jaundice, epistaxis, bilirubin urea, and body weight loss. The dying and the dead animals were necropsied within 24 hours after death.

2.4 Clinical Biochemistry

Total protein, albumin, AST, ALT were examined.

2.5 Organ Weight

At necropsy, liver, spleen, kidney were weighed.

2.6 Histopathology and Image Analysis

Liver tissues were fixed in 10% neutral buffered formalin, embedded, sectioned, stained with H&E staining and Masson's trichrome staining and examined with image analysis software(Image-pro plus 4.0, Media Cybernetics LP).

2.7 Probe

Genomic DNA of human UCB-MSCs was extracted with the DNeasy tissue kit(Qiagen, Hilden, Germany). The PCR primers were positioned in the most conserved areas of the human *Alu* sequences and produced a PCR product of 224 base pairs(bp).[31-33] The following primers were used to expand the *Alu* sequences via PCR: *Alu*-sense, 5'-ACG CCT GTA ATC CCA GCA CTT-3'; *Alu*-anti-sense, 5'-TCG CCC AGG CTG GAG TGCA-3'. PCR was carried out under the following conditions: 95°C for 10 minutes; 25 cycles of 95°C for 30 seconds, 58°C for 45 seconds, and 72°C for 45 seconds; and 72°C for 10 minutes. The PCR product was electrophoresed on a 2% agarose gel and stained with ethidium bromide(10 ng/mL). A DNA band of 224 bp was then eluted with the Qiaquick gel extraction kit(Qiagen). The PCR product was DIG-labeled with the PCR DIG probe synthesis kit(Roche Diagnostics, Basel Switzerland). This PCR was performed with 50 ng of eluted DNA using the same PCR protocol as described above. The labeled probe was purified by ethanol precipitation according to the protocol of the PCR DIG probe synthesis kit.

2.8 *In Situ* Hybridization

The *in situ* hybridization for the *Alu* gene was performed in order to confirm whether the MSCs were homed to the damaged liver or not. Sections were deparaffinized in xylene and rehydrated in PBS just prior to application to *in situ*

hybridization. Following incubation with PBS containing 0.3% Triton X-100, the slides were incubated with TE buffer containing 2 mg/mL proteinase K for 30 minutes at 37°C and rinsed again three times for 5 minutes. The slides were acetylated with TEA buffer containing 0.25%(v/v) acetic anhydride(Sigma-Aldrich, St. Louis) twice for 5 minutes to reduce the nonspecific background staining. After prehybridization with hybridization buffer(50% formamide(Sigma-Aldrich(in 5x SSC, 0.1% sodium-lauroylsarcosine(Sigma-Aldrich), 0.02% SDS(Sigma-Aldrich), and 2% blocking reagent(Roche) for 3 hours at 85°C, the slides were incubated with fresh hybridization buffer containing the denatured DIG-labeled DNA probe(10-200 ng/mL) for a further 10 minutes at 94°C. The slides were then transferred to ice for 10 minutes and incubated overnight at 42°C. The prehybridization and hybridization steps were performed in a moist chamber containing 50% formamide. After hybridization, the slides were briefly rinsed in 2x SSC at room temperature and three times in 0.1x SSC for 15 minutes at 42°C. Visualization of the DIG-labeled DNA probe was performed according to the protocol of the DIG nucleic acid detection kit(Roche). The slides were blocked for 30 minutes with blocking buffer(1% blocking reagent(Roche) in maleic acid buffer(0.1 M maleic acid, 0.15 M NaCl, pH 7.5)) and then incubated for 1 hour with an alkaline phosphates/conjugated antibody solution(anti-sheep, 1:2000 in blocking buffer containing 0.1% Triton X-100). Following four washes with maleic acid buffer for 15 minutes, the slides were equilibrated for 5 minutes in Tris buffer, pH 9.5. The color development was performed with freshly prepared substrate solution(nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Roche) in Tris buffer, pH 9.5). After 2-6 hours theenzymatic reaction was terminated with stop buffer(10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The slides were washed three times for 15 minutes and counterstained with 0.1% nuclear Fast Red (Sigma-Aldrich) for 2 minutes. The slides were then rinsed again three times with PBS.

2.9 Statistics

The data obtained from at least three separate experiments were represented as the mean±SD(standard deviation). The statistical comparison was performed using Tukey test after the ANOVA test. $P<0.05$ was considered significant.

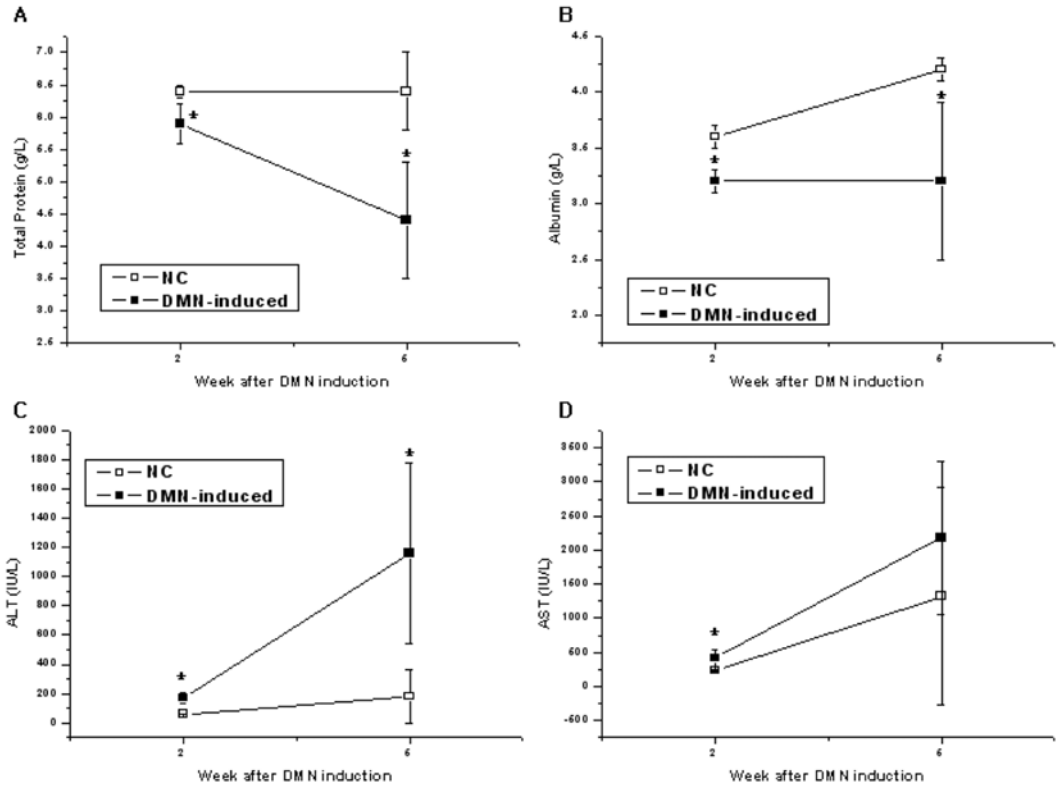

Figure 2. Total protein(g/L), albumin(g/L), ALT(IU/L), AST(IU/L) on the 2nd and 5th week after DMN induction. Total protein and albumin level of the DMN-induced group(A,B) were decreased on the 2nd and 5th week, however, ALT and AST increased(C,D). NC is negative control group. ALT: alanine transaminase, AST: aspartic acid transaminase. Values are the means ±SD from three different animals. Asterisk indicates a significant difference from the values of NC group($P<0.05$).

3. Results

3.1 DMN-induced Liver Fibrosis of SD Rats

3.1.1 General Examination. The clinical signs, including ascites, jaundice, epistaxis, bilirubin urea and body weight loss, were the primary indicators of the liver fibrosis monitored throughout the experiment. The rats that had the clinical signs typically showed low total protein and albumin levels and high AST and ALT levels. The number of dead rats in the DMN-induced group increased from the 6th week and then all of the remaining rats died during the 8th week.

3.1.2 Clinical Biochemistry. Total protein and albumin, which represent the synthetic capabilities of the liver, decreased in the DMN-induced group on the 2nd and 5th week, significantly (Fig 2A, 2B). AST and ALT, which reflect the level of hepatocellular necrosis, increased in the DMN-induced group on the 2nd and 5th week(Fig 2C, 2D).

3.1.3 Organ Weight. The weight of liver, spleen and kidney were monitored. No significant weight differences were observed in the spleen and kidney(data not shown). However, the liver weight per body weight(LW/BW) of the DMN-induced group decreased about 50% of that of the NC on the 5th week(Fig 3D).

3.1.4 Histopathology and Image Analysis. The results of the H&E staining showed that the DMN-induced group exhibited dilatation and thickening of portal triads, arterioles and bile ducts. Thick scar tissue and fibrosis bridging were also observed(data not shown). The results of the Masson's trichrome staining showed that the DMN-induced group(Fig 3B) exhibited a heavy increase of the collagen fiber(stained as blue color) in comparison to the NC on the 5th week(Fig 3A). The images from three slides of each group were analyzed using the Image Pro Plus software to measure the area of the collagen fiber to the other tissue. The per-area(%) represents the percentage of the selected area that is regarded as the collagen fiber in comparison to the entire area. The per-area of the DMN-induced group reached about 9 % on the 5th week(Fig 3C).

3.2 MSCs Administration to the Liver Fibrosis-induced Rats

3.2.1 Clinical Biochemistry. Total protein and albumin levels of all groups started to increase after the cell injection. Total protein and albumin levels of the UCB-MSC group were higher

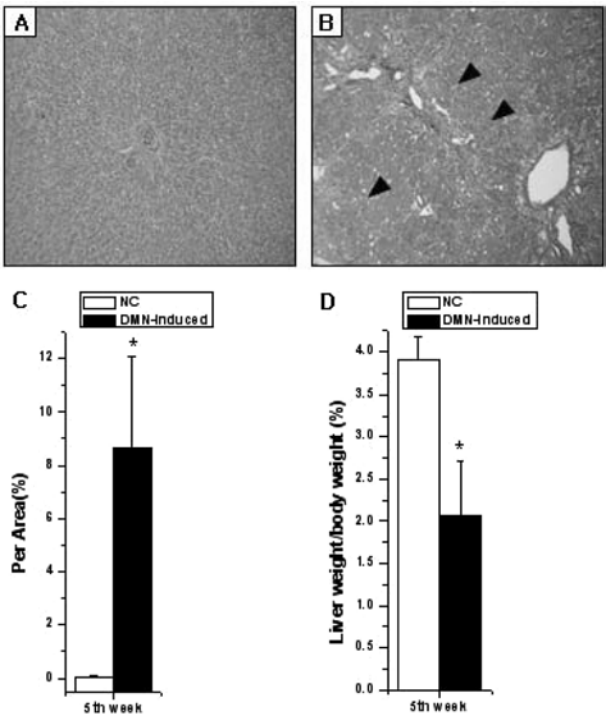

Figure 3. Masson's trichrome staining of the liver on the 5th week after DMN induction. NC (A) and DMN-induced group (B). Collagen fiber deposition, severe hepatocellular necrosis, bile duct proliferation and bridging necrosis(black arrow head) were observed (B)(Original magnification ×100). Image analysis of Masson's trichrome stained liver on the 5th week after DMN induction. The per-area of the DMN-induced group (C) reached about 9%. The liver weight per body weight(LW/BW) of DMN-induced group (D) was about 50% of that of the NC on the 5th week. NC is negative control group. Values are the means±SD from three different animals. Asterisk indicates a significant difference from the values of NC group($P<0.05$).

than those of the AD-MSC and VC groups during the 4th week after the cell injection(Fig 4A, 4B). AST and ALT started to decrease after the cell injection(Fig 4C, 4D). However, there was no significant difference between MSCs-administered and VC groups of each week. The clinical biochemistry data from the UCB-MSC group showed the best recovery potential on the 4th week after the cell injection.

3.2.2 Organ Weight. The LW/BW was measured on the 1st and 4th week after the cell injection. The LW/BW was increased only in the AD-MSC group on the 1st week after the cell injection. The VC and UCB-MSC groups showed a high increase, while, the AD-MSC group demonstrated a decrease on the 4th week after the cell injection(Fig 5). There was no significant difference between MSCs-administered and VC

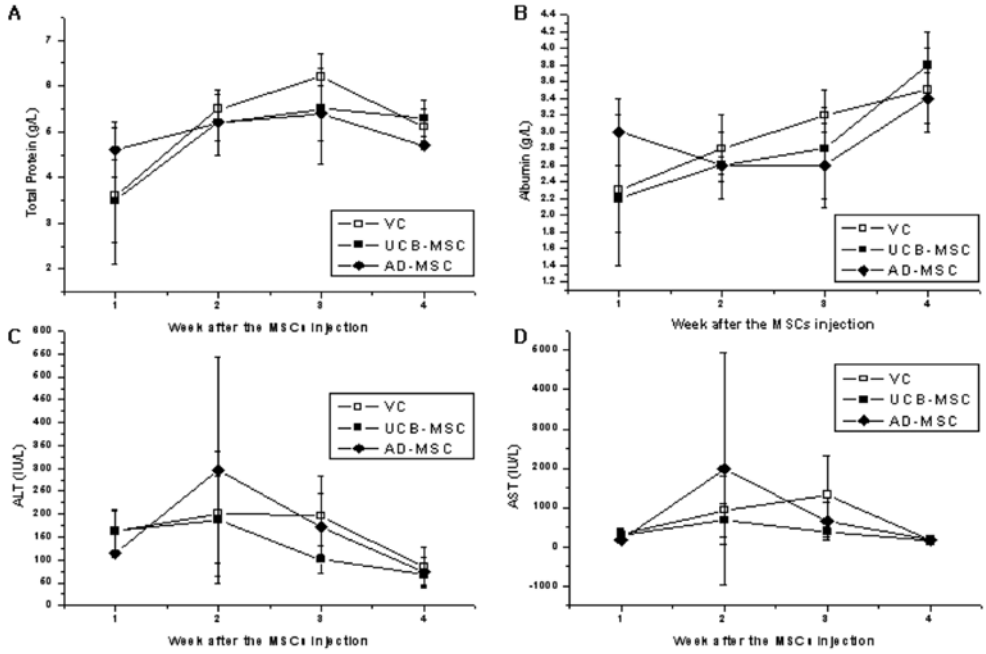

Figure 4. Total protein(g/L), albumin(g/L), alanine transaminase(IU/L), aspartic acid transaminase(IU/L). Blood samples were taken from the abdominal vein during the exsanquination on the 1st and 4th week after the cell injection. Blood samples were taken from the ophthalmic venous plexus to monitor the individuals on the 2nd and 3rd week. On the 4th week after the cell injection, the total protein levels of UCB-MSC group surpassed those of the AD-MSC and VC groups (A). On the 4th week after the cell injection, the albumin levels of UCB-MSC group surpassed those of the AD-MSC and VC groups (B). The ALT level of UCB-MSC and AD-MSC groups started to decrease after the cell injection (C). The AST level of UCB-MSC and AD-MSC groups started to decrease after the cell injection (D). VC is only cell-vehicle control group. ALT : alanine transaminase, AST : aspartic acid transaminase. Values are the means±SD from three different animals(two animals on 4th week of AD-MSC group). There was no significant difference between MSCs-treated groups and VC of each week.

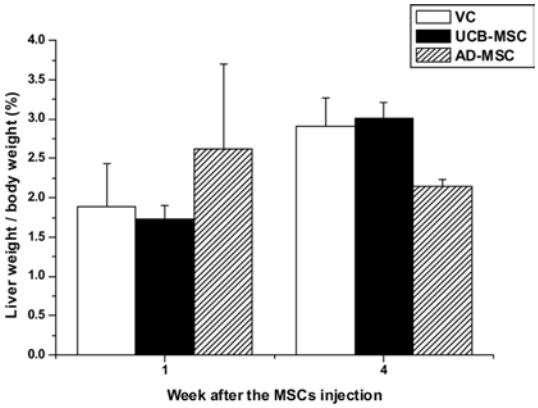

Figure 5. Liver weight per body weight(LW/BW). The LW/BW was measured on the 1st, 4th week after the cell injection. The LW/BW was increased only in the AD-MSC group on the 1st week after the cell injection. On the 4th week after the cell injection, VC and UCB-MSC groups showed a steep increase but the AD-MSC group showed decrease, compared to the level of each group on 1st week after the cell injection. VC is only cell-vehicle control group. Values are the means±SD from three different animals(two animals on 4th week of AD-MSC group). There was no significant difference between MSCs-administered groups and VC of each week.

groups of each week.

3.2.3 Histopathology and Image Analysis. The results of the H&E staining showed that the collagen fiber in the MSCs-administered group was thinner than that in the VC group. The results of the Masson's trichrome staining showed that the UCB- and AD-MSC groups(Fig 6C, 6D) exhibited a significant decrease in the collagen fiber, whereas the VC group(Fig 6B) showed a lesser decrease on the 1st week after the cell injection. The significant decrease in the collagen fiber was maintained until the 4th week after cell injection in the UCB- and AD-MSC groups(Fig 7C, 7D). The images from three slides of each group(two slides of the AD-MSC group on the 4th week after the cell injection) were analyzed using the Image Pro Plus software to measure the area of the collagen fiber to the other tissue. The per-area of the UCB- and AD-MSC groups was reduced to 6% and 2% on the 1st week after the cell injection, respectively. The per-area of the UCB- and AD-MSC groups bottomed down to 1% and 2% on the 4th week after the cell injection, respectively (Fig 8). These results agreed with those of the histopathological

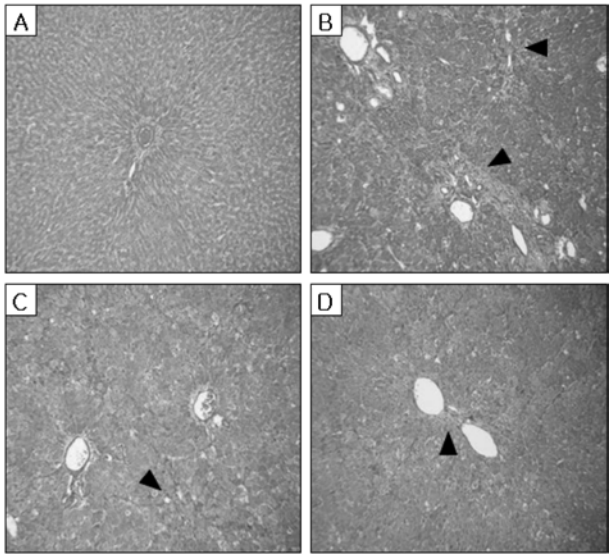

Figure 6. Masson's trichrome staining of the liver on the 1st week after the cell injection. Normal liver of rat (A). Collagen fiber deposition and bridging necrosis(black arrow head) were observed in many parts in the VC group (B). Reduced collagen fiber and few fibrosis bridging(black arrow head) were observed in the UCB-MSC group (C) and in the AD-MSC group (D) (Original magnification, x100). VC is only cell-vehicle control group.

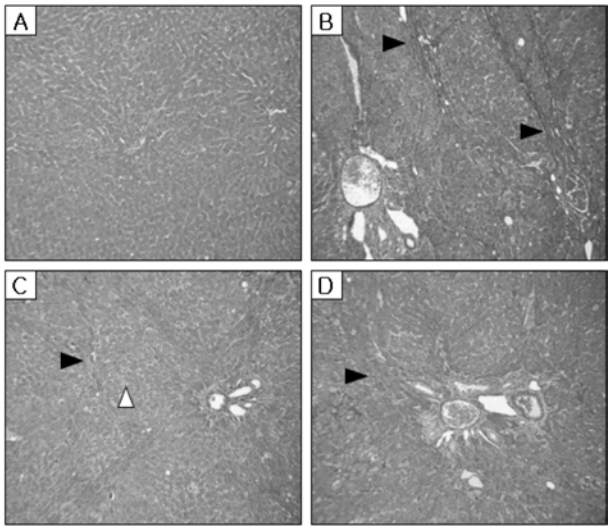

Figure 7. Masson's trichrome staining of the liver on the 4th week after the cell injection. Normal liver of rat (A). Collagen fiber deposition and bridging necrosis(black arrow head) were remained in many parts in the VC group (B). Reduced collagen fiber, few remaining bridging(black arrow head) and the surrounding normal hepatocytes (blank arrow head) were observed in the UCB-MSC group (C). Reduced collagen fiber and remaining bridging(black arrow head) were observed in the AD-MSC group (D)(Original magnification, x100). VC is only cell-vehicle control group.

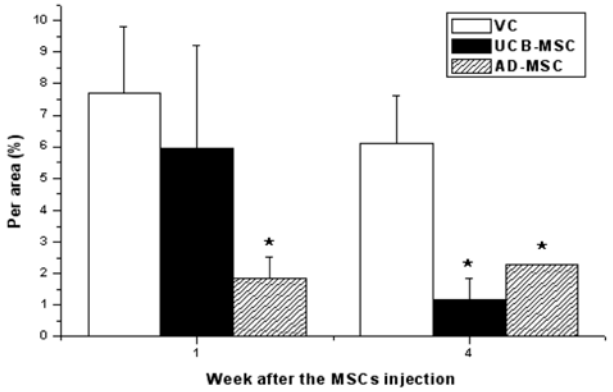

Figure 8. Image analysis of Masson's trichrome stained liver on the 1st and 4th week after the cell injection. The per-area(%) represents the percentage of the selected area that is regarded as the fiber and collagen in comparison to the entire area. On the 1st week after the cell injection, the per-area of the UCB- and AD-MSC groups decreased to 6% and 2%, respectively. On the 4th week after the cell injection, the per-area of the UCB- and AD-MSC groups bottomed down to 1% and 2%, respectively. VC is only cell-vehicle control group. Values are the means±SD from three different slides(two animals on 4th week of AD-MSC group). Asterisk indicates a significant difference from the values of VC group of each week($P<0.05$).

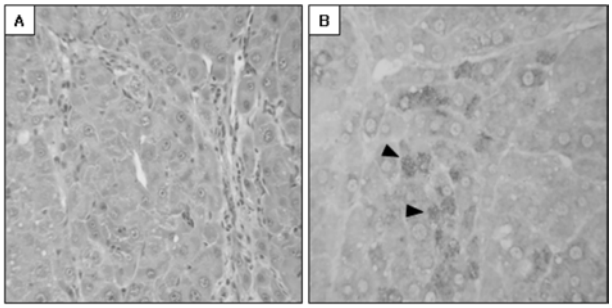

Figure 9. *In situ* hybridization. The serially sectioned H&E slide of the UCB-MSC group on the 1st week after the cell injection (A). Positive signals(black arrow head) were detected in the UCB-MSC group on 1st week after the cell injection (B)(Original magnification, x 200).

analysis.

3.2.4 *In Situ* Hybridization. The *in situ* hybridization for the human specific *Alu* gene was performed accompanied by the H&E staining of serially sections in order to confirm the homing of the UCB- and AD-MSC to the damaged liver tissue. Positive signals(black arrow head) were detected in the UCB-MSC group on the 1st week after the cell injection(Fig 9). Weak or no signals were detected in the AD-MSC group of the 1st week and

in the both groups of the 4th week after the cell injection.

4. Discussion

It should be fully confirmed when the liver fibrosis was induced in rat after the treatment of DMN before the MSCs were administered. In this study, liver fibrosis was induced during the 5th week after the DMN induction. It was a week later than the time of previous report.[28,29] The number of dead rats in the DMN-induced group started to increase from the 5th week and sharply increased during 6th week. So, it was thought that MSCs could be administered between the 5th and 6th week. Therefore, DMN induction was terminated on the 5th week and MSCs were then administered.

Xenotransplantation of the human UCB- and AD-MSCs to rats, intravenously, was examined by the histopathology and clinical biochemistry. In the previous report,[34] the xenotransplanted recipient usually did not survive longer than one week and had many complication from transplantation. However, in this experiment, though human MSCs were transplanted, the rats survived(tree rats in the UCB-MSC, two rats in the AD-MSC) until the 4th week after the cell injection. This was thought to be the result of the immunomodulatory effects of the MSCs.[26] Adult MSCs persisted for up to 13 months after systemic *in utero* transplantation in sheep, before and after the development of immunologic competence.[25] MSCs also showed HLA-DR negative, which is related to graft versus host rejection.[17]

As for the clinical biochemistry, a high level of the variance is thought to refer to the stress of taking blood from the ophthalmic venous plexus without anesthesia. Such variation in test is typical for all of the serum-base tests currently available.[35,36]

The results of the histopathological analysis showed that the MSCs-administered groups exhibited a major reduction in collagen fiber. However, the VC group showed no comparable reduction. It was suggested that the MSCs could replace the damaged hepatocytes of the recipient and could decompose the surrounding collagen fiber through cytokines, such as insulin-like growth factor-1(IGF-1) as previously reported.[37] The mechanism involved should be further investigated.

The *in situ* results showed that the UCB-MSC group exhibited better recovery than the AD-MSC group. It was demonstrated that the preterm, as compared with term, cord blood is richer in MSCs and HSCs.[38,39] Therefore, the earlier the sample wastaken near birth, the richer the MSCs resided in the peripheral blood. This suggests that UCB-MSC could be more primitive than other adult MSC. That is, the primitive UCB-MSC might be homed to the damaged site and engrafted better than AD-MSC might, easily overcoming the graft versus host rejection.

The paracrine effect of some cytokines related to the MSCs proliferation and differentiation were demonstrated.[40] The mechanism how MSCs effect to the nearby cells or themselves is not clearly understood.

Taken together, it is suggested that human MSCs might not only replace the damaged hepatocytes by themselves but also might be responsible for the reduction of liver fibrosis.

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