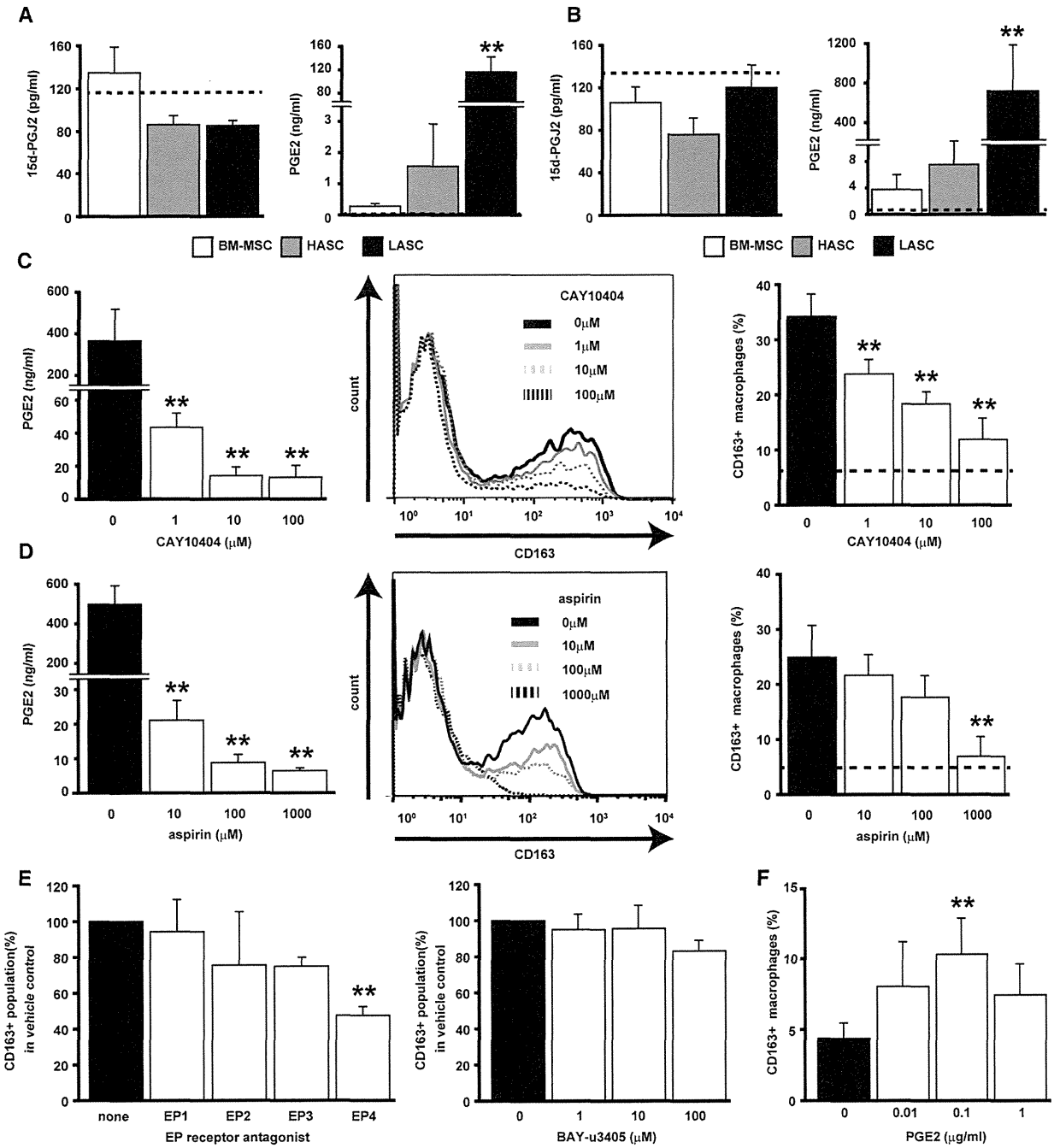

Figure 6. LASC-derived PGE2 is an essential soluble factor that promotes polarization of macrophages toward immunoregulatory cells *in vitro*. (A and B) The concentrations of 15d-PGJ2, a metabolite of PGD, and PGE2 in culture supernatants of MSCs cultured alone (A) or with peritoneal macrophages at a 1:2 ratio (B) are measured. Dotted line indicates the value in medium (A) and in macrophage culture supernatant (B). LASCs constitutively secrete a higher amount of PGE2 than BM-MSCs or HASCs do, and this is enhanced in coculture with macrophages (n=4 in each group). (C and D) The Cox-2 inhibitor CAY10404 (C) and aspirin (D) impaired PGE2 excretion by LASCs cultured with macrophages in a dose-dependent manner. Reduction of LASC-mediated macrophage polarization by CAY10404 (C) and aspirin (D) is also evaluated by flow cytometry as macrophage CD163 expression. Representative histograms and percentage of $CD163^+$ macrophages incubated with CAY10404 or aspirin at indicated concentrations are shown. Dotted line indicates value in cultures containing macrophages alone. (E) The decrease in the $CD163^+$ population is evaluated as the percentage of $CD163^+$ cells cultured with each EP or PGD receptor antagonist compared with $CD163^+$ cells cultured with the respective vehicle control (black

and tubular injury were significantly reduced by LASC transfer, there was no difference between the LASC- and HASC-treated groups with respect to the number of interstitial $CD163^+$ macrophages. Therefore, the renoprotective effect of LASCs against tubulointerstitial damage in anti-GBM GN is not dependent on macrophage phenotypic conversion, but rather on LASC-derived humoral factors such as HGF[32] or on LASC-mediated amelioration of glomerular injury.

From results obtained using a mouse sepsis model, Németh *et al.* reported that BM-MSC–derived PGE2 is essential for IL-10 production by macrophages that downregulate the systemic inflammatory response.[12] They demonstrated production of PGE2 by BM-MSCs after LPS stimulation only when the BM-MSCs were cocultured with macrophages after the LPS exposure. Our real-time observations of peritoneal macrophages cocultured with MSCs demonstrated that macrophages are attracted to and contact LASCs, and subsequently express CD163 on the cell surface. Of note, LASC-mediated PGE2 generation and subsequent conversion of M2 macrophages did not require LPS stimulation, indicating that LASCs possess enhanced therapeutic potential compared with BM-MSCs. Moreover, a moderate level of CD163 expression on macrophages was observed in the trans-well culture system in which macrophages and LASCs were not in direct contact, suggesting that humoral factors also contribute to macrophage polarization. This cell contact-independent effect of MSCs upon macrophages was largely absent with BM-MSCs in the sepsis model.[12]

Compared with HASCs, LASCs generated significant amounts of PGE2. Production of PGE2 by BM-MSCs was minimal. Interestingly, PGE2 levels were further enhanced when LASCs were cocultured with macrophages, implying that physical interaction between LASCs and macrophages affects LASC-derived PGE2 synthesis. Ablation of LASC-derived PGE2 production significantly diminished CD163 expression on macrophages *in vitro*, and impeded efficacy of LASCs against rat anti-GBM GN *in vivo*. On the other hand, phenotypic conversion of macrophages to $CD163^+$ cells after a single treatment with PGE2 was not as striking as that resulting from LASC treatment. Therefore, additional signals may be required for induction of M2 polarization of macrophages by LASCs. In addition to its proinflammatory effects, MSC-derived IL-6 has been shown to exhibit anti-inflammatory functions, such as inhibition of DC differentiation and production of reactive oxygen species by neutrophils.[11,47] More recently, it was shown that tumor-derived IL-6 and PGE2 promote M2 polarization.[48] We found that LASC-derived IL-6 and PGE2 induce $CD163^+$ macrophages in our *in vitro* coculture system. Although IL-6 could not be detected in sera of diseased animals, regardless of whether they were subjected to LASC transfer (unpublished data), LASC-derived IL-6 in inflamed sites of the kidneys may be effective for M2 cell polarization. Furthermore, limiting MSC-mediated immunomodulation to inflamed sites would be more attractive and beneficial for the reduction of adverse effects than would systemic administration of a therapeutic agent.

In conclusion, although previous studies involving disease models and clinical trials have demonstrated that BM-MSC administration can be beneficial,[7,49–51] we could not demonstrate the therapeutic efficiency of BM-MSC treatment in anti-GBM GN, because the immunomodulatory ability of MSCs may be context dependent. Further investigations into the functional differences between ASCs and BM-MSCs will be valuable at better defining the suitability of one cell type versus the other for clinical applications. Immunosuppressive therapy with corticosteroids and cyclophosphamide remains the prevailing approach for treating CGN, but the potential for adverse effects such as infection and cytotoxicity restrict the use of these agents. Our study suggests that LASC administration may be a desirable and feasible therapeutic alternative to improve the prognosis of anti-GBM GN patients.

CONCISE METHODS

Animals

WKY/NCrj female rats were purchased from Charles River Inc. (Yokohama, Japan); CAG-EGFP-transgenic Lewis rats were kindly provided by Mito Otsuki (Kyoto University, Kyoto, Japan). All experimental animals were housed at a constant temperature and humidity, with a 12-hour light/dark cycle, and had unrestricted access to a standard diet and tap water in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For 16-hour collection of urine, animals were housed in metabolic cages on days 3, 5, and 14 after disease induction. The experimental protocols were in accordance with the Animal Experimentation Guidelines of Nagoya University Graduate School of Medicine.

Cell Preparation

Ex Vivo *Expansion of MSCs from Rat Bone Marrow and Adipose Tissue*

Isolation and expansion of BM-MSCs and ASCs from WKY/NCrj rats were performed as previously described.[14,15,52] Adipose-derived stromal cells cultured in conventional high-serum (20% v/v) and low-serum (4% v/v) media were designated HASCs and LASCs, respectively. In addition, rat BM-MSC cultures were established in culture media containing 20% FBS. All three MSC types expressed CD44 (homing-associated cell adhesion molecule), CD54 (intercellular adhesion molecule-1), and CD90 (Thy-1), as previously demonstrated for rat MSCs,[14] whereas they lacked surface expression of CD34 for hematopoietic stem cells and CD45 (leukocyte common antigen), a

column). (F) Macrophage polarization into $CD163^+$ cells after a single stimulation with PGE2. CD163 expression on macrophages incubated for 48 hours with synthetic PGE2 at the indicated concentrations is determined by flow cytometry (n=4–5 per group). All data are mean ± SD. $^{**}P<0.05$ as determined by ANOVA.

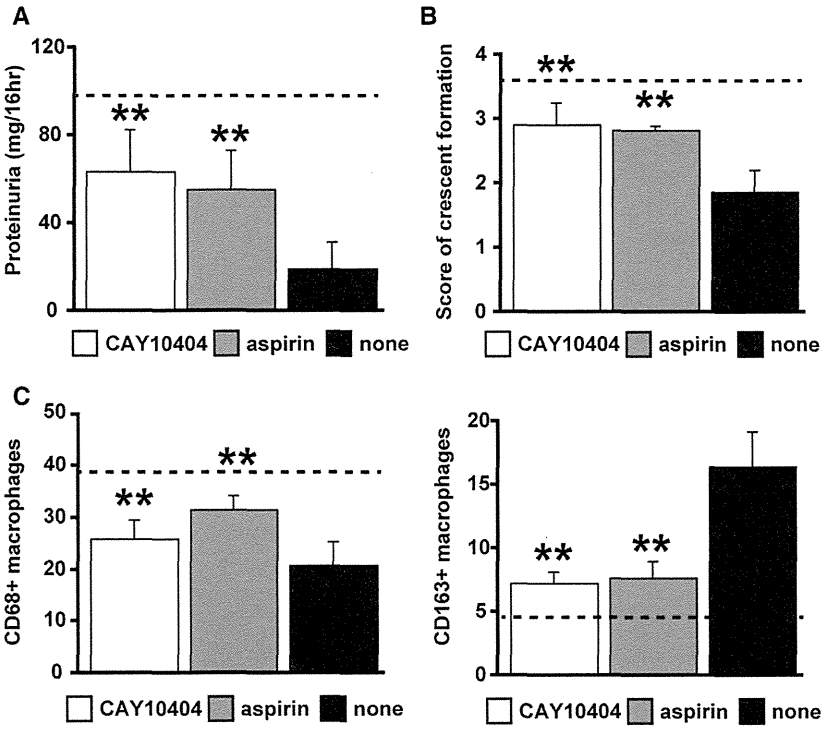

Figure 7. Pharmaceutical ablation of PGE2 synthesis abrogated the therapeutic potency of LASCs. (A–C) LASCs pretreated with 50 μM CAY10404 or 1000 μM aspirin are transferred into anti-GBM GN rats. The 16-hour proteinuria level at day 5 (A), histologic score of glomerular crescent formation at day 7 (B), and glomerular accumulation (C) of $CD68^+$ (left) and $CD163^+$ (right) macrophages at day 7 are evaluated as described in the legend for Figure 2. Dotted lines indicate value in anti-GBM GN rats not subjected to LASC transfer (n=10). All data are mean ± SD. $^{**}P<0.05$ compared with untreated LASC group as determined by ANOVA (n=7–10 per group).

common marker for leukocytes (Supplemental Figure 1A). Rat BM-MSCs barely proliferated under low-serum (4%) culturing conditions (data not shown), whereas adipose-derived MSCs cultured under these conditions proliferated at rates comparable to BM-MSCs cultured under high-serum (20%) conditions (Supplemental Figure 1B). Notably, LASCs produced substantial amounts of VEGF and HGF (Supplemental Figure 1C).

Differentiation of MSCs into a Mesenchymal Lineage

Cultures of BM-MSCs, HASCs, and LASCs were examined using differentiation kits for adipocytes, chondrocytes, and osteoblasts (Invitrogen-Gibco, Carlsbad, CA). Adipocytes, chondrocytes, and osteoblasts were identified by oil red O, Alcian Blue, and alkaline phosphatase staining, respectively.[1,15] All MSCs exhibited multipotency because they were competent for adipogenesis, osteogenesis, and chondrogenesis, as previously shown for human MSCs (Supplemental Figure 1D).[15]

Isolation of Peritoneal Macrophages

Peritoneal lavages as a source of rat macrophages were obtained by intraperitoneal injection of 50 ml of sterile saline. The abdomen was gently massaged before retrieval of the lavage. Mononuclear cells within the pellet resulting from centrifugation (400×g, 30 minutes) of the lavage were isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO), and were then transferred to culture dishes. After overnight incubation at 37°C in a 5% CO_2 atmosphere, floating cells were depleted from culture dishes and $CD11b/c^+$ adhesive cells were isolated as macrophages.

Cell Culture Using a Trans-Well Plate System

A trans-well plate system (0.4-μm pore size; Costar, Boston, MA) was used to prevent close contact between LASCs and macrophages. The upper chamber contained 1×10^6 LASCs on an inserted membrane, and the bottom chamber contained 2×10^6 macrophages. Trans-well plates were incubated for 48 hours at 37°C in humidified air containing 5% CO_2.

Flow Cytometry Analyses

Identification of BM-MSCs, HASCs, and LASCs

All antibodies were obtained from BD Biosciences Pharmingen (San Diego, CA) unless otherwise indicated. FITC mouse anti-rat CD34 (Santa Cruz Biotechnology, Santa Cruz, CA), PE mouse anti-rat CD45, FITC mouse anti-rat CD44H, PE mouse anti-rat CD54 (AbD Serotec, Oxford, UK), and FITC mouse anti-rat CD90 were used for identification of MSCs. Respective isotype controls, including PE mouse IgG1 for CD34 (Santa Cruz Biotechnology), PE mouse IgG1κ for CD45, FITC mouse IgG2aκ for CD44H, PE mouse IgG1 for CD54 (AbD Serotec), and FITC mouse IgG2aκ, were used as negative controls. Cells were acquired using a FACS Canto II flow cytometer (BD Biosciences).

Assessment of Functional Polarization of Macrophages

After 48-hour coculturing of 3×10^6 macrophages and 1.5×10^6 BM-MSCs, HASCs, or LASCs, adherent cells were subjected to flow cytometry analysis. Infiltrating macrophages and immunoregulatory macrophages were defined using mouse FITC anti-rat CD68 IgG1 (ED1; AbD Serotec) or mouse PE anti-rat CD163 IgG1 (ED2; AbD Serotec), and rabbit anti-rat CD206 IgG (mannose receptor; Abcam, Cambridge, MA), respectively, in cells gated by APC mouse anti-rat CD11b/c IgG2aκ (BioLegend, San Diego, CA). Intracellular staining using BUF09 (AbD Serotec) was essential for CD68 detection. The fluorescent PE signal for CD163 was enhanced using a FASER kit-PE (Miltenyi Biotec, Bergisch Gladbach, Germany). In all experiments, the Fcγ receptor was blocked using purified mouse anti-rat CD32 IgG1κ. Populations of $CD68^+$ and $CD163^+$ macrophages (M2) were evaluated as a percentage of $CD11b/c^+$ cells.

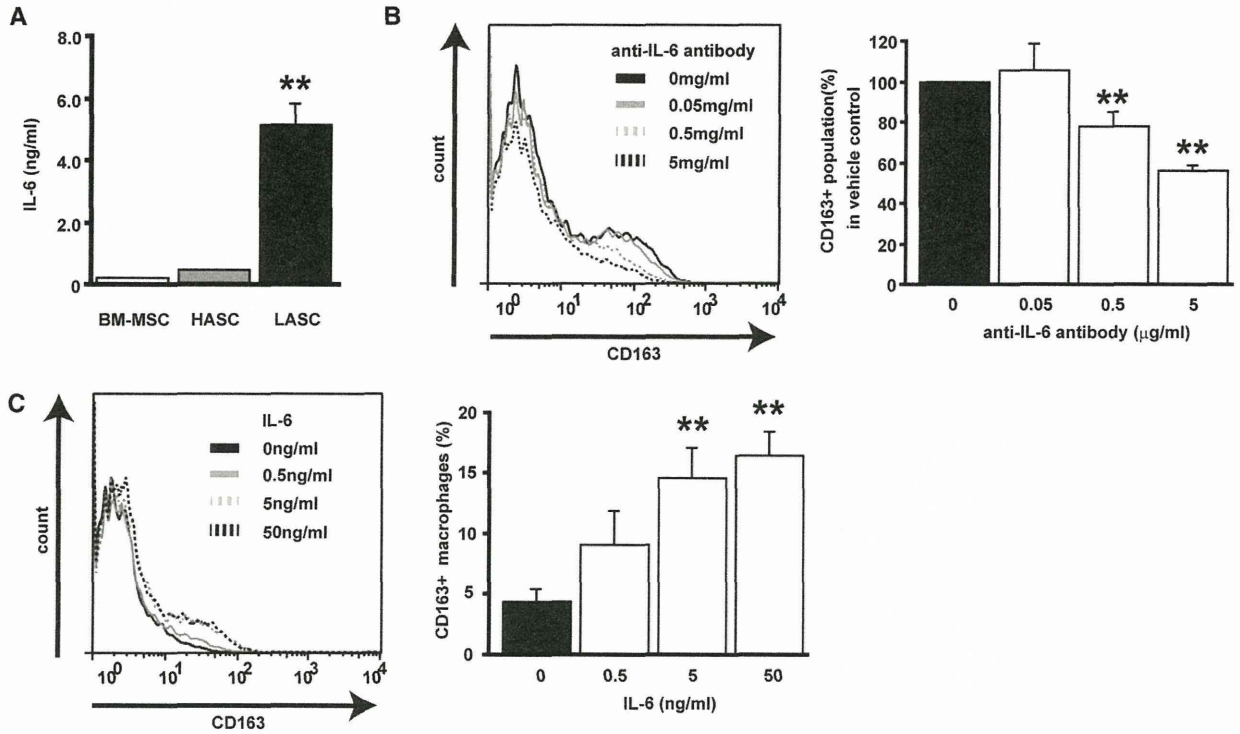

Figure 8. LASC-derived IL-6 is another soluble factor that promotes polarization of macrophages into CD163$^+$ cells *in vitro*. (A) IL-6 concentration in the culture supernatants of BM-MSCs, HASCs, and LASCs incubated for 24 hours. (B) Neutralization of IL-6 using an anti-IL-6 antibody results in a dose-dependent decrease in the population of LASC-induced CD163$^+$ macrophages compared with cultures treated with normal goat IgG (black column). (C) Recombinant IL-6 alone promotes the phenotypic switch of macrophages into CD163$^+$ cells (n=5 per group). All data are mean ± SD. $^{**}P<0.05$ as determined by ANOVA.

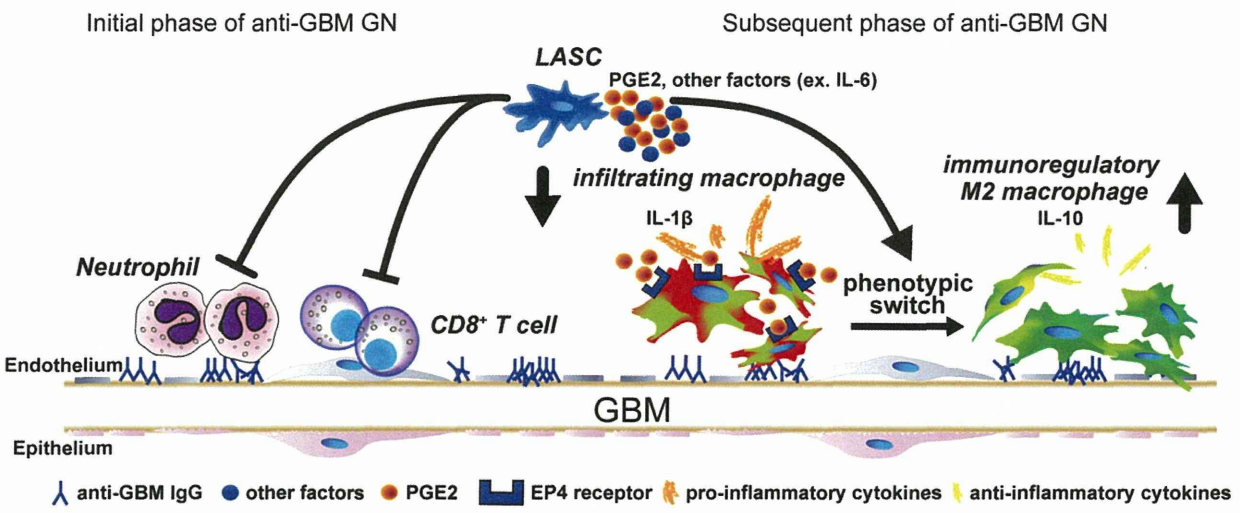

Figure 9. Model for LASC-mediated amelioration of anti-GBM GN. LASCs impair recruitment of neutrophils and CD8$^+$ T cells into the glomerulus during the initial phase of anti-GBM GN and promote PGE2-EP4 receptor-dependent phenotypic conversion of infiltrating macrophages to immunoregulatory macrophages during subsequent phases of the disease. Other soluble factors, including IL-6, are also required for this process. The accumulation of a significant number of immunoregulatory macrophages in the glomeruli protect against development of proteinuria and glomerular crescent formation, which is critical for a positive prognosis in anti-GBM GN.

Induction and Treatment of Rat Anti-GBM GN

Using a previously described method, we established a mouse monoclonal IgG clone designated TF78, which specifically binds to α4(IV) NC1 of the rat GBM to stably induce CGN.[53] Intraperitoneal administration of TF78 induced the disease in WKY/NCrj rats in a dose-dependent manner (Supplemental Figure 7A), and treatment with 100 μg of TF78 led to progressive elevations in BUN, sCr, and proteinuria (Supplemental Figure 7B). At various time points, blood and urine samples were sent to Mitsubishi BCL Co. Ltd (Tokyo, Japan) and then BUN, sCr, and urine protein were measured by Mitsubishi BCL Co. Ltd. Histologic analyses of rat kidneys treated with TF78 demonstrated severe crescent formation in glomeruli together with linear IgG deposition in the GBM (Supplemental Figure 7, C and D). Unless otherwise indicated, 100 μg of TF78 was administered to each animal.

Assessment of Mouse IgG Deposition on the Rat GBM

Deposition of TF78 on the rat GBM was confirmed using frozen kidney sections and polyclonal rabbit FITC-labeled anti-mouse IgG (H+L) (Invitrogen). Deposition of IgG was semiquantitatively assessed by determining the end point positive titer for detection of staining using serial dilutions of anti-mouse IgG antibody ranging from 1:125 to 1:4000[27,54] (Supplemental Figure 2).

Transfer of BM-MSCs, HASCs, or LASCs for Treating Rat Anti-GBM GN

We administered 2×10^6 third to fifth passage BM-MSCs, HASCs, or LASCs in 2 ml of sterile PBS to each rat in the respective treatment group *via* the tail vein on days 0–5 after TF78 injection. Diseased rats in the control group received 2 ml of sterile PBS lacking cells.

Histologic Assessment of Glomerular Crescent Formation and Leukocyte Accumulation

Histologic evaluation of glomerular crescent formation was determined semiquantitatively on paraffin-embedded tissue sections using the periodic acid–Schiff staining method. The percentage of area occupied by crescents in each glomerulus was estimated and assigned one of the following scores: 0, no crescent; 1, 0%–25%; 2, 25%–50%; 3, 50%–75%; and 4, 75%–100% crescent occupation. The mean score was then calculated as the crescent score.[55] Tubulointerstitial injury was defined as tubular dilation or atrophy, denudation of the tubular basement membrane, or tubular necrosis. Tubular injury scores were evaluated on a scale of 0–4 as follows: 0, no tubulointerstitial injury; 1, <25% injury; 2, 25%–50% injury; 3, 51%–75% injury; and 4, >75% injury. Buffered (1:100) formalin-fixed tissues were immunostained using mouse anti-rat CD68 monoclonal IgG1 (clone ED-1; BMA Biomedicals, Augst, Switzerland) as a marker for infiltrating macrophages or dendritic cells, mouse anti-rat CD163 monoclonal IgG1 (clone ED-2; BMA Biomedicals) as a marker for alternatively activated M2 macrophages, and rabbit anti-rat CD8 monoclonal IgG (Epitomics, Burlingame, CA) as a marker for cytotoxic T cells. Infiltration of glomerular neutrophils was assessed using the naphthol-AS-D chloroacetate (Sigma-Aldrich) esterase reaction, as described elsewhere.[23] For each animal, crescent formation and the number of stained cells were evaluated in >40 glomeruli per renal cross-section. To evaluate the colocalization of $CD163^+$ and $CD206^+$ or IL-10 in glomerular cells, immunohistochemical staining using mouse biotin anti-rat CD163 IgG1 (ED2; AbD Serotec) and rabbit anti-rat CD206 IgG (mannose receptor; Abcam) or goat anti-rat IL-10 polyclonal IgG (R&D Systems, Minneapolis, MN) was performed after blocking endogenous biotin using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). For the double stain of CD163 and CD206, the anti-CD163 antibody was detected by staining with Alexa488 streptavidin (Molecular Probes, Eugene, OR) followed by biotinylated anti-streptavidin (Vector Laboratories) to amplify the signal, and anti-CD206 antibody was detected by staining with Alexa555 goat anti-rabbit IgG (Molecular Probes). For the double stain of cytosolic IL-10 and CD163 or CD206, anti-IL-10 antibody was detected by staining with biotinylated donkey anti-goat IgG followed by FITC-conjugated avidin (Vector Laboratories) and anti-CD163 antibody or anti-CD206 antibody was detected by staining with Alexa555 streptavidin (Molecular Probes) or Alexa555 goat anti-rabbit IgG, respectively as described above. Biotinylated CD4 mAb (LifeSpan Biosciences, Seattle, WA) was used to stain $CD4^+$ T cells according to the same method used for detection of CD163.

Determination of Growth Factor and Cytokine Concentrations

The concentrations of growth factors and cytokines were determined using ELISA kits, each used according to the manufacturer's instructions. ELISA analyses of VEGF (IBL, Gunma, Japan), HGF (Institute of Immunology Co. Ltd., Tokyo, Japan), and IL-6 (Thermo Scientific, Rockford, IL) were performed on cultured MSCs at the fifth passage.[14,15] For cytokine profiles in the kidney, the concentrations of IL-1β, IL-12 p70, and IL-10 were measured by ELISA (Invitrogen) in renal cortex homogenates. Secretion of IL-10 from 2×10^6 peritoneal macrophages cocultured with 0.1×10^6 MSCs was assessed for functional evaluation of M2 cells. Generation of PGE2 and 15d-PGJ2 by MSCs cultured with peritoneal macrophages at a 2:1 ratio or without peritoneal macrophages were determined by ELISA (Enzo Life Sciences, Farmingdale, NY).

Tracking of Intravenously Injected ASCs in Rat Organs

Rats were administered 2×10^6 LASCs stained with CFSE (Molecular Probes) on days 0–4 *via* the tail vein, and tissue samples were taken on days 5, 14, and 28. Uniform CFSE staining of LASCs was confirmed by flow cytometry analysis before injection (Supplemental Figure 5J). Cryostat tissue sections were stained with goat polyclonal anti-CFSE IgG (Molecular Probes) followed by a conjugate of rabbit anti-goat IgG and horseradish peroxidase–labeled polymer (Histofine Simple Stain; Nichirei, Tokyo, Japan) as a secondary reagent. For each animal, $CFSE^+$ cells were counted in at least 100 glomeruli per renal cross-section.

Time-Lapse Recording of MSC-Mediated Polarization of Macrophages to CD163-Presenting Cells

BM-MSCs or LASCs from GFP transgenic rats were plated with GFP-negative macrophages from WKY/NCrj rats at a 1:2 ratio. Cells were harvested with culture medium containing mouse Alexa568-conjugated anti-rat CD163 IgG and maintained on a microscope slide for 30 hours at 37°C in a 5% CO_2 atmosphere in a recording chamber. Isotype mouse IgG (BD Pharmingen) was used as a negative control. Both phase and

fluorescent images were captured every 15 minutes using an LCV110 incubator microscope system (Olympus, Tokyo, Japan).

Western Blot Analyses

The concentration of each purified protein was measured using a protein quantification kit (Thermo Scientific). Protein from each sample (3 μg) was separated by SDS-PAGE on a NuPAGE 4%–12% Bis-Tris gel electrophoresed at 200 V using a mini-cell gel apparatus (Invitrogen). Separated proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA) at 30 V for 720 minutes using a semidry transfer module. Nonspecific binding was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan). Blots were then incubated for 60 minutes at room temperature with the appropriate primary antibody in antibody buffer containing 5% Blocking One in PBST (0.05% v/v Tween 20 in PBS), washed three times with PBST, and then incubated for 1 hour with a peroxidase-conjugated secondary antibody in the antibody buffer. After washing, the blots were developed for visualization using an enhanced chemiluminescence detection kit (ImmunoStar LD; Wako, Osaka, Japan). The primary antibodies and their titers were as follows: CD163, 1:10,000 (AbD Serotec); and β-actin, 1:500,000 (Sigma-Aldrich).

Pharmaceutical Ablation, Blocking, and Stimulation of PGE2 or IL-6

For the pharmaceutical ablation of PGE2 synthesis in LASCs, 1–100 μM selective COX-2 inhibitor (CAY10404; Cayman Laboratories, Ann Arbor, MI) or 10–1000 μM of aspirin (Cayman Laboratories) dissolved in ethanol was added to the cell culture medium. The duration of the effect of CAY10404 on LASCs is shown in Supplemental Figure 8. For *in vivo* analysis of PGE2-ablated LASCs, LASCs were incubated with 50 μM CAY10404 or 1 mM aspirin for 48 hours and then injected into anti-GBM GN rats as described above. For the evaluation of CD163 expression by macrophages induced by LASC-derived IL-6 and PGE2, IL-6 present in coculture medium along with LASCs and macrophages was neutralized by addition of 0.05–5 mg/ml of anti-IL-6 antibody (R&D Systems), or 0.5–50 ng/ml IL-6 (R&D Systems) and 0–1 μg/ml of synthetic PGE2 (Sigma-Aldrich) were added to the culture medium containing macrophages only and incubated for 48 hours.

Statistical Analyses

Statistical analyses were performed using SPSS 18.0 statistical software (SPSS Inc, Chicago, IL). The means and SDs were calculated for all parameters determined in this study. Statistical significance was evaluated by ANOVA to determine the significance of differences between experimental groups. When a statistically significant difference was indicated by ANOVA, further analysis was performed using Tukey's test to determine the significance of differences between any pair of groups. A significant difference was defined as a P value <0.05.

ACKNOWLEDGMENTS

The authors thank Ms. N. Asano, Ms. Y. Sawa, and Mr. N. Suzuki for their excellent technical assistance. We also thank Dr. Tanya N. Mayadas (Brigham and Women's Hospital, Harvard Medical School, Boston, MA) for insightful suggestions and for editing the manuscript. The authors thank Ono Pharmaceutical Co., Ltd. (Osaka, Japan) for supplying the EP1, EP3, and EP4 receptor antagonists.

This work was supported by a grant-in-aid for scientific research (C) from the Ministry of Education, Culture, Sports, Science, and Technology (T.O., N.T., and S.M.), and by a grant-in-aid for progressive renal diseases research, research on intractable diseases, from the Ministry of Health, Labour, and Welfare of Japan (Seiichi Matsuo and Shoichi Maruyama). This work was also supported by the Aichi Kidney Foundation (K.F., N.T., T.O., and S.M.) and the NAGONO Medical Promotion Foundation (K.F.), as well as a Molecular Nephrology Forum Award Japan (K.F.) and a Study for Renal Disease and Hypertension Award supported by the Research Foundation for Community Medicine, Japan (K.F.).

DISCLOSURES

None.

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This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2012030264/-/DCSupplemental.

Cell Transplantation, Vol. 22, pp. 287–297, 2013
Printed in the USA.

0963-6897/13 $90.00 + .00
DOI: http://dx.doi.org/10.3727/096368912X655019
E-ISSN 1555-3892
www.cognizantcommunication.com

Low Serum Cultured Adipose Tissue-Derived Stromal Cells Ameliorate Acute Kidney Injury in Rats

Takayuki Katsuno, Takenori Ozaki, Yosuke Saka, Kazuhiro Furuhashi, Hangsoo Kim, Kaoru Yasuda, Tokunori Yamamoto, Waichi Sato, Naotake Tsuboi, Masashi Mizuno, Yasuhiko Ito, Enyu Imai, Seiichi Matsuo, and Shoichi Maruyama

Department of Nephrology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Current studies suggest that mesenchymal stromal cells (MSCs) improve acute kidney injury (AKI) via paracrine/endocrine effects. We established human adipose tissue-derived stromal cells (hASCs) cultured in low (2%) serum (hLASCs), which have great potential of tissue regeneration. The present study was performed to investigate the therapeutic effects of hLASCs on AKI and to clarify the mechanisms involved. In low serum, hASCs proliferated well, while human bone marrow-derived stromal cells (hBMSCs) did not. hLASCs secreted higher levels of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) than did hASCs cultured in high (20%) serum (hHASCs) or hBMSCs cultured in high serum (hHBMSCs). AKI was induced in nude rats by folic acid, and hLASCs, hHASCs or control medium were administered into the renal subcapsules. hLASCs significantly attenuated acute renal damage, while hHASCs showed far less effect. Furthermore, interstitial fibrosis observed on day 14 was less pronounced in the hLASCs group. Cell tracking experiment showed no evidence of transdifferentiation. Intravenous injection of hLASCs or hHBMSCs or subcapsular injection of hHBMSCs did not ameliorate AKI. Concerning the mechanisms, our in vivo experiments showed that HGF knockdown by siRNA impaired the ability of hLASCs to protect the kidney from acute injury whereas VEGF knockdown did not. In conclusion, hLASCs, but not hHASCs or hHBMSCs, ameliorated AKI via paracrine effects, and HGF is one of the key mediators.

Key words: Acute renal failure; Stem cells; Cell transfer; Interstitial fibrosis

INTRODUCTION

The importance of acute kidney injury (AKI) has been well recognized because AKI is associated with significantly increased mortality and the patients' outcomes are related directly to the severity of AKI. Prevention and effective treatment of AKI are a high priority (7). However, kidney targeted interventions beyond supportive therapy are currently not available.

Previous studies have demonstrated that administration of mesenchymal stromal cells (MSCs) accelerates the recovery of tissue injury in several organs including heart, liver, neuron, and pancreas (12,23,29,30). Administration of bone marrow-derived stromal cells (BMSCs) has also been shown to protect the kidney from AKI induced by cisplatin, glycerol, and ischemia-reperfusion injury (4,13,14,21,26,35).

Recently, it has been demonstrated that MSCs can be obtained from adipose tissue (41). Like BMSCs, adipose tissue-derived stromal cells (ASCs) have the potential to differentiate into various types of cells and tissues (10,33). Previous studies suggest that ASCs may have an advantage over BMSCs. Firstly, adipose tissue is abundant and can be obtained repeatedly with minimal invasive procedure. Secondly, the number of stem cells in the fat is greater than that in the bone marrow (18). Lastly, in general ASCs grow faster than BMSCs (18,22,39).

In a previous study, we reported a novel culture system for human ASCs (hASCs) using low serum medium (17). hASCs cultured in low (2%) serum (hLASCs) secreted higher levels of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) than in high (20%) serum (hHASCs). hLASCs also showed significantly greater therapeutic effects on rat hind limb ischemia than hHASCs. We have also demonstrated that ASCs cultured in low serum (LASCs) have great potential for transdifferentiation and immunosuppression (31,40).

The precise mechanism that attenuates kidney injury after MSCs injection remains to be clarified (3). The process might involve recruitment of MSCs to the site of injury, transfer of microvesicles, or most likely paracrine/endocrine effect (15). Several reports suggest that the renal protective properties of MSCs may be attributed to

Received August 5, 2011; final acceptance March 30, 2012. Online prepub date: September 7, 2012.
Address correspondence to Shoichi Maruyama, M.D., Department of Nephrology, Nagoya University Graduate School of Medicine, 65 Tsurumaicho, Showaku, Nagoya, Japan 466-8550. E-mail: marus@med.nagoya-u.ac.jp

the action of insulin-like growth factor-1(IGF-1), HGF, and VEGF (16,36,37). In the present study, we hypothesized that hLASCs may have a great potential as a novel resource for cell therapy of AKI. Investigation of renoprotective factors of hLASCs would possibly provide new insight for the management of AKI.

The aim of the present study was to examine whether there are therapeutic advantages to using hLASCs rather than hHASCs or human BMSCs (hBMSCs) in an AKI model induced by folic acid injection in rats. In addition, we aimed to investigate the mechanisms involved in the treatment of AKI, focusing on HGF and VEGF.

MATERIALS AND METHODS

Culture Conditions

The basal culture medium was prepared as previously described (17). Cells were cultured under the following two conditions: a low serum culture medium containing 2% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 10 ng/ml human fibroblast growth factor-2 (FGF-2) (Peprotech, Inc., Rocky Hill, NJ, USA) and a high serum culture medium containing 20% FBS and 10 ng/ml human FGF-2.

Preparation of hASCs and hBMSCs

Human abdominal subcutaneous adipose tissue was obtained with the written informed consent from patients undergoing surgery. All tissue samples were used with approval and according to the guidelines of the ethical committee at the Nagoya University Medical School (approval No. 505-2), and the study was performed according to the guidelines of the Declaration of Helsinki. hASCs were prepared as previously described (17). hBMSCs were purchased from LONZA (Basel, Switzerland).

Growth Speeds of hASCs and hBMSCs

hASCs from five patients or hBMSCs from three patients (2×10^5) were cultured for 30 days. Growth kinetics was calculated at each time point and shown as mean ± SD as described (17).

Measurement of HGF and VEGF Secreted From hASCs and hBMSCs

Secretion of HGF and VEGF was studied as described (17). Briefly, hLASCs and hHASCs obtained from five patients, and hBMSCs cultured in high serum (hHBMSCs) from three patients were expanded, and cells at passages 4 through 7 were used. The 24-h conditioned media were analyzed using HGF Otsuka enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical Co., Tokyo, Japan) or Quantikine Human VEGF Immunoassay (R&D Systems, Minneapolis, MN, USA). Data are expressed as nanograms of the secreted factor per 10^6 cells at the time of harvest.

Animals

Male F344/NJcl-rnu/rnu rats were purchased from CLER, Inc. (Tokyo, Japan).

Animals were housed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Animal Experimentation Guidelines of Nagoya University Graduate School of Medicine.

Folic Acid-Induced Acute Kidney Injury

Eight-week-old male F344/NJcl-rnu/rnu rats weighing approximately 160–180 g were subjected to right hemineprectomy. Seven days later (day 0), AKI was induced in immunodeficient rats by the intravenous injection of folic acid (Sigma-Aldrich, St. Louis, MO, USA) at the dose of 200 mg/kg.

In Vivo Experimental Protocol 1: Subcapsular Administration of hASCs

Subcapsular injection of 6×10^6 of hLASCs, hHASCs, or control medium (Dulbecco's modified Eagle's medium, DMEM; Sigma-Aldrich) (each group $n=8$) was given to the left kidney of AKI rats, immediately after folic acid injection. Blood samples were collected on days 0, 1, 2, 6, and 14, and blood urea nitrogen (BUN) and serum creatinine levels were measured by Mitsubishi Chemical Medience Co. Ltd (Tokyo, Japan). Kidney samples were taken on day 14. AKI was induced in another set of rats, and hLASCs, hHASCs, or control medium (each group $n=8$) were injected in the same way. Rats were euthanized on day 2, renal cortical microcirculation was assessed using CCD video microscope (28), and kidney samples were taken for the study.

Histology and Immunohistochemistry

Kidney sample was processed for routine histology and immunohistology as described previously (32). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using in situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan) as described (34). Immunostaining for monocytes/macrophages (ED-1), α-smooth muscle actin (α-SMA), and type III collagen was performed on buffered formalin-fixed tissues. Antibodies used were mouse anti-rat monocyte/macrophage antibody (ED-1, BMA Biomedicals, Augst, Switzerland), mouse anti-α-SMA antibody (Dako, Glostrup, Denmark), rabbit anti-type III collagen antibody (Cosmo Bio, Tokyo, Japan), a conjugate of polyclonal goat anti-mouse IgG antibody or anti-rabbit IgG antibody, and horseradish peroxidase-labeled polymer (Histofine Simple Stain, Nichirei, Tokyo, Japan).

Morphological Analysis

To evaluate tubulointerstitial injury, periodic acid Schiff (PAS)-stained kidney sections were analyzed using a

quantitative grading as described previously (24). The numbers of TUNEL-positive cells and ED-1-positive cells were counted in 10 nonoverlapping fields (200× magnification) of the cortex and the numbers were expressed per field. Renal fibrosis was assessed by quantification of Masson's trichrome (MT), α-SMA or type III collagen-positive area using the MetaMorph 6.3 image analysis program (Universal Imaging, West Chester, PA, USA) as described (20).

Measurement of HGF and VEGF Concentrations in the Renal Cortex

Renal cortex samples taken on day 2 were homogenized with RIPA Buffer (Pierce, Rockford, IL, USA) and centrifuged, and the supernatants were collected. The levels of growth factors were measured using Human HGF Otsuka ELISA kit (Otsuka), Quantikine Human VEGF Immunoassay (R&D Systems), Rat HGF EIA kit (Institute of Immunology, Tokyo, Japan), and Rat VEGF ELISA kit (Invitrogen, Carlsbad, CA, USA).

In Vivo Experimental Protocol 2: hLASCs Tracking Study

Subcapsular injection of 6×10^6 hLASCs was given to rats with folic acid-induced AKI and kidney samples were removed on day 2 or day 14 (each group $n=6$). Frozen sections were stained with mouse monoclonal anti-Lamin A/C antibody (Novocastra/Vision BioSystems, Norwell, MA, USA) followed by goat anti-mouse IgG antibody (Histofine).

In Vivo Experimental Protocol 3: Intravenous Injection of hLASCs

Tail vein injection of 6×10^6 of hLASCs or PBS (each group $n=6$) was given to rats with AKI immediately after folic acid injection. Blood samples were taken on days 0, 1, and 2 for the measurement of BUN and serum creatinine.

In Vivo Experimental Protocol 4: Subcapsular or Intravenous Injection of hHBMSCs

Subcapsular administration of hHBMSCs (6×10^6) or control medium (each group $n=6$), or intravenous injection of hHBMSCs (6×10^6) or PBS (each group $n=6$) was performed in rats with AKI immediately after folic acid injection. Blood samples were taken on days 0, 1, and 2 for the measurement of BUN and serum creatinine.

Transfection of siRNA Into hLASCs

ON-TARGET plus SMART pool siRNA targeted to human HGF or human VEGF was purchased (Dharmacon, Lafayette, CO, USA) and tested at concentrations of 5, 10, and 50 nM. Forward transfection procedure was used into hLASCs in a 24-well format. hLASCs (2×10^4) were seeded onto 24-well plates (BD Falcon, Franklin Lakes, NJ, USA) in 500 μl of low serum medium without antibiotics. The next day, diluted siRNA duplex with the diluted Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM I Reduced Serum Medium (Gibco, Carlsbad, CA, USA) was transfected into hLASCs. After 6 h of incubation, medium change was performed. Knockdown efficiency was detected on the protein level in the cultured medium measured by ELISA kits; HGF Otsuka ELISA kit (Otsuka), Quantikine Human VEGF Immunoassay (R&D Systems) at 48 and 96 h after transfection. Controls consisted of hLASCs treated with ON-TARGET plus Non-targeting pool (negative control siRNA) (Dharmacon), Lipofectamine RNAiMAX transfection agent only, and untreated cells.

In Vivo Experimental Protocol 5: Subcapsular Administration of HGF or VEGF Knockdown hLASCs

In the folic acid-induced rat AKI model, 2×10^6 of hLASCs transduced with HGF siRNA (hLASCs-siHGF), VEGF siRNA (hLASCs-siVEGF), negative control siRNA (hLASCs-siCtrl) or control medium (each group $n=9$) were injected into the subcapsular space of the kidney at 2 days after transfection. Blood samples were collected on days 0, 1, 2, and BUN and creatinine levels were measured.

Statistical Analysis

Statistical analysis was performed using the software program, Stat View 5.0 (SAS Institute, Cary, NC, USA). Student's t test was used to determine the significant differences between two groups. Two-way analysis of variance (ANOVA) was employed to determine the significant difference among three or four groups. When a statistical difference was indicated by ANOVA, further analysis was performed using Scheffe to determine the difference between any pair of groups. A value of $p<0.05$ was considered statistically significant. All values are expressed as mean ± SD.

RESULTS

Growth Kinetics of hASCs and hBMSCs

The cell proliferation speeds of hASCs and hBMSCs were compared under low serum (2%) and high serum (20%) culture conditions. hHASCs grew the fastest, followed by hLASCs. Of note, hLASCs grew faster than hHBMSCs. Low serum cultured hBMSCs (hLBMSCs) proliferate too slowly to be used in the following experiments (Fig. 1A).

Growth Factor Secretion by hASCs and hBMSCs

Since HGF and VEGF are known to promote recovery from ischemic tissue injury (17,25,27), secretion of these molecules was analyzed. hLASCs secreted higher levels of HGF and VEGF than did the hHASCs or hHBMSCs at passages 4 through 7 while no difference was observed

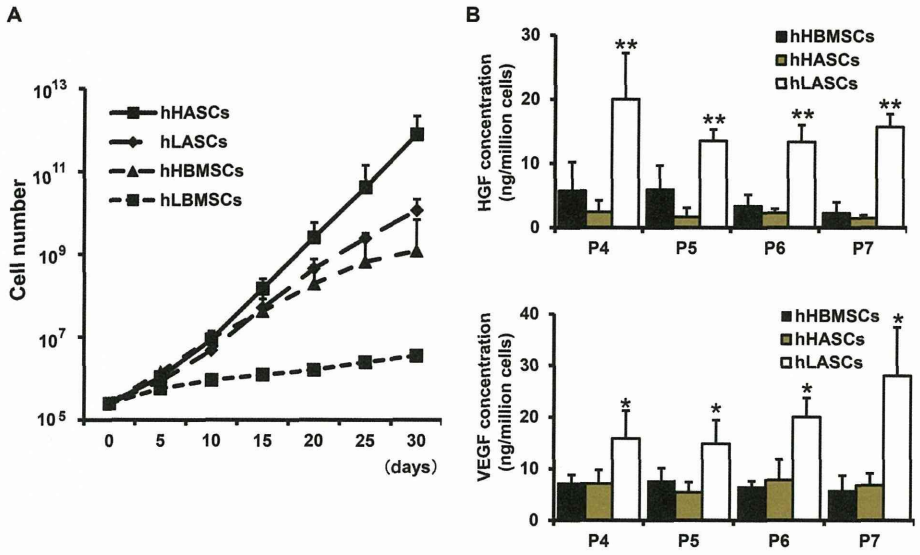

Figure 1. (A) Growth speeds of human adipose tissue-derived stromal cells (hASCs) and human bone marrow-derived stromal cells (hBMSCs) under low serum (2%) or high serum (20%) culture conditions. High serum cultured human adipose tissue-derived stromal cells (hHASCs) grew the fastest, followed by low serum cultured human adipose tissue-derived stromal cells (hLASCs), and human bone marrow-derived stromal cells cultured in high serum (hHBMSCs). Low serum cultured human bone marrow-derived stromal cells (hLBMSCs) did not proliferate well. (B) Secretion of growth factors by hLASCs, hHASCs, and hHBMSCs. The levels of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) were significantly higher in the hLASCs-conditioned medium than those in the hHASCs or hHBMSCs-conditioned medium. *$p<0.05$ versus hHASCs or hHBMSCs, **$p<0.01$ versus hHASCs or hHBMSCs.

between the levels of HGF or VEGF secreted by hHASCs and hHBMSCs (Fig. 1B).

Effects of Subcapsular Injection of hLASCs and hHASCs

Renal Function. An experimental model of AKI was induced in immunodeficient rats, and hLASCs, hHASCs, or control medium were injected into the subcapsular space of the kidney immediately upon folic acid injection. Rats treated with control medium demonstrated a marked rise in BUN and serum creatinine on days 1 and 2, whereas rats given hHASCs showed preserved renal function on day 2. Interestingly, hLASCs further suppressed the increase of BUN and serum creatinine on days 1 and 2 (Fig. 2).

Tubular Injury. Examination of PAS-stained kidney sections taken from AKI rats treated with control medium showed severe tubular cell degenerative changes with necrosis and luminal casts on day 2 (Fig. 3A). hLASCs

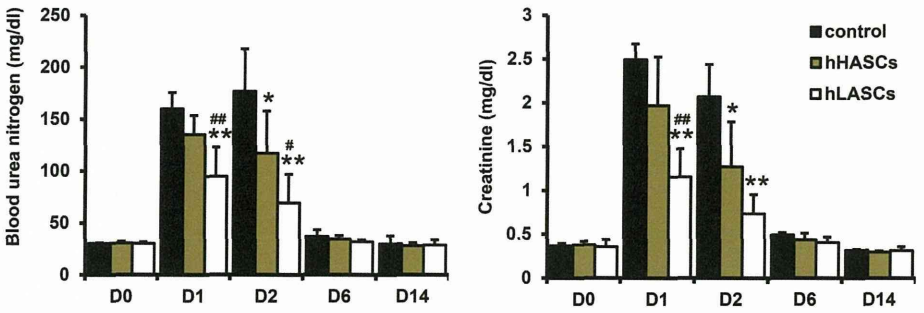

Figure 2. Renoprotection by subcapsular administration of low serum cultured human adipose tissue-derived stromal cells (hLASCs). Immunodeficient rats were given intravenous injection of folic acid on day 0, followed by subcapsular administration of hLASCs, high serum cultured human adipose tissue-derived stromal cells (hHASCs), or control medium. Blood urea nitrogen (BUN) and serum creatinine were measured on days 0, 1, 2, 6, and 14. hLASCs markedly attenuated renal injury, as reflected by the significantly lower BUN and serum creatinine levels on days 1 and 2, whereas hHASCs showed less renoprotection. *$p<0.05$ versus control, **$p<0.01$ versus control, #$p<0.05$ versus hHASCs, ##$p<0.01$ versus hHASCs. Hpf, high power field.

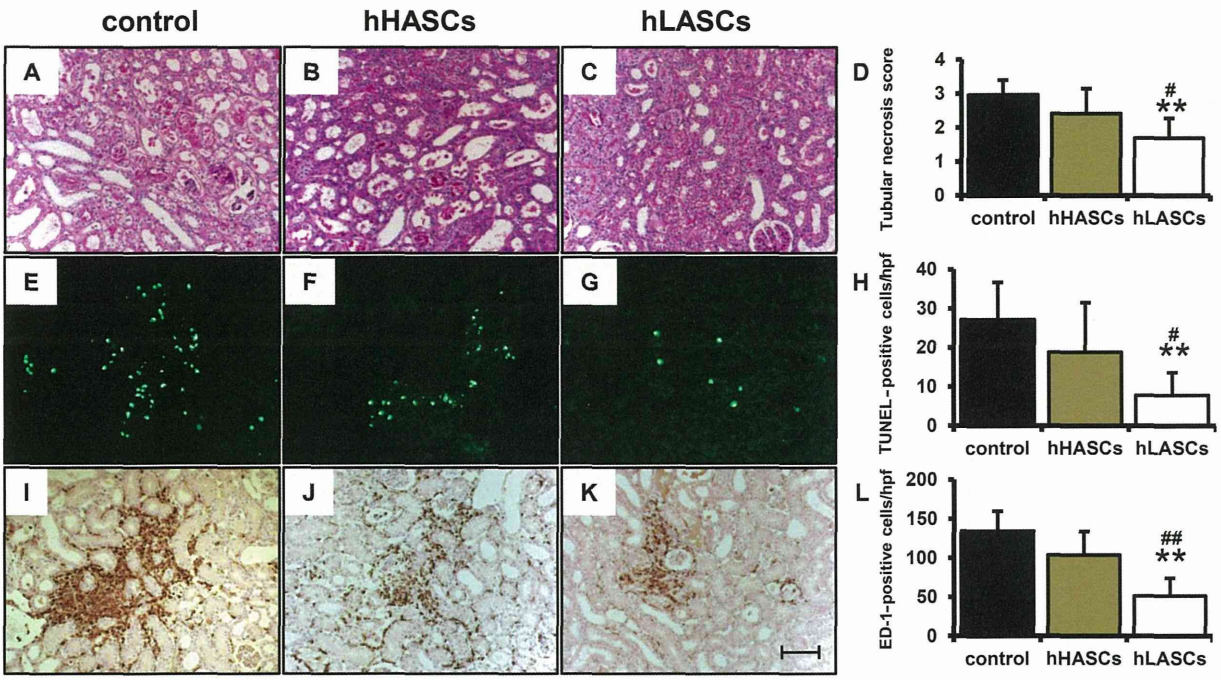

Figure 3. Tubular damage and macrophage infiltration. (A–C) Representative micrographs show the kidney sections taken on day 2 from AKI rats treated with control medium (A), AKI rats treated with high serum cultured human adipose tissue-derived stromal cells (hHASCs) (B), and those treated with low serum cultured human adipose tissue-derived stromal cells (hLASCs) (C), and followed by periodic acid Schiff (PAS) staining. The hLASCs group showed significantly better tubular necrosis scores than the other groups (D). (E–G) Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining performed on the kidney sections taken on day 2 from the control group (E), the hHASCs group (F), and the hLASCs group (G). hLASCs significantly decreased the number of apoptotic cells as compared to the other groups (H). (I–K) ED-1 staining performed on the kidney sections taken on day 2 from the control group (I), the hHASCs group (J), and the hLASCs group (K). The numbers of ED-1-positive cells were significantly lower in the hLASCs group than the other groups (L). Original magnification: 200× (in all micrographs). Scale bar: 100 μm (A–C, E–G, I–K). $^{**}p<0.01$ versus control, $\#p<0.05$ versus hHASCs, $\#\#p<0.01$ versus hHASCs.

greatly attenuated the tubular injury, while hHASCs partially attenuated the tubular damage (Fig. 3B, C). The severity of the tubular damage, including tubular dilatation, degeneration, and cast formation was scored (24). Treatment with hLASCs resulted in significantly better scores than the control or hHASCs group. In contrast, the hHASCs-treated group failed to show significantly better scores than the control group (Fig. 3D).

Tubular Cell Apoptosis. TUNEL assay was performed on the kidney sections obtained on day 2 (Fig. 3E–G). Transplantation of hLASCs significantly decreased the numbers of TUNEL-positive cells as compared to control or hHASCs, while hHASCs did not (Fig. 3H).

Macrophage Infiltration. Macrophage infiltration was evident in the interstitium of the AKI rats treated with control medium (Fig. 3I). hHASCs treatment slightly suppressed macrophage infiltration (Fig. 3J). Further suppression was observed in the hLASCs group (Fig. 3K). Quantification of ED-1-positive cells showed a significant reduction in the number of macrophages in the hLASCs group while no significant suppression was observed in the hHASCs group (Fig. 3L).

Growth Factors in the Kidney. Since hLASCs produced significantly higher levels of HGF and VEGF than hHASCs in vitro, the concentrations of these growth factors in the renal cortex were measured on day 2. In the hLASCs group, the human HGF and VEGF levels were significantly higher than in the hHASCs group (Fig. 4A). The rat HGF level was significantly higher in the hLASCs group compared with the control group, while the hHASCs group did not show a significant difference. In contrast, no differences were observed in the rat VEGF levels among the three groups (Fig. 4B).

Direct Visualization of the Renal Cortical Capillaries. The effects of hLASCs or hHASCs on renal cortical microcirculation were examined by analyzing the direct images obtained with a CCD video microscope system on day 2. The blood flow velocity was significantly faster and the blood flow volume was greater in the hLASCs group than in the control or the hHASCs groups (Fig. 4C).

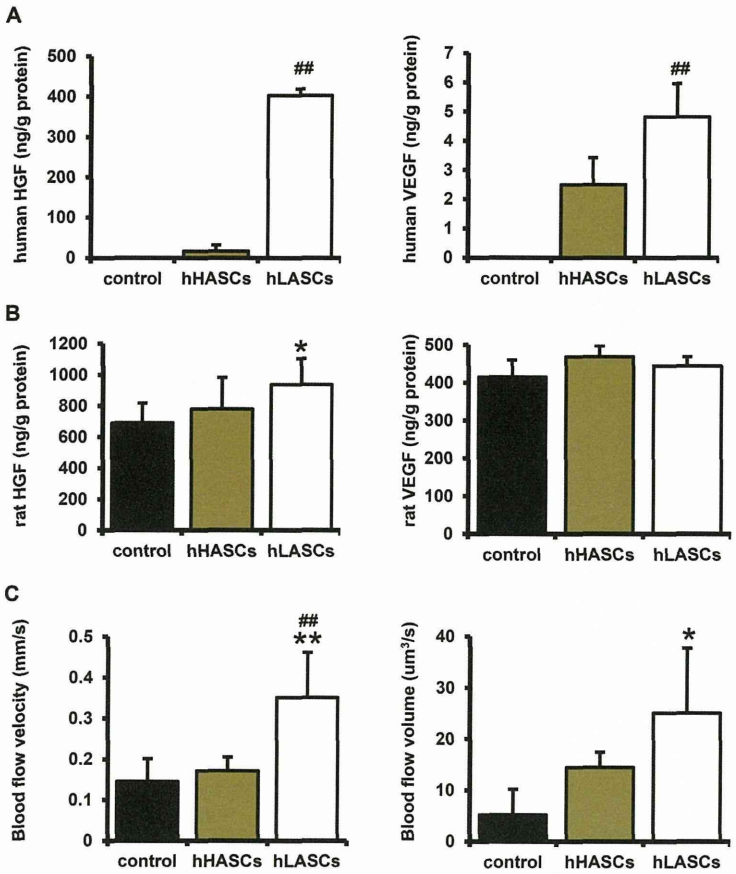

Figure 4. (A, B) Levels of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) in the renal cortex. HGF and VEGF concentrations were measured in the homogenates of the renal cortex samples taken on day 2 from AKI rats given subcapsular injection of low serum cultured human adipose tissue-derived stromal cells (hLASCs), high serum cultured human adipose tissue-derived stromal cells (hHASCs), or control medium. Levels of human HGF and VEGF were significantly higher in the hLASCs group as compared to the hHASCs group (A). In the hLASCs group, rat HGF concentration was significantly higher than in the control group. No differences were observed in the levels of rat VEGF among three groups (B). (C) Renal cortical microcirculation. Velocity of the capillary blood flow and the capillary blood flow volume were significantly higher in AKI rats given subcapsular injection of hLASCs than those given the control medium, while hHASCs did not show significant effects. *$p<0.05$ versus control, **$p<0.01$ versus control, ##$p<0.01$ versus hHASCs.

Subsequent Interstitial Fibrosis. MT staining was performed on the kidney sections obtained on day 14 (Fig. 5A–C). Quantitative analysis with the use of the Meta Morph 6.3 image computer program demonstrated that the MT-positive area was significantly reduced in the hLASCs group but not in the control group (Fig. 5D). α-SMA staining was performed on the kidney sections obtained on day 14 (Fig. 5E–G). Administration of hLASCs significantly decreased the interstitial α-SMA-positive area as compared to the control medium or hHASCs (Fig. 5H). Type III collagen staining was also performed (Fig. 5I–K). Quantification of type III collagen-positive area on day 14 showed a significant reduction in the hLASCs group while no significant change was observed in the hHASCs group (Fig. 5L).

Fate of Transplanted hLASCs

Human lamins A and C immunostaining was performed on the kidney sections from rats with AKI on days 2 and 14 after hLASCs transplantation into the subcapsular space. hLASCs were detected only in the subcapsule of the kidney on day 2 (Fig. 6A), and the transplanted cells remained alive in the subcapsule on day 14 (Fig. 6B).

Therapeutic Effects of Intravenous Injection of hLASCs

In order to study whether or not intravenous injection of hLASCs is as effective, AKI was induced by folic acid injection followed by intravenous administration of hLASCs. The increase in the levels of BUN and serum creatinine was not statistically significant (Fig. 7A).

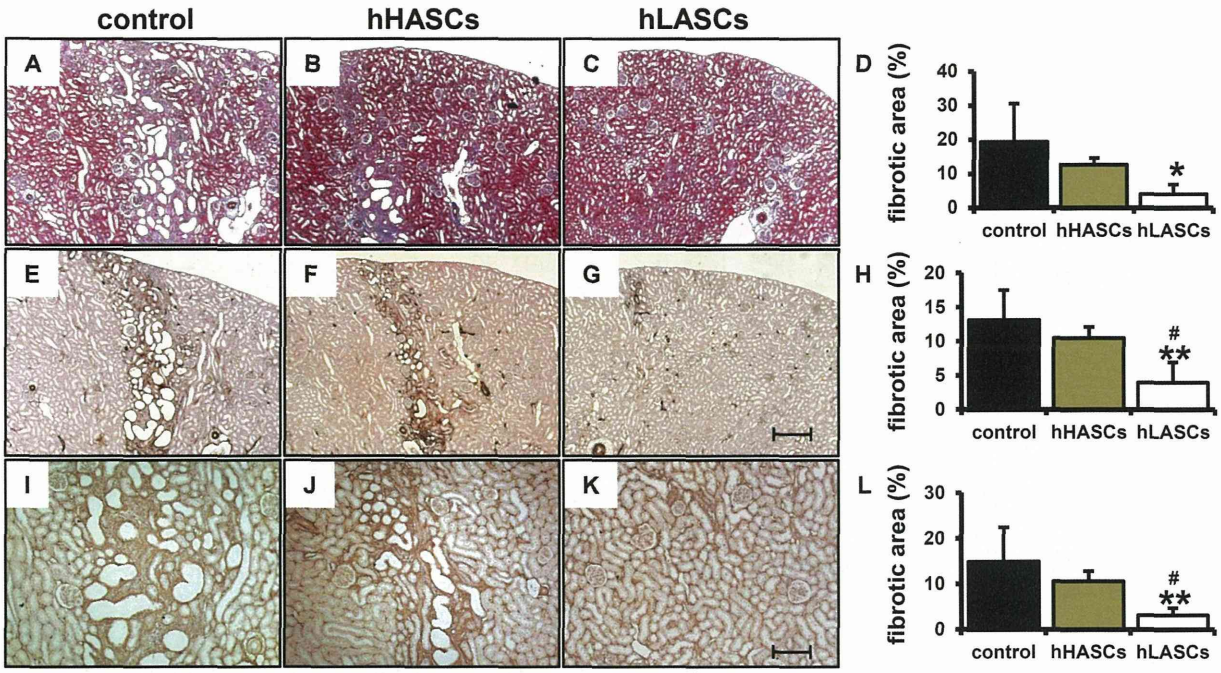

Figure 5. (A–L) Subcapsular administration of low serum cultured human adipose tissue-derived stromal cells (hLASCs) decrease subsequent renal fibrosis. Representative micrographs show the kidney sections taken on day 14 from rats given control medium (A, E, I), high serum cultured human adipose tissue-derived stromal cells (hHASCs) (B, F, J), or hLASCs (C, G, K). Masson's trichrome (MT) (A–C), α-smooth muscle actin (α-SMA) (E–G), and type III collagen (I–K) staining were performed. (D, H, L) Quantitative analysis of fibrotic area with the use of the Meta Morpho demonstrated that renal fibrosis detected by MT (D), α-SMA (H), and type III collagen (L) was reduced in the hLASCs group. Original magnifications: 50× (in A–G) and 100× (in I–K). Scale bars: 400 μm (A–C, E–G) and 200 μm (I–K). *$p<0.05$ versus control, **$p<0.01$ versus control, #$p<0.05$ versus hHASCs.

Therapeutic Effects of Subcapsular or Intravenous Administration of hHBMSCs

To investigate whether hHBMSCs are renoprotective in AKI, hHBMSCs were injected into the subcapsular space of the kidney or the tail vein, immediately after folic acid injection. Subcapsular administration of hHBMSCs showed a tendency to reduce BUN and serum creatinine elevation, which was not statistically significant (Fig. 7B).

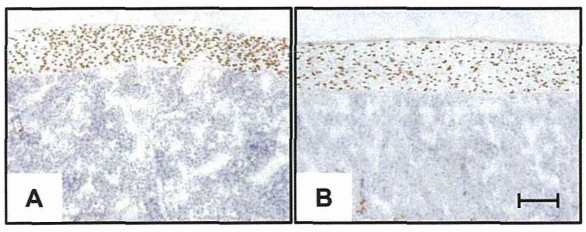

Figure 6. Representative micrographs of low serum cultured human adipose tissue-derived stromal cells (hLASCs) transplanted into the subcapsule of the kidney of AKI rats. hLASCs stained by anti-human Lamins A and C were detected in the subcapsule of the kidney on days 2 (A) and 14 (B). Original magnification: 100×. Scale bar: 200 μm (A, B).

Intravenous injection of hHBMSCs did not decrease the elevation of BUN and serum creatinine (Fig. 7C).

Knockdown of HGF or VEGF in hLASCs by siRNA

Knockdown efficiency was determined on the protein levels in the culture media supernatants of hLASCs at 48 and 96 h after transfection. Treatment with either HGF or VEGF siRNA at a concentration of 10 nM proved to be the most effective. In the hLASCs-siHGF and hLASCs-siVEGF, greater than 80% knockdown level of HGF or VEGF expression was observed at 48 and 96 h after transfection compared to the hLASCs-siCtrl. HGF or VEGF concentrations in the supernatants of untreated hLASCs, hLASCs treated with transfection agent only, or hLASCs-siCtrl were similar (Fig. 8)

Effects of HGF or VEGF Knockdown hLASCs on Renal Function

The in vivo effects of hLASCs-siHGF or hLASCs-siVEGF on renal function were studied in the rat AKI model. In the hLASCs-siCtrl goup, renal injury assessed by BUN and serum creatinine levels on days 1 and 2 was significantly attenuated compared to the control.

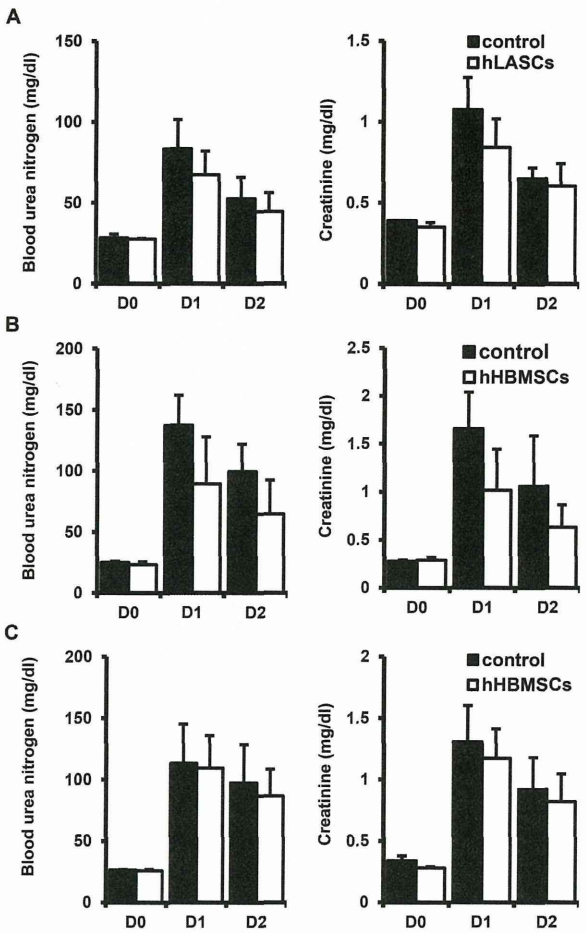

Figure 7. Renal function after the intravenous injection of low serum cultured human adipose tissue-derived stromal cells (hLASCs), subcapsular injection of high serum cultured human bone marrow-derived stromal cells (hHBMSCs), or intravenous injection of hHBMSCs. Immunodeficient rats were given folic acid on day 0, followed by intravenous injection of hLASCs (A), subcapsular injection of hHBMSCs (B), or intravenous injection of hHBMSCs (C). Blood urea nitrogen (BUN) and serum creatinine levels were measured on days 0, 1, and 2. No significant difference was observed in any set of experiments.

hLASCs-siHGF significantly reduced the renoprotection exerted by hLASCs-siCtrl. hLASCs-siVEGF also reduced renoprotective effects, which however was not significant (Fig. 9).

DISCUSSION

The present study provides clear evidence that administration of hLASCs into the renal subcapsule protects the kidney from acute injury induced by folic acid as well as subsequent fibrosis more efficiently than does administration of hHASCs. In contrast, hHBMSCs failed to offer significant renoprotection.

hBMSCs have been shown to secrete a variety of growth factors, which promote tissue regeneration (1,6,11,35). In a previous study, we have shown that hLASCs secrete higher levels of HGF and VEGF and promote ischemic tissue recovery more efficiently than hHASCs (17). In the present study, we further compared hASCs and hBMSCs regarding the proliferation and growth factor secretion and found that hASCs showed better growth rate than did hBMSCs and that a high serum medium resulted in a greater proliferation rate than a low serum medium. Of note, hLASCs proliferated better than hHBMSCs. Concerning the growth factors, hLASCs secreted higher levels of HFG and VEGF than did hHASCs or hHBMSCs.

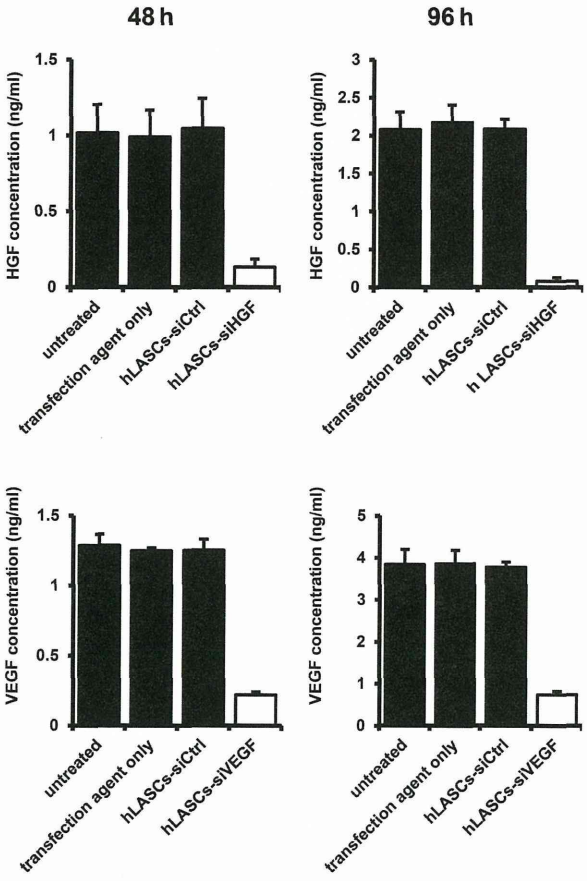

Figure 8. Knockdown of human hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) in low serum cultured human adipose tissue-derived stromal cells (hLASCs) in vitro. Human HGF and VEGF protein concentrations in the cell culture supernatants of hLASCs treated with specific siRNA at 48 and 96 h after transfection. Either hLASCs transduced with HGF siRNA (hLASCs-siHGF) or hLASCs transduced with VEGF siRNA (hLASCs-siVEGF) yielded a greater than 80% knockdown. hLASCs, hLASCs treated with transfection agent only, and hLASCs transduced with negative control siRNA (hLASCs-siCtrl) showed almost equivalent HGF and VEGF concentrations.

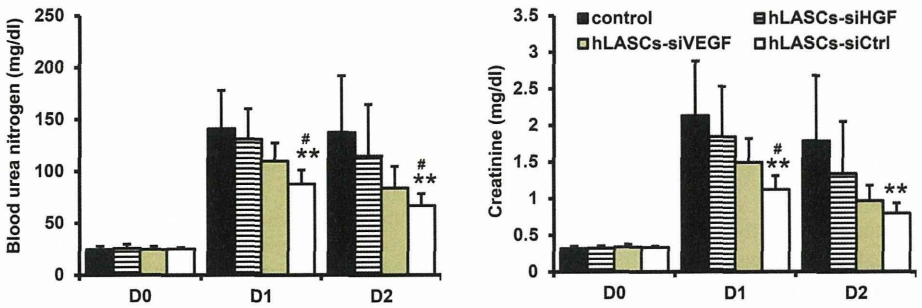

Figure 9. Hepatocyte growth factor (HGF) knockdown reduced renoprotective effects of low serum cultured human adipose tissue-derived stromal cells (hLASCs) in vivo. Immunodeficient rats were given intravenous injection of folic acid on day 0, followed by subcapsular administration of hLASCs transduced with HGF siRNA (hLASCs-siHGF), hLASCs transduced with vascular endothelial growth factor (VEGF) siRNA (hLASCs-siVEGF), hLASCs transduced with negative control siRNA (hLASCs-siCtrl), or a control medium. Blood urea nitrogen (BUN) and serum creatinine values were higher in the hLASCs-siHGF-treated group than in the hLASCs-siCtrl-treated groups on days 1 and 2. $^{**}p<0.01$ versus control, $\#p<0.05$ versus hLASCs-siHGF.

These results indicate that hLASCs are a useful and powerful tool in regenerative cell therapy.

In order to deliver MSCs to the target organ efficiently, we tried direct subcapsular injection (8,19). When we administered hLASCs at 5 h after induction of AKI, a significant therapeutic effect was not obtained (data not shown). The tubular damage in this model may have already been established by this time. We then administered hLASCs immediately after folic acid injection. And we found that administration of hLASCs showed significant renoprotection while hHASCs showed only moderate effects. Our result—that a subcapsular delivery of hHBMSCs failed to show significant therapeutic effects—suggests a clear advantage for hLASCs over hHBMSCs. Since hLASCs secreted high levels of HGF and VEGF, the levels of these molecules in the kidney samples were measured. We found that the concentrations of HGF and VEGF were significantly higher in the renal cortex of the hLASCs group than the hHASCs group, suggesting that hLASCs produced and secreted high levels of HGF and VEGF in vivo. Moreover, we demonstrated that rat HGF concentration in the renal cortex was significantly higher in the hLASCs group. It was previously reported that HGF promotes the expression of HGF itself (38). Therefore, we assume that the human HGF produced by hLASCs promoted further rat HGF expression, thus attenuating tubular injury. Of interest, only a single delivery of hLASCs at day 0 reduced the subsequent fibrosis evaluated on day 14.

To date, the way MSCs promote tissue regeneration remains controversial. Previous studies have suggested that the renoprotective effects are attributable to the replacement of damaged tubular cells by transdifferentiation of MSCs (9,26). However, recent studies have suggested that almost no MSCs are transdifferentiated into renal cells when applied to the AKI model (5). In our study, hLASCs remained alive in the renal subcapsule at days 2 and 14 after injection, showing no evidence of migration and transdifferentiation. A recent study has demonstrated that intravenous injection of either BMSCs or ASCs protected against cisplatin-induced AKI via an endocrine effect since even administration of conditioned culture medium alone ameliorated renal injury (4). Based on these findings, we then tried intravenous administration of hLASCs and hHBMSCs and found that neither of them showed significant therapeutic effects in our model. These results suggest that hLASCs exert renoprotection mainly via a paracrine effect.

In order to specifically evaluate the contribution of HGF or VEGF toward hLASCs mediated renoprotection, we conducted a HGF or VEGF knockdown experiment using siRNA. It has already been reported that knockdown of VEGF by siRNA lessened the therapeutic effect of MSCs in the ischemia-reperfusion AKI model (37). However, there are no reports regarding the effects of HGF knockdown. In our study, VEGF knockdown decreased the effectiveness of hLASCs, which however was not statistically significant. In contrast, we showed for the first time that knocking down HGF in hLASCs significantly reduced their therapeutic activity, suggesting that HGF is a predominant mediator of renoprotection in the folic acid-induced AKI model.

There are several possible explanations concerning the difference between the VEGF and HGF knockdown effects. hLASCs secreted 6–10 times more HGF than did hHASCs, whereas the level of VEGF secretion by hLASCs was only 2–4 times higher. The effects of VEGF knockdown may have been offset by a high level of HGF. Another explanation is that HGF was particularly effective in the model employed in our study. VEGF has been shown to protect endothelial cells from injury, while HGF is known to protect not only endothelial cells but also renal tubular cells. The contribution of VEGF

may be greater in the ischemic renal injury model where endothelial cells are severely damaged. In folic acid-induced AKI, where renal tubular cells, not endothelial cells, are the primary target of tissue injury, the contribution of HGF may be greater. Furthermore, the superior anti-inflammatory effect shown by hLASCs may also be attributed to the action of HGF.

Of note, HGF knockdown did not fully cancel the renoprotection of hLASCs, indicating that all of the paracrine effects of hLASCs cannot be attributed to a single growth factor. Cell therapy, as a new strategy, exerts the combined actions of several factors to simultaneously reduce tissue damage and is therefore potentially more effective than any single growth factor or drug therapy.

What is interesting is the fact that a clear difference exists in the potency of renoprotection between hLASCs and hHASCs even derived from the same donor patient. We propose that these ASCs are of great use as tools in future research. Studying the molecular differences between hLASCs and hHASCs and examining the function, for instance, by using a siRNA system as employed in this study, will further reveal molecular mechanisms, leading to the development of novel therapies.

That hHBMSCs did not show significant renoprotection in the present study may be attributable to the model employed. In order to evaluate chronic fibrosis, we used folic acid-induced AKI (2), while previous studies used AKI models induced by cisplatin, glycerol, or ischemia-reperfusion injury (4,13,14,21,26,35). Moreover, we used nude rats whose immune reaction is compromised. Therefore, it was difficult to evaluate the immunosuppressive effects of ASCs or BMSCs in this study.

In conclusion, we demonstrate that subcapsular administration of hLASCs protects the kidney from acute tubular injury. Our data suggest that HGF is an important mediator in the renoprotective effects of hLASCs and provide evidence that hLASCs will be considered a new source for cell therapy with providing a mixture of several humoral factors. Moreover, hLASCs and hHASCs are useful for a deeper investigation of the molecular mechanisms involved in organ protection.

ACKNOWLEDGMENTS: The authors thank N. Asano, Y. Sawa, and N. Suzuki for excellent technical assistance. This study was supported in part by a Grant-in-Aid for Progressive Renal Diseases Research, Research on Intractable Disease, from the Ministry of Health, Labor, and Welfare of Japan. The authors declare no conflict of interest.

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Hirosaki Med. J. 64(Suppl.) : S1—S3, 2013

PRETREATMENT OF RENAL SUPSCAPULAR ADMINISTRATION OF ADIPOSE TISSUE-DERIVED STEM CELLS AMELIORATE ISCHEMIA-REPERFUSION-INDUCED ACUTE KIDNEY INJURY

Tokunori Yamamoto, Funahashi Yasuto, Yoshihisa Mastukawa, Masashi Kato, Yasushi Yoshino, and Momokzu Gotoh

Abstract

Background

Acute renal ischemic injury (AKI) represents a major clinical problem with renal arterial clamp at partial nephrectomy. The use of therapy using adipose tissue-derived stem cells(ASCs) has been suggested as a potential modality to attenuate the ischemic renal damage.

Methods

We investigated the possible reno-protection of pretreatment of ASCs before and after in a rat ischemia-reperfusion (I-R) model of AKI. Twenty-four hours post-ischemia, blood flow in peritubular capillaries (PTC) was measured using intravital videomicroscopy.

Results

We demonstrated that ADRC therapy significantly reduced serum creatinine and BUN. Histological analysis further validated a significantly attenuated tubular damage. Intravital videomicroscopy and measurement of red blood cell velocity in peritubular capillaries showed ASCs-injected kidneys displayed significant hemodynamic improvement.

Conclusions

The subscapular administration of ASCs to the kidney attenuates I/R renal injury though anti-inflammation, anti-apotoyic effect and peritubular capillary microcirculation. The present study suggests that ASCs would be a useful tool in preventing ischemic kidney damage in the clinical setting.

Hirosaki Med. J. 64, Supplement : S1—S3, 2013

Key words: ADIPOSE TISSUE-DERIVED STEM CELLS; ISCHEMIA-REPERFUSION-INDUCED ACUTE KIDNEY INJURY; RENAL PROTECT; CYTOKINES

Background

Previous studies have demonstrated that administration of mesenchymal stromal cells (MSCs) accelerates the recovery of tissue injury in several organs including heart, liver, neuron, and pancreas. Administration of bone marrow-derived stromal cells (BMSCs) has also been shown to protect the kidney from AKI induced by cisplatin, glycerol, and ischemia-reperfusion injury. Recently, it has been demonstrated that MSCs can be obtained from adipose tissue. Like BMSCs, adipose tissue-derived stromal cells (ASCs) have the potential to differentiate into various types of cells and tissues. Previous studies suggest that ASCs may have an advantage over BMSCs. Firstly, adipose tissue is abundant, and can be obtained repeatedly with minimal invasive procedure. Secondly, the number of stem cells in the fat is greater than that in the bone marrow. Lastly, in general ASCs grow faster than BMSCs.

In a previous study, we reported renoprotection on and low serum cultured and non cultured ASCs and a transplanted endothelial cell for folic acid[2] and cisplathin induced[5] AKIs and acute

Department of Urology, Nagoya University
Corresponding Author: Tokunoti Yamamoto,
Department of Urology, Nagoya University
Tel: 052-744-2985
Fax: 052-744-2319
Email: toku@med.nagoya-u.ac.jp