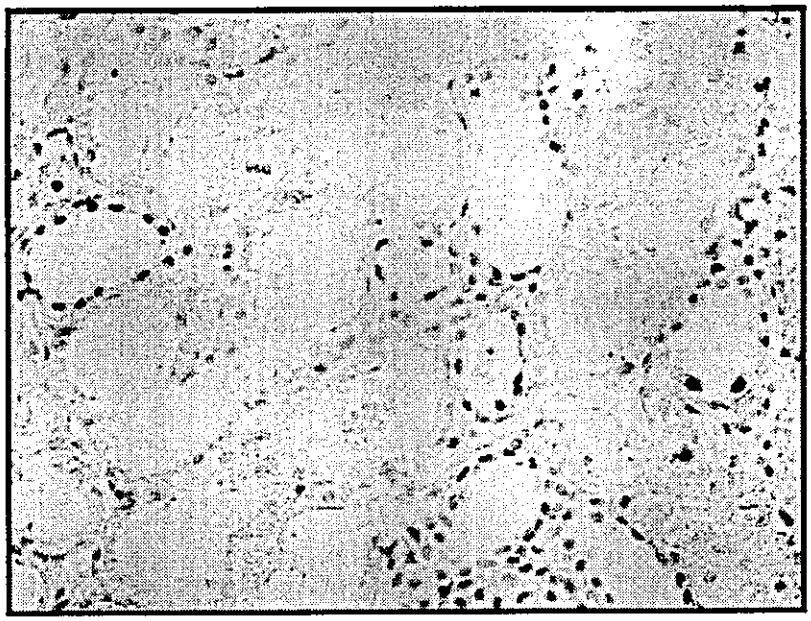

Fig. 7. A tissue section with acute rejection stained with a 2 terminal deoxynucleotidyl transferase (TdT)/3,3′-diaminobenzidine (DAB) kit for apoptosis detection in situ, and methylene green counterstaining. Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) + signals (brown) were almost confined to the distal tubules, and were unrelated to interstitial cell infiltration (magnification ×100).

cell types during the acute and recovery phase of acute renal failure [42]. This may account for the frequently observed apoptosis in the distal but not the proximal tubules in acute rejection. OPN has antiapoptotic effect. In a recent study, ischemic kidneys from OPN knockout mice showed significantly enhanced apoptosis [43]. OPN expression did not correlate with the observed number of apoptotic cells, indicating that OPN is probably an irrelevant regulator of apoptosis in acute rejection.

The available data on the significance of OPN expression in human renal diseases are still limited. In IgA nephropathy, OPN has been shown to have a negative impact on the prognosis [16]. In membranous nephropathy, tubular expression of OPN was significantly higher in patients with progressive disease [15]. In these two kinds of chronic glomerulonephritis, the adverse prognostic significance of OPN expression is probably caused by its significant association with interstitial fibrosis. Overload of tubular cells with filtered proteins has been shown to induce OPN expression in the proximal tubular epithelium in rat remnant kidney model in vivo, suggesting that proteinuria may be a strong inducer of tubular OPN expression [44]. However, the correlation of proteinuria with OPN expression in human renal diseases is rather conflicting [16, 45]. We could not find significant correlation between OPN expression and renal function or urinary protein excretion. This discrepancy between the clinical and pathogenic significance of OPN expression may be resulted from the antirejection treatment, the biopsy timing, or because of the diverse biologic functions of OPN. The diversity of its function may limit the prospect of being a promising target for immunosuppressive therapy.

In the present study, we demonstrated the up-regulated tubular expression of OPN at both the protein and mRNA levels in biopsies from renal allograft with acute rejection. OPN expression was correlated with interstitial macrophage infiltration and cell proliferation in both the tubular and interstitial compartments, supporting the role of OPN in macrophage recruitment and the subsequent proliferation and regeneration of tubulointerstitial cells in acute rejection.

ACKNOWLEDGMENTS

This work was supported in part by a Health and Labor Science Research Grants for Research on Specific Diseases from the Ministry of Health, Labor and Welfare (14164701, to F. Gejyo) and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (16390242, to I. Narita). A part of this work has been presented at the 18th Niigata Symposium of Nephrology (Niigata, Japan, 2003). We thank Dr. Kota Takahashi and Dr. Kazuhide Saito from the Department of Urology, Niigata University for their kind support.

Reprint requests to Bassam Alchi, M.D., Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medicine and Dental Sciences, 1–757 Asahimachi-dori, Niigata 951–8510, Japan. E-mail: bassamalchi@hotmail.com

REFERENCES

1. Denhardt DT, Guo X: Osteopontin: A protein with diverse functions. *FASEB J* 7:1475–1482, 1993
2. Liaw L, Almeida M, Hart CE, *et al*: Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. *Circ Res* 74:214–224, 1994
3. Scatena M, Almeida M, Chaisson ML, *et al*: NF-kappaB mediates alphavbeta3 integrin-induced endothelial cell survival. *J Cell Biol* 141:1083–1093, 1998

4. Hudkins KL GC, Cui Y, Couser WG, *et al*: Osteopontin expression in fetal and mature human kidney. *J Am Soc Nephrol* 10:444–457, 1999
5. Madsen KM, Zhang L, Abu Shamat AR, *et al*: Ultrastructural localization of osteopontin in the kidney: induction by lipopolysaccharide. *J Am Soc Nephrol* 8:1043–1053, 1997
6. Xie Y, Sakatsume M, Nishi S, *et al*: Expression, roles, receptors, and regulation of osteopontin in the kidney. *Kidney Int* 60:1645–1657, 2001
7. Giachelli CM, Lombardi D, Johnson RJ, *et al*: Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo. *Am J Pathol* 152:353–358, 1998
8. Pichler R, Giachelli CM, Lombardi D, *et al*: Tubulointerstitial disease in glomerulonephritis. Potential role of osteopontin (uropontin). *Am J Pathol* 144:915–926, 1994
9. Panzer U, Thaiss F, Zahner G, *et al*: Monocyte chemoattractant protein-1 and osteopontin differentially regulate monocytes recruitment in experimental glomerulonephritis. *Kidney Int* 59:1762–1769, 2001
10. Okada H MK, Kalluri R, Takenaka T, *et al*: Osteopontin expressed by renal tubular epithelium mediates interstitial monocyte infiltration in rats. *Am J Physiol Renal Physiol* 278:110–121, 2000
11. Yu XQ, Wu LL, Huang XR, *et al*: Osteopontin expression in progressive renal injury in remnant kidney: Role of angiotensin II. *Kidney Int* 58:1469–1480, 2000
12. Kelly DJ, Wilkinson-Berka JL, Ricardo SD, *et al*: Progression of tubulointerstitial injury by osteopontin-induced macrophage recruitment in advanced diabetic nephropathy of transgenic (mRen-2)27 rats. *Nephrol Dial Transplant* 17:985–991, 2002
13. Yu XQ, Nikolic-Paterson DJ, Mu W, *et al*: A functional role for osteopontin in experimental crescentic glomerulonephritis in the rat. *Proc Assoc Am Physicians* 110:50–64, 1998
14. Hudkins KL GC, Ettner F, Couser WG, *et al*: Osteopontin expression in human crescentic glomerulonephritis. *Kidney Int* 57:105–116, 2000
15. Mezzano SA, Droguett MA, Burgos ME, *et al*: Overexpression of chemokines, fibrogenic cytokines, and myofibroblasts in human membranous nephropathy. *Kidney Int* 57:147–158, 2000
16. Okada H, Moriwaki K, Konishi K, *et al*: Tubular osteopontin expression in human glomerulonephritis and renal vasculitis. *Am J Kidney Dis* 36:498–506, 2000
17. Ophascharoensuk V, Giachelli CM, Gordon K, *et al*: Obstructive uropathy in the mouse: role of osteopontin in interstitial fibrosis and apoptosis. *Kidney Int* 56:571–580, 1999
18. Xie Y, Nishi S, Iguchi S, *et al*: Expression of osteopontin in gentamicin-induced acute tubular necrosis and its recovery process. *Kidney Int* 59:959–974, 2001
19. Verstrepen WA, Persy VP, Verhulst A, *et al*: Renal osteopontin protein and mRNA upregulation during acute nephrotoxicity in the rat. *Nephrol Dial Transplant* 16:712–724, 2001
20. Padanilam BJ, Martin DR, Hammerman MR: Insulin-like growth factor I-enhanced renal expression of osteopontin after acute ischemic injury in rats. *Endocrinology* 137:2133–2140, 1996
21. Persy VP, Verstrepen WA, Ysebaert DK, *et al*: Differences in osteopontin up-regulation between proximal and distal tubules after renal ischemia/reperfusion. *Kidney Int* 56:601–611, 1999
22. Lewington AJ, Padanilam BJ, Martin DR, Hammerman MR: Expression of CD44 in kidney after acute ischemic injury in rats. *Am J Physiol Regul Integr Comp Physiol* 278:247–254, 2000
23. Kon S, Maeda M, Segawa T, *et al*: Antibodies to different peptides in osteopontin reveal complexities in the various secreted forms. *J Cell Biochem* 77:487–498, 2000
24. Hall PA, Greenwood RA, d'Ardenne AJ, Levison DA: In situ demonstration of renal tubular regeneration using the monoclonal antibody Ki67. *Nephron* 49:122–125, 1988
25. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493–501, 1992
26. Nitta H, Kishimoto J, Grogan TM: Application of automated mRNA in situ hybridization for formalin-fixed, paraffin-embedded mouse skin sections: Effects of heat and enzyme pretreatment on mRNA signal detection. *Appl Immunohistochem Mol Morphol* 11:183–187, 2003
27. Hudkins KL, Le QC, Segerer S, *et al*: Osteopontin expression in human cyclosporine toxicity. *Kidney Int* 60:635–640, 2001
28. Racusen LC, Solez K, Colvin RB, *et al*: The Banff 97 working classification of renal allograft pathology. *Kidney Int* 55:713–723, 1999
29. Kleinman JG, Worcester EM, Beshensky AM, *et al*: Upregulation of osteopontin expression by ischemia in rat kidney. *Ann NY Acad Sci* 760:321–323, 1995
30. Nagasaki T, Ishimura E, Shioi A, *et al*: Osteopontin gene expression and protein synthesis in cultured rat mesangial cells. *Biochem Biophys Res Commun* 233:81–85, 1997
31. Yu XQ FJ, Nikolic-Paterson DJ, Yang N, *et al*: IL-1 up-regulates osteopontin expression in experimental crescentic glomerulonephritis in the rat. *Am J Pathol* 154:833–841, 1999
32. Kirk AD, Bollinger RR, Finn OJ: Rapid, comprehensive analysis of human cytokine mRNA and its application to the study of acute renal allograft rejection. *Hum Immunol* 43:113–128, 1995
33. Al-Lamki RS, Wang J, Skepper JN, *et al*: Expression of tumor necrosis factor receptors in normal kidney and rejecting renal transplants. *Lab Invest* 81:1503–1515, 2001
34. Noronha IL, Eberlein-Gonska M, Hartley B, *et al*: In situ expression of tumor necrosis factor-alpha, interferon-gamma, and interleukin-2 receptors in renal allograft biopsies. *Transplantation* 54:1017–1024, 1992
35. Nambi P, Gellai M, Wu HL, Prabhakar U: Upregulation of osteopontin in ischemia-induced renal failure in rats: a role for ET-1? *Biochem Biophys Res Commun* 241:212–214, 1997
36. Wagrowska-Danilewicz M, Danilewicz M: Immunohistochemical study of endothelin-1 (ET-1) in human acute renal allograft rejection. *Pol J Pathol* 54:95–100, 2003
37. Chareandee C, Herman WH, Hricik DE, Simonson MS: Elevated endothelin-1 in tubular epithelium is associated with renal allograft rejection. *Am J Kidney Dis* 36:541–549, 2000
38. Divate SA: Acute renal allograft rejection: progress in understanding cellular and molecular mechanisms. *J Postgrad Med* 46:293–296, 2000
39. Denhardt DT, Noda M, O'Regan AW, *et al*: Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J Clin Invest* 107:1055–1061, 2001
40. Ashkar S, Weber GF, Panoutsakopoulou V, *et al*: Eta-1 (osteopontin): An early component of type-1 (cell-mediated) immunity. *Science* 287:860–864, 2000
41. Wever PC, Aten J, Rentenaar RJ, *et al*: Apoptotic tubular cell death during acute renal allograft rejection. *Clin Nephrol* 49:28–34, 1998
42. Ueda N, Kaushal GP, Shah SV: Apoptotic mechanisms in acute renal failure. *Am J Med* 108:403–415, 2000
43. Persy VP VA, Ysebaert DK, De Greef KE, De Broe ME: Reduced postischemic macrophage infiltration and interstitial fibrosis in osteopontin knockout mice. *Kidney Int* 63:543–553, 2003
44. Abbate M, Zoja C, Corna D, *et al*: In progressive nephropathies, overload of tubular cells with filtered proteins translates glomerular permeability dysfunction into cellular signals of interstitial inflammation. *J Am Soc Nephrol* 9:1213–1224, 1998
45. Kaimori JY, Takenaka M, Nagasawa Y, *et al*: Quantitative analyses of osteopontin mRNA expression in human proximal tubules isolated from renal biopsy tissue sections of minimal change nephrotic syndrome and IgA glomerulonephropathy patients. *Am J Kidney Dis* 39:948–957, 2002

JASN Express. Published on March 2, 2005 as doi: 10.1681/ASN.2004050367

Intrarenal Injection of Bone Marrow–Derived Angiogenic Cells Reduces Endothelial Injury and Mesangial Cell Activation in Experimental Glomerulonephritis

Hideki Uchimura,* Takeshi Marumo,†‡ Osamu Takase,* Hiroshi Kawachi,§ Fujio Shimizu,§ Matsuhiko Hayashi,* Takao Saruta,* Keiichi Hishikawa,†‡ and Toshiro Fujita†‡

**Department of Internal Medicine, Keio University School of Medicine, Tokyo; †Department of Clinical Renal Regeneration and ‡Division of Nephrology and Endocrinology, Department of Internal Medicine, University of Tokyo, Tokyo; and §Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*

Loss of glomerular endothelial cells has been suggested to contribute to the progression of glomerular injury. Although therapeutic angiogenesis induced by administration of bone marrow–derived endothelial progenitor cells has been observed in disease models of endothelial injury, the effects on renal disease have not been clarified. Whether administration of culture-modified bone marrow mononuclear cells would mitigate the glomerular endothelial injury in anti-Thy1.1 nephritis was investigated. After cultivation under conditions that promote endothelial progenitor cell growth, bone marrow mononuclear cells were labeled with CM-DiI, a fluorescence marker, and injected into the left renal artery of Lewis rats with anti-Thy1.1 glomerulonephritis. The decrease in glomerular endothelial cells was significantly attenuated in the left kidney, as compared with the right, in nephritic rats that received the cell infusion. Glomerular injury score, the area positive for mesangial α-smooth muscle actin, and infiltration of macrophages were significantly decreased in the left kidney. CM-DiI–positive cells were distributed in glomeruli of the left kidney but not in those of the right kidney. Among CM-DiI–labeled cells incorporated into glomeruli, 16.5 ± 1.2% of cells were stained with an endothelial marker, rat endothelial cell antigen-1. Culture-modified mononuclear cells secreted 281.2 ± 85.0 pg of vascular endothelial growth factor per 10^5 cells per day. In conclusion, intra-arterial administration of culture-modified bone marrow mononuclear cells reduced endothelial injury and mesangial activation in anti-Thy1.1 glomerulonephritis. Incorporation into the glomerular endothelial lining and production of angiogenic factor(s) are likely to contribute to the protective effects of culture-modified mononuclear cells against glomerular injury.

J Am Soc Nephrol 16: ???-???, 2005. doi: 10.1681/ASN.2004050367

Endothelial injury in the kidney has been shown to correlate with the progression of glomerular and tubulointerstitial damage (1). The observations that administration of vascular endothelial growth factor enhances capillary repair and improves renal function in several experimental models (2–4) suggest that therapy that is aimed at stimulating capillary repair may slow the progression of renal diseases.

Recent evidence indicates that bone marrow–derived endothelial progenitor cells contribute to tissue vascularization after ischemic events such as hindlimb arterial occlusion and myocardial infarction (5). On the basis of their roles in the response to injury, bone marrow–derived endothelial progenitor cells have been isolated and transplanted for therapeutic applications, and enhancement of re-endothelialization and restoration of organ function have been observed in cardiac and hindlimb ischemia and carotid artery injury (5,6).

A recent report showed endothelial progenitor cells mobilized from the bone marrow also to contribute to glomerular endothelial repair in anti-Thy1.1 glomerulonephritis (7), a model characterized by mesangial and glomerular endothelial injury (8). However, the therapeutic potential of exogenously administered bone marrow–derived cells, including endothelial progenitor cells, for endothelial injury in the kidney has not been clarified. We, therefore, investigated whether administration of culture-modified bone marrow mononuclear cells (CMMC), which had been prepared using a method designed to obtain endothelial progenitor cells, were capable of attenuating endothelial damage and mesangial activation in anti-Thy1.1 glomerulonephritis. Because recent evidence suggests that the production of angiogenic factors also contributes to re-endothelialization by exogenously administered endothelial progenitor cells (9–11), we further investigated whether CMMC secrete vascular endothelial growth factor (VEGF), an angiogenic factor that re-

Received May 10, 2004. Accepted January 1, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

Address correspondence to: Dr. Takeshi Marumo, Department of Clinical Renal Regeneration, Division of Nephrology and Endocrinology, Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyouku, 113-8655 Tokyo, Japan. Phone: 81-3-3815-5411, ext. 35717; Fax: 81-3-5800-9738; E-mail: tmarumo-npr@umin.ac.jp

ISSN: 1046-6673/1604-0001

portedly enhances glomerular endothelial repair in experimental glomerulonephritis (3).

Materials and Methods

Cell Culture

CMMC were isolated and cultured according to a previously described method used for isolation of endothelial precursor cells from bone marrow (12,13). In brief, bone marrow mononuclear cells were isolated from the femurs and tibias of 6-wk-old male Lewis rats (CLEA Japan, Tokyo, Japan) by density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences). The obtained cells were cultured in DMEM supplemented with 10% FBS, 50 μg/ml heparin, 10 ng/ml recombinant human VEGF (R&D Systems, Minneapolis, MN), 5 ng/ml fibroblast growth factor-2 (R&D Systems), 100 μg/ml streptomycin, and 500 μg/ml penicillin, on fibronectin-coated dishes.

Adherent cells after 5 or 6 d of culture were infused into nephritic rats, as CMMC. The cells were labeled with a fluorescence marker, CM-DiI (Molecular Probes, Eugene, OR), by incubation in PBS that contained 3 μg/ml CM-DiI for 5 min at 37°C and 15 min at 4°C. After being detached from the dishes by addition of 3 mM EDTA solution and a minimal amount of trypsin, cells were passed through a cell strainer (Falcon) and injected into the nephritic rats.

For the determination of endothelial lineage characteristics (5,14), CMMC that were cultured for 6 d were evaluated for the uptake of acetylated LDL, the binding of Bandeiraea simplicifolia lectin (BS-1), and VEGF receptor 2 (VEGF-R2) expression. After incubation with 10 μg/ml DiI-labeled acetylated LDL (Biogenesis Ltd., Poole, UK) for 1 h, cells were fixed in 2% paraformaldehyde, stained with 100 μg/ml FITC-labeled BS-1 (Sigma, St. Louis, MO), and examined by fluorescence microscopy. For measuring VEGF-R2 expression, CMMC were fixed in ice-cold methanol and stained with a rabbit polyclonal antibody against VEGF-R2 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:200) as the primary antibody. After incubation with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) as the secondary antibody at a dilution of 1:200, the cells were analyzed by confocal laser-scanning microscopy (Leica DMIR/E2 TCS SL; Leica GmbH, Wetzlar, Germany). Negative controls included nonimmune rabbit IgG (Cedarlane Laboratories, ON, Canada) instead of the primary antibody.

Experimental Protocol

Anti-Thy1.1 glomerulonephritis was induced in 6-wk-old female Lewis rats by injection of 250 μg/100 g body wt monoclonal antibody 1-22-3 (15) into the tail vein. Control rats received a saline injection instead of the antibody ($n = 6$). One day after the injection of monoclonal antibody 1-22-3, cultured CMMC ($1.0 \pm 0.2 \times 10^6$) in 1 ml of PBS were injected into the left renal artery ($n = 6$). Four nephritic rats were monitored in parallel without CMMC injection for analysis of renal function. Twenty-four-hour urine collections were performed on day 5 after the antibody injection. Urinary protein concentrations were determined with a pirogarol-red method kit (Wako Chemistry Co., Ltd., Tokyo, Japan). Seven days after the induction of glomerulonephritis, the kidneys were perfused with saline and removed from anesthetized animals. Blood was collected when the rats were killed for determination of serum creatinine concentrations. For morphologic analysis and immunohistochemical staining for α-smooth muscle actin (α-SMA) and ED-1, coronal sections of renal tissue were immersion-fixed in 10% neutral-buffered formalin and embedded in paraffin. For detection of CM-DiI–labeled cells and for immunohistochemical staining for rat endothelial cell antigen-1 (RECA-1) and OX-7, coronal sections of renal tissue were immersion-fixed in 4% buffered paraformaldehyde for 12 h; washed with 10, 15, and 20% sucrose in PBS for 4 h each time; embedded in OCT compound; and snap-frozen in liquid nitrogen. Animal care and treatment complied with the standards described in the Guidelines for the Care and Use of Laboratory Animals of Keio University School of Medicine.

Renal Morphology

For assessing morphologic changes, 4-μm paraffin sections were stained with periodic acid-Schiff (PAS) reagent and hematoxylin and examined by normal light microscopy. Semiquantitative morphologic studies of glomerular lesions were performed on 25 glomeruli randomly selected from each specimen by one of the authors, each author being unaware of the origins of the slides. The degree of glomerular mesangial cell and/or matrix expansion was graded according to the percentage of glomerular involvement as described previously (16) using the following criteria: 0 = normal glomerular cellularity with no significant mesangial expansion; 1+ = mesangial cell and/or matrix expansion involving <25% of the glomerular area; 2+ = mesangial cell and/or matrix expansion involving 26 to 50% of the glomerular area; 3+ = mesangial cell and/or matrix expansion involving 51 to 75% of the glomerular area; and 4+ = diffuse (>75% of the glomerular area) mesangial cell and/or matrix expansion or a glomerulus showing basement membrane disruption and/or mesangiolysis.

Immunohistochemical Staining and Quantitative Analysis

Glomerular endothelial injury was evaluated by immunofluorescent staining for RECA-1 and analysis of glomerular capillary density, according to a previously described method (17), with minor modification. Frozen sections, 6 μm thick, were stained with mouse anti–RECA-1 monoclonal antibody (Serotec Ltd., Oxford, UK; dilution 1:20) as the primary antibody. Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) was used as the secondary antibody at a dilution of 1:200. Sections then were analyzed by confocal laser-scanning microscopy (Leica DMIR/E2 TCS SL). Glomerular capillaries, identified by positive staining for RECA-1, were counted, and the glomerular tuft area was calculated by image analysis using Adobe Photoshop 7.0 and Scion Image. After evaluation of 20 glomeruli from each kidney in a blinded manner, the glomerular capillary density was normalized to the number of capillaries per square millimeter.

For the detection of α-SMA, deparaffinized sections, 4 μm thick, were stained with mouse anti–α-SMA monoclonal antibody (1A4; DakoCytomation Co. Ltd.; 1:50 dilution), according to a previously described method (18), with minor modification. Immobilized antibody was detected by the biotin-avidin-immunoperoxidase technique using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3–3′-diaminobenzidine as the chromogen. Sections then were counterstained with Mayer's hematoxylin and examined by light microscopy. The ratio of the glomerular α-SMA–positive area to the glomerular tuft area was calculated by image analysis using Nikon ACT-1 version 220, Adobe Photoshop 7.0, and Scion Image. The mean value of the α-SMA–positive area was calculated by evaluating 20 glomeruli from each kidney in a blinded manner.

Glomerular infiltration of macrophages was determined by counting the number of ED-1–positive cells according to a previously described method (19), with minor modification. Deparaffinized sections, 4 μm thick, were stained with mouse anti-rat macrophage monoclonal antibody, clone ED-1 (Chemicon International, Temecula, CA) at a dilution of 1:100. Immobilized antibody was detected by the biotin-avidin-immunoperoxidase technique using an LSAB kit (DakoCytomation Co. Ltd.) and 3–3′-diaminobenzidine as the chromogen. Sections then were counterstained with Mayer's hematoxylin and examined by light microscopy. The number of ED-1–positive cells in glomerular cross-sec-

tions was counted in a blinded manner in at least 30 glomeruli for each section, and mean values per kidney were calculated.

Incorporation of CMMC was investigated using fluorescence microscopy. The rate of glomeruli positive for CM-DiI–labeled cells was calculated by dividing the number of glomeruli that contained CM-DiI–labeled cells by the total number of glomeruli examined. The number of CM-DiI–labeled cells per glomerulus, in glomeruli with these cells, was also determined. At least 50 glomeruli per kidney were examined, and the mean number was obtained from six kidneys.

For evaluating the possibility of differentiation of CMMC into mesangial cells, frozen sections, 6 μm thick, were stained with mouse anti-CD90 monoclonal antibody, clone MRC OX-7 (Serotec Ltd; dilution 1:800) followed by Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) at a dilution of 1:1000. For the determination of possible differentiation of CMMC into macrophages, deparaffinized sections, 4 μm thick, were stained with anti-rat macrophage antibody, clone ED-1. The sections then were incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) at a dilution of 1:200. After staining of nuclei with TO-PRO-3 (Molecular Probes), sections were analyzed by confocal laser-scanning microscopy. At least 20 DiI-positive cells per kidney were evaluated for staining with anti–RECA-1 antibody, OX-7, or ED-1. The mean positive rate was obtained by examining five rats. Negative controls for immunohistochemical staining included substitution of the primary antibody with an irrelevant mouse IgG or PBS.

ELISA Assay

Culture supernatants of freshly obtained bone marrow mononuclear cells were collected after incubation in the presence of endothelial growth factors for 4 d. After removal of the nonadherent cells, CMMC were further incubated, and the culture supernatants from days 6 to 9 then were collected. Rat VEGF content in the culture supernatants was determined with a murine VEGF ELISA kit (R&D Systems), using rat VEGF (R&D Systems) as a standard. Cross-reactivity to human VEGF was negligible under our assay conditions. VEGF production was expressed as the amount of VEGF produced per day divided by the cell number at the start of incubation. Cell number on day 6 was obtained by counting trypsinized cells that had been treated in a manner identical to that of cells that were subjected to further incubation.

Statistical Analyses

All data are expressed as means ± SEM. Multiple parametric comparisons were performed using ANOVA, followed by Fisher protected least significant difference test. Comparisons between two groups were performed by t test. $P < 0.05$ was considered statistically significant.

Results

Characterization of CMMC

After a 6-d culture in the presence of endothelial growth factors, >90% adherent bone marrow cells had incorporated acetylated LDL and stained positive for BS-1 lectin (Figure 1). Endothelial progenitor cells from various sources have been shown to have these characteristics (5,13,14,20,21). In contrast, before culture, <5% of bone marrow mononuclear cells showed incorporation of acetylated LDL (data not shown). CMMC also expressed VEGF-R2, a marker for endothelial-lineage cells (5).

Attenuation of Glomerular Endothelial Cell Injury by Administration of CMMC

The effect of CMMC on glomerular endothelial injury in anti-Thy1.1 nephritis was investigated by injecting 1.0 ± 0.2 × 10^6 CM-DiI–labeled cells into the left renal arteries of rats (n = 6) 1 d after anti-Thy1.1 antibody injection. In agreement with previously published observations (17,22,23), glomerular RECA-1–positive capillary density was decreased at 7 d after anti-Thy1.1 antibody injection (Figure 2). Compared with the right kidney, the decrease in glomerular capillary density was significantly attenuated in the left kidneys of nephritic rats that had received CMMC infusions into the left renal artery.

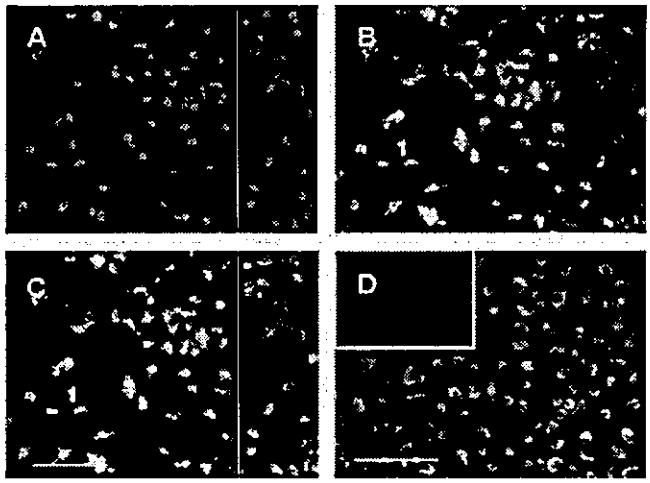

Figure 1. Uptake of acetylated LDL (A), binding of Bandeiraea simplicifolia lectin (BS-1 lectin; B), and expression of vascular endothelia growth factor receptor 2 (VEGF-R2; D) by culture-modified bone marrow mononuclear cells. Bone marrow mononuclear cells were cultivated in the presence of endothelial growth factors for 6 d and evaluated for the uptake of acetylated LDL labeled with CM-DiI (A; red), binding of BS-1 lectin labeled with FITC (B; green), and VEGF-R2 expression (D; small frame: isotype control). The overlay image of A and B indicates double positive cells in yellow (C). Bars = 100 μm.

Attenuation of Mesangial Activation by Administration of CMMC

Glomerular architecture evaluated by PAS staining 7 d after anti-Thy1.1 antibody injection showed mesangial hypercellularity, focal mesangiolysis, and marked expansion of the extracellular matrix. Glomeruli in the left kidney, into which CMMC had been injected 1 d after the injection of anti-Thy1.1 antibody, showed less expansion of the mesangial area than those in the right kidney, as judged from semiquantitative histologic analysis (Figure 3). The increase in expression of glomerular α-SMA, a marker of activated mesangial cells (24), was also significantly reduced by administration of CMMC. Glomerular infiltration of macrophages, as assessed by counting the number of glomerular ED-1–positive cells, induced in nephritic rats was also attenuated by injection of CMMC, as shown in Figure 4.

Incorporation of CMMC into Glomeruli and Expression of RECA-1

Incorporation of CMMC was investigated by fluorescence microscopic examination of the left kidney obtained 6 d after injection of the cells into rats with anti-Thy1.1 nephritis (Figure

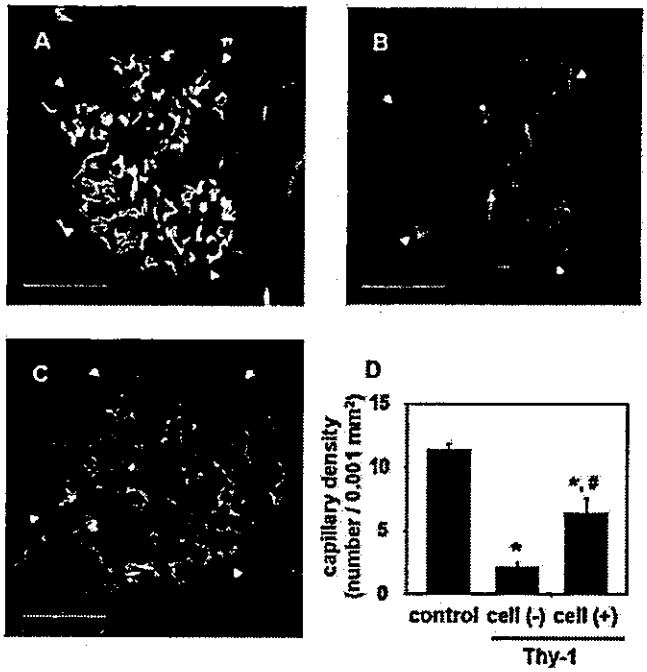

Figure 2. Inhibition of glomerular endothelial injury by administration of culture-modified bone marrow mononuclear cells (CMMC) to nephritic rats. Representative photomicrographs of glomerular immunostaining for rat endothelial cell antigen-1 (RECA-1) in kidney sections obtained from normal rats (A) and right (B) and left (C) kidney sections obtained from nephritic rats in which CMMC had been injected into the left renal artery. The number of RECA-1–positive glomerular capillaries from six kidneys in each group was counted and divided by the glomerular tuft area (D). Control, normal rat kidneys; cell (−), right kidneys from nephritic rats that had been given CMMC injections into the left renal artery; cell (+), left kidneys from nephritic rats with CMMC injected into the left renal artery. The data shown in D are means ± SEM. $*P < 0.05$ *versus* control group; $\#P < 0.05$ *versus* cell (−) group. Arrowheads indicate the margins of the glomerular tuft. Bars = 40 μm.

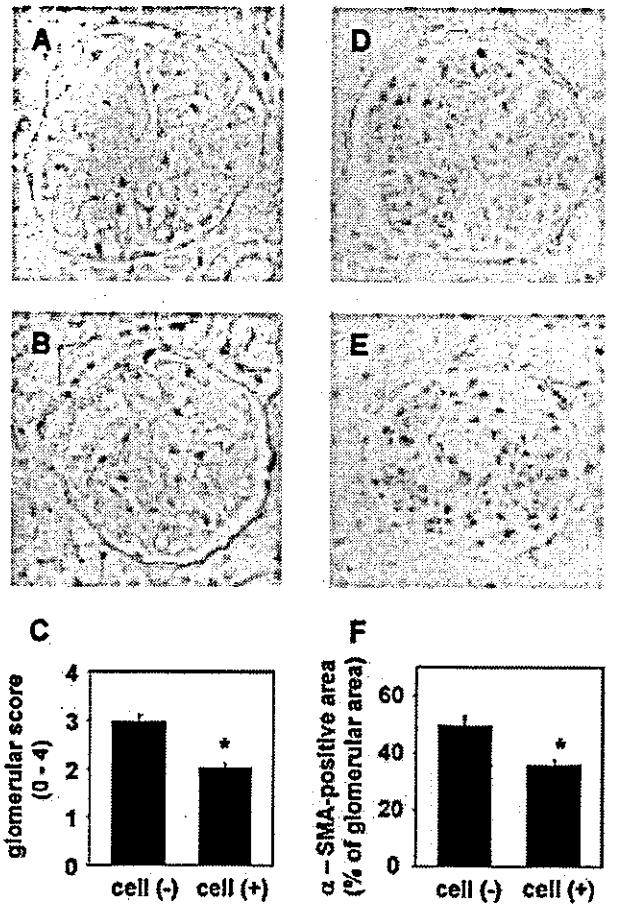

Figure 3. Inhibition of glomerular mesangial cell and/or matrix expansion and expression of glomerular α-smooth muscle actin (α-SMA) by administration of CMMC to nephritic rats. Representative photomicrographs of periodic acid-Schiff staining (A and B) and immunohistochemical staining for glomerular α-SMA (D and E) of right (A and D) and left (B and E) kidney sections obtained from nephritic rats in which CMMC had been injected into the left renal artery. Glomerular score (C) and α-SMA–positive areas per glomerular tuft area (F) were obtained from six rats. The data shown in C and F are means ± SEM. $*P < 0.05$ *versus* cell (−) group. Groups are the same as in Figure 2. Magnification, ×400

5). Labeled cells were identified in 14.0 ± 4.2% of glomeruli and only occasionally in the interstitial area. The average number of CM-DiI–labeled cells incorporated into glomeruli that were positive for these cells was 1.67 ± 0.15. No labeled cells were observed in right kidneys that were obtained from the same rats (data not shown). Among the CM-DiI–labeled cells that were incorporated into glomeruli, 16.5 ± 1.2% of cells were stained with RECA-1. As shown in Figure 6, A through C, some RECA-1–positive CMMC participated in capillary formation together with the adjacent endothelial cells. In addition to the CMMC showing strong RECA-1 staining, there were some CMMC that stained only weakly with RECA-1 (Figure 6, D through F). The latter cells, however, seemed to be forming connections with adjacent endothelial cells. A few labeled cells that were positive for RECA-1 were also observed in the interstitial space (data not shown).

To evaluate whether CMMC may have differentiated into glomerular components other than endothelial cells, we stained the sections with OX-7 and ED-1, markers of mesangial cells and macrophages, respectively. Although CM-DiI–positive cells were negative for OX-7 (Figure 6, G through I), 21.8 ± 7.0% of cells were stained with ED-1 (Figure 6, J through L). The CM-DiI signals in ED-1–positive cells, however, were mostly granular in character and not homogeneously distributed, as in labeled CMMC just before injection.

Proteinuria and Serum Creatinine Concentrations

Proteinuria was mildly increased in Lewis rats that received anti-Thy1.1 antibody as compared with control rats and was not significantly altered by CMMC injection, although a tendency for reduction was observed (normal control rats, 1.2 ± 0.6 mg/24 h; nephritic rats, 14.4 ± 3.9 mg/24 h; nephritic rats

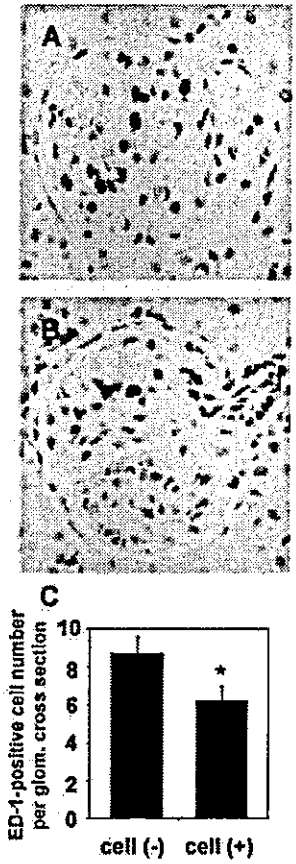

Figure 4. Inhibition of glomerular infiltration of macrophages by administration of CMMC to nephritic rats. Representative photomicrographs of immunohistochemical staining for glomerular ED-1 of right (A) and left (B) kidney sections obtained from nephritic rats in which CMMC had been injected into the left renal artery. The mean number of glomerular ED-1–positive cells per glomerular cross section (C) was obtained from six rats. The data shown in C are means ± SEM. $^*P < 0.05$ *versus* cell (−) group. Groups are the same as in Figure 2. Magnification, ×400

treated with CMMC, 12.9 ± 2.1 mg/24 h). Serum creatinine concentrations were not significantly changed on day 7 after the injection of anti-Thy1.1 antibody under our experimental conditions (normal control rats, 0.30 ± 0.02 mg/dl; nephritic rats, 0.28 ± 0.04 mg/dl).

VEGF Production by CMMC

To explore the possible contribution of angiogenic factors produced by CMMC to their renoprotective effects, we investigated whether CMMC secrete VEGF *in vitro*. CMMC did produce VEGF, and the amount of VEGF secreted was markedly increased as compared with that produced by freshly derived bone marrow mononuclear cells (Figure 7), suggesting that cells with angiogenic potential had been selectively grown under our culture conditions.

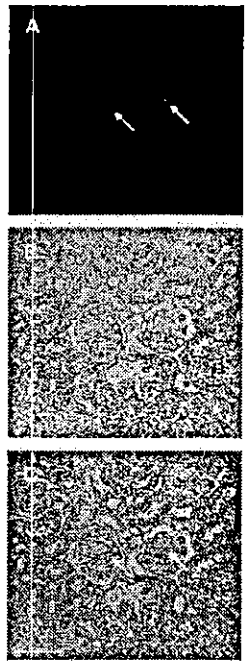

Figure 5. Glomerular localization of CMMC incorporated into the kidneys of nephritic rats. Representative photomicrographs of fluorescence (A), phase contrast (B), and overlay images (C) of renal cortex obtained from kidneys that were injected with CMMC. One day after the induction of anti-Thy1.1 nephritis by intravenous injection of monoclonal antibody 1-22-3, CMMC were labeled with CM-DiI (red) and injected into the left renal artery. Sections of the left kidney obtained 7 d after the induction of nephritis were examined by confocal laser-scanning microscopy. Labeled cells in glomeruli of left kidney are visualized in red by fluorescence microscopy (A). Arrows in A and C indicate the localization of injected cells. Bar = 100 μm.

Discussion

This study demonstrated intrarenal injection of CMMC to ameliorate glomerular endothelial injury in anti-Thy1.1 glomerulonephritis. Loss of glomerular endothelial cells has been observed in several models of progressive renal disease and correlates with the development of glomerulosclerosis (1,17,25). Because an imbalance between pro-angiogenic and anti-angiogenic factors in the intrarenal microenvironment has been suggested to play a role in endothelial loss (1), administration of angiogenic factors was expected to have a protective effect against progressive renal diseases. Indeed, VEGF and hepatocyte growth factor have been shown to accelerate repair of glomerular endothelial cells and stabilize renal function when injected into models of glomerular injury (2–4,26). Our data demonstrate that injection of CMMC is another approach to maintaining glomerular endothelial cells in glomerulonephritis.

Protective effects of CMMC were also observed in the mesangial area. The observation that glomerular expression of α-SMA was inhibited by injection of CMMC indicates that activation of mesangial cells was attenuated. In accordance with the results of α-SMA staining, expansion of the mesangial

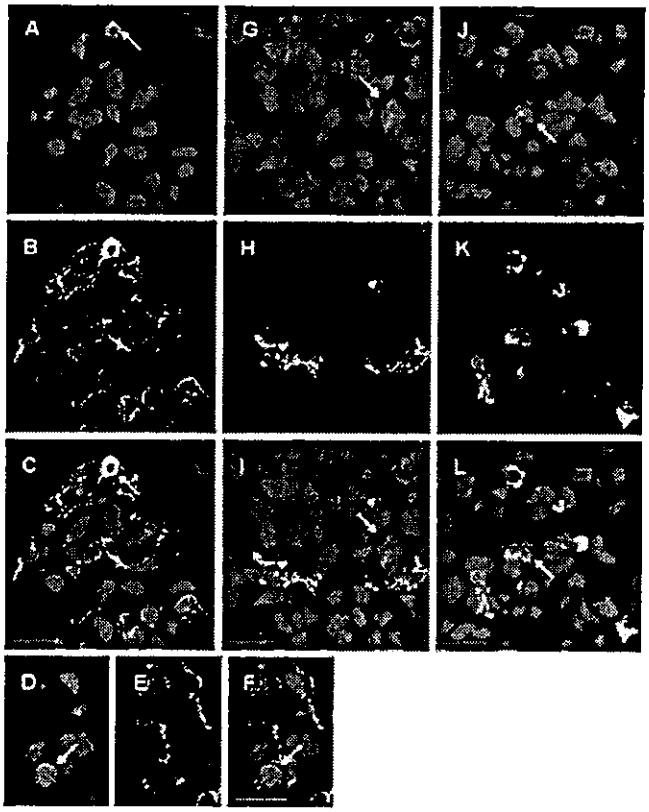

Figure 6. Immunohistochemical staining of glomeruli with CM-DiI–positive cells for markers of endothelial and mesangial cells and macrophages. Representative photomicrographs of fluorescence images of CM-DiI signals in glomeruli (A, D, G, and J); immunohistochemical staining for RECA-1 (B and E), OX-7 (H), and ED-1 (K); and overlay images (C, F, I, and L) of renal cortex obtained from kidneys that were injected with CMMC. One day after the induction of anti-Thy1.1 nephritis by intravenous injection of monoclonal antibody 1-22-3, CMMC were labeled with CM-DiI (red) and injected into the left renal artery. Sections of the left kidney obtained 7 d after the induction of nephritis were examined by confocal laser-scanning microscopy. RECA-1, OX-7, and ED-1 stains identify endothelial and mesangial cells and macrophages (green), respectively. The overlay images (C and F) indicate positive RECA-1 staining of the injected cells. Whereas the overlay image in I indicates negative OX-7 staining of the injected cells, L indicates a CM-DiI-positive cell stained for ED-1. Arrows indicate CM-DiI-positive cells in glomeruli. Nuclei (blue) are stained with TO-PRO-3 in A, C, D, F, G, I, J, and L. Bars = 10 μm.

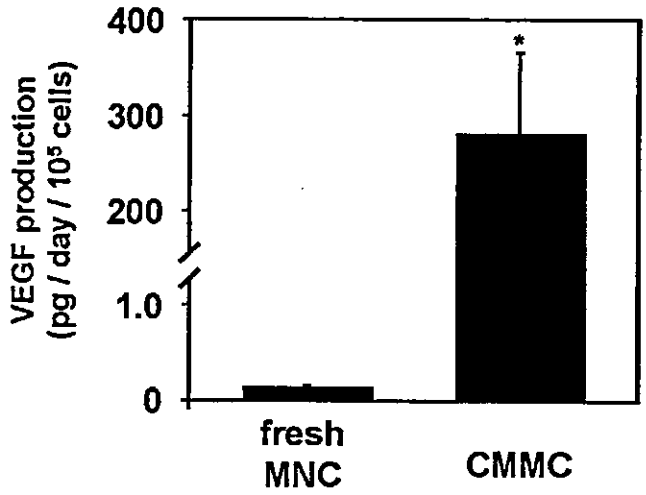

Figure 7. Production of VEGF by freshly isolated bone marrow mononuclear cells (fresh MNC) and CMMC was determined by ELISA. The data shown are means ± SEM ($n = 3$). *$P < 0.05$ *versus* fresh MNC.

area, as assessed by PAS staining, was also attenuated by administration of CMMC. Preservation of the endothelial population by cell therapy is likely to contribute to attenuating mesangial cell activation, possibly through preventing direct exposure of mesangial cells to the microcirculation and via the production of anti-inflammatory factors. In addition, direct effects of CMMC on mesangial cells may be involved. In addition to endothelial injury and mesangial activation, glomerular infiltration of macrophages was also attenuated by CMMC treatment. This finding is in accordance with previous reports demonstrating the protective effects of angiogenic factors against the progression of glomerulonephritis (3,26) and supports the notion that protecting glomerular endothelial cells may attenuate the inflammation in glomerulonephritis. Although we observed improvement in glomerular histologic changes, proteinuria was not significantly reduced by CMMC injection. Treatment of just one kidney with CMMC may not have been sufficient for a significant reduction in proteinuria.

CMMC had some of the characteristics of endothelial-lineage cells (5), including uptake of acetylated LDL, binding to BS-1 lectin, and expression of VEGF-R2. After administration to nephritic rats, CM-DiI–labeled CMMC were incorporated into the glomerular microvasculature and a significant number, although not all, cells stained positive for RECA-1, a mature endothelial cell marker. These observations suggest that some CMMC act as endothelial progenitor cells, although fusion of the injected CMMC with glomerular endothelial cells cannot be ruled out. The finding that CMMC stained for RECA-1 with different intensities, together with the observation that no CMMC before injection showed strong positive staining for RECA-1 (data not shown), suggests that CMMC 6 d after injection may be at various stages of maturation into RECA-1–positive endothelial cells in response to local cues in the injured glomeruli.

Glomerular capillary density in kidneys that were treated with CMMC was approximately three times higher than that in kidneys without cell infusion. This improvement cannot be explained solely by the direct incorporation of CMMC into the glomerular endothelial lining, considering the proportion of RECA-1–positive CMMC incorporated into glomeruli. In addition to differentiating into mature endothelial cells, exogenously administered endothelial progenitor cells, prepared by culturing bone marrow–derived mononuclear cells under conditions of endothelial differentiation, have been suggested to exert their angiogenic effects by producing angiogenic factors

(9–11). Recent evidence also suggests that the beneficial effects of hematopoietic stem cell therapy on cardiac function after ischemia may be due to the angiogenic activities of the injected cells rather than transdifferentiation, originally thought to be important (27,28). Because an *in vitro* experiment revealed that CMMC produced a significant amount of VEGF, VEGF secreted by CMMC in glomeruli is likely to stimulate endothelial growth in a paracrine manner and contribute to the prevention of endothelial cell loss. The amount of VEGF produced by CMMC (281.2 ± 85.0 pg/10^5 cells per d) was comparable to that reportedly produced by cells that were used for angiogenic cell therapy (40 to 260 pg/10^5 cells per d) (11,29). Local production of VEGF by CMMC incorporated in injured glomeruli may also induce recruitment of endogenous endothelial progenitor cells, because this angiogenic factor is known to promote mobilization of endothelial progenitor cells (5). Other angiogenic factors such as hepatocyte growth factor, the mRNA of which was detected by reverse transcription–PCR in CMMC (data not shown), may also be involved in the angiogenic activities of CMMC. The roles of these angiogenic factors, however, are still only speculative and remain to be clarified by experiments on specific inhibitory approaches.

Although VEGF has been shown to be effective for prevention and treatment of renal injury in various models, systemic administration of angiogenic factors may exacerbate pathologic angiogenesis, *e.g.*, diabetic retinopathy and tumors (1). Local infusion of angiogenic cells is expected to circumvent such detrimental effects by acting in the microenvironment where the cells are incorporated. By infusing CMMC into the left renal artery, selective delivery and incorporation of CMMC in the left but not the right kidney in the same animal was achieved. This finding suggests the potential of this therapy for selective modification of the local environment, although it remains to be determined whether such a selective delivery system would work even in the presence of pathologic angiogenesis.

The lack of CMMC staining with OX-7 indicates that CMMC did not contain progenitors of mesangial cells under our experimental conditions. Because some CM-DiI–positive cells stained positive for ED-1, these cells may have differentiated into macrophages. However, because the CM-DiI signals obtained were mostly granular, these signals probably represent, in large part, the debris of injected CMMC that did not survive and were phagocytosed by macrophages. In addition, because macrophages have been shown to induce glomerular matrix expansion in anti-Thy1.1 glomerulonephritis (30), the finding that CMMC attenuated expansion of the mesangial area (Figure 3) indicates that functionally active macrophages possibly derived from CMMC were, if present, minimal.

In conclusion, injection of bone marrow mononuclear cells, after culture under conditions that promote endothelial progenitor cell growth, into the renal artery attenuated glomerular endothelial injury and mesangial activation in anti-Thy1.1 glomerulonephritis. Injected cells exerted angiogenic effects in injured glomeruli probably by being incorporated into the glomerular endothelial lining and by producing angiogenic factor(s). Injecting bone marrow–derived angiogenic cells into the kidney may represent a novel therapeutic approach for glomerular injury associated with endothelial loss and mesangial activation.

Acknowledgments

This work was supported by Mochida Pharmaceutical Co. Ltd.; a Grant-in-Aid for Scientific Research for the Young Investigators B from the Japan Society for the Promotion of Science; and a Grant of "Research on Measures for Intractable Diseases" from the Ministry of Health, Labor and Welfare.

References

1. Kang DH, Kanellis J, Hugo C, Truong L, Anderson S, Kerjaschki D, Schreiner GF, Johnson RJ: Role of the microvascular endothelium in progressive renal disease. *J Am Soc Nephrol* 13: 806–816, 2002
2. Kang DH, Hughes J, Mazzali M, Schreiner GF, Johnson RJ: Impaired angiogenesis in the remnant kidney model: II. Vascular endothelial growth factor administration reduces renal fibrosis and stabilizes renal function. *J Am Soc Nephrol* 12: 1448–1457, 2001
3. Masuda Y, Shimizu A, Mori T, Ishiwata T, Kitamura H, Ohashi R, Ishizaki M, Asano G, Sugisaki Y, Yamanaka N: Vascular endothelial growth factor enhances glomerular capillary repair and accelerates resolution of experimentally induced glomerulonephritis. *Am J Pathol* 159: 599–608, 2001
4. Suga S, Kim YG, Joly A, Puchacz E, Kang DH, Jefferson JA, Abraham JA, Hughes J, Johnson RJ, Schreiner GF: Vascular endothelial growth factor (VEGF121) protects rats from renal infarction in thrombotic microangiopathy. *Kidney Int* 60: 1297–1308, 2001
5. Rafii S, Lyden D: Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med* 9: 702–712, 2003
6. Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M, Nickenig G: Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. *Circ Res* 93: e17–e24, 2003
7. Rookmaaker MB, Smits AM, Tolboom H, Van 't Wout K, Martens AC, Goldschmeding R, Joles JA, Van Zonneveld AJ, Grone HJ, Rabelink TJ, Verhaar MC: Bone-marrow-derived cells contribute to glomerular endothelial repair in experimental glomerulonephritis. *Am J Pathol* 163: 553–562, 2003
8. Kriz W, Hahnel B, Hosser H, Ostendorf T, Gaertner S, Kranzlin B, Gretz N, Shimizu F, Floege J: Pathways to recovery and loss of nephrons in anti-Thy-1 nephritis. *J Am Soc Nephrol* 14: 1904–1926, 2003
9. Gulati R, Jevremovic D, Peterson TE, Witt TA, Kleppe LS, Mueske CS, Lerman A, Vile RG, Simari RD: Autologous culture-modified mononuclear cells confer vascular protection after arterial injury. *Circulation* 108: 1520–1526, 2003
10. Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S: Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. *Circulation* 108: 2511–2516, 2003
11. Rehman J, Li J, Orschell CM, March KL: Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* 107: 1164–1169, 2003

12. Edelberg JM, Tang L, Hattori K, Lyden D, Rafii S: Young adult bone marrow-derived endothelial precursor cells restore aging-impaired cardiac angiogenic function. *Circ Res* 90: E89–E93, 2002
13. Griese DP, Achatz S, Batzlsperger CA, Strauch UG, Grumbeck B, Weil J, Riegger GA: Vascular gene delivery of anticoagulants by transplantation of retrovirally-transduced endothelial progenitor cells. *Cardiovasc Res* 58: 469–477, 2003
14. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM: HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest* 108: 391–397, 2001
15. Kawachi H, Orikasa M, Matsui K, Iwanaga T, Toyabe S, Oite T, Shimizu F: Epitope-specific induction of mesangial lesions with proteinuria by a MoAb against mesangial cell surface antigen. *Clin Exp Immunol* 88: 399–404, 1992
16. Grande JP, Walker HJ, Holub BJ, Warner GM, Keller DM, Haugen JD, Donadio JV Jr, Dousa TP: Suppressive effects of fish oil on mesangial cell proliferation in vitro and in vivo. *Kidney Int* 57: 1027–1040, 2000
17. Wada Y, Morioka T, Oyanagi-Tanaka Y, Yao J, Suzuki Y, Gejyo F, Arakawa M, Oite T: Impairment of vascular regeneration precedes progressive glomerulosclerosis in anti-Thy 1 glomerulonephritis. *Kidney Int* 61: 432–443, 2002
18. Kasuga H, Ito Y, Sakamoto S, Kawachi H, Shimizu F, Yuzawa Y, Matsuo S: Effects of anti-TGF-β type II receptor antibody on experimental glomerulonephritis. *Kidney Int* 60: 1745–1755, 2001
19. Ostendorf T, van Roeyen CR, Peterson JD, Kunter U, Eitner F, Hamad AJ, Chan G, Jia XC, Macaluso J, Gazit-Bornstein G, Keyt BA, Lichenstein HS, LaRochelle WJ, Floege J: A fully human monoclonal antibody (CR002) identifies PDGF-D as a novel mediator of mesangioproliferative glomerulonephritis. *J Am Soc Nephrol* 14: 2237–2247, 2003
20. Nagaya N, Kangawa K, Kanda M, Uematsu M, Horio T, Fukuyama N, Hino J, Harada-Shiba M, Okumura H, Tabata Y, Mochizuki N, Chiba Y, Nishioka K, Miyatake K, Asahara T, Hara H, Mori H: Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. *Circulation* 108: 889–895, 2003
21. Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M, Nickenig G: Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation* 109: 220–226, 2004
22. Iruela-Arispe L, Gordon K, Hugo C, Duijvestijn AM, Claffey KP, Reilly M, Couser WG, Alpers CE, Johnson RJ: Participation of glomerular endothelial cells in the capillary repair of glomerulonephritis. *Am J Pathol* 147: 1715–1727, 1995
23. Notoya M, Shinosaki T, Kobayashi T, Sakai T, Kurihara H: Intussusceptive capillary growth is required for glomerular repair in rat Thy-1.1 nephritis. *Kidney Int* 63: 1365–1373, 2003
24. Johnson RJ, Iida H, Alpers CE, Majesky MW, Schwartz SM, Pritzi P, Gordon K, Gown AM: Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. Alpha-smooth muscle actin is a marker of mesangial cell proliferation. *J Clin Invest* 87: 847–858, 1991
25. Shimizu A, Kitamura H, Masuda Y, Ishizaki M, Sugisaki Y, Yamanaka N: Rare glomerular capillary regeneration and subsequent capillary regression with endothelial cell apoptosis in progressive glomerulonephritis. *Am J Pathol* 151: 1231–1239, 1997
26. Mori T, Shimizu A, Masuda Y, Fukuda Y, Yamanaka N: Hepatocyte growth factor-stimulating endothelial cell growth and accelerating glomerular capillary repair in experimental progressive glomerulonephritis. *Nephron Exp Nephrol* 94: e44–e54, 2003
27. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ: Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 428: 664–668, 2004
28. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC: Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* 428: 668–673, 2004
29. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL: Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 109: 1292–1298, 2004
30. Westerhuis R, van Straaten SC, van Dixhoorn MG, van Rooijen N, Verhagen NA, Dijkstra CD, de Heer E, Daha MR: Distinctive roles of neutrophils and monocytes in anti-Thy-1 nephritis. *Am J Pathol* 156: 303–310, 2000

Gene Therapy (2005), 1–9

www.nature.com/gt

RESEARCH ARTICLE

Exploring RNA interference as a therapeutic strategy for renal disease

Y Takabatake[1], Y Isaka[1], M Mizui[1], H Kawachi[2], F Shimizu[2], T Ito[1], M Hori[1] and E Imai[1]

[1]*Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine (A8), Suita, Japan; and* [2]*Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*

The short synthetic interfering RNA duplexes (siRNAs) can selectively suppress gene expression in somatic mammalian cells without nonselective toxic effects of double-stranded RNA (dsRNA). However, a selective in vivo delivery of siRNA transfer has not been reported in kidney. Here, we investigated whether injection of synthetic siRNAs via renal artery followed by electroporation could be effective and therapeutic in silencing specific gene in glomerulus. We investigated the effect of siRNA in rat cultured mesangial cells (MCs) and showed that siRNA sequence-specific suppression of transgene expression was over a 1000-fold more potent than that by antisense oligodeoxynucleotide (ASODN). Transfection of siRNA targeting luciferase into rat kidneys significantly inhibited expression of a cotransfected luciferase expression vector in vivo. The delivery of siRNA targeting enhanced green fluorescent protein (EGFP) in the transgenic 'green' rat reduced endogenous EGFP expression, mainly in glomerular MCs. Furthermore, RNAi targeting against TGF-β1 significantly suppressed TGF-β1 mRNA and protein expression, thereby ameliorated the progression of matrix expansion in experimental glomerulonephritis. In addition, vector-based RNAi also inhibited TGF-β1 expression in vitro and in vivo. In conclusion, siRNA-directed TGF-β1 silencing may be of therapeutic value in the prevention and treatment of fibrotic diseases.

Gene Therapy advance online publication, 24 February 2005; doi:10.1038/sj.gt.3302480

Keywords: *RNA interference (RNAi); short interfering RNA (siRNA); antisense oligonucleotide; electroporation; glomerulonephritis; enhanced green fluorescent protein (EGFP)*

Introduction

RNA interference (RNAi), which is initiated by the introduction of double-stranded RNA (dsRNA) into the cell, leads to the sequence-specific destruction of endogenous RNA.[1,2] RNAi-induced gene-specific silencing has been proven successful in organisms such as *Caenorhabditis elegans* and plants. However, the application of long dsRNA in vertebrates is limited because it induces a generalized suppression of protein synthesis and cell death by activating the interferon pathway.[3] Tuschl *et al*[4] made a crucial breakthrough that short synthetic interfering RNA duplexes (siRNAs) can selectively silence the expression of complementary genes in somatic mammalian cells without the nonselective toxic effects of long dsRNAs. These observations in mammals not only produced the next generation of genetic research, but also allowed the development of new therapies.

An important step in realizing the potential of RNAi as a therapeutic tool is determining if this mechanism can be triggered *in vivo*. Recently, we developed an electroporation-mediated gene transfer system targeting the kidney: DNA is injected via the renal artery, and is followed by electric pulses with tweezers-type electrodes.[5] This system allows us to deliver DNA mainly to the glomeruli, a region central to the inflammatory response in the initiation and progression of various kidney diseases. Therefore, we investigated whether electroporation-mediated introduction of synthetic siRNA could be effective in silencing transgene and endogenous gene expression in glomeruli. Furthermore, to prove the therapeutic application of transfected siRNA in an animal disease model, we targeted TGF-β1, a potent cytokine which plays an important role in the fibrogenic phase of various diseases, including glomerulonephritis.[6] Also, we examined the efficacy of a siRNA-producing DNA-based vector system. Here, we showed that electroporation-mediated siRNA transfer inhibited glomerular gene expression, and that RNAi targeting TGF-β1 could be therapeutic in renal disease.

Correspondence: Dr Y Isaka, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine (A8), Suita 565-0871, Japan
Received 7 August 2004; accepted 27 December 2004

Results

Effect of synthetic siRNA in vitro

We investigated whether siRNA is effective in rat mesangial cells (MCs) and compared its effect with that of antisense oligodeoxynucleotides (ASODNs). In MCs treated with siRNA targeting luciferase GL-3 (siGL-3),[4] expression of *Photinus pyralis* luciferase was inhibited 50–95% in a dose-dependent fashion (0.01–50 nM), when compared with scrambled siRNA (siSCR) (Figure 1a). In contrast, ASODN targeting GL-3 (ASGL-3)[7] inhibited

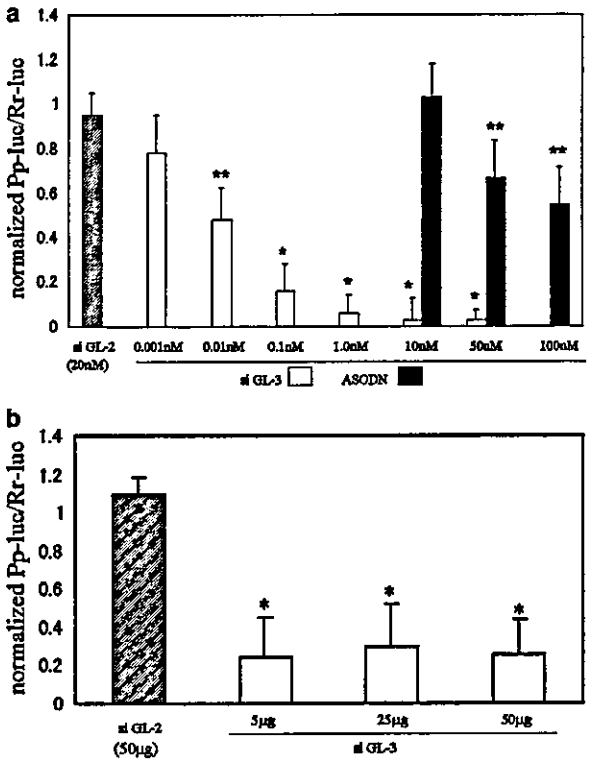

*Figure 1 siRNA-mediated silencing of luciferase in vitro and in vivo. siRNA-mediated silencing of luciferase in rat primary MCs (a). The average of three independent experiments is shown; error bars indicate standard deviation. *P<0.001 compared with siGL-2. **P<0.01 compared with siGL-2 or SGL-3. siRNA-mediated silencing of luciferase in rat glomeruli (b). The average of three independent experiments is shown (n=4); error bars indicate standard deviation. *P<0.01 compared with siGL-2.*

luciferase activities by only 30 and 40% at the concentration of 50 and 100 nM, respectively. We observed no significant suppression in MCs transfected with siRNA targeting luciferase GL-2 , a variant of luciferase GL-3 (siGL-2).[4] Expression of *Renilla reniformis* luciferase was also unaffected by siGL-3. These observations suggest that inhibition by siRNA is specific to the target gene and is over a 1000 times more effective than ASODN.

Effect of synthetic siRNA in vivo

We then tested whether electroporation-mediated siRNA transfection could silence gene expression effectively in kidney *in vivo*. For *in vivo* studies, luciferse plasmid combinations (pGL-3 and pRL-TK) were co-transfected with siGL-3 or siGL-2 by electroporation into the kidney. In rats receiving siGL-3, glomerular expression of *P. pyralis* luciferase was inhibited by about 70%, when compared with siSCR-treated rats (Figure 1b). Treatment with siGL-2 did not affect *P. pyralis* luciferase activity. These results demonstrate that siRNA can be delivered to the glomeruli using electroporation method and, subsequently, gene expression is inhibited in a sequence-specific manner.

To identify the cellular localization of siRNA delivered to the kidney, we employed siRNA targeting enhanced

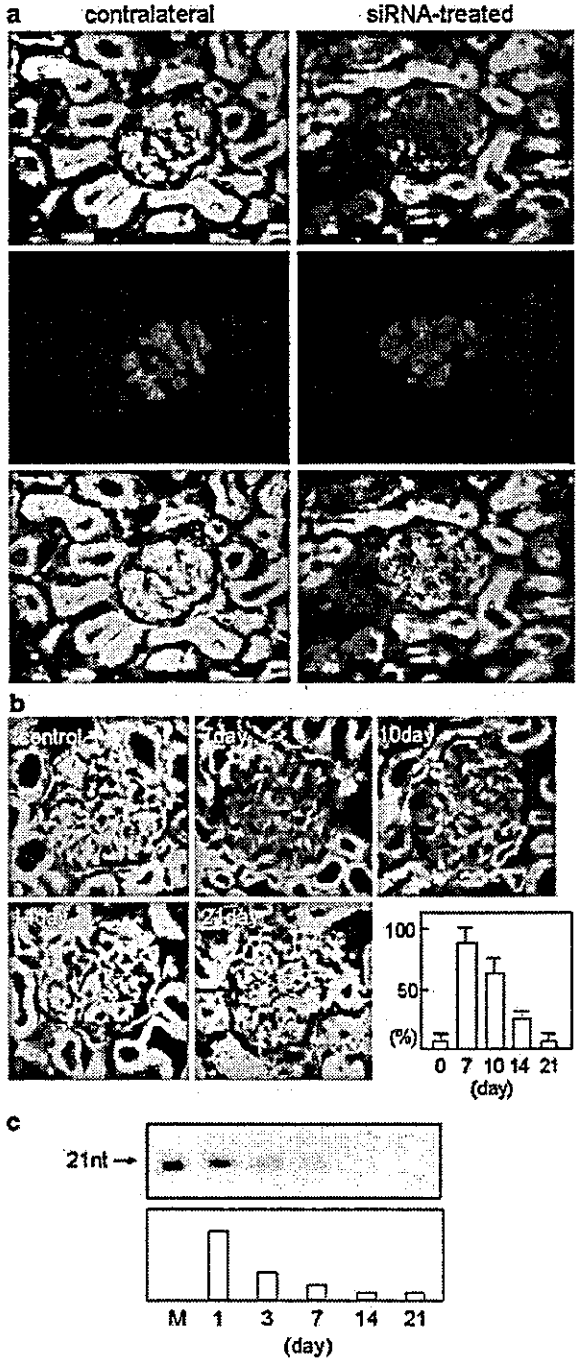

Figure 2 siRNA-mediated silencing of EGFP expression in the glomerular cells. Representative fluorescence micrographs of glomeruli in the siEGFP-transfected (right) and contralateral (left) kidney, which were taken 7 days after transfection (a, upper). Sections were stained with Texas red-labeled OX-7 antibody, a marker of MCs (a, middle), and merged photos are shown (a, lower). Original magnifications are ×400. Representative fluorescence micrographs of siEGFP-transfected gromeruli at various time point (b, upper) and ratios of EGFP-knocked down MCs were presented (b, lower). Total RNA extracted from transfected glomeruli was subjected to Northern blot analysis to detect siEGFP using 5′ end-labeled oligodeoxynucleotide probe (c). Synthetic siEGFP (0.2 ng) was used as a positive control and a size-marker (M).

green fluorescent protein (EGFP) (siEGFP), and transferred siEGFP to the kidney of EGFP transgenic rats.[8] At 7 days after transfection, EGFP expression was diminished substantially in almost all of the glomeruli (>95%), while it was unchanged in the tubules (Figure 2a). This inhibition seemed nearly complete in MCs stained red with Texas red-labeled OX-7 antibody (Figure 2a, lower panel), whereas in other glomerular cells, endothelial and epithelial cells, no inhibition of EGFP expression was observed. These results demonstrate that siRNA effectively inhibits the expression of a transgene expressed from the genome, at least in glomerular MCs. The reduction of mesangial EGFP expression was observed for up to 2 weeks, but recovered completely 3 weeks after transfection (Figure 2b, upper panel). Quantitative data were also summarized (Figure 2b, lower panel). Moreover, to examine the fate of transfected siRNA in glomeruli, total glomerular RNA was subjected to Northern blot analysis detecting the amount of remained siEGFP in glomeruli (Figure 2c). In consistent with the morphological effect, transfected siRNA was degraded gradually and signal was almost undetectable three weeks after transfection.

Effect of synthetic siRNA targeting TGF-β1

To investigate the therapeutic potential of siRNA, we targeted TGF-β1. First, we determined the optimal siRNA sequence to target TGF-β1 using cultured rat MCs. Three different TGF-β1-specific siRNAs (siTGFA, siTGFB and siTGFC) were synthesized. Northern blot analysis demonstrated siSCR or siTGFC transfection did not affect the FCS-induced increase in TGF-β1 mRNA levels (Figure 3a). In contrast, TGF-β1 mRNA levels in MCs transfected with siTGFA, siTGFB decreased to 0.40- and 0.36-fold respectively, compared to siSCR-transfected cells. As the treatment with siTGFB reduced the levels of TGF-β1 mRNA expression most effectively, we employed siTGFB for *in vivo* experiments.

Next, we tested whether electroporation-mediated delivery of synthetic siTGFB into MCs *in vivo* could suppress glomerular TGF-β1 expression using the anti-Thy-1 model of glomerulonephritis, in which TGF-β1 is upregulated in the glomerular MCs. At 24 h after injection of mAb 1-22-3, 50 μg of siTGFB was transfected into the left kidney. Northern blot analysis showed that treatment with siTGFB reduced glomerular TGF-β1 mRNA expression 0.42-fold, as compared to the contralateral right kidney of siTGFB-treated rats or siSCR-transfected kidney (ratios of TGF-β1 signal to GAPDH signal) (Figure 3b). Western blot analysis demonstrated that siTGFB significantly reduced glomerular TGF-β1 protein expression when compared with the contralateral right kidney (Figure 3c). No difference in TGF-β1 protein expression was observed between siSCR-transfected left kidneys and contralateral right kidneys. Immunohistochemical examination also showed that TGF-β1 induction was significantly reduced in the siTGFB-treated kidney compared to contralateral nephritic kidney (Figure 4a–e).

To determine the effect on pathological changes in nephritic kidneys, histological analysis was performed using PAS staining. PAS staining showed that siTGFB transfection reduced extracellular matrix (ECM) accumulation (Figure 4f, h) compared with the contralateral right kidneys of siTGFB-treated rat (Figure 4g, i). We

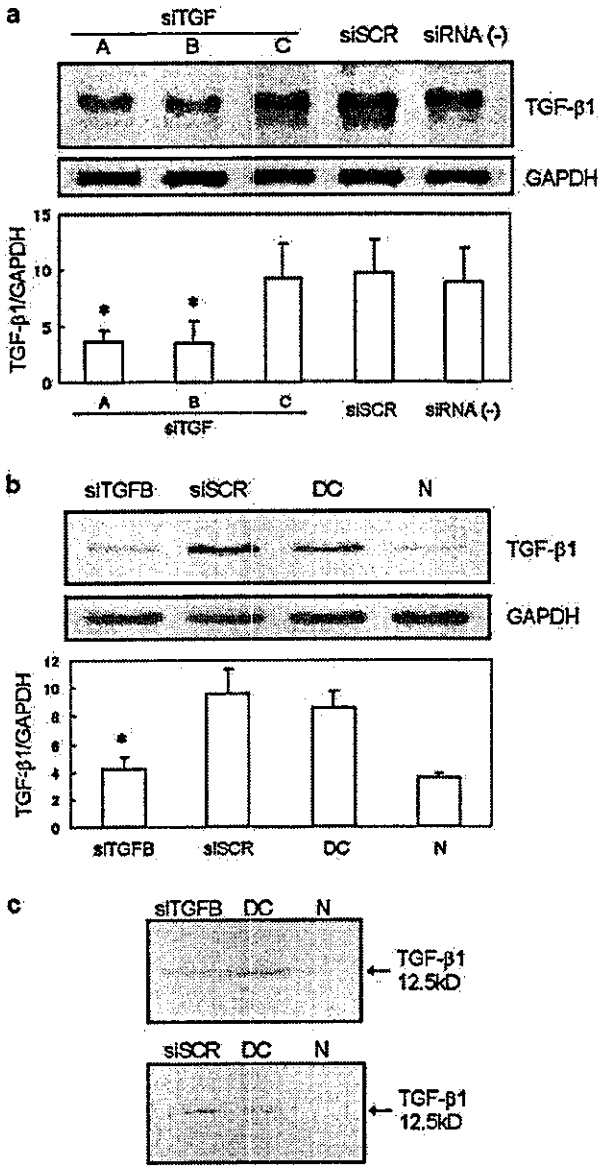

*Figure 3 siRNAs-mediated silencing of TGF-β1 expression in vitro and in vivo. Northern blot analysis showed the siRNA-mediated silencing of FCS-induced TGF-β1 expression in cultured rat MCs (a). The band density in three experiments expressed as the mean+s.d. is illustrated (*P<0.01 compared with siSCR). Northern blot analysis (b) and Western blot analysis (c) demonstrated siRNA-mediated silencing of TGF-β1 expression in glomeruli of an anti-Thy-1 nephritis model. Treatment with siTGFB reduced TGF-β1 mRNA (b) and protein (c) expression as compared to the untransfected contralateral right kidney (DC) or siSCR-transfected kidney. Normal (N) kidneys were also represented. The band density expressed as the mean+s.d. is illustrated (*P<0.01 compared with siSCR and DC).*

observed strong SMαA expression in the glomeruli of contralateral right kidneys (Figure 4k). However, immunostaining of SMαA was weak in siTGFB-treated kidneys (Figure 4j). Computerized image analysis shows that SMαA expression was significantly suppressed in siTGFB-treated nephritic kidneys compared with contralateral or siSCR-treated kidneys (Figure 4l).

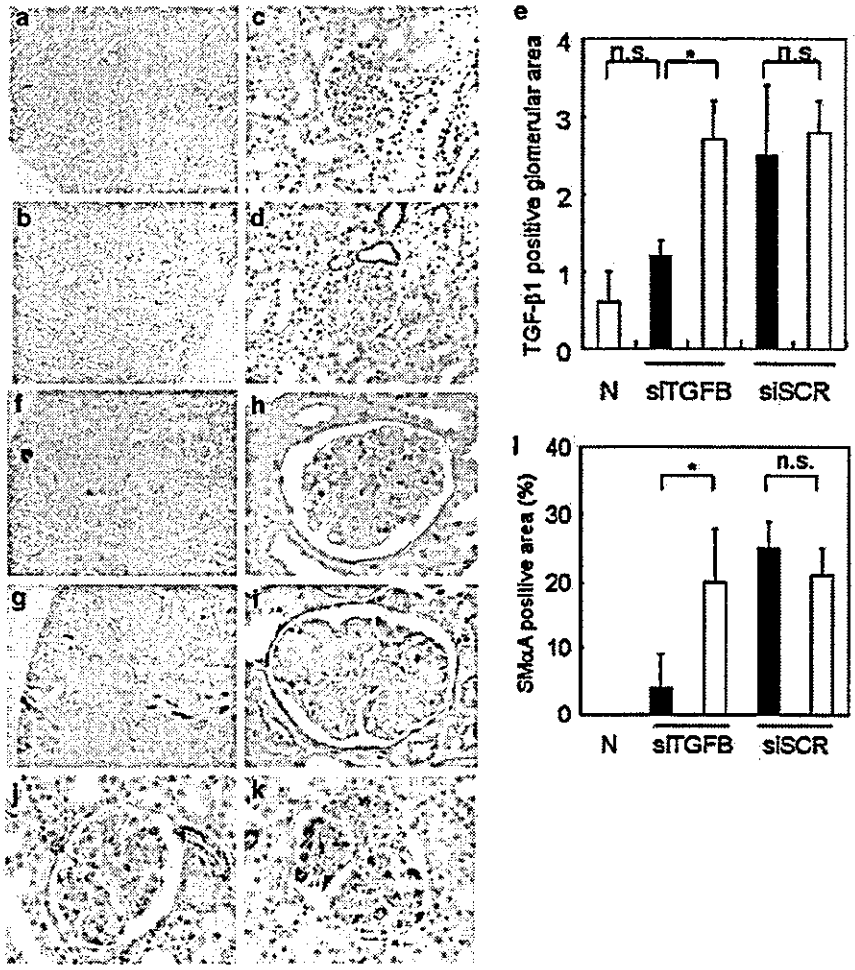

*Figure 4 Morphological and immunohistochemical analysis of an siRNA-treated kidney (a, c, f, h, j) and contralateral nephritic kidney (b, d, g, i, k). Representative microphotographs of glomeruli stained with TGF-β1 (a–d), PAS (f–i), SMαA (j, k) on day 4 post-transfection were presented. Original magnifications are ×100 (a, b, f, g) and ×400 (c, d, h, i, j, k). Histological improvement of the disease manifestations was (semi-)quantitatively estimated (e, l). Glomerular expression of TGF-β1 was graded semiquantitatively according to the scoring system (e). Glomerular expression of SMαA was evaluated by computerized image analysis (l). In both figures treated kidneys (solid bars) were compared to untreated contralateral right kidney (open bars) or normal kidneys (N) (*P<0.01).*

Inhibition of gene expression by short hairpin RNA

To overcome the shortcomings of chemically synthesized siRNA, several groups have developed DNA-vector-mediated mechanism to express substrates that can be converted into siRNA *in vitro*. In this study, we used the p*Silencer* 2.0-U6 siRNA expression vector, featuring the human U6 RNA pol III promoter, and constructed pSU6TGFB to encode small hairpin RNA (shRNA) targeting the same sequence as siTGFB and tested its efficacy (Figure 5a). By transferring this expression vector into cultured MCs or rat kidney, we verified the transcripts *in vitro* and *in vivo* by Northern blot analysis (Figure 5b). shRNA, an intermediate in this system, was not detected, probably due to immediate processing by Dicer-like nuclease.

First, we examined shRNA-mediated silencing of TGF-β1 mRNA expression in rat MCs. After transfecting pSU6TGFB or pSU6SCR, TGF-β1 mRNA levels in cultured MCs were estimated by Northern blot analysis (Figure 5c). Densitometric analysis showed pSU6TGFB significantly reduced the FCS-induced increase of TGF-β1 mRNA levels compared with pSU6SCR-treatment or untreatment. Next, we checked shRNA-mediated silencing of TGF-β1 expression in glomeruli of the anti-Thy-1 nephritis model. TGF-β1 expression of isolated glomeruli was estimated using Northern blot analysis (Figure 5d) and Western blot analysis (Figure 5e). pSU6TGFB efficiently inhibited mRNA and protein expression of TGF-β1 *in vivo* when compared with pSU6SCR. According to these observations, pSU6TGFB ameliorated the pathological changes of the nephritic kidney as efficiently as siTGFB (data not shown).

Discussion

We first demonstrated in this study that siRNA successfully inhibited exogenous and endogenous gene expression in kidney using an electroporation method, thereby validating the potential of siRNA as a therapeutic tool.

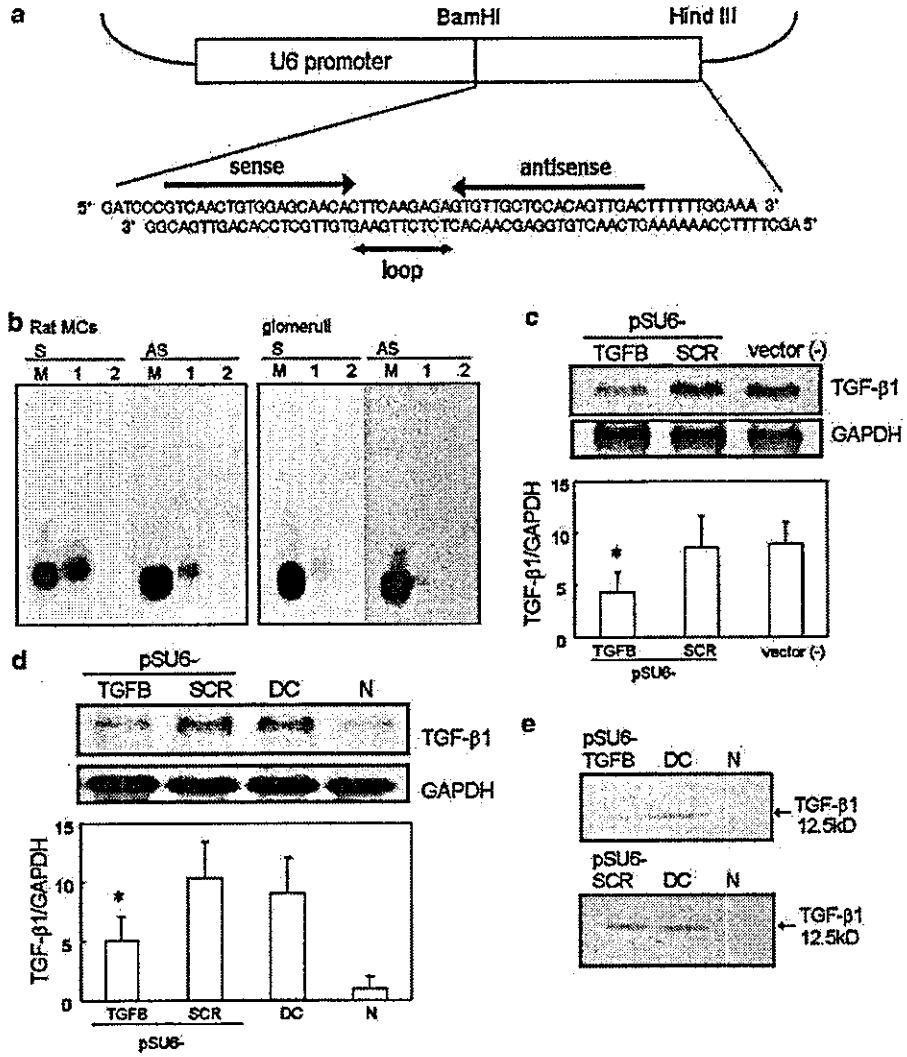

*Figure 5 shRNA-mediated silencing of TGF-β1 expression. Schematic drawing of the construction of pSU6TGFB (a). Two oligodeoxynucleotides containing sense and antisense 21-nt sequences of siTGFB, a 9-nt loop sequence, and a transcription termination signal of six thymidines were annealed and inserted downstream of the U6 promoter. Identification of transcripts expressed from pSU6TGFB in cultured MCs (left) and glomeruli (right) (b). Transcripts expressed from pSU6TGFB (lane 1) or pSU6SCR (lane 2) in rat MCs or glomeruli were probed with either ^{32}P-labeled sense (S) or antisense (AS) 19-nt oligonucleotide. Synthetic siTGFB (0.2 ng) was used as a size-marker (M). shRNA-mediated silencing of TGF-β1 mRNA expression in rat MCs (c). After transfecting pSU6TGFB or pSU6SCR, TGF-β1 (upper) and GAPDH (lower) mRNA levels in MCs were estimated by Northern blot analysis. Densitometric analysis was performed for three independent experiments. The band density expressed as the mean+s.d. is illustrated (*P<0.01 compared with pSU6SCR). shRNA-mediated silencing of TGF-β1 expression in vivo (d, e). TGF-β1 expression of isolated glomeruli was estimated using Northern blot analysis (d) and Western blot analysis (e). Untransfected contralateral (DC) and normal (N) kidney were also represented. The band density expressed as the mean+s.d. is illustrated (*P<0.01 compared with pSU6SCR and contralateral kidney).*

In this study, we showed a selective *in vivo* delivery of siRNA into kidney. Several researchers tried the *in vivo* delivery of siRNA in a variety of organs. Using a hydrodynamic-based delivery technique, several groups have demonstrated sequence-specific silencing mainly in liver either by siRNA or by siRNA expression vectors.[9,10] Virus-vector-mediated RNAi *in vivo* has also been described using recombinant adenovirus[11] and baculovirus.[12] Successful application of RNAi for gene therapy depends on the effective delivery of RNA molecules exclusively to their intended site of action. To our knowledge, however, kidney-targeted siRNA transfer has not been reported. Recently, we developed a new gene transfer system by electroporation *in vivo*, involving DNA injection via the renal artery followed by electric pulses with tweezers-type electrodes.[5] This technique allows us to deliver DNA mainly to the glomeruli, a region central to the inflammatory response in the initiation and progression of various kidney diseases.

Here, we verified the delivery of synthetic siRNA into rat kidneys by electroporation-mediated gene transfer. Dual luciferase assays showed that siRNA targeting against luciferase decreased the level of glomerular *P. pyralis* luciferase expression up to 30%, compared with scrambled siRNA-transfected rats. We did not observe the dose-dependency of siGL-3 *in vivo* (5–50 μg). The observation from our *in vitro* experiment suggests that less amount of siRNA (<5 μg) might be

effective in *in vivo* gene suppression. We observed no significant inhibition in rats injected with the control siRNA against luciferase GL-2, a variant of luciferase GL-3, suggesting that the inhibition of siRNA was specific to the target gene. In addition, we transfected siRNA targeting EGFP to EGFP-transgenic rats to investigate whether siRNA could be effective in silencing transgene expressed from the genome and to identify its precise site of action in the kidney. At 7 days after transfection, EGFP expression was diminished substantially in almost all of glomeruli, while it remained unchanged in the tubules. This inhibition seemed nearly complete in MCs, whereas no inhibition was observed in other glomerular cells, endothelial and epithelial cells. As a similar transduction pattern occurred when FITC-conjugated ODN was transfected by electroporation,[5] this observation may result from the differences in transfection efficiency between glomerular cell types rather than from the nature of siRNA itself. These results demonstrate that siRNA is effective in inhibiting the expression of a transgene expressed from the genome, at least in a majority of MCs.

In order to prove the therapeutic application of transfected siRNA in an animal disease model, we selected TGF-β1 as the target gene. TGF-β1 is a profibrotic cytokine that plays a pivotal role in fibrosis.[6] We showed that siRNA targeting against TGF-β1 could be therapeutic in anti-Thy-1 nephritis, in which TGF-β1 is upregulated in the glomerulus.[13] In addition to the kidney, inhibition of TGF-β1 activity has been successfully demonstrated in several organs, for example, lung, skin, brain, and arterial wall.[6] In each case, blocking the activity of TGF-β1 dramatically decreased the excessive deposition of ECM, but did not interfere with normal healing of tissue repair. Our observation in this study is applicable to other conditions with TGF-β1 involvement, and the use of siRNA for TGF-β1 may be suitable for the treatment of fibrotic diseases.

In the present study, we demonstrated shRNA expression vector suppressed the target gene expression as efficiently as synthetic siRNAs *in vitro* and *in vivo*. A major concern about RNAi is the nonspecific effect, which may be induced by siRNA or shRNA, especially, the interferon responses. Recently, some synthetic siRNAs or shRNA expression vectors were reported to induce interferon responses.[14,15] This is of critical importance in exploring RNAi for clinical application. However, we did not observe the increase of apoptotic cells nor induction of nonspecific gene inhibition in siRNA- or shRNA-treated MCs *in vitro* or *in vivo* glomeruli *in vivo*. Another concern in applying RNA to gene therapy is the longevity of silencing target gene expression *in vivo*. It is reported that gene silencing by synthetic siRNAs is transient:[4] levels of target protein in siRNA-treated cells recover 5 or 7 days after transfection, probably due to dilution accompanied with cell division and the nonrenewable nature of siRNAs in mammals. In our study a single transfection of siRNA for EGFP (siEGFP) or its expression vector (data not shown) into kidney resulted in the reduction of mesangial EGFP expression for up to 2 weeks. Of interest is transfection of siEGFP or its expression vector into the anterior tibial muscle by electroporation silenced EGFP expression unexpectedly for more than 90 days (data not shown). These data suggest that the longevity of RNAi may be dependent on the resistance to nuclease attack in individual cells. Therefore, we examined the expression of *eri-1*, which was recently reported to be a kind of 'siRNase'.[16] The expression of *eri-1* was higher in brain and kidney than in muscle (data not shown), which may explain the difference in the duration of silencing. We also showed that the amount of siRNAs derived from its expression vectors as detected by Northern analysis was rather small, especially *in vivo* (Figure 5b). Considering the high efficacy of synthetic siRNAs transfection *in vitro* (>80% in MCs) and *in vivo* (nearly all glomeruli were transfected with siRNA, compared to 75% at the most with DNA vector[5]), synthetic siRNAs still have some advantages over plasmid-based gene silencing, even in the practical application at present.

In conclusion, siRNA transfer into the renal artery followed by electroporation was effective and siRNA-directed TGF-β1 silencing may be of therapeutic value in the prevention and treatment of fibrotic diseases.

Materials and methods

Cell culture

Rat MCs were isolated and cultured as described previously[17] and were maintained in RPMI 1640 (SIGMA, St Louis, MO, USA) containing 20% fetal bovine serum (FBS) equilibrated with 5% CO_2–95% air at 37°C. MCs at passage 6–12 were used for *in vitro* transfection.

Animal studies

The transgenic 'green' rat line, Rat SD TgN (act-EGFP) Osb4,[8] and 6-week-old male Sprague–Dawley (SD) rats, weighing approximately 150 g, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Anti-Thy-1 glomerulonephritis was induced by a single injection of 700 μg of monoclonal antibody (mAb) 1-22-3.[18] In all animal experiments, rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and handled in a humane fashion in accordance with the guidelines of the Animal Committee of Osaka University.

Preparations of siRNA and ASODNs

siRNAs (21 nucleotides long) were designed to target luciferase GL-2 (siGL-2),[4] GL-3 (siGL-3),[4] EGFP (siEGFP),[19] TGF-β1 (siTGF), and the scrambled genes (siSCR). siRNAs were chemically synthesized as 2′ bis (acetoxyethoxy)-methyl ether-protected oligonucleotides, deprotected, annealed, and purified by Dharmacon Research (Lafayette, CO, USA). The sense and antisense strands of three different siTGFs were: siTGF (sequence A; siTGFA), 5′-ACGGAAGCGCAUCGAAGCCdTdT-3′ (sense), 5′-GGCUUCGAUGCGCUUCCGUdTdT-3′ (antisense); siTGF(sequence B; siTGFB), 5′-GUCAACUGUG GAGCAACACdTdT-3′ (sense), 5′-GUGUUGCUCCACA GUUGACdTdT-3′ (antisense); siTGF(sequence C; siTGFC), 5′-GGUCCUUGCCCUCUACAACdTdT-3′ (sense), 5′-GU UGUAGAGGGCAAGGACCdTdT-3′ (antisense). Antisense and sense phosphorothioate-modified oligonucleotides (phosphodiester protected by 2 phosphorothioates in 3′ and 2 phosphorothioates in 5′) targeting luciferase GL-3′ (ASGL-3, SGL-3, respectively) were synthesized and purchased from Bex (Tokyo, Japan).

siRNA-mediated inhibition of luciferase activity in vitro and in vivo

To assess the silencing effect of siRNA in the kidney, co-transfection assays were performed *in vitro* and *in vivo*. First, reporter plasmids were co-transfected with synthetic siRNAs or ASODN into rat primary MCs by Lipofectamine 2000 reagent (Invitrogen Corp., Carsbad, CA, USA). Reporter plasmid combinations of 1 μg of pGL-3 (Promega, Madison, WI, USA), containing the *P. pyralis* luciferase gene and 0.1 μg of pRL-TK (Promega) containing the *R. reniformis* luciferase gene (a quantitative control), were co-transfected into subconfluent rat MCs in 6 cm dishes along with 0.001–50 nM of siGL-3, 10–100 nM of ASGL-3, or 20 nM of siGL-2, an siRNA targeting against a variant of *P. pyralis* luciferase gene. Scrambled siRNA (siSCR) or sense ODN (SGL-3) at equivalent concentrations was used as a control for siGL-3 or ASGL-3, respectively. At 24 h after transfection, we examined the effect of siRNA by dual luciferase assay (Promega). In all cases, the ratios of *P. pyralis* luciferase (Pp-luc) to *R. reniformis* luciferase (Rr-luc) activities were normalized by those observed in equivalent amount of siSCR or SGL-3 treated MCs to compensate for differences in transfection efficiencies.

For *in vivo* studies, reporter plasmid combinations (200 μg of pGL-3 and 10 μg of pRL-TK) were co-transfected with 5–50 μg of siGL-3 or 50 μg of siGL-2 by electroporation into the kidney as previously described ($n=4$ in each group).[5] At 4 days after transfection, rat glomeruli were collected by a graded sieving technique[20] and prepared homogenates were screened for the activity of target *P. pyralis* luciferase and control *R. reniformis* luciferase by dual luciferase assay.

siRNA-mediated inhibition of EGFP expression in glomeruli

To identify transfected cells in the kidney, we transfected 50 μg of siEGFP to the kidney of EGFP-transgenic rats via the renal artery, followed by electroporation ($n=4$). At 7 days after transfection, kidneys were examined by immunofluorescence. Kidneys were fixed in ice-cold neutral-buffered 4% paraformaldehyde (PFA) solution and embedded in O.C.T. compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA, USA). The cryostat sections (4 μm thick) were stained with the monoclonal antibody OX-7, a specific marker for MCs (a kind gift from Dr Ken-ichi Isobe and Professor Seichi Matsuo, Nagoya University, Nagoya, Japan). After blocking in 5% normal horse serum in PBS, sections were incubated with OX-7 (1:200) for 1 h at room temperature, followed by Texas red-conjugated anti-mouse IgG (1:200) (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature. The green fluorescence of EGFP and red fluorescence were photographed on the same film by double exposure. Contralateral kidneys were used as normal controls. To examine the reduction of EGFP expression quantitatively, we selected 20 glomeruli from each sample at various time point and the red staining area on merged image (showing the EGFP-knocked down MS area) relative to that on nonmerged image (showing total MS area) was calculated automatically as a percentage using Mac SCOPE program. Moreover, to elucidate the fate of transfected siRNA, Northern blot analysis detecting siEGFP was performed using total RNA extracted from siEGFP-transfected glomeruli. 5′ end-labeled oligodeoxynucleotide (5′-GGCTACGTC CAGGAGCGCA-3′) that is complementary to antisense strand of siEGFP was used as a probe. Synthetic siEGFP (0.2 ng) was used as a positive control and a size-marker.

siRNA-mediated inhibition of TGF-β1 in vitro and in vivo

To target TGF-β1, we examined the optimal sequence for siRNAs using cultured MCs. Three different TGF-β specific siRNAs (siTGFA, siTGFB, and siTGFC) were synthesized. At 12 h after transfection of siTGFA, siTGFB, siTGFC or siSCR, at a final concentration of 20 nM, into subconfluent rat MCs using Lipofectamine 2000, serum-free medium was replaced with complete growth medium containing 20% FCS to stimulate TGF-β1 expression. After incubating further for 48 h, the effect of the siRNAs was assessed by Northern blot analysis.

As siTGFB appeared to be the most effective, this siRNA was further used in *in vivo* studies. At 24 h after induction of anti-Thy-1 nephritis, 50 μg of siTGFB or siSCR was transfected into rat glomeruli ($n=6$ in each group). TGF-β1 expression in the glomeruli was monitored by Northern blot analysis, Western blot analysis and immunohistochemistry 4 days after transfection.

Northern blot analysis

We examined the effect of siRNA on TGF-β1 mRNA expression *in vitro* and in *vivo* by Northern blot analysis. For *in vitro* studies, transfected rat MCs were rinsed twice with ice-cold PBS and scraped with 1 ml of Trizol reagent (Invitrogen). For *in vivo* studies, isolated glomeruli were homogenized with Polytron homogenizer (Kinematica, Switzerland) in Trizol reagent 4 days after siRNA transfection. In both studies, total RNA was extracted according to the manufacturer's protocol. TGF-β1 and GAPDH cDNA were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Biosciences) by the random priming method (Rediprime II, Amersham Biosciences) and hybridization was carried out as described previously.[21] Autoradiographs were obtained and the density of each band was quantified using the laser densitometry (Scanning Imager; Molecular Dynamics, Sunnyvale, CA, USA). These experiments were repeated three times and the density of each band relative to that of GAPDH was calculated.

Western blot analysis

To study the effect of siRNA on glomerular TGF-β1 protein expression, Western blot analysis was performed. Collected glomeruli were homogenized in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet-P40, 10% glycerol, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 0.5 mM sodium orthovanadate by tissue grinders (Iwaki, Tokyo, Japan) at 4°C. The soluble lysates were mixed 1:2 with 3 × Laemmli buffer and heated for 10 min at 95°C. Lysates (20 μg) were loaded per lane, resolved by 15% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Hybond-P PVDF Membrane, Amersham Biosciences). Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline (TBS)–Tween 20 (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20 (vol/vol)) for 30 min at room temperature and

then immunoblotted with polyclonal antibodies against TGF-β1 (Anti-TGFβ1 pAb, 1:1000, Promega) in blocking buffer for 1 h at room temperature. The primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG and visualized with SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL, USA) according to the manufacturer's directions. The blots were exposed to X-ray film (Hyperfilm, Amersham Biosciences).

Morphological and immunohistochemical studies

Nephritic kidneys treated with siTGFB or siSCR were fixed in 4% PFA and paraffin-embedded. Contralateral nephritic kidneys were used as disease controls. For renal morphological studies, 4 μm sections were stained with the periodic acid-Schiff (PAS) reagent. Immunohistochemical stains for TGF-β1 and SMαA were performed using a streptavidin biotin-staining method (Vector ABC kit; Vector Laboratories). For TGF-β1 staining, sections were autoclaved in 0.01 M citrate buffer (pH 6.0) for 10 min at 121°C to retrieve the antigen. Rabbit anti-human TGF-β1 polyclonal antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), which crossreacts with rat tissue, and mouse IgG anti-SMαA monoclonal antibody (Immunotech, Marseilles, France) were used.

Pathological changes in anti-Thy-1 nephritis model are heterogeneous among different glomeruli. Histological improvement of the disease manifestations was quantitatively estimated. For each samples, approximately 30 cortical glomerular cross sections containing vascular pole were evaluated in a blind fashion. Glomerular expression of TGF-β1 was graded semiquantitatively according to the score described elsewhere.[22] It ranged from 0 to 4; 0 = glomerulus without any positive staining, 1 = glomerulus with up to 25% positive staining, 2 = glomerulus with 26–50% positive staining, 3 = glomerulus with 51–75% positive staining, 4 = glomerulus with 76–100% positive staining. Glomerular expression of SMαA was evaluated by computerized image analysis using Mac SCOPE program. In brief, selected glomeruli from each sample were photographed and positive areas were highlighted on the captured images. The area of positive staining relative to each glomerular area was automatically calculated as a percentage with determined threshold settings.

siRNA expression vectors

Oligodeoxynucleotides (64 nt) encoding hairpin siRNAs (shRNAs) targeting TGF-β1 (sequence B) were synthesized by Bex, annealed and ligated into *Bam*HI and *Hind*III site of p*Silencer* 2.0-U6 siRNA expression vector, featuring human U6 RNA pol III promoter (Ambion, Inc., Austin, TX, USA) (pSU6TGFB). p*Silencer* containing scrambled sequences (pSU6SCR) was used as a negative control.

First, Northern blot analysis was performed to identify the transcripts of pSU6TGFB *in vitro* and *in vivo*. Total RNA was isolated from rat MCs and glomeruli transfected by pSU6TGFB. In total, 20 μg of total RNA was separated by electrophoresis on 15% (wt/vol) polyacrylamide-8 M urea gel and transferred onto a nylon membrane (Hybond-N+). Sense (5′-GTCAACTGTGGAG CAACAC-3′) or antisense (5′-GTGTTGCTCCACAGTT GAC-3′) oligodeoxynucleotide (19nt) was labeled by the 5′-end labeling method using [γ-^{32}P] ATP (3000 Ci/mmol; Amersham Biosciences) and T4 polynucleotide kinase (New England Biolabs, Inc., Beverley, MA, USA). The blots were hybridized with γ-P^{32}-labeled sense or antisense probe at 37°C overnight. They were washed in 2 × SSC and 0.1% SDS twice for 15 min at room temperature, twice for 15 min at 37°C and subjected to autoradiography. Synthetic siTGFB (0.2 ng) was used as size-marker.

We then examined whether shRNA could inhibit TGF-β expression *in vitro* and *in vivo*. We transfected 10 μg of pSU6TGFB into cultured MCs in 6 cm dishes or 200 μg of pSU6TGFB into nephritic kidneys ($n = 6$). Next, we assessed the effect of shRNA by Northern blot analysis and Western blot analysis.

Statistical analyses

All values are expressed as means ± s.d. Statistical significance was evaluated using the one-way analysis of variance (ANOVA). A P-value of <0.05 was considered significant.

References

1 Fire A *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998; 391: 806–811.
2 Hannon GJ. RNA interference. *Nature* 2002; 418: 244–251.
3 Billy E *et al.* Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc Natl Acad Sci USA* 2001; 98: 14428–14433.
4 Elbashir SM *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; 411: 494–498.
5 Tsujie M *et al.* Electroporation-mediated gene transfer that targets glomeruli. *J Am Soc Nephrol* 2001; 12: 949–954.
6 Border W, Noble N. Transforming growth factor in tissue fibrosis. *N Engl J Med* 1994; 331: 1286–1292.
7 Noah DL, Blum MA, Sherry B. Transfection of primary cardiac myocyte cultures with DNA and anti-sense oligonucleotides using FuGENE 6 transfection reagent. *Biochemica* 1998; 2: 38–40.
8 Ito T *et al.* Bone marrow is a reservoir of repopulating mesangial cells during glomerular remodeling. *J Am Soc Nephrol* 2001; 12: 2625–2635.
9 McCaffrey AP *et al.* RNA interference in adult mice. *Nature* 2002; 418: 38–39.
10 Song E *et al.* RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med* 2003; 9: 347–351.
11 Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nat Biotechnol* 2002; 20: 1006–1010.
12 Valdes VJ, Sampieri A, Sepulveda J, Vaca L. Using double-stranded RNA to prevent *in vitro* and *in vivo* viral infections by recombinant baculovirus. *J Biol Chem* 2003; 278: 19317–19324.
13 Border WA *et al.* Suppression of experimental glomerulonephritis by antiserum against transforming growth factor beta 1. *Nature* 1990; 346: 371–374.
14 Bridge AJ *et al.* Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* 2003; 34: 263–264.
15 Sledz CA *et al.* Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 2003; 5: 834–839.
16 Kennedy S, Wang D, Ruvkun G. A conserved siRNA-degrading RNase negatively regulates RNA interference in C. *elegans*. *Nature* 2004; 427: 645–649.
17 Sugiura T *et al.* Group II phospholipase A2 activates mitogen-activated protein kinase in cultured rat mesangial cells. *FEBS Lett* 1995; 370: 141–145.

18 Kawachi H *et al.* Epitope-specific induction of mesangial lesions with proteinuria by a MoAb against mesangial cell surface antigen. *Clin Exp Immunol* 1992; 88: 399–404.
19 Novina CD *et al.* siRNA-directed inhibition of HIV-1 infection. *Nat Med* 2002; 8: 681–686.
20 Akagi Y *et al.* Inhibition of TGF-beta1 expression by antisense oligonucleotides suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int* 1996; 50: 148–155.
21 Nakamura H *et al.* Introduction of DNA enzyme for Egr-1 into tubulointerstitial fibroblasts by electroporation reduced interstitial alpha-smooth muscle actin expression and fibrosis in unilateral ureteral obstruction (UUO) rats. *Gene Therapy* 2002; 9: 495–502.
22 Daniel C *et al.* Antisense oligonucleotides against thrombospondin-1 inhibit activation of TGF-beta in fibrotic renal disease in the rat *in vivo*. *Am J Pathol* 2003; 163: 1185–1192.

日本経済新聞
5月19日
月曜日
発行所 日本経済新聞社
東京本社〒100-8066㊎(03)3270-0251
東京都千代田区大手町1-9-5
大阪本社〒540-8588㊎(06)6943-7111
大阪市中央区大手前1-1-1
名古屋支社〒460-8366㊎(052)243-3311
名古屋市中区栄4-16-33
西部支社〒812-8666㊎(092)473-3300
福岡市博多区博多駅東2-16-1
札幌支社〒060-8621㊎(011)281-3211
札幌市中央区北1条西7-3

腎臓細胞増殖に関与

動物の「体内時計」

阪大チームが解明

臓器の修復治療に期待

睡眠や目覚めなど生物の一日のリズムを作り出す「体内時計」が、腎臓の細胞の増殖を制御していることを、大阪大学と産業技術総合研究所が動物実験で突き止めた。体内時計をうまく調節できれば、腎臓の細胞の増殖を活発にして傷んだ臓器を修復するといった治療法につながる可能性がある。

阪大医学系研究科の伊藤孝仁博士らの成果で、二十二日から東京で開く日本腎臓学会で発表する。

研究はまず、ラット（大型ネズミ）の腎臓の尿細管という部分で、細胞が活発に増殖する時間帯を調べた。普通のラットは朝方に増殖が活発になる。

ところが体内時計の昼夜を人工的に逆転させると、増殖が活発な時間帯も逆転し、体内時計が細胞の増殖にかかわっていることが分かった。

腎臓の細胞の増殖に関係するうえ、体内時計と連動して働く遺伝子も突き止めた。この遺伝子が体内時計の指示を受けて、細胞が増殖するタイミングを決めている可能性が高いという。体内時計が常に「朝」を示す状態を医薬品などで作り出し、腎炎などで傷んだ腎臓の細胞増殖を活発にして修復することもできると期待している。

研究グループは今後、人間にも同様の仕組みがあるかどうかを調べる。