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ORIGINAL ARTICLE

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Association of a single-nucleotide polymorphism in the immunoglobulin μ-binding protein 2 gene with immunoglobulin A nephropathy

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Abstract Immunoglobulin A (IgA) nephropathy is the most common form of primary glomerulonephritis worldwide. The pathogenesis of IgA nephropathy is unknown, but it is certain that some genetic factors are involved in susceptibility to the disease. Employing a large-scale, case–control association study using gene-based single-nucleotide polymorphism (SNP) markers, we previously reported four candidate genes. We report here an additional significant association between IgA nephropathy and an SNP located in the gene encoding immunoglobulin μ-binding protein 2 (IGHMBP2) at chromosome 11q13.2–q13.4. The association ($\chi^2 = 17.1$, $p = 0.00003$; odds ratio of 1.85 with 95% confidence interval of 1.39–2.50 in a dominant association model) was found using DNA from 465 affected individuals and 634 controls. The SNP (G34448A) caused an amino acid substitution from glutamine to lysine (E928K). As the gene product is involved in immunoglobulin-class switching and patients with the A allele revealed higher serum levels of IgA ($p = 0.048$), the amino acid change might influence a class switch to increase serum IgA levels, resulting in a higher risk of IgA nephropathy.

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Introduction

Immunoglobulin A (IgA) nephropathy, originally described by Berger and Hinglasis (1968), is now recognized as the most common form of primary glomerulonephritis worldwide. This disease is defined as a process of proliferative changes in glomerular mesangial cells and increases in mesangial matrices associated with deposition of IgA. At first, IgA nephropathy was considered to be a mild clinical manifestation with a relatively good prognosis. However, since recent reports have indicated that 20–30% of patients develop end-stage renal disease within 10–30 years after the onset of the disease (Koyama et al. 1997), its prognosis is now regarded as more serious than previously thought.

The pathogenesis of IgA nephropathy is unknown, but accumulating evidence suggests that genetic factors are involved in disease susceptibility (Donadio and Grande 2002). For example, the prevalence of IgA nephropathy seems to reflect demographic and ethnic characteristics of the populations studied; furthermore, several cases of familial IgA nephropathy have been reported (Scolari 2003).

Recent approaches to identify disease-susceptibility genes have focused on genome-wide analysis of associations using single-nucleotide polymorphisms (SNPs). SNPs are likely to be useful as markers for identification of genes involved in complex diseases because they are the most common type of genetic variation in human DNA (Kruglyak 1999). We have been screening gene-based SNPs on a genome-wide scale to detect possible associations with susceptibility to IgA nephropathy and have already reported that specific SNP alleles in the L- and E-selectin (*SELL* and *SELE*) genes on chromosome 1q24–25 (Takei et al. 2002), the *HLA-DRA* locus at 6p21.3 (Akiyama et al. 2002), and the polymeric immunoglobulin receptor (*PIGR*) gene at 1q31–q41 (Obara et al. 2003) were candidate susceptibility factors for IgA nephropathy in the Japanese population.

Through an extension of those genome-wide analyses, we have now identified an additional association with IgA nephropathy in the Japanese population involving SNPs in the immunoglobulin μ-binding protein 2 (*IGHMBP2*) gene.

Materials and methods

DNA samples

This study enrolled 465 Japanese individuals with IgA nephropathy who were referred to several hospitals in Japan (Department of Medicine, Kidney Center, Tokyo Women's Medical University; Department of Urology, Iwate Medical University; Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences; Iwate Prefectural Ofunato Hospital; Sanai Hospital; Osaka City General Hospital; Toride Kyodo General Hospital; and Osaka Rosai Hospital). IgA nephropathy was diagnosed on the basis of clinical manifestations as well as renal-biopsy findings. Patients with Henoch–Schönlein purpura and secondary IgA nephropathy, such as hepatic glomerulosclerosis, were excluded from the analysis. Peripheral blood samples were obtained for DNA extraction. As controls, we analyzed DNA from 634 volunteers from the general Japanese population. Written informed consent was obtained from all participants, and DNA was prepared from each blood sample according to standard protocols. We are given approval for the study using human materials from our committee dealing with ethics for medical research.

Multiplexed polymerase chain reactions

We amplified multiple genomic fragments using 20 ng of genomic DNA for each polymerase chain reaction (PCR), as described previously (Ohnishi et al. 2001). Sequences of the primers used in this study are available from the JSNP database (http://snp.ims.u-tokyo.ac.jp/). Each PCR was performed in a 20-µl solution containing 50 pmol of each primer, 10U Ex-*Taq* DNA polymerase (TaKaRa), and 0.55 µg *Taq*Start (Clontech Laboratories) in a GeneAmp PCR system 9700 (Applied Biosystems). Initial denaturation was performed at 94°C for 2 min, followed by 37 cycles of amplification at 94°C for 15 s and annealing at 60°C for 45 s, with final extension for 2 min at 72°C.

Genotyping

For a new genome-wide association study, we selected 88,148 SNPs described in our JSNP database (Haga et al. 2002). All genotyping was done by the Invader assay, which combines a structure-specific cleavage enzyme with a universal fluorescent resonance energy transfer (FRET) system (Mein et al. 2000). FRET probes were labeled with either FAM or VIC corresponding to each allele. Signal intensity was indicated as the ratio of FAM or VIC to ROX, an internal reference. Each 5-µl reaction contained 0.25 µl of signal buffer, 0.25 µl of each FRET probe, 0.25 µl of the structure-specific cleavage enzyme, 0.25 µl of allele-specific probe mix, and 2 µl of the PCR product diluted 1:10. Samples were incubated in a GeneAmp PCR system 9700 (95°C for 5 min and then at 63°C for 15 min) and were analyzed on an ABI Prism 7700 sequence detector.

Statistical analysis

The genotype distribution and allele frequencies of each selected SNP were compared between cases and controls by means of chi^2 tests. Significance was judged according to the guidelines of Lander and Kruglyak (1995). Fisher's exact test was used when criteria for the chi-squared test could not be applied. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by Woolf's method. Hardy–Weinberg equilibrium was assessed by chi-squared statistics (Nielsen et al. 1998). Linkage disequilibrium (LD) coefficients were calculated and expressed as $D' = D/D_{max}$ (Devlin and Risch 1995). Clinical data were recorded as means ± SD. Statistically significant differences were assessed with Student's t tests using Stat View SE.

Results

We performed this genome-wide association study in a stepwise manner. First, we genotyped 94 Japanese patients with IgA nephropathy for 88,148 gene-based SNPs selected from our database (Haga et al. 2002) by means of the high-throughput multiplex PCR-Invader assay method (Ohnishi et al. 2001). A comparison of the allelic frequencies among these 94 patients with frequencies calculated for 634 members of the general Japanese population identified 2,109 SNPs that revealed p values of 0.01 or smaller. We then genotyped these 2,109 SNPs using DNAs of 94 additional patients (Fig. 1) and found that 581 of them still revealed P values of 0.01 or smaller.

When these 581 polymorphisms were analyzed for all 465 cases, 13 of the SNPs, including those reported previously in the HLA-DRA gene, revealed p values of 0.0001 or smaller. One of the SNPs showing relatively more significant associations was located in exon 14 of the immunoglobulin μ-binding protein 2 (IGHMBP2) locus on chromosome 11q13.2–q13.4. This SNP, G34448A, was associated with IgA nephropathy with $\chi^2 = 17.1$, $P = 0.00003$ (odds ratio = 1.85, 95% CI = 1.39–2.50) by a dominant association model (Table 1). The G to A substitution at nucleotide 34448 changed glutamine to lysine (E928K) (Fig. 2).

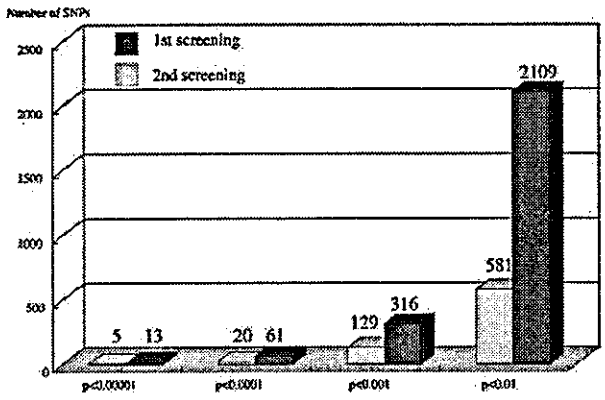

Fig. 1 Results of the first and second screening experiments. First screening (94 cases), $P < 0.01$: 2,109/88,148 SNPs; second screening (188 cases), $P < 0.01$: 581/2,109 SNPs. By the increase of the number of the cases examined, most of the SNPs lost their association

We subsequently carried out LD mapping of 21 SNPs with minor allele frequencies of >20% that were located in the *IGHMBP2* region; this genomic fragment included six other genes (*MTL5*, *CPT1A*, *MRPL21*, *MRGPRD*, *MRGPRF*, *and TPCN2*). Mapping procedures separated these 21 SNPs into three LD blocks; the candidate SNP was located in the LD block containing two genes, *MRPL21* and *IGHMBP2*. When we genotyped patients with IgA nephropathy for the eight SNPs present in that LD block, G34448A in exon 14 of *IGHMBP2* showed the most significant association with the disease (Fig. 3).

Since individuals having the AA or GA genotype (homozygotes for the minor allele, or heterozygotes) of G34448A were likely to have a higher risk of IgA nephropathy ($P = 0.00003$) in a dominant manner, we compared available clinical data at the time of biopsy for patients with A alleles (AA and GA genotype) with those with the GG genotype (Table 2). We found no significant differences with respect to gender, age, levels of serum creatinine or 24-h urinary excretion of protein, but observed higher serum IgA levels in patients with AA and GA genotypes than in subjects with the GG genotype ($P = 0.048$).

Table 1 Genotype frequencies and association tests of the SNP in the *IGHMBP2* gene (465 cases of IgA versus 634 control)

	IgA number (%)	Control number (%)	
Major allele	798 (85.8%)	1162 (91.6%)	
Minor allele	132 (14.2%)	106 (8.3%)	
Total	930 (100%)	1268 (100%)	
Major homozygous	342 (73.5%)	531 (83.8%)	
Heterozygous	114 (24.5%)	100 (15.8%)	
Minor homozygous	9 (1.9%)	3 (0.47%)	
Total	465 (100%)	634 (100%)	
	χ^2	P	Odds ratio (95%CI)
Allele frequency (minor versus major)	18.9	0.00001	1.81 (1.38–2.38)
Other versus major homozygous	17.1	0.00003	1.85 (1.39–2.50)
Minor homozygous versus other	5.3	0.0353[a]	3.90 (1.03–14.77)

[a]Fisher's exact test

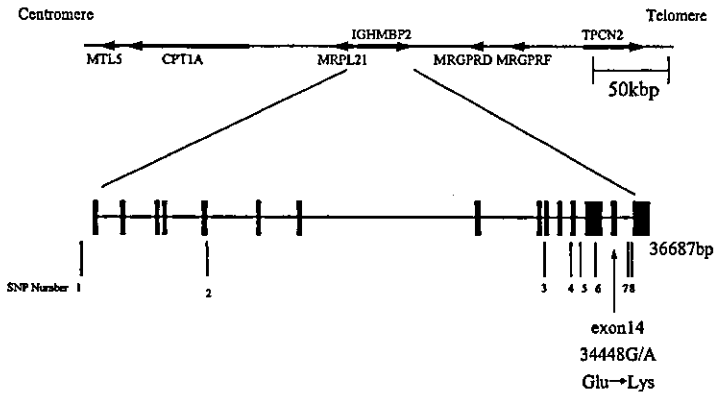

Fig. 2 Genomic structure of chromosome 11q13.2–q13.4 around the *IGHMBP2* gene. *IGHMBP2* gene contains 36687 bp and 15 exons. The 34448G/A SNP lies in exon 14 of *IGHMBP2* (*upward arrow*). The G to A substitution at nucleotide 34448 changed an amino acid from glutamine to lysine (E928K); exons are indicated by *black rectangles*. The SNP number is the same as Fig. 3

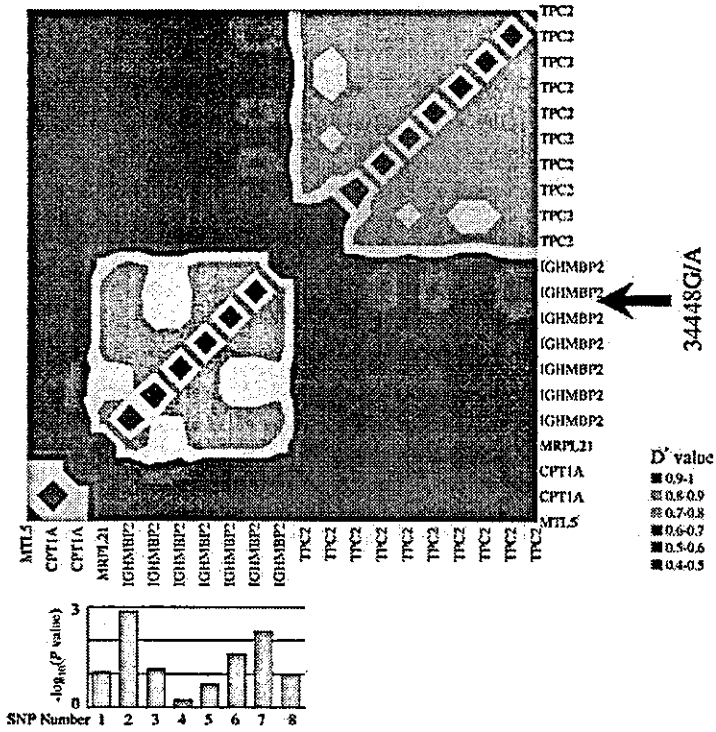

Fig. 3 Pairwise linkage disequilibrium between SNPs in the *IGHMBP2* region, as measured by *D'* in the case population and case–control association plots [$-\log_{10}(P$ value)]. The candidate SNP was located in the LD block containing two genes, *MRPL21* and *IGHMBP2*. G34448A in exon 14 of *IGHMBP2* showed the most significant association with the disease in this block. The SNP number is the same as Fig. 2

Table 2 Clinical data at the time of biopsy. Mean ± SD, *NS* not significant

	GG	GA + AA	*P* value
Gender (M/F)	140/122	60/38	NS
Age (years)	35.9 ± 14.2	38.9 ± 14.9	NS
Serum IgA (mg/dl)	319.4 ± 117.6	352.4 ± 162.9	0.048
Serum creatinine (mg/dl)	1.06 ± 0.66	1.08 ± 0.67	NS
Proteinuria (g/day)	1.16 ± 1.33	1.04 ± 1.33	NS

Discussion

Our genome-wide SNP system offers at least two advantages. First, our SNP database is an extensive catalog of gene-based SNPs located within exons, introns, and promoter regions (Haga et al. 2002). Second, using a combination of multiplex PCR and the Invader assay, our method allows high-throughput, low-cost screening that requires as little as 0.1 ng of genomic DNA per single SNP. Through a genome-wide SNP analysis, we found a significant association between IgA nephropathy and an SNP in the *IGHMBP2* gene. This polymorphism, G34448A, substitutes lysine for glutamine in the gene product; the allele encoding lysine was more frequently observed in individuals with IgA nephropathy than in the general Japanese population. *IGHMBP2* is ubiquitously expressed and consists of 15 exons encoding a protein of 993 amino acids that possesses a putative DNA helicase region, an R3H motif, and a zinc-finger AN1-like domain.

Although the physiological function of IGHMBP2 is not known in detail, the protein is suspected to be involved in immunoglobulin-class switching, pre-mRNA processing, and regulation of transcription through DNA-binding or interaction with TATA-binding proteins (Fukita et al. 1993; Molnar et al. 1997; Zhang et al. 1999; Miao et al. 2000; Liepinsh 2003). Fukita et al. (1993) first showed that IGHMBP2 binds to single-stranded DNA with 5′-phosphorylated guanine-rich sequences related to the immunoglobulin μ-chain switch (Sm) region. Immunoglobulin switch (S) regions are essential targets of S–S recombination, which changes the expressed class (isotype) of the immunoglobulin heavy chain (H) constant region (C) from μ to γ, ε, or α while maintaining the antigen specificity determined by DNA sequences in the variable region. S regions are located 5′ to each C_H gene, except for C_δ.

Increases in serum IgA levels have been observed in 50–70% of patients with IgA nephropathy, along with selective deposition of IgA1 in the kidney (Conley et al. 1980; Trascasa et al. 1980). We found that patients with the A allele of G34448A had higher serum IgA levels ($P=0.048$) and suspected that the E928K amino acid substitution might affect the class-switch event thereby increasing serum IgA levels, elevating the risk of IgA deposition, and causing the disease. However, the biological role of the zinc-finger AN1-like domain, where the E928K substitution lies, is still unknown. To clarify the relationship between E928K amino acid substitution and mesangial IgA deposition, examination of the frequency of this SNP in another nephritis with the same deposition, such as Henoch–Schönlein purpura, might be effective, but we do not have any available sample.

Gharavi et al. (2000) reported a genome-wide analysis of familial cases, but no familial history of the disease was observed in most Japanese patients with IgA nephropathy. This disease is a complex disorder, the etiology of which involves immunological, environmental, and genetic factors (Hsu et al. 2000). The fact that mesangial IgA deposition has been observed in 16% of allografts in non-blood-related Japanese kidney donors (Suzuki et al. 2003) indicates that latent mesangial IgA deposition is a relatively common phenomenon. Furthermore, patients with IgA nephropathy vary significantly with respect to progression and prognosis; some have a relatively good prognosis while others lose renal function, sometimes very rapidly. Thus, a variety of mechanisms may lay down deposits of IgA in glomerular mesangial areas, causing subsequent glomerular injuries and clinically active renal disease.

So far, we have identified five candidate genes that may be related to susceptibility to IgA nephropathy. On the basis of that information, we propose the potential mechanisms of IgA nephropathy indicated in Fig. 4, although it might be too preliminary, and more extensive biological and clinicopathological analysis would be required to substantiate this hypothesis. The onset of IgA nephropathy could be associated with antigens such as viruses, fungus, bacteria, or foods that are processed

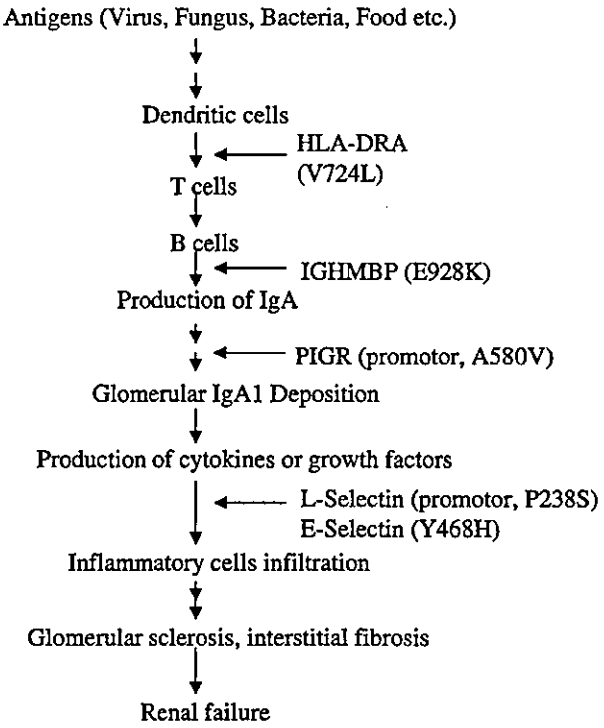

Fig. 4 Potential mechanism of IgA nephropathy based on results of case–control association studies using SNP markers for four candidate genes

and presented to T cells. HLA-DR, which regulates immune responses against protein antigens, is of great importance in the selection and activation of CD4-positive T cells; we identified the gene encoding HLA-DR earlier as a candidate susceptibility gene (Akiyama et al. 2002). HLA-DR molecules with the V724L substitution might account for individual differences in immune responses of T cells, which activate antibody-producing B cells. For its part, as noted above, the E928K variant of IGHMBP2 might influence a class-switch leading to increased serum IgA levels.

The third of our candidates, PIGR, is an integral membrane secretory component localized on the basolateral surface of secretory epithelial cells where it is thought to mediate the transepithelial transport of polymeric IgA. We showed earlier that a genetic variation in the promoter region of the PIGR gene caused an A580V substitution associated with IgA nephropathy and suggested that the V allele might affect binding of polymeric IgA to PIGR and cause deposition of mesangial IgA (Obara et al. 2003). IgA deposits in the kidney can trigger production of a variety of cytokines and growth factors by renal cells and by circulating inflammatory cells, leading to the characteristic histopathological features of mesangial-cell proliferation and depositions of immunoglobulin and complement in mesangial regions.

SELL and *SELE* genes encode cell–cell adhesion molecules involved in the leukocyte–endothelial cell

interaction required for extravasation at sites of tissue injury. SELE is expressed predominantly in cytokine-activated endothelium, and SELL is present in circulating leukocytes. We reported that Y468H in the *SELE* gene, as well as P238S-*SELL* and an SNP in the promoter region of *SELL*, were strongly associated with IgA and suggested that these substitutions could affect the quality and/or quantity of gene products and possibly play a significant role in inflammatory changes leading to renal fibrosis and ultimately renal failure (Takei et al. 2002).

Although functional studies must be undertaken to determine how these genetic variations, now including E928K-IGHMBP2, can affect the onset and development of IgA nephropathy, the results of our genetic studies have suggested several potential mechanisms for investigation.

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

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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.

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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-

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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.

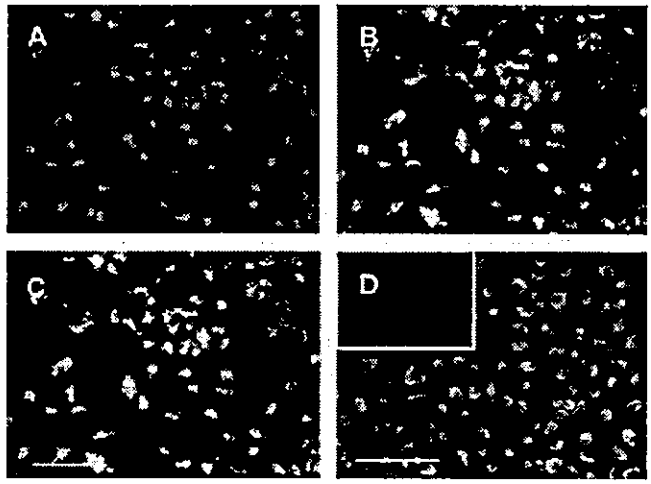

Figure 1. Uptake of acetylated LDL (A), binding of Bandeiraea simplicifolia lectin (BS-1 lectin; B), and expression of vascular endothelia growth factor receptor 2 (VEGF-R2; D) by culture-modified bone marrow mononuclear cells. Bone marrow mononuclear cells were cultivated in the presence of endothelial 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

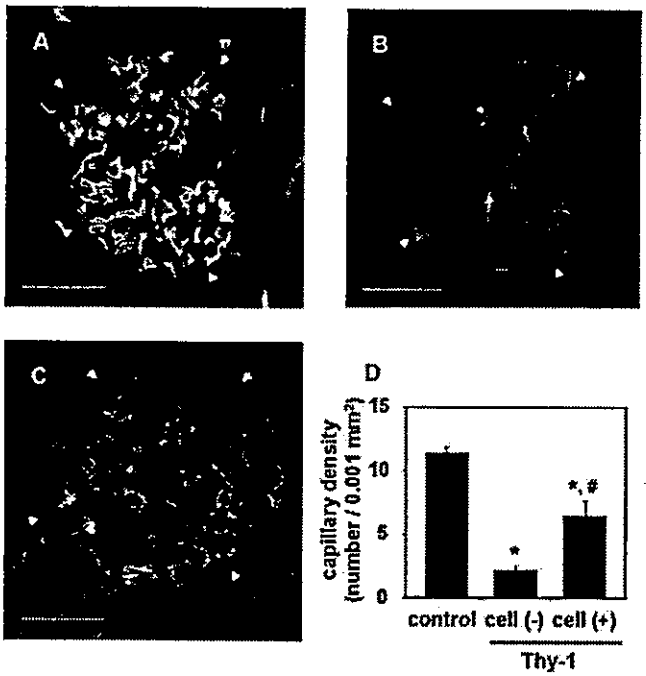

Figure 2. Inhibition of glomerular endothelial injury by administration of culture-modified bone marrow mononuclear cells (CMMC) to nephritic rats. Representative photomicrographs of glomerular immunostaining for rat endothelial cell antigen-1 (RECA-1) in kidney sections obtained from normal rats (A) 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.

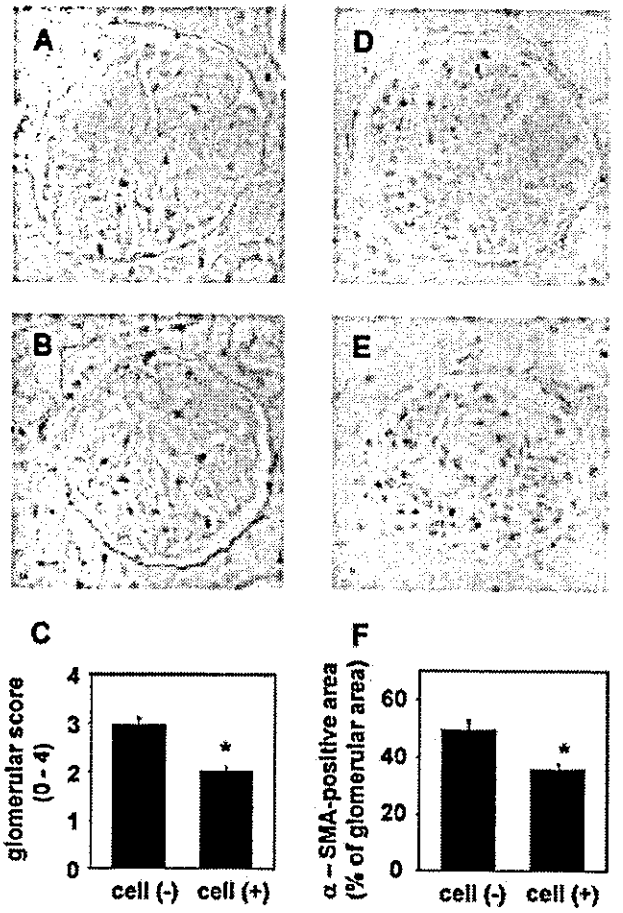

Figure 3. Inhibition of glomerular mesangial cell and/or matrix expansion and expression of glomerular α-smooth muscle actin (α-SMA) by administration of CMMC to nephritic rats. Representative photomicrographs of periodic acid-Schiff staining (A and B) and immunohistochemical staining for glomerular 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

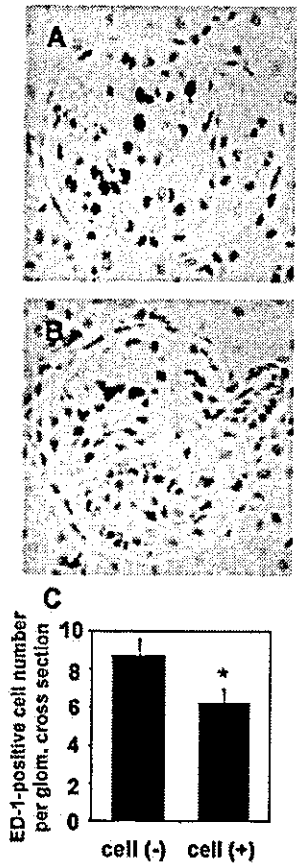

Figure 4. Inhibition of glomerular infiltration of macrophages by administration of CMMC to nephritic rats. Representative photomicrographs of immunohistochemical staining for glomerular ED-1 of right (A) and left (B) kidney sections obtained from nephritic rats in which CMMC had been injected into the 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.

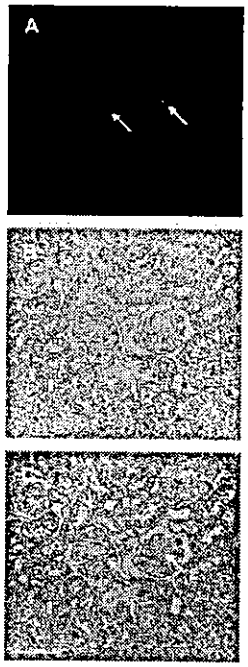

Figure 5. Glomerular localization of CMMC incorporated into the kidneys of nephritic rats. Representative photomicrographs of fluorescence (A), phase contrast (B), and overlay images (C) of renal cortex obtained from kidneys that were injected with CMMC. One day after the induction of anti-Thy1.1 nephritis 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

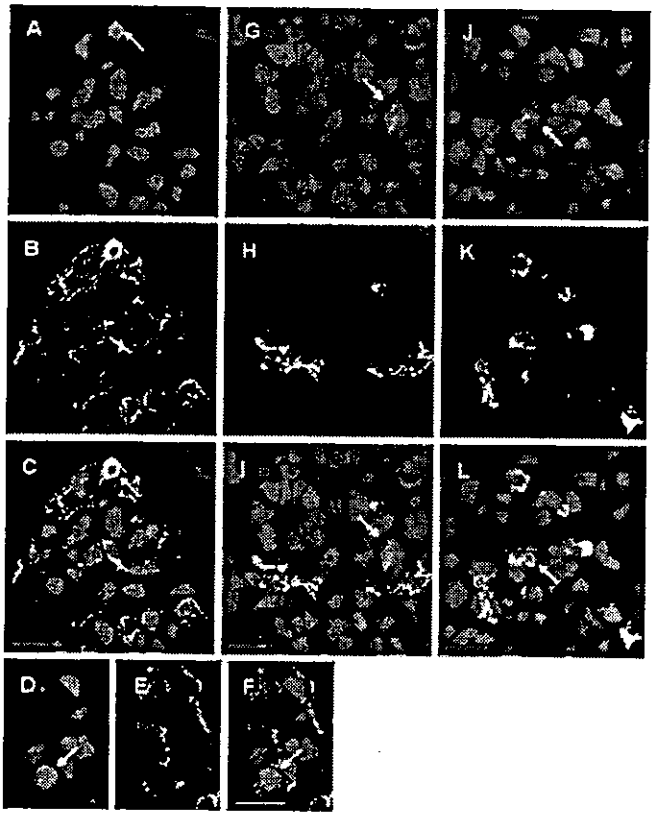

Figure 6. Immunohistochemical staining of glomeruli with CM-DiI–positive cells for markers of endothelial and mesangial cells and macrophages. Representative photomicrographs of fluorescence images of CM-DiI signals in glomeruli (A, D, G, and J); immunohistochemical staining for RECA-1 (B and E), OX-7 (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.

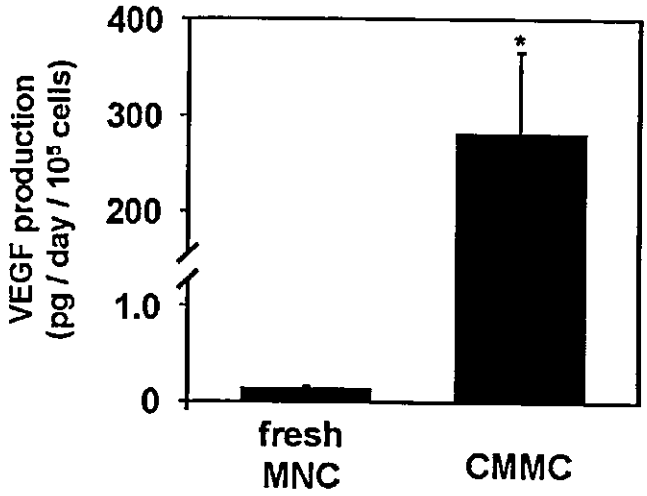

Figure 7. Production of VEGF by freshly isolated bone marrow mononuclear cells (fresh MNC) and CMMC was determined by ELISA. The data shown are means ± SEM ($n = 3$). *$P < 0.05$ *versus* fresh MNC.

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

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Human mesenchymal stem cells in rodent whole-embryo culture are reprogrammed to contribute to kidney tissues

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The use of stem cells has enabled the successful generation of simple organs. However, anatomically complicated organs such as the kidney have proven more refractory to stem-cell-based regenerative techniques. Given the limits of allogenic organ transplantation, an ultimate therapeutic solution is to establish self-organs from autologous stem cells and transplant them as syngrafts back into donor patients. To this end, we have striven to establish an *in vitro* organ factory to build up complex organ structures from autologous adult stem cells by using the kidney as a target organ. Cultivation of human mesenchymal stem cells in growing rodent embryos enables their differentiation within a spatially and temporally appropriate developmental milieu, facilitating the first step of nephrogenesis. We show that a combination of whole-embryo culture, followed by organ culture, encourages exogenous human mesenchymal stem cells to differentiate and contribute to functional complex structures of the new kidney.

organogenesis | regeneration

Organ regeneration has recently attracted considerable attention as a new therapeutic strategy. The potential for regenerative medicine has been gradually realized with the discovery of tissue stem cells and the reported therapeutic benefits of their implantation or systemic delivery for the regeneration of several tissues such as neurons (1), β-islet cells (2), myocytes (3) and vessels (4). However, success using such strategies to date has been limited to cells and simple tissues. Anatomically complicated organs such as the kidney and lung, which are comprised of several different cell types and have a sophisticated 3-dimensional organization and cellular communication, have proven more refractory to stem cell-based regenerative techniques. Allogenic tissue transplantation by using a scaffold is an alternative strategy to replace whole organs. However, the scarcity of suitable organs has prevented organ transplantation from becoming a practical solution in most cases of organ failure. Furthermore, chronic rejection of the allograft remains a common cause of graft failure after organ transplantation despite life-long administration of immunosuppressive agents (5). One of the ultimate therapeutic aims is therefore to establish self-organs from autologous tissue stem cells and transplant the *in vitro*-derived organ as a syngraft back into the donor individual.

Human mesenchymal stem cells (hMSCs) found in adult bone marrow were recently shown to maintain plasticity and to differentiate into several different cell types, depending on their microenvironment (6). In contrast to embryonic stem cells, adult MSCs can be isolated from autologous bone marrow and applied for therapeutic use without any serious ethical issues or immunologic consequences (7). Primary hMSCs were obtained from the bone marrow of healthy volunteers and used throughout this study.

The kidney was selected as the target organ for this study, because it represents a complicated organ, comprising several different cell types, has a sophisticated 3D organization, and its embryonic development has been well researched. Kidney development is initiated when the metanephric mesenchyme at the caudal portion of the nephrogenic cord (8) induces the nearby Wolffian duct to produce a ureteric bud (9). Development proceeds as a result of reciprocal epithelial-mesenchymal signaling between the ureteric bud and metanephric mesenchyme (10). To test whether hMSCs could participate in kidney development, they were initially cocultured with either rodent Wolffian duct extracted at the embryonic stage immediately before formation of the kidney primordia, or with established metanephric rudiment. However, this procedure was not sufficient to achieve kidney organogenesis or even integration of hMSCs into the developing rodent metanephros (T.Y., unpublished data). This finding suggests that hMSCs must be placed in a specific, defined embryonic niche to allow for exposure to the repertoire of nephrogenic signals required to generate the organ. This outcome can best be achieved by implanting hMSCs into the nephrogenic site of a developing embryo. However, it is difficult to implant cells prenatally at the exact site of organogenesis by a transuterus approach. Equally, once embryos are removed for cell implantation, they cannot be returned to the uterus for further development. Therefore, embryos were isolated from uteri for cell implantation, after which they were further developed *in vitro*, using whole-embryo culture. Here, we show that by using this culture combination, hMSCs develop into morphologically identical cells to endogenous renal cells and are able to contribute to complex kidney structures.

Methods

Animals. Wild-type Sprague–Dawley rats were purchased from Sankyo Lab Services (Tokyo). A breeding colony of Fabry mice was established at the Laboratory Animal Center of the Jikei University School of Medicine from breeding pairs that were kindly donated by R. O. Brady (National Institutes of Health, Bethesda). The midpoint on the day when a vaginal plug was observed was designated as day 0.5. Animals were housed in a ventilated (positive airflow) rack and bred and maintained under pathogen-free conditions. All experimental procedures were

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Abbreviations: hMSC, human mesenchymal stem cell; GDNF, glial cell line-derived neurotrophic factor; En, embryonic day n; α-gal A, α-galactosidase A; Gb3, globotriaosylceramide; AQP-1, aquaporin-1; PTH, parathyroid hormone; NBC-1, Na^+-HCO_3^- cotransporter 1; GLEPP-1, glomerular epithelial protein 1; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine.

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approved by The Committee for Animal Experiments of the Jikei University School of Medicine.

Culture and Manipulation of hMSCs. Bone marrow-derived hMSCs that were confirmed to be CD105-, CD166-, CD29-, and CD44-positive and CD14-, CD34-, and CD45-negative, were purchased from Cambrex Bio Science Walkersville (Walkersville, MD) and cultured according to the manufacturer's instructions. The hMSCs were used within five cell passages to avoid phenotypic changes. A replication-defective recombinant adenovirus carrying human glial cell line-derived neurotrophic factor (GDNF) cDNA (AxCAhGDNF) was generated and purified as described (11). Packaging cells (ψ-crip) that produce a recombinant retrovirus bearing the bacterial LacZ gene, MFG-LacZ, were a gift from H. Hamada (Sapporo Medical University, Sapporo, Japan). Adenoviral and retroviral infection were performed as described (12, 13). The cells were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) (Molecular Probes) at 0.25% (wt/vol) in 100% dimethylformamide and injected by using micropipettes at the site of ureteric bud sprouting.

Whole-Embryo Culture and Organ Culture. Whole embryos were cultured *in vitro* according to a method described (14), with several modifications. Using a surgical microscope at low-power magnification, uteri were dissected from anaesthetized mothers. Stage embryonic day (E)11.5 rat embryos and stage E9.5 mouse embryos were freed from the uterine wall, decidua, and the outer-membrane layer, including Reichert's membrane. The yolk sac and amnion were opened to enable injection but the chorioallantoic placenta was left intact. Successfully injected embryos were immediately cultivated in 15-ml culture bottles containing 3 ml of culture media consisting of 100% centrifuged rat serum supplemented with glucose (10 mg/ml), penicillin G (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The culture bottles were allowed to rotate in an incubator (model no. RKI10–0310, Ikemoto, Tokyo). *Ex vivo* development of the rat embryos was assessed after 24- and 48-h cultivation periods and compared with E12.5 and E13.5 rat embryos. Forty-eight hours later, embryos were assessed for heartbeat, whole-body blood circulation, and general morphology. Kidney rudiments were dissected and cultured as described (15). To enhance the accumulation of globotriaosylceramide (Gb3) in the kidney primordia, the cultivated metanephroi were cultured in the presence of ceramide trihexoside (1 nmol, Sigma) (16). α-galactosidase A (α-gal A) enzymatic activity in metanephroi was fluorometrically assessed as described (17).

Histology. Two-color staining of metanephroi were performed essentially as described (17) by using mouse anti-β-gal (Promega) and rabbit anti-human WT-1 (Santa Cruz Biotechnology) as the primary antibodies. A monoclonal mouse anti-Gb3 antibody (Seikagaku, Tokyo) was also used. Whole-mount *in situ* hybridization with digoxigenin UTP-labeled *c-ret* riboprobes was performed as described (15). *In situ* hybridization was also performed on histological sections by using biotin-labeled human genomic *Alu*I/II probes (Invitrogen) according to the manufacturer's protocol. An X-gal assay was used to assess expression of the LacZ gene as described (13).

Identification of hMSC-Positive Cells. Metanephroi generated by relay culture were digested in collagenase type I (1 mg/ml) for 30 min and labeled with fluorescein digalactoside (Molecular Probes) by using transient permeabilization by hypotonic shock as described by Fiering *et al.* (18) (FACS-galactosidase assay). LacZ-positive cells were sorted by using a cell sorter (Becton Dickinson). Total RNA was extracted and subjected to RT-PCR to analyze expression of aquaporin-1 (AQP-1), parathyroid hormone (PTH) receptor 1, 1α hydroxylase, Na^+-HCO_3^- co-transporter 1 (NBC-1), nephrin, podocine, and glomerular epithelial protein 1 (GLEPP-1). A list of primer sequences and reaction conditions used can be found in Table 1, which is published as supporting information on the PNAS web site. For the analysis of cell ploidy, cells were stained with propidium iodide, and DNA content was assessed by using a flow cytometer.

Statistical Analysis. Data were expressed as the mean ± SE. Statistical analysis was performed by using the two-sample *t* test to compare data in different groups. $P < 0.05$ was taken to be statistically significant.

Results and Discussion

The whole-embryo culture system was first optimized to allow a defined concentration of oxygen to be supplied continuously to rotating culture bottles, thus improving embryonic development *ex utero* (14). Using this system, rat embryos (E11.5), together with the yolk sac, amnion, and chorioallantoic placenta were cultured in the media consisting of 100% freshly centrifuged rat serum supplemented with glucose (10 mg/ml) at 37°C in the culture bottle (see Movie 1, which is published as supporting information on the PNAS web site). Forty-eight hours later, embryos were assessed for heartbeat, whole-body blood circulation, and general morphology. Based on the resultant somite number and general morphology, the devel-

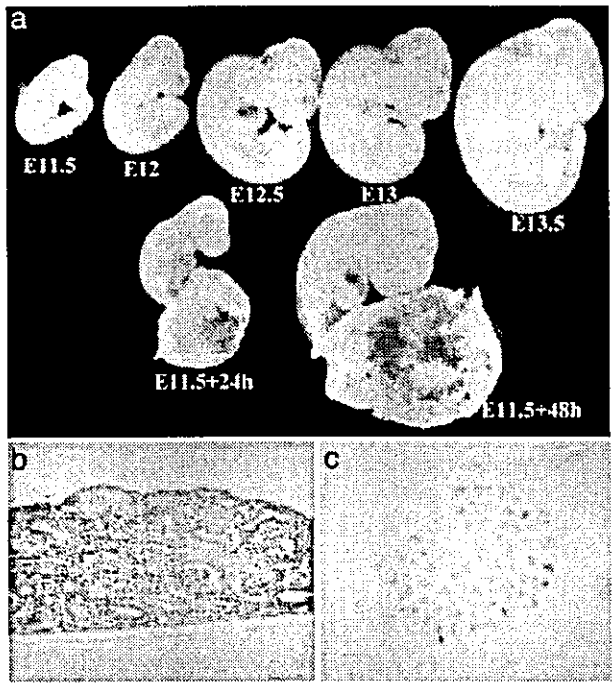

Fig. 1. *Ex utero* development of kidney primordia by using the relay culture system. Rat embryos (E11.5) just before ureteric bud sprouting were cultured *in vitro*, using a whole-embryo culture system for 48 h. After 24 and 48 h in culture, *ex utero* development of the rat embryos was assessed by comparing them with those that had grown in the uterus for E11.5, E12.0, E12.5, E13.0 and E13.5. Embryos that were developed in the culture bottle reached stages of development that were consistent with E13 embryos that had developed *in utero*. (*a*). At the end of the 48-h culture period, kidney rudiments were isolated and subjected to metanephric organ culturing for 6 days. To confirm the extent of tubulogenesis and ureteric bud branching, hematoxylin/eosin staining (*b*) and whole-mount *in situ* hybridization for *c-ret* (*c*) were performed. Fine tubulogenesis and ureteric bud branching can be observed. Experiments were performed in triplicate and representative pictures are shown.

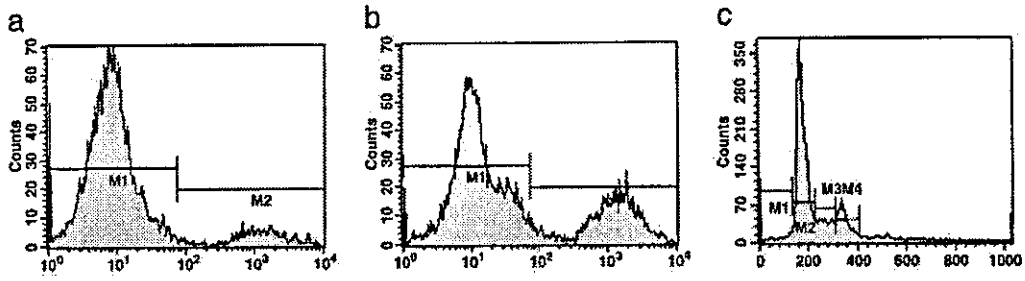

Fig. 2. Proportion of donor-derived cells in culture-derived metanephroi and assessment of their DNA ploidy. hMSCs expressing the LacZ gene were retrovirally transfected with GDNF (*b*) or without GDNF (*a*) and injected into rodent embryos at the site of budding. After relay culture, the neogenerated metanephroi were digested with collagenase, and single cells were subjected to a FACS-galactosidase assay. M, the informative peak. The use of GDNF was found to significantly increase the number of hMSCs that were incorporated into the developing metanephroi. (*c*) LacZ-positive cells were sorted, and their DNA content was assessed by using propidium iodide intensity. These cells were found to be euploid, and thus did not represent transplanted hMSCs that had fused with host metanephric cells. Ten thousand cells were subjected to flow cytometric analysis. Experiments were performed in quadruplicate and representative figures are shown.

opmental age of rat embryos cultured in this way appeared consistent with E13 embryos that had developed *in utero* (Fig. 1*a*). At this stage, ureteric buds were elongated and initial branching was completed (data not shown), indicating that during culture, the metanephric mesenchyme had been stimulated to undertake the initial step of commitment toward nephrogenesis. However, embryos could not develop further and died soon after 48 h because of insufficient development of the placenta *in vitro* (19). To overcome this limitation, whole-embryo culture was followed by organ culture. After whole-embryo culture for 48 h, metanephroi were dissected from embryos and subjected to organ culture for 6 days. Using this combination, which will be referred to as relay culture, kidney rudiments continued to grow *in vitro*, as assessed by the observation of fine tubulogenesis and ureteric bud branching (Fig. 1 *b* and *c*). Thus, the metanephros can complete devel-

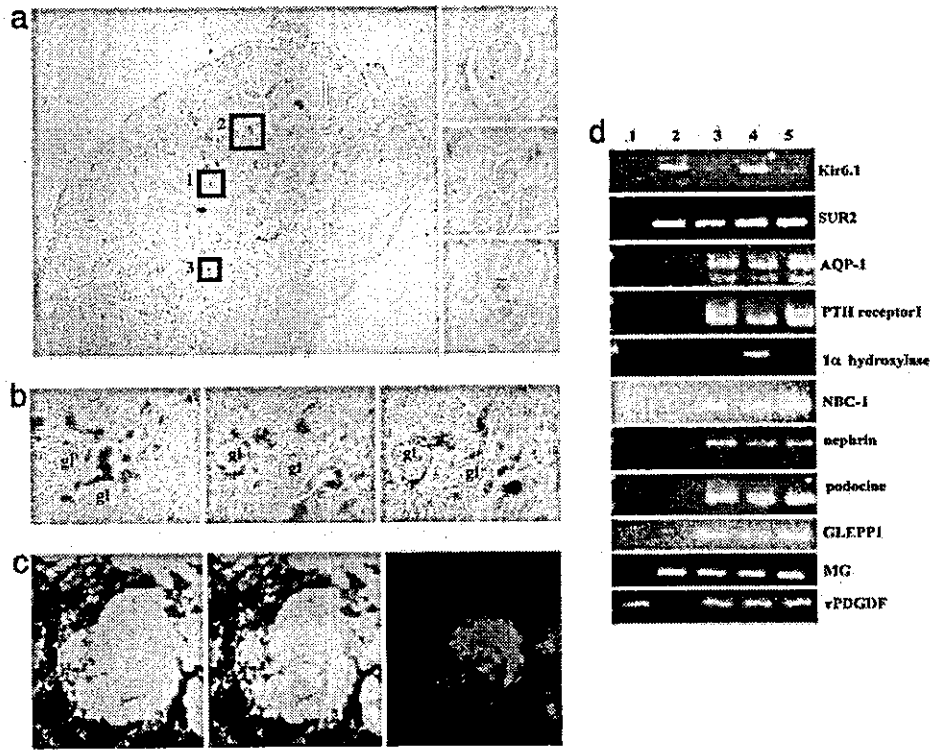

Fig. 3. Differentiation of transplanted hMSCs into organized, resident renal cells. (*a*) After relay culturing, the resulting metanephros was subjected to an X-gal assay to trace the transplanted hMSCs. The morphology of these LacZ-positive cells (shown under high magnification; ×400) and the renal structures to which they contributed, were consistent with them being glomerular epithelial cells (lane 1), tubular epithelial cells (lane 2), and interstitial cells (lane 3). (*b*) Serial sections were examined by light microscopy. Glomerular epithelial cells were linked to tubular epithelial cells (arrow head), and some of these cells formed a continuous tubular extension toward the medulla (arrow). gl, glomerulus. (*c*) Tissue sections were subjected to two-color immunofluorescent staining for β-gal (*Left*) and WT-1 (*Right*). (*Center*) A merged image is presented. (*d*) After relay culture, the resulting metanephroi were digested, and single cells were subjected to the FACS-galactosidase assay. LacZ-positive cells were sorted and subjected to RT-PCR for expression analysis of Kir6.1, SUR2, AQP-1, PTH receptor 1, 1α hydroxylase, NBC-1, nephrin, podocin, GLEPP1, human-specific β_2 microglobin (MG) and rat GAPDH. Lane 1, control rat metanephros; lane 2, hMSCs; lanes 3–5, regenerated metanephros from three individual experiments. Representative photographs are shown.

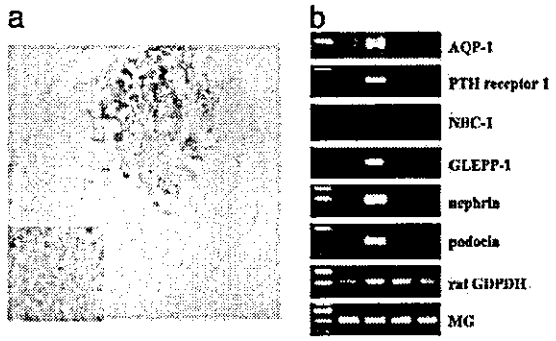

Fig. 4. Injection and culture of hMSCs in isolated metanephroi. hMSCs expressing the LacZ gene were retrovirally transfected with GDNF and injected into the cultured metanephroi (E13). (*a*) After 6 days of organ culture, the resulting metanephroi were subjected to an X-gal assay. (*Inset*) LacZ-positive cells at a high magnification are shown. Note that hMSCs-derived cells remain aggregated and do not form recognizable kidney structures. (*b*) RNAs were extracted and subjected to RT-PCR. Neogenerated kidney rudiment before (lane 2) and after (lane 3) organ culturing is shown. Mixture of metanephroi and hMSCs before (lane 4) and after (lane 5) organ culture is shown. Note that only the hMSCs differentiated in the whole embryo are able to express kidney-specific gene after organ culture. Lane 1, maker (ϕX174/*Hae*III).

opment *ex utero*, even if the embryo is dissected before the stage at which the ureteric bud sprouts.

Using this system, hMSCs were injected into rodent embryos at the site of organogenesis. To distinguish the donor-derived cells from host cells, hMSCs were labeled with the LacZ gene and DiI. A total of 1×10^3/embryo of labeled cells were then injected into the intermediate mesoderm between the somite and the lateral plate at the level of somite 29 for rat and somite 26 for mouse, which we previously estimated by *in situ* hybridization for *c-ret*, to be the ureteric budding sites (15). Successful injection was confirmed by *in situ* hybridization for human genomic *Alu*I/II, which identifies exclusively human cells, and injected hMSCs-derived cells were detected along the Wolffian duct (see Fig. 6, which is published as supporting information on the PNAS web site). After relay culture, LacZ-positive cells were detected in the metanephros (5.0 ± 4.2%), as measured by a FACS-galactoside assay of single cells derived from a dissected metanephros (Fig. 2*a*). No LacZ-positive cells were detected in the isolated kidney if the injection site was altered by >1 somite in length. In control embryos, injection of labeled mouse fibroblasts instead of hMSCs resulted in only a negligible number of LacZ-positive cells detected in the metanephros (data not shown). To enhance the number of integrated donor-derived cells, the hMSCs were further modified before injection to temporally express GDNF by using the adenovirus AxCAh-GDNF (11). GDNF is normally expressed in metanephric mesenchyme at this stage, and the interaction between GDNF and its receptor, c-ret, is required for epithelial-mesenchymal signaling to occur (10). The FACS-galactosidase assay revealed a significant increase in the number of LacZ-positive cells that were detected after transient GDNF expression (29.8 ± 9.2%, Fig. 2*b*). Importantly, 68.8 ± 11.4% of LacZ-positive cells in the neogenerated metanephros were euploid (Fig. 2*c*). The number of LacZ-positive cells were significantly increased ($2.84 \pm 0.49 \times 10^5$/metanephros) compared with the starting number of injected cells (1×10^3/embryo), suggesting that the remaining polyploid cells were mostly undergoing cell division. Furthermore, FISH, using the human and rat Y chromosome showed that a negligible number of cells were doubly positive for the Y chromosome (≈0.1%, see Fig. 7, which is published as supporting information on the PNAS web site). These data strongly suggest that, if any, only a small percentage of hMSC cells undergo cell fusion during differentiation.

During metanephric organ culture, DiI-positive cells migrated toward the medulla and dispersed in the kidney primordia (see Fig. 8, which is published as supporting information on the PNAS web site), suggesting that the transplanted cells become integrated in the host kidney. To confirm that these cells contribute to renal structures, the kidney primordia was subjected to an X-gal assay. LacZ-positive cells were scattered throughout the metanephric rudiment and were morphologically identical to the resident glomerular epithelial cells, tubular epithelial cells, and interstitial cells (Fig. 3*a*). Serial sections of metanephric rudiment showed glomerular epithelial cells linked to tubular epithelial cells (Fig. 3*b*, arrowhead). Some β-gal-positive cells of the S-shaped bodies were also positive for WT-1, which is strongly expressed in glomerular podocytes at this stage (20) (Fig. 3*c*). RT-PCR of FACS-sorted metanephric cells revealed that LacZ-positive cells expressed podocyte-specific genes (nephrin, podocine, and GLEPP-1) and tubular epithelial cell-specific genes (AQP-1, 1α hydroxylase, PTH receptor 1, and NBC-1) (Fig. 3*d*). In contrast to endogenous renal cells, ATP-sensitive K^+ channel subunit, Kir6.1/SUR2 (21), which is expressed in hMSCs, was still expressed after relay culture. Furthermore, when hMSCs were injected into cultured metanephroi (E13), cell dispersal was not observed, and the hMSCs remained aggregated. After 6 days in organ culture, the hMSCs failed to contribute to kidney structures (Fig. 4*a*), and did not express kidney-specific genes (Fig. 4*b*). These data suggest that during whole-embryo culture, hMSCs complete an initial step essential for commitment to a renal fate and that during organ culture, they further undergo a mesenchyme-to-epithelium transition or stromogenic differentiation.

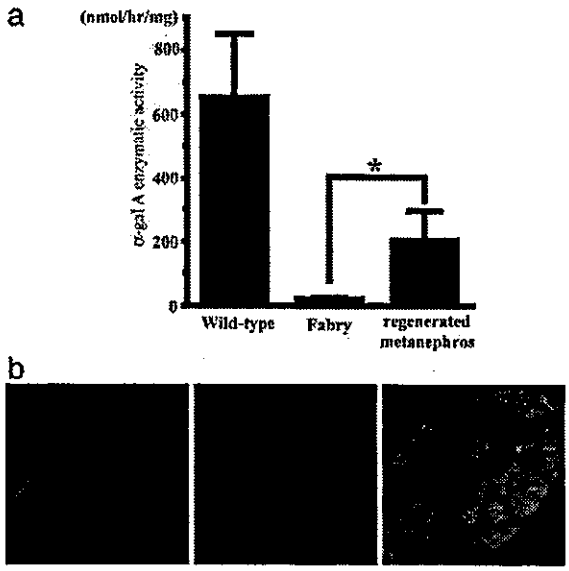

Fig. 5. Therapeutic kidney regeneration in an α-Gal A-null Fabry mouse. hMSCs were transplanted into E9.5 embryos of Fabry mice lacking the α-gal A gene and subjected to relay culture. (*a*) The α-gal A enzymatic bioactivity of resulting metanephroi was fluorometrically assessed as described (19). For the controls, metanephros from wild-type mice (*Left*) and Fabry mice (*Right*) were also subjected to the same protocol. The data are shown as the means ± SE. Asterisks indicate statistically significant differences ($P < 0.05$) between the two groups. (*b*) To confirm the potency of Gb3 clearance in resulting metanephroi, organ culture was performed in the presence of Gb3, and accumulation in the metanephroi was assessed by immunostaining for Gb3. Control metanephroi from wild-type mice (*Left*) and Fabry mice (*Right*) were subjected to the same analysis.

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To examine whether the hMSC-positive nephrons are viable, hMSCs were developed in the Fabry mouse (22), which does not express the gene encoding α-gal A enzyme. This defect leads to the abnormal accumulation of glycosphinogolipid mainly in glomerular podocytes and tubular epithelial cells, leading to renal failure after birth. The hMSCs were injected into E9.5 Fabry mouse embryos and subjected to relay culturing to regenerate the kidney. Compared with the wild-type mouse (655.0 ± 199.6 nmol per mg per hour), the basal level of α-gal A bioactivity in the metanephros from the Fabry mouse is low (19.7 ± 5.5 nmol per mg per hour), whereas chimeric kidney primordia expressed significantly higher amounts of α-gal A bioactivity (204.2 ± 98.8 nmol per mg per hour, $P < 0.05$, Fig. 5*a*). Furthermore, accumulation of the glycosphinogolipid Gb3 within the ureteric buds and S-shaped bodies (Fig. 5*b Right*) in the metanephros of the Fabry mouse was markedly resolved by integration of the hMSC-derived nephrons that possess α-gal A and act to normalize substrate metabolism in the surrounding host cells (Fig. 5*b Center*). This result indicates that the neogenerated nephrons were biologically viable.

In this study, we show that allowing hMSCs to grow in a specific organ location in whole-embryo culture can commit them to the fate of that organ. Injection of GDNF-transfected hMSCs into embryos followed by relay culture enables the generation of chimeric kidneys. In some cases, entire nephrons are hMSC-derived. These hMSC-derived cells are functional as tested by their ability to metabolize Gb3.

Thus, hMSCs could be reprogrammed for other fates and organ structures, depending on the embryonic environment into which they are. An added advantage of using hMSCs is that although they are mesodermal in origin, they have the potential to differentiate into cell types that are normally derived from ectoderm or endoderm (23). Thus, it might be possible to reconstitute organs such as liver and pancreas that, unlike the kidney, are derived from the endodermal germ layer. Furthermore, by changing the conditions of organ culture after the initiation of organ development during whole-embryo culture, a specific cell or simple-structured tissue, such as an endocrine gland, may be generated from autologous MSCs. Importantly, the host immune system is not sufficiently developed during this stage of whole-embryo culture, thus facilitating the tolerance to xenogenic cells. Here, we have demonstrated a system that might provide the means to generate self-organs from autologous MSCs by using the inherent developmental system of an immunocompromised xenogeneic host.

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