Anemia with Chronic Renal Disorder and Disrupted Metabolism of Erythropoietin in ICR-derived Glomerulonephritis (ICGN) Mice

Misuzu YAMAGUCHI-YAMADA[1], Noboru MANABE[1]*, Kozue UCHIO-YAMADA[1], Naotsugu AKASHI[1], Yasufumi GOTO[1], Youhei MIYAMOTO[1], Masaya NAGAO[2], Yoshie YAMAMOTO[3], Atsuo OGURA[4] and Hajime MIYAMOTO[1]

[1]*Unit of Anatomy and Cell Biology, Department of Animal Sciences,* [2]*Laboratory of Biosignals and Response, Department of Applied Molecular Biology, Kyoto University, Kyoto 606–8502,* [3]*Department of Veterinary Sciences, National Institute of Infectious Diseases, Tokyo 162–8640 and* [4]*Bioresource Center, RIKEN, Tsukuba 305–0074, Japan*

ABSTRACT. The ICR-derived glomerulonephritis (ICGN) mouse, a new inbred mouse strain with a hereditary nephrotic syndrome, is considered to be a good model of human idiopathic nephrotic syndrome and notably exhibits proteinuria and hypoproteinemia from the neonatal stage. In chronic renal disorder (CRD), anemia is a major subsequent symptom (renal anemia). The precise cause of renal anemia remains unclear, primarily owing to the lack of appropriate spontaneous animal models for CRD. To establish adequate animal models for anemia with CRD, we examined the hematological-biochemical properties and histopathological characteristics. With the deterioration of renal function, ICGN mice developed a normochromic and normocytic anemia, and exhibited normochromic and microcytic at the terminal stage. The expression of erythropoietin (EPO) mRNA both in the kidneys and liver and the EPO leak into the urine were observed in ICGN mice, indicating a disrupted metabolism of EPO in ICGN mice. In addition, a lack of iron induced by the hemolysis in the spleen and the leak of transferrin into urine as proteinuria aggravated the anemic condition. In conclusion, the ICGN mouse is a good model for anemia with CRD.
KEY WORDS: erythropoietin (EPO), hereditary nephrotic ICR-derived glomerulonephritis (ICGN) mouse, kidney, liver, renal anemia.

The ICR-derived glomerulonephritis (ICGN) mouse established in the National Institute of Infectious Diseases (NIID; Tokyo, Japan) is a novel inbred mouse strain with a hereditary nephrotic syndrome of unknown etiology, and considered to be a good model of human idiopathic nephrotic syndrome [13–16]. Homozygous ICGN mice show proteinuria at a young age, later develop hypoproteinemia, hyperlipidemia, severe anemia and systemic edema, and eventually die as a result of chronic renal disorder (CRD) [15]. Our previous studies [20–23, 25] showed that most of the renal tubules expanded and many kinds of extracellular matrix (ECM) components, both interstitial and basement membrane components, abnormally accumulated in glomeruli and tubulointerstitium of ICGN mouse kidneys. The progress of fibrotic degeneration in ICGN mouse kidneys may be caused by overproduction of ECM components, inhibition of ECM breakdown, and decreased activities of matrix metalloproteinases [22]. Apoptotic cells or proliferating cells were detected only in the kidneys of ICGN mice but not those of normal ICR mice [25]. In the kidneys of ICGN mice, apoptotic cells with large round nuclei were observed only in the tubulointerstitium, and proliferating cells were detected in epithelial cells of distal renal tubules. Age-dependent increases in apoptotic cell and proliferating cell densities were also noted. Such irregular cellular kinetics may cause renal dysfunction in ICGN mice.

Anemia with CRD (renal anemia) is a major subsequent symptom in patients with CRD. The hematocrit levels are less than 30% in CRD patients and the condition of renal anemia directly affects their quality of life [8, 18]. The lack of appropriate animal models for CRD means that the precise cause of renal anemia is unknown, however, it has been established that the lack of erythropoietin (EPO), a 30–34 kDa glycoprotein and classified as part of the hematopoietic cytokine family [6, 10, 17], is one of the conditions leading to anemia. *In vitro* studies have revealed that EPO regulates the proliferation, differentiation and survival of erythroid progenitor cells in bone marrow through the EPO receptor (EPOR)-mediated pathway [9]. Administration of recombinant human EPO (rhEPO) significantly improved the anemic condition in patients with CRD [8].

Based on our previous findings [13, 15], we believe that ICGN mice are an appropriate hereditary model to elucidate the precise cause of renal anemia compared with artificial models, i.e., drug-induced, antibody-induced and/or nephrectomized animal models. In the present study, to establish a hereditary animal model for renal anemia, we examined the hematological and biochemical properties of ICGN mice and the expression of EPO mRNA in the kidneys and the liver of ICGN mice.

MATERIALS AND METHODS

Animals and tissue preparation: Homozygous ICGN mice (10- to 30-week-age) were prepared by mating homozygous males (*nep/nep*) and heterozygous females (*nep/–*), and age- and sex-matched ICR mice, which were

* CORRESPONDENCE TO: MANABE, N., Department of Animal Sciences, Kyoto University, Kyoto 606–8502, Japan.

purchased from Clea Japan (Tokyo, Japan) and used as healthy control. They were housed in autoclaved metal cages and were given a standard diet (CM; Oriental Yeast, Tokyo, Japan) and tap water *ad libitum* in an air-conditioned room ($23 \pm 1°C$) under controlled lighting conditions (12 hr light/12 hr dark). They received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" (Kyoto University Animal Care Committee according to NIH No. 86–23; revised 1999). At 2 hr before sacrifice, all mice were intraperitoneally administered 5-bromo-2'-deoxy-yuridine (BrdU; 100 mg/kg body weight; Sigma Aldrich Chemicals, St. Louis, U.S.A.). After collecting urine samples from each mouse, blood samples were obtained from the cervical vein under ether anesthesia and used for hematological and biochemical analyses and for enzyme-linked immunosorbent assay (ELISA) for EPO. Consequently, the animals were sacrificed under deep ether anesthesia, and then the kidneys, liver, spleen, and thigh bone were rapidly removed. A part of each kidney, liver, spleen and thigh bone sample was fixed in 10% neutral-buffered formalin, pH 7.4, for histopathological and histochemical analyses. For reverse transcription-polymerase chain reaction (RT-PCR) analysis, the remaining part of each kidney, liver and spleen sample was frozen in liquid nitrogen.

Hematological and biochemical analyses and ELISA for EPO: Hematological analysis was performed with an automatic counter (K-4500; Sysmex Co., Tokyo, Japan) according to the manufacturer's protocol. Reticulocytes were counted on glass slides after preparing blood smears were stained with brilliant cresyl blue (Wako Pure Chemicals, Osaka, Japan), and the rate of reticulocytes was calculated. Serum biochemical analysis was performed with an automatic analyzer (Fuji Drichem 3500V; Fuji Film, Tokyo, Japan) according to the manufacturer's instruction. Serum iron, total iron-binding capacity (TIBC), and unsaturated iron-binding capacity (UIBC) were measured by a Fe-B test kit (Wako) according to the manufacturer's instruction. As previously reported, serum and urine EPO levels were determined by sandwich-ELISA method using R2- and R6-monoclonal antibodies [12].

Histopathological and histochemical analyses: Formalin-fixed samples were dehydrated through a graded ethanol series and embedded in Histosec (Merck, Darmstadt, Germany). Serial sections 4 μm thick were mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Sigma), deparaffinized with xylene and rehydrated through a graded ethanol series. For histopathological evaluation, each section was stained with hematoxylin and eosin. The degree of ECM deposition was assessed on the kidney sections stained with Sirius red solution (saturated picric acid in distilled water containing 0.1% Sirius red F3B; BDH Chemicals, Poole, UK) [11]. To assess hemolysis in the spleen, the spleen sections were stained with Berlin blue (Wako), which can detect the residues after erythrophagia (hemosiderin/ferric iron). After the staining, the slides were mounted with Entellan (Merck) and examined by light microscopy (at least 3 sections of each tissue/mouse). As described previ-

ously [25], proliferating cells in the bone marrow of thigh bone were histochemically detected by BrdU labeling technique using an anti-BrdU monoclonal antibody (Sigma).

Western immunoblotting: Protein concentrations in urine samples were measured by Bradford methods. Equal amounts of protein (300 μg/m*l*) were separated by 5–20% gradient SDS-PAGE gels (Bio-Rad Laboratories, Hercules, U.S.A.) and then transferred onto PVDF membranes (Millipore, Billerica, U.S.A.). Western immunoblotting was performed by the use of rabbit polyclonal anti-transferrin (1:3,000 dilution; Inter-cell Technologies, Hopewell, U.S.A.), and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2,000 dilution; Dako Cytomation, Copenhagen, Denmark) was used to identify the sites of primary antibody binding. The chemiluminescence was visualized by an ECL system (Amersham Biosciences, Piscataway, U.S.A.) according to the manufucture's protocols, and recorded with a digital fluorescene recorder (LAS-1000; Fuji Film). Each chemiluminescence intensity was quantified with Image-Gauge software (Fuji Film) on a Macintosh computer.

RT-PCR for EPO mRNA: The expression of EPO mRNA in the kidneys, liver, spleen of ICR and ICGN mice was examined by RT-PCR according to Masuda *et al.* [12]. Briefly, total RNA was extracted from each tissue sample with an RNeasy mini kit (Qiagen, Chastsworth, U.S.A.), and then the RNA was reverse-transcribed by the use of a Ready-to-go T-primed first-strand kit (Amersham Biosciences) to synthesize first strand cDNA according to the manufacturer's protocols. The first strand cDNA prepared from each tissue sample was mixed with the PCR reaction mixture (Pratinum PCR supermix; Gibco BRL, Grand Island, U.S.A.) containing $1 \times$ PCR buffer, 0.1 mM dNTP mixture, 1.5 mM $MgCl_2$, 0.5 μM each primer pair and 0.025 units/μl Platinum Taq DNA polymerase. Specific primer pairs for partial cDNA sequences of the EPO (NCBI: #M12930) were used as follows, forward: 5'-TCCTT GCTAC TGATT CCTCT GG-3' and reverse: 5'-AAGTA TCCGC TGTGA GTGTT CG-3'; expected PCR product size, 451 bp. As an intrinsic control, β-actin (NCBI: #M12481) was amplified with the following primers, forward: 5'-TCCTA TGTGG GTGAC GAGGC-3' and reverse: 5'-TACAT GGCTG GGGTG TTGAA-3'; expected PCR product size, 245 bp. The mixtures were subjected to PCR on a thermal cycler (GeneAmp PCR Systems 2400; PE Applied Biosystems, Foster City, U.S.A.). Hot start-PCR conditions for EPO were 94°C for 5 min, 40 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 1 min, and then 72°C for 5 min. Those for β-actin were 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and then 72°C for 5 min. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide (Wako). After electrophoresis, the gels were recorded with a digital recorder (LAS-1000). To confirm the expression of EPO mRNA, the DNA sequence of the PCR product was determined with an automatic DNA sequencer (ABI prism 310 Genetic Analyzer; PE Applied Biosystems) according to the

manufacturer's protocol.

Statistical analysis: ANOVA with Fisher's least significant differences test was performed for biochemical and hematological data with StatView IV (Abacus Concepts, Berkely, U.S.A.) on a Macintosh computer. Each biochemical and hematological value represents mean ± SE. Differences of $P<0.01$ and 0.05 were considered significant.

RESULTS

Hematological and biochemical data and serum EPO level: No significant correlation between serum creatinine level (Cre) and hematocrit (Ht) was seen in ICR mice, but negative correlation between them was seen in ICGN mice (Fig. 1A), indicating ICGN mice develop a marked anemia with the deterioration of renal function. Aged ICGN mice with high Cre (higher than 0.60 mg/d*l*) showed edema as a terminal symptom, and their ears and tails looked pale.

Based on Cre, ICGN mice were categorized into three stages: latent, progressing and terminal stages. ICGN mice with less than 0.34 mg/d*l* (mean plus standard deviation of ICR mice: 0.27 plus 0.07) of Cre were categorized into the latent stage, and those with 0.35–0.54 mg/d*l* (twice the mean of ICR mice) and those with higher than 0.55 mg/d*l* were classified into progressing and terminal stages, respectively.

There was no distinct correlation between Ht and serum EPO levels in ICGN mice. Serum EPO levels according to Ht were dispersed both in the ICR and ICGN mice (Fig. 1B). However, serum EPO levels tend to decrease in terminal stage-ICGN mice (Table 1).

As shown in Table 2, significant decreases of Ht (35.8, 34.7 and 32.0% in latent, progressing and terminal stages, respectively), hemoglobin concentration (11.6, 11.3 and 10.5 g/d*l*, respectively) and red blood cell count (669.0, 646.0 and 625.5 × 10^4/μ*l*, respectively) were shown in ICGN mice when compared with those in the ICR mice, indicating that ICGN mice develop a distinct anemia as the deterioration of renal function progressed. An increased rate of reticulocytes was seen in ICGN mice. The mean corpuscular volume (MCV) remained stable in both the latent
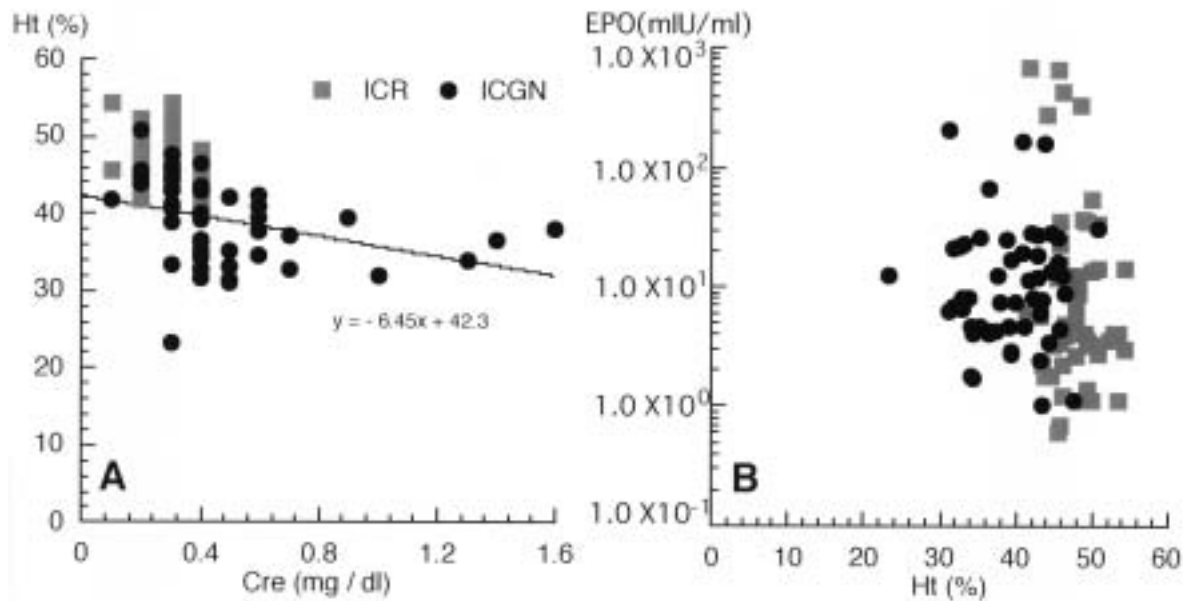

Fig. 1. Correlation between serum creatinine levels (Cre) and hematocrit values (Ht) (A), and Ht and peripheral serum erythropoietin concentration (EPO) (B) in ICR (closed squares) and ICGN (closed circles) mice. A: In ICGN mice, Cre is correlated with Ht. Increased Cre indicates the decline of renal function, and decreased Ht indicates the development of anemia. In ICR mice, steady levels of Cre and Ht were seen. B: Serum EPO is variable in both ICR and ICGN mice, and no significant correlation between Ht and EPO was seen.

Table 1.   Biochemical features in the serum of ICR and ICGN mice

Parameters	ICR mice (n=46)	ICGN mice (n=49)		
		Latent stage	Progressing stage	Terminal stage
Serum Cre (mg/d*l*)	0.26 ± 0.01	0.27 ± 0.01	0.43 ± 0.01*	0.84 ± 0.09*
Serum EPO (mIU/m*l*)	55.4 ± 22.1	30.7 ± 11.0	22.8 ± 10.6	8.6 ± 1.7

Results of biochemical testing of 12-week-old ICR mice and ICGN mcie. Data in ICR mice and ICGN mice represent mean value ± SE. *: $P<0.01$, versus ICR group.

Table 2. Hematological features in the serum of ICR and ICGN mice

Parameters[a]	ICR mice (n=9)	ICGN mice (n=9)		
		Latent stage	Progressing stage	Terminal stage
Ht (%)	45.9 ± 1.48	35.8 ± 1.22**	34.7 ± 0.80*	32.0 ± 2.00**
RBC ($10^4/\mu l$)	832.1 ± 25.0	669.0 ± 27.6**	646.0 ± 2.0*	625.5 ± 24.5**
Hemoglobin (g/dl)	14.1 ± 0.45	11.6 ± 0.49*	11.3 ± 0.25*	10.5 ± 0.35**
MCV (fl)	55.2 ± 0.69	53.5 ± 0.69	53.8 ± 0.25	51.1 ± 1.2
MCH (pg)	16.9 ± 0.24	17.3 ± 0.12	17.5 ± 0.05	16.7 ± 0.10
MCHC (g/dl)	30.7 ± 0.28	32.4 ± 0.34*	32.4 ± 0.00	32.8 ± 0.95*
Reticulocytes (%)	0.27 ± 0.27	0.40 ± 0.40	0.95 ± 0.56	3.27 ± 0.00*
WBC ($10^2/\mu l$)	62.0 ± 13.0	86.0 ± 15.7	89.0 ± 3.0	70.0 ± 11.0
Platelets ($10^4/\mu l$)	113.6 ± 9.4	134.8 ± 9.5	131.9 ± 8.7	155.1 ± 23.3

Results of hematological testing of 12-week-old ICR mice and ICGN mice. Data in ICR and ICGN mice represent mean value ± SE. * and **: $P<0.05$ and 0.01, respectively, versus ICR group. a) Ht=hematocrit; RBC=red blood cells; MCV=mean corpuscular volume; MCH=mean corpuscular hemoglobin; MCHC=mean corpuscular hemoglobin concentration; WBC=white blood cells.

stage and progressing stage, but decreased in terminal stage-ICGN mice. The mean corpuscular hemoglobin (MCH) remained unchanged in ICGN mice. The mean corpuscular hemoglobin concentration (MCHC) significantly increased in ICGN mice. Thus, the anemia in ICGN mice was diagnosed as normochromic and normocytic anemia. In addition, the iron concentration in the serum was decreased in ICGN mice at the terminal stage (not significantly), when the anemia tended to be normochromic and microcytic.

Histopathology and histochemistry in the kidney, bone marrow and spleen: In the kidney sections of ICGN mice, expansion of renal tubules and mesangium, appearance of cysts, and glomerular-fibrosis (Fig. 2) were observed in correspondence with the progression of renal disorder (increased Cre) as previously reported [21–23, 25]. No obvious differences in histological findings of bone marrow were demonstrated between ICR and ICGN mice (Fig. 3A and B, respectively). A lot of proliferating cells detected by BrdU-labeling were observed in the bone marrow of the ICR mice, but fewer proliferating cells were detected in those of the terminal stage-ICGN mice (Fig. 3C and D, respectively).

Some positive staining of Berlin blue was demonstrated in the spleen sections of ICR mice, but excessive positive staining was seen in those of ICGN mice at terminal stage (Fig. 4A and B, respectively). Unexpectedly, positive staining for Berlin blue was found in the kidneys of ICGN mice (Fig. 4D and E) but not ICR mice (Fig. 4C). A number of the positive granular blue spots were concentrated in the tubulointerstitium, the glomeruli and the epithelial cells of intact proximal tubules. The kidneys of progressing stage-ICGN mice showed stronger reactions than those of terminal stage-ICGN mice.

Biochemical data of iron metabolism and protein leaks into urine: ICGN mice exhibit severe proteinuria in comparison with ICR mice. As shown in Table 3, decreases of serum total protein (6.1, 3.7 and 3.8 g/dl, in latent, progressing and terminal stages, respectively) and serum albumin (2.5, 1.6 and 1.7 g/dl, respectively) were observed. Increases of total protein in urine (3.3, 9.8 and 9.3 g/dl,

respectively) were also observed. Interestingly, urine EPO was detected only in progressing and terminal-stage ICGN mice, though it was not detected thoroughly in ICR mice and latent-stage ICGN mice.

Disturbance of iron metabolism was also observed in ICGN mice. The level of serum iron decreased (0.47, 0.36 and 0.31 mg/dl, in latent, progressing and terminal stages-ICGN mice, respectively), and total iron-binding capacity decreased as well (0.76, 0.80 and 0.52 mg/dl, in latent, progressing and terminal stages-ICGN mice, respectively). Transferrin in urine was observed only in ICGN mice by immunoblotting (Fig. 5), and the intensity of the bands increased as the renal functions deteriorate.

Changes in EPO mRNA expression in the kidney and liver: In ICR mice and latent stage-ICGN mice, EPO mRNA was detected in the kidneys alone (Fig. 6). Interestingly, lower levels of serum EPO, less than 5 mIU/ml, were shown in the ICR mice and latent stage-ICGN mice, in which no EPO mRNA expression in the kidneys was seen. Higher levels, more than 20 mIU/ml, were noted in the ICR mice and latent stage-ICGN mice, in which EPO mRNA expression in the kidneys was detected. In progressing stage-ICGN mice, three types of EPO mRNA-expression profile were noted as follows: EPO mRNA was detected in the kidneys alone, both in the kidneys and the liver, and in the liver alone. Finally, no detectable EPO mRNA was shown in the kidneys or liver of the terminal stage-ICGN mice, in which trace level of serum EPO was seen. No expression of EPO mRNA was detected in the spleen of ICR or ICGN mice.

DISCUSSION

It is a frequent and consistent complication that anemia develops during the course of human CRD [8, 18]. ICGN mice with a hereditary and spontaneous nephrotic syndrome exhibit proteinuria, hypoproteinemia, hyperlipidemia, and systemic edema [13–15]. In the present study, we divided ICGN mice into three categories according to the increased level of serum Cre, and then hematological and histopathological analyses were performed. Anemia developed mark-
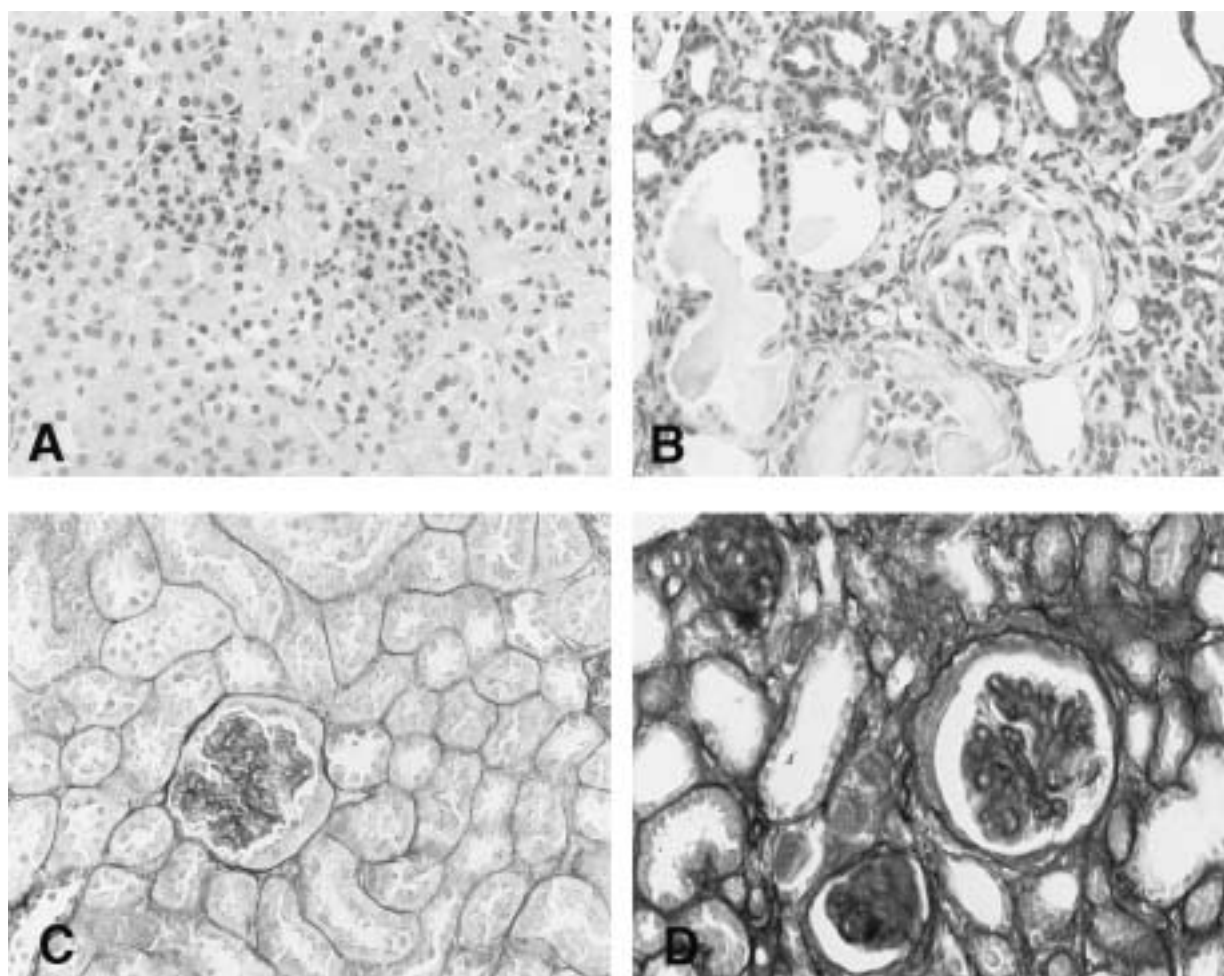
Fig. 2.   Kidney sections prepared from 10-week-old ICR (A and C) and 30-week-old ICGN mice in terminal stage (B and D). A and B: Kidney sections were stained with hematoxylin and eosin. In the terminal stage-ICGN mice, sclerotic glomeruli and highly expande d or occluded renal tubules were observed (× 200). C and D: Extracellular matrix (ECM) components were stained with Sirius red. ECM abnormally accumulates in both interstitial and basement membrane components, in glomeruli and tubulointerstitium (× 200).

edly in ICGN mice with the deterioration of renal function. Hematological data confirmed that ICGN mice at the progressing and terminal stages had normochromic and normocytic anemia, which is consistent with clinical reports on patients with renal anemia [8, 18]. Moderate anemia shown in ICGN mice with latent renal disorder is considered to have a protective role in renal function, in other words, to decrease the blood pressure of glomerular filtration. However, the anemia becomes a harmful subsequent symptom as renal injury progresses in progressing stage-ICGN mice, probably induced by disturbing the blood pressure and oxygen sensing system which regulates the EPO production in the kidneys.

EPO, a blood-circulating cytokine, controls erythropoiesis in the bone marrow, and regulates the proliferation, differentiation and survival of erythroid progenitor cells through EPOR-mediated signal transduction [6, 8–10, 17]. Due to its strong erythropoietic effect, rhEPO has been gen-

erally used as therapeutic agent for patients with renal anemia. In patients with anemia, EPO mRNA is up-regulated, which is induced by hypoxic conditions. EPO mRNA in anemic or hypoxic conditions is up-regulated two hundred-fold higher than that in the normal state [2], and EPO levels in peripheral blood serum drastically increased, a few hundred-fold, in patients with acute anemia [3, 28]. Thus, EPO production is stringently regulated, and EPO is abruptly produced in large quantities only when required. In patients with CRD and chronic renal anemia, wide dispersion of serum EPO levels was reported [3, 7, 27]. Wide dispersion of serum EPO levels was observed in ICGN mice at latent and progressing stages, and higher levels of serum EPO was shown in ICGN mice with moderate renal anemia. However, the maximum levels of serum EPO in ICGN mice were much lower than those of ICR mice. In addition, EPO were detected only in the urine samples of progressing and terminal-stage ICGN mice, though it was not detected thoroughly
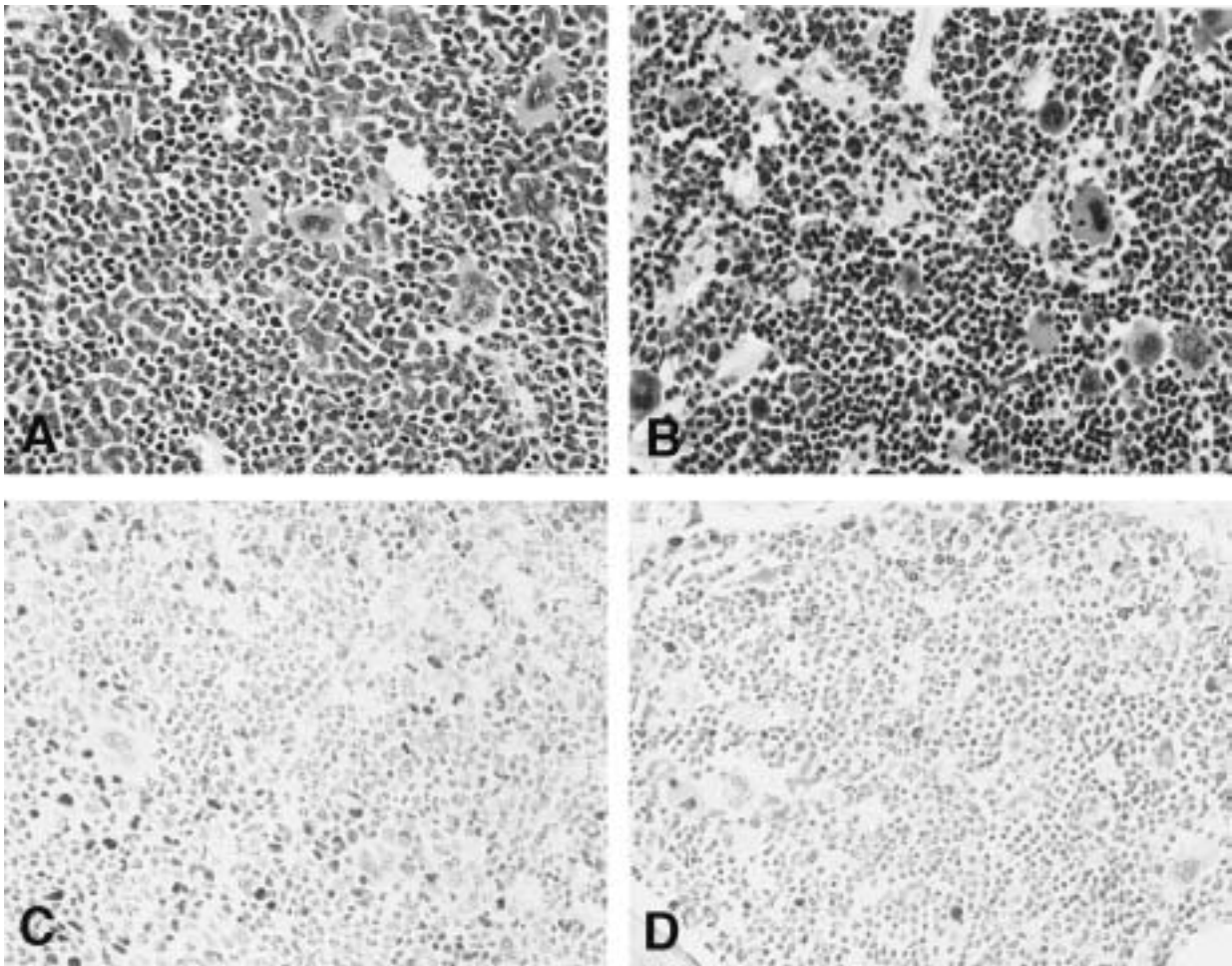
Fig. 3. Bone marrow sections prepared from ICR (A and C) and terminal stage-ICGN mice (B and D). A and B: No obvious difference in the bone marrow sections stained with hematoxylin and eosin was observed between ICR and ICGN mice (× 400). C and D: Proliferating cells were histochemically demonstrated by 5-bromo-2'-deoxyuridine (BrdU) labeling. A lot of BrdU-positive proliferating cells were observed in the bone marrow sections of ICR mice, but fewer proliferating cells were detected in those of terminal stage-ICGN mice (× 400).

in ICR mice and latent-stage ICGN mice. We surmise that increased levels of serum EPO in ICGN mice is insufficient not to ameliorate their anemia, and leak of serum EPO into urine disturbs the amelioration.

We hypothesize two possible causes of renal anemia in ICGN mice. One is a deficiency of the EPO production system in the kidneys. The other is an abnormality in the EPO receiving system, including EPOR, and the EPOR-mediated intracellular signal transduction pathway in the bone marrow erythroblastic cells. Our preliminary results showed that a subcutaneous injection of rhEPO (5 IU/mouse daily for 5 times; Sigma) to ICGN mice with moderate anemia increased Ht and improved their anemic symptoms (data not shown). In the present study, moreover, we demonstrated normal histopathological and histochemical observations in the bone marrow of ICGN mice. These findings indicate that ICGN mice have no abnormality in the EPO receiving

system in the erythroblastic cells. The kidney is a principal organ of EPO production, accounting for more than 90% under normal conditions, with extra-renal sources estimated at less than 10% [2, 19, 29]. The liver is the primary site of EPO synthesis in embryos and a major organ of extra-renal EPO production, and the brain, spleen, testis and uterus also produce EPO [5, 19, 26]. In progressing stage-ICGN mice, EPO mRNA was expressed not only in the kidneys but also in the liver, and no expression of EPO mRNA was detected in either the kidneys or liver in terminal stage-ICGN mice. Interestingly, the ICR mice with EPO mRNA expression in the kidneys showed higher serum EPO levels, indicating that higher serum EPO levels can be maintained by the production of EPO in kidneys alone. The progressing stage-ICGN mice with the EPO mRNA expression in the kidneys and liver showed similar levels of serum EPO in ICR mice. We surmise that sustained levels of serum EPO in progress-
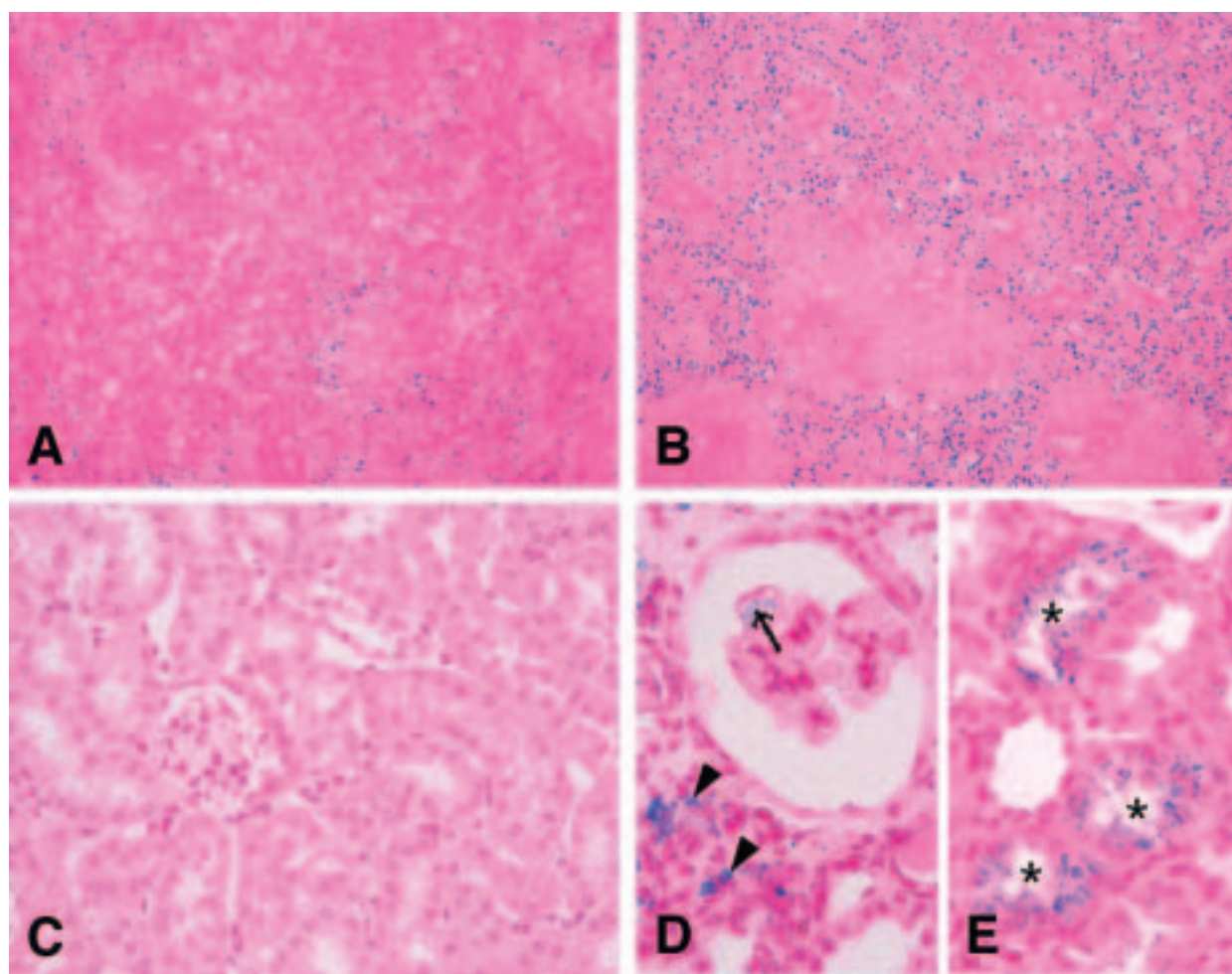
Fig. 4. Spleen and kidney sections prepared from ICR (A and C) and progressed stage-ICGN mice in (B, D and E) stained with Berlin blue. A and B: Few Berlin blue-positive granules were seen in the spleen sections of ICR mice, but excessive positive granules were noted in those of terminal stage-ICGN mice ($\times$ 100). C, D and E: Positive reactions for Berlin blue were also found in the kidney sections of the ICGN mice (D and E) but not of ICR mice (C). A number of Berlin blue-positive granules were concentrated consecutively in the glomeruli (arrow in D), the tubulointerstitium (arrowheads in D) and epithelial cells of intact proximal tubules (asterisks in E) ($\times$ 400).

Table 3. Hematological features in the serum and urine of ICR and ICGN mice

Parameters[a]	ICR mice (n=10)	ICGN mice (n=11)		
		Latent stage	Progressing stage	Terminal stage
Fe (mg/dl)	0.40 ± 0.03	0.47 ± 0.07	0.36 ± 0.04	0.31 ± 0.11
TIBC (mg/dl)	0.71 ± 0.04	0.76 ± 0.12	0.80 ± 0.02	0.52 ± 0.05
UIBC (mg/dl)	0.35 ± 0.04	0.29 ± 0.12	0.44 ± 0.05	0.21 ± 0.06
Serum TP (g/dl)	4.7 ± 0.10	6.1 ± 0.55	3.7 ± 0.49	3.8 ± 0.50
Serum Alb (g/dl)	1.9 ± 0.14	2.5 ± 0.45	1.6 ± 0.13	1.7 ± 0.30
Serum EPO (mIU/ml)	83.7 ± 62.6	19.5 ± 8.0	49.1 ± 21.3	5.2 ± 2.3
Urine TP (g/dl)	6.6 ± 1.3	3.3 ± 2.0	9.8 ± 3.5	9.3 ± 5.5
Urine EPO (mIU/ml)	ND	ND	3.4 ± 1.3	3.8 ± 3.8

Results of hematological testing of 12-week-old ICR mice and ICGN mice. Data in ICR and ICGN mice represent mean value ± SE. ND: Not detected. a) TIBC=total iron-binding capacity; UIBC=unsaturated iron-binding capacity; Cre=creatinine; TP=total protein; Alb=albumin; EPO=erythropoietin.

ing stage-ICGN mice may be the result of EPO production in the liver, or extra-renal EPO production, and that the decrease of serum EPO level in terminal stage-ICGN mice is related to the degradation of the EPO-producing cells both
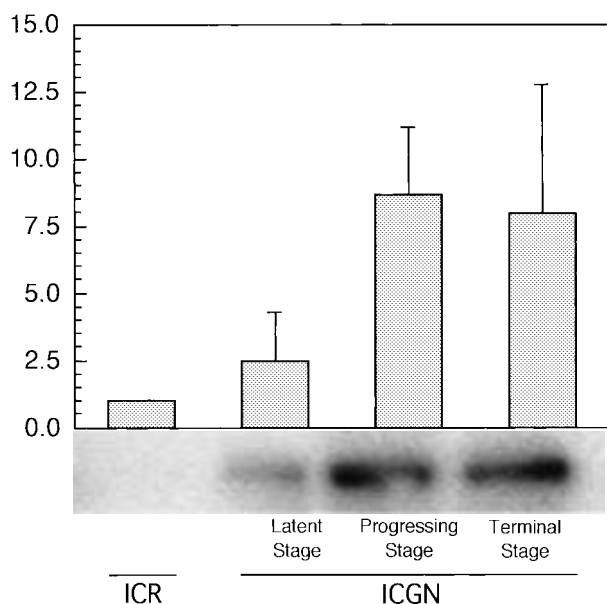
Fig. 5.   The expressions of transferrin in urine detected by West-
ern immunoblotting. The clear bands were detected only in
ICGN mice and the intensity increased as the renal function
deteriorates.

anemia. These histopathological findings on the spleen and
increased rate of peripheral reticulocytes indicated that the
fragility of erythrocytes increased and the life span of eryth-
rocytes shortened in ICGN mice with anemia. Interestingly,
a number of Berlin blue-positive granules were concen-
trated consecutively in glomeruli, tubulointerstitium and
epithelial cells of proximal tubules in the kidney sections of
progressing stage-ICGN mice. We suppose that the noxious
effect of the excluded ferric iron into tubule fluid causes
tubular degeneration in the kidneys of ICGN mice. Recent
studies pointed out the relation between the urinary iron and
the formation of free radicals in the kidneys of patients with
chronic renal failure [1, 24]. The iron is enabled to catalyze
the Haber-Weiss reaction with the formation of reactive
oxygen species [1]. To avoid the toxicity by the oxygen spe-
cies, iron is usually presented in a nonreactive form as trans-
ferrin-bound iron in the blood, or ferritin and hemosiderin in
the tissues. In patients with nephrotic syndrome, excess
protein excretion, including transferrin excretion into urea,
is a serious problem [4]. Transferrin leaked into tubule fluid
dissociates iron below at pH 6.0 as in acidic urine [4], and
the reactive formed ferric iron consequently induces free
radical formation. ICGN mice at the progressing and termi-
nal stages exhibit acidic urine (below pH 6.0; data not
shown). We detected transferrin only in the urine of ICGN
mice by immunoblotting, and this indicates that increasing
levels of transferrin leaked from serum into urine spurs on
the anemic conditions of ICGN mice. We presume that the
consecutive Berlin blue positive-depositions in the intact
proximal tubules in the kidneys of ICGN mice are due to the
reabsorption of ferric iron from urea, and that reabsorbed
ferric iron may causes tubulo-interstitial cell injury in those
mice. Further study is necessary to elucidate the iron distur-
bance and noxious effect in ICGN mice.

in the kidneys and liver.

Aged erythrocytes are degraded in the spleen, and ferrous
iron (Fe^{2+}) contained in the erythrocytes is removed as
hemosiderin and reused to produce new erythrocytes in the
bone marrow. Hemolysis in the spleen was histopathologi-
cally assessed by Berlin blue staining, which specifically
detects ferric iron (Fe^{3+}) and is an appropriate technique to
assess hemosiderin deposition in the spleen. Many strong
Berlin blue-positive depositions were observed in the spleen
of terminal stage-ICGN mice, indicating accelerated hemol-
ysis occurs in those mice. With the result that the serum iron
concentration decrease in the terminal stage-ICGN mice,
hemolysis is considered to be harmful factor for developing

Due to the lack of suitable model animals to study hered-
itary renal diseases and renal anemia, most studies have
been performed by the use of artificial models, for example,
drug-induced nephrosis models, antibody-induced nephro-
sis models, and surgically nephrectomized models. How-
ever, the artificial models did not display the same

Fig. 6.   The expressions of erythropoietin (EPO) and β–actin mRNAs in the kidneys (K) and livers (L) of control ICR mice, progressing
stage-ICGN mice and terminal-stage ICGN mice. Each mRNA was amplified by RT-PCR, and the PCR product was separated by 2%
agarose gel electrophoresis and visualized by ethidium bromide staining.

conditions leading to chronic renal anemia as those in patients. As described above, idiopathic nephrotic syndrome and severe renal anemia develop in ICGN mice with aging, and thus we conclude that the ICGN mouse is an appropriate model to investigate the precise cause of renal anemia.

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