FULL PAPER *Anatomy*

Dysfunction of Erythropoietin-Producing Interstitial Cells in the Kidneys of ICR-derived Glomerulonephritis (ICGN) Mice

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ABSTRACT. Anemia is a major secondary symptom in chronic renal disorder (CRD), but the precise cause of insufficient production of erythropoietin (EPO) remains unclear owing to the controversial localization of EPO-producing cells in the kidneys. The ICR-derived glomerulonephritis (ICGN) mouse, a new hereditary nephrotic mouse, is an appropriate model of anemia associated with CRD. By using an amplified *in situ* hybridization technique, we detected and counted the renal EPO-producing cells under both normoxic and hypoxic conditions. The expression levels of renal EPO mRNA were quantified and oxygen gradients were also assessed immunohistochemically. Amplified *in situ* hybridization clarified that EPO-producing cells were peritubular interstitial cells in the middle region of renal cortex in both ICR and ICGN mice. Hypoxia (7% O_2) induced low oxygen tension in proximal tubular epithelial cells of renal cortex, and increased the expression of EPO mRNA and the number of EPO-producing cells in both ICR and ICGN mice. However, hypoxia did not increase the serum EPO levels in ICGN mice. The ICGN mouse is a good model for anemia associated with CRD, and the suppression of EPO protein production in the renal EPO-producing cells is considered to be a potential cause of anemia associated with CRD.
KEY WORDS: amplified *in situ* hybridization, anemia with chronic renal disorder (CRD), erythropoietin (EPO)-producing cell, hereditary nephrotic ICR-derived glomerulonephritis (ICGN) mouse, kidney.

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Anemia associated with chronic renal disorder (CRD) is a major secondary symptom that appears in the early stages of renal disease. This anemia is initially mild; however, ultimately, severe anemia directly affects the quality of life (QOL) [25, 37]. Anemia associated with CRD is normochromic and normocytic, and, based on case studies, insufficient serum levels of erythropoietin (EPO) are considered to be a major cause [6, 25, 37].

EPO, a 30–34 kDa glycoprotein, is classified as part of the hematopoietic cytokine family [5, 14, 24], controls the erythropoiesis in the bone marrow, and regulates the proliferation, differentiation and survival of erythroid progenitor cells through EPO receptor (EPOR)-mediated signal transduction [5, 12, 14, 24]. The kidney is a principal organ of EPO production, accounting for more than 90% under normal conditions, with extra-renal sources estimated to produce than 10% [3, 26]. Due to its strong erythropoietic effect, recombinant human EPO (rhEPO) is generally used as a therapeutic agent for anemia associated with CRD [7, 19]. However, the precise cause of the insufficient serum levels of EPO in anemia associated with CRD remains unclear, primarily owing to the lack of knowledge of the identity of the EPO-producing cells and how they are regulated in the kidneys.

Studies on anemia associated with CRD have been mainly accomplished by using artificial models of acute renal failure, i.e. drug-induced, antibody-induced and/or nephrectomized animal models. Compared with these experimental animals, the ICR-derived glomerulonephritis (ICGN) mouse established in the National Institute of Infectious Diseases (NIID; Tokyo, Japan) is a superior nephrotic model exhibiting chronic renal failure without any special management. The ICGN mouse is a novel inbred mouse strain with a hereditary nephrotic syndrome of unknown etiology, and is considered to be a good model of human idiopathic nephrotic syndrome [20–22]. Homozygous ICGN mice exhibit proteinuria at a young age, later develop hypoproteinemia, hyperlipidemia, severe anemia and systemic edema, and eventually die as a result of CRD [20, 21]. In the kidneys, most of the renal tubules expand and many kinds of extracellular matrix (ECM) components accumulate abnormally in glomeruli and the tubulointerstitium because of the overproduction and stabilization of ECM components, and the inhibition of ECM breakdown [9, 29, 31–33]. Irregular cellular kinetics are also noted in ICGN mice, and age-dependent increases in apoptotic cell and proliferating cell densities have been reported [34].

Our recent studies demonstrated that the ICGN mouse is an appropriate model for anemia associated with CRD [35]. ICGN mice develop a normochromic and normocytic anemia as the renal function deteriorates. Administration of rhEPO significantly improves the anemic condition in ICGN mice without histopathological changes in bone marrow or kidneys [36]. Markedly insufficient levels of serum EPO and urinary leakage of EPO are observed in ICGN

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mice. Urinary leakage of transferrin and hemolysis are accessory factors for aggravation of anemia as well. In the present study, we identified the EPO-producing cells in the kidneys by using an amplified *in situ* hybridization technique and measured the EPO mRNA in the kidneys. The findings about the EPO-producing competence of ICR-control mice compared to ICGN-anemic mice will contribute to elucidating the mechanism of the regulation of EPO production in the kidneys.

MATERIALS AND METHODS

Animals: Homozygous ICGN mice (10- to 30-week-old, n=61) were prepared by mating homozygous males (*nep/nep*) and heterozygous females (*nep/–*), and age- and sex-matched ICR mice (n=61) as healthy controls were purchased from Clea Japan (Tokyo, Japan). 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 ± 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). According to our previous report [35], ICGN mice were classified into three stages based on serum creatinine (Cre) levels: latent, progressing and terminal stages. Briefly, ICGN mice with less than 0.34 mg/d*l* Cre were categorized into the latent stage, while those with 0.35–0.54 mg/d*l* and those with higher than 0.55 mg/d*l* were classified into the progressing and terminal stages, respectively.

Hypoxic exposure: Hypoxic stimulation was induced by using an airtight cabinet with a flow of premixed gas containing 7% O_2 and 93% N_2. Both ICR mice and progressing-stage ICGN mice were placed into the hypoxic cabinet for 2 hr.

Tissue preparation: One hr before sacrifice, all mice were intraperitoneally administered pimonidazole hydrochloride; 60 mg/kg body weight (Chemicon International, Temecula, U.S.A.). 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. Subsequently, the animals were sacrificed under deep ether anesthesia, and then the kidneys were rapidly removed. A part of each kidney was fixed in 4% (v/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.2, for histopathological and histochemical analyses. For real-time RT-PCR analysis, the remaining part was frozen in liquid nitrogen.

Hematological and biochemical analyses and ELISA for EPO: Hematological analysis was performed using an automatic counter (K-4500; Sysmex Co., Kobe, Japan) according to the manufacturer's protocol. Serum biochemical analysis was performed using an automatic analyzer (Drichem 3500V; Fuji Film, Tokyo, Japan) according to the manufacturer's instruction. As previously reported [8, 35, 36], serum and urine EPO level were determined by the sandwich-ELISA method using R2- and R6-monoclonal antibodies.

Histopathological and immunohistochemical analyses: After fixation, kidney 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 Aldrich Chemicals, St. Louis, U.S.A.), deparaffinized with xylene and rehydrated through a graded ethanol series. For histopathological evaluation, each section was stained with periodic acid-Schiff (PAS) and Masson's trichrome stain. ECM deposition was stained dark blue. To assess oxygen gradients in the kidneys, immunohistochemical analysis was performed by the use of a Hypoxyprobe^{TM}-1 kit (Chemicon) according to the manufacturer's instructions. Positive reactions were visualized using 3,3-diaminobenzidine (DAB) solution (Dako Cytomation Co., Glostrup, Denmark) and positive cells were considered to suffer from hypoxia (pO_2<10mmHg). After the staining, the slides were mounted with Entellan (Merck) and examined by light microscopy.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR for EPO mRNA: The expression of EPO mRNA in the kidneys of ICR and ICGN mice was examined by RT-PCR and real-time RT-PCR. Briefly, total RNA was extracted from each tissue sample using an RNeasy mini kit (Qiagen, Chastsworth, U.S.A.), and then the 3 μg/organ of total RNA was reverse-transcribed to synthesize the first strand cDNA by using a Ready-to-go T-primed First-strand kit (Amersham Biosciences, Piscataway, U.S.A.). For RT-PCR, the first strand cDNA prepared from each tissue sample was mixed with the PCR reaction mixture (Platinum PCR supermix, Invitrogen, Carlsbad, U.S.A.) containing 1 × 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. The mixtures were subjected to PCR in a thermal cycler (GeneAmp PCR Systems 2400; Applied Biosystems, Foster City, U.S.A.). The following primer pair specific for the partial cDNA sequence of EPO (NCBI #M12930) was used, forward: 5'-TCTGC GACAG TCGAG TTCTG-3' and reverse: 5'-GGAGG AAGTT GGCGT AGACC-3'; expected PCR product size, 431 bp. As an intrinsic control, ribosomal protein 23 (Rpl 23; NCBI #AF287271) was adopted on the basis of microarray data, in which the ratio of this mRNA in ICR mice to that in ICGN mice was 1:1.1 (unpublished data; CodeLink Uniset Mouse 20K I Bioarray, Amersham Biosciences). It was amplified using the following primers, forward: 5'-GGCAT GACCT TCATG ACCTT-3' and reverse: 5'-GGGCA TTGAG ACATG AACCT-3'; expected PCR product size, 125 bp. The mRNA levels of EPO and Rpl 23 as intrinsic control were also quantified by using a Light-Cycler system (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions. The same primer pairs were utilized. Briefly, PCRs were performed in 20 μl of a reaction mixture (Light-Cycler-DNA master SYBR green I; Roche) containing each primer pair, nucle-

otides, Taq polymerase, and $MgCl_2$ optimized for each primer pair. PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide (Wako Pure Chemicals, Osaka, Japan). The quantification analysis was performed using Light-Cycler analysis software (Roche) on an IBM computer. The abundance of EPO mRNA was normalized to the abundance of Rpl 23 mRNA. To confirm the expression of EPO mRNA, the DNA sequences of the conventional and real-time RT-PCR products were determined using an automatic DNA sequencer (ABI Prism 310 Genetic Analyzer; Applied Biosystems) according to the manufacturer's protocol.

Synthesis of digoxigenin (DIG)-labeled cRNA probes for amplified in situ hybridization: The first strand cDNA prepared from each tissue sample was mixed with the PCR reaction mixture (Platinum PCR supermix; Invitrogen). To generate templates for the sense or antisense cRNA probes of partial EPO mRNA, the T7 phage RNA polymerase promoter was added to the same primers used for real-time RT-PCR, and consequently each product size was 453 bp. The mixtures were subjected to PCR in a thermal cycler (GeneAmp PCR Systems 2400). The DNA sequence of the PCR product was determined with an ABI Prism 310 Genetic Analyzer. This PCR product was purified by using a MiniElute PCR Purification Kit (Qiagen) and then DIG-labeled by using a DIG-RNA-labeling Kit (Roche), followed by the ethanol precipitation.

Amplified in situ hybridization for detecting EPO-producing cells: According to the protocol of *in situ* hybridization using biotinylated tyramide [10, 27], amplified *in situ* hybridization was performed to detect the localization of EPO mRNA. Four-micrometer-thick paraffin sections mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Matsunami Glass, Osaka, Japan) were deparaffinized with xylene and rehydrated through a graded ethanol series. After washing with phosphate buffered saline (PBS), the sections were immersed in 0.2 N HCl for 10 min. Post-fixation was performed with 4% PFA for 5 min, followed by digestion with 10 μg/m*l* proteinase K (Roche) for 20 min at 37°C. After sufficient rinsing with PBS, acetylation for 10 min, and immersion in 3% H_2O_2 for 1 hr to inactivate endogeneous peroxidase, the sections were dehydrated through a graded ethanol series. DIG-labeled sense or antisense cRNA probes for EPO mRNA were diluted to 2 μg/m*l* in Dako mRNA *in situ* hybridization solution (Dako) containing 60% formamide. Hybridization was performed at 50°C for 18 hr. After the wash in 2 × standard saline citrate (SSC) buffer containg 50% formamide for 30 min at 55°C, the sections were rinsed in three decreasing concentrations of SSC buffer: 2 × SSC, 0.2 × SSC, 0.1 × SSC, each for 20 min at 55°C. After a rinse with Tris buffered saline (TBS), the sections were preincubated with 0.5% casein (Wako) and incubated with peroxidase-conjugated rabbit anti-DIG antibody (Dako; diluted 1:400 with 0.5% casein) for 1 hr at room temperature (22–25°C). Following a rinse with TBS, the sections were incubated with 0.7 μM biotinyl tyramide (Sigma) supplemented with 1% H_2O_2 for 15 min at room temperature to amplify the reaction. After washes with TBS, the sections were incubated with peroxidase-conjugated streptavidin (Dako; diluted 1:500 with 0.5% casein) for 15 min at room temperature. DAB solution was used for visualization and the sections were counterstained with methylgreen (Dako). After a wash with distilled water, the slides were mounted with Entellan and examined by light microscopy (at least 6 sections per mouse). Positive cells in the whole kidney sections were counted under a light microscope in each microscopic field to calculate the EPO-producing cell densities. The relative area of the whole kidney was estimated by tracing around the kidney on paper and then cutting and weighing the paper. As negative controls, serial sections were hybridized with DIG-labeled sense cRNA probe for EPO mRNA or without probe, or incubated without anti-DIG antibody. As a positive control, the sections were hybridized with DIG-labeled oligo-DNA probe for 28S RNA as previously reported [11].

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

RESULTS

Histopathology of the kidneys, hematological and biochemical data and serum EPO level: As previously reported [9, 29, 31–36], sclerotic glomeruli, highly expanded or occluded renal tubules, and appearance of cysts were observed in the kidneys of the progressing stage of ICGN mice compared to ICR control mice. The brush border of the expanded proximal tubules showed weakened intensity of PAS staining, whereas the glomeruli showed stronger intensity (Fig. 1A and B). Masson's trichrome stain revealed that ECM components were abnormally accumulated both in glomeruli and the tubulointerstitium, indicating the progression of the renal disorder (Fig. 1C and D).

As described in our previous report [35], ICGN mice were categorized into three stages based on serum Cre levels: latent, progressing and terminal stages. ICR mice exhibited fairly uniform levels of serum Cre (mean value; 0.26 mg/d*l*); however, ICGN mice exhibited a significant increase in serum Cre (0.27, 0.43 and 0.84 mg/d*l*, in latent, progressing and terminal stages, respectively), indicating the deterioration of renal function (Table 1). In addition to the increased levels of serum Cre, hematocrit (Ht) was decreased significantly in ICGN mice (42.6, 37.4 and 37.1% in latent, progressing and terminal stages, respectively). Progressing-stage ICGN mice began to show a significant Ht decrease and edema, and had pale ears and tails.

The levels of serum EPO showed wide variation both in ICR and in ICGN mice, with the wide range of 0.6 to 690.0 mIU/m*l* in ICR mice. ICGN mice generally exhibited low serum EPO as the deterioration of renal function progressed

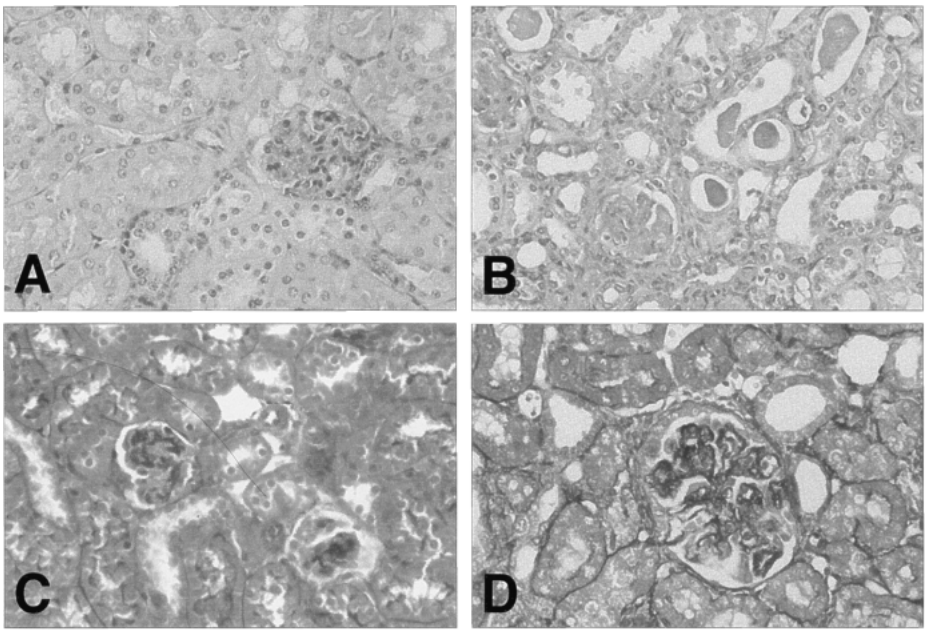

Fig. 1. Kidney sections prepared from 10-week-old ICR (A and C) and 15-week-old ICGN mice in progressing stage (B and D). A and B: Kidney sections were stained with periodic acid-Schiff (PAS). In the progressing-stage ICGN mice, sclerotic glomeruli and highly occluded renal tubules were observed (× 400). C and D: Extracellular matrix (ECM) components were stained with Masson's trichrome stain. ECM was abnormally accumulated both in glomeruli and in the tubulointerstitium (× 400).

Table 1. Hematological and biochemical features of the serum of ICR and ICGN mice

Parameters[a)]	ICR mice (n=61)	ICGN mice (n=61)		
		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*
Ht (%)	47.5 ± 0.41	42.6 ± 1.22*	37.4 ± 1.00*	37.1 ± 0.84*
serum EPO (mIU/m*l*)	50.4 ± 17.7	28.2 ± 10.1	23.0 ± 8.8	8.6 ± 1.7

Results of biochemical testing of 12-week-old ICR mice and ICGN mice. Data in ICR mice and ICGN mice represent mean value ± SE. * : $P<0.01$, versus ICR group. a) Ht = hematocrit; Cre = creatinine; EPO = erythropoietin.

(Table 1).

Quantification of renal EPO mRNA and serum EPO levels in ICR and ICGN Mice: The RT-PCR method was adopted to choose the appropriate samples for quantification of EPO mRNA in the kidneys. Without any hypoxic stimulation (normoxia), less than 40% of ICR mice and latent-stage ICGN mice expressed EPO mRNA, while 100% of progressing-stage ICGN mice expressed EPO mRNA in the kidneys. We selected the samples certainly expressing renal EPO mRNA even in the normoxic condition (n=6 and n=7, ICR mice and progressing-stage ICGN mice, respectively) for quantitative analysis. Quantification of renal EPO mRNA was performed by real-time RT-PCR, and the abundance of EPO mRNA was normalized to the abundance of Rpl 23 mRNA, a structural constituent of the ribosome.

The average relative levels of EPO mRNA expression under normoxia are shown in Fig. 2A. Normoxic progressing-stage ICGN mice exhibited a 1.6 times higher level of EPO mRNA than normoxic ICR mice. Consistent with the higher EPO mRNA expression, the serum EPO level of normoxic ICGN mice was 1.8 times higher than that of normoxic ICR mice (Fig. 2C).

To induce EPO production in the kidneys, ICR and ICGN mice (n=6 and n=5, respectively) were exposed to reduced oxygen tension of 7% O_2 (hypoxia) for 2 hr. In ICR mice, hypoxia induced a 2.4-fold increase of EPO mRNA and a 5.4-fold ($P<0.05$) increase of serum EPO, in comparison to normoxia. In ICGN mice, hypoxia also increased the EPO mRNA expression (4.9 times); however, there was little increase in the serum EPO level (1.1 times), in comparison to normoxia. During the 2 hr-hypoxia, EPO was rarely detected in the urine of ICGN mice.

Localization and density of renal EPO-producing cells: As shown in Fig. 2A, hypoxia enabled us to induce the higher and constant expression of renal EPO mRNA, which ensured the detection of EPO-producing cells. According to

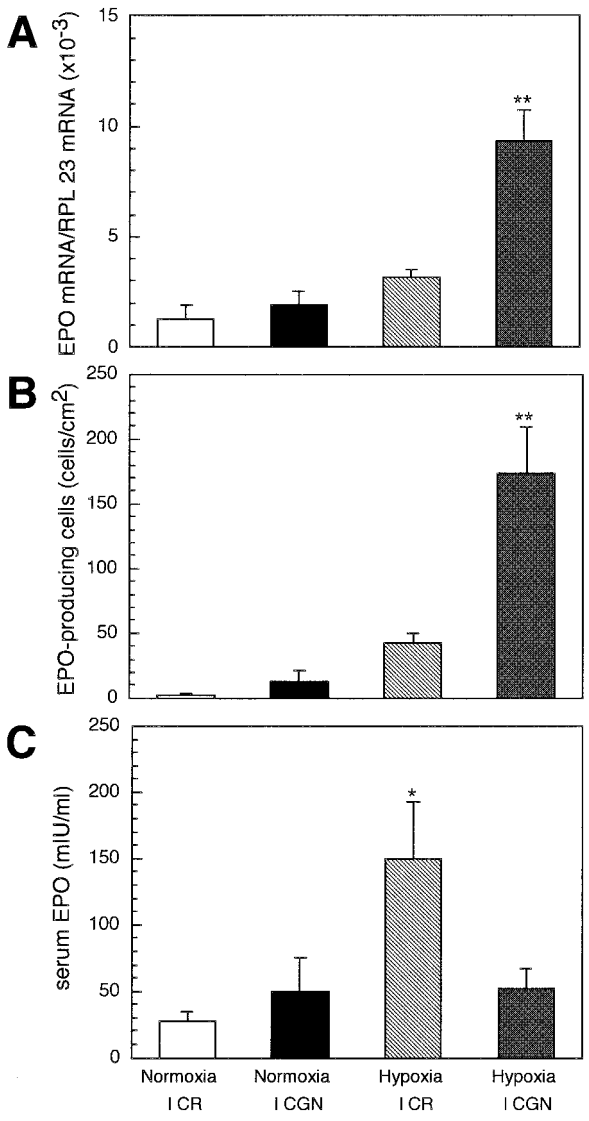

Fig. 2. Open and closed columns indicate ICR (n=6) and progressing-stage ICGN mice (n=7) under normoxia, respectively. Striped and grayed columns indicate ICR (n=6) and ICGN mice (n=5) under hypoxia, respectively. A: Ratios of erythropoietin (EPO) mRNA relative to ribosomal protein 23 (Rpl 23) mRNA in the kidneys under normoxia and hypoxia. B: Density of EPO-producing cells in the kidney. C: Peripheral serum EPO concentration measured by amplified ELISA. All data are shown as means ± SE. * and **: $P<0.05$ and 0.01 versus normoxic ICR mice, respectively.

Chikuma *et al.* [4], 2-hr hypoxia induced the highest expression level of EPO mRNA in the kidney. The expression of renal EPO mRNA was confirmed by RT-PCR in all of the ICR and ICGN mice exposed to hypoxia before their kidneys were used for *in situ* hybridization. Masson's trichrome stain revealed that no histopathological change was induced by short-term hypoxia.

Amplified *in situ* hybridization with DIG-labeled cRNA probe specific to EPO mRNA allowed the identification of EPO-producing cells precisely without any background. In both ICR and ICGN mice exposed to hypoxia, EPO-producing cells were fibroblast-like interstitial cells between tubules in the renal cortex (Fig. 3B and D). No positive cells were found in the glomerular or tubular epithelial cells of the renal cortex, or in the renal medulla. Most of the EPO-producing cells had slender elliptical nuclei and were present singly or in a small cluster in the middle of the cortex, outside of the basement of proximal and distal tubules and blood capillaries. Hypoxia increased the number of EPO-producing cells and up-regulated the EPO mRNA expression both in ICR and ICGN mice (Fig. 2B). Compared to normoxic ICR mice, ICR and ICGN mice under hypoxia showed 4.7 and 66.1-fold increases in the number of EPO-producing cells.

We also detected the renal EPO-producing cells in normoxia. Consistent with the findings under hypoxia, EPO-producing cells were peritubular fibroblast-like interstitial cells in the middle of the renal cortex both in normoxic ICR and ICGN mice (Fig. 3A and C), and no positive cells were observed in the renal medulla. EPO-producing cells were rare, especially in normoxic ICR mice (Fig. 2B).

Oxygen gradient in the kidneys of ICR and ICGN mice: Oxygen gradients in the kidneys were assessed immunohistochemically by using a Hypoxyprobe™-1 kit, which included pimonidazole hydrochloride and an antibody against this compound. Under hypoxia, numerous positive cells were observed in the epithelium of proximal tubules in the renal cortex in both ICR and ICGN mice (Fig. 4B and D, respectively). No positive reactions were found in the glomeruli or the interstitium of the cortex, or in the medulla. Interestingly, normoxic ICGN mice showed low oxygen tension only in the expanding proximal tubular epithelial cells of the renal cortex, while no cells showed low oxygen tension in ICR mice (Fig. 4A and C). The proximal tubular epithelial cells of the inner cortex showed a stronger positive reaction than the surface of the cortex.

DISCUSSION

In the course of CRD, anemia is a frequent symptom that directly affects the QOL of patients. In patients with CRD and chronic renal anemia, a wide range of serum EPO levels is reported [6, 25, 37] and insufficient serum levels of EPO are now considered to be a major cause of the anemia. Pharmacological administration of rhEPO to compensate for the deficiency of endogenous EPO has significant therapeutic value when performed with careful monitoring of Ht and hemoglobin concentration [6, 19, 25]. However, the etiology of anemia with CRD remains unclear, primarily owing to the lack of suitable model animals for anemia associated with CRD, and the mechanism of the regulation of renal EPO production remains unclear due to the lack of information about EPO-producing cells.

ICGN mice, which have a hereditary and spontaneous nephrotic syndrome, exhibit proteinuria, hypoproteinemia,

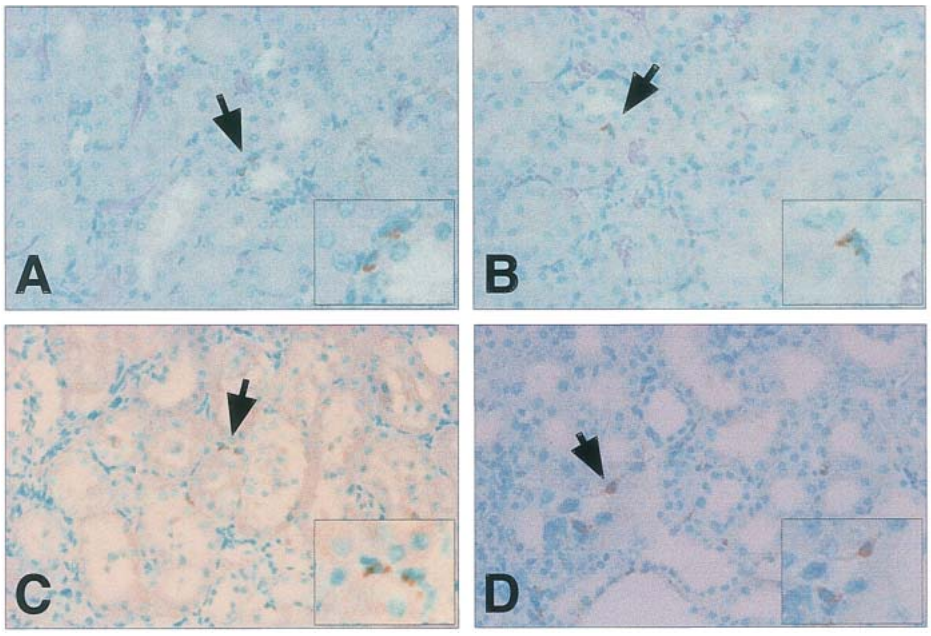

Fig. 3. EPO-producing cells in the kidneys detected by amplified *in situ* hybridization. Paraffin-embedded kidney sections were prepared from normoxic ICR (A) and progressing-stage ICGN (C) mice, and hypoxic ICR (B) and progressing-stage ICGN mice (D). To induce the expression of renal EPO mRNA, both ICR and ICGN mice were exposed to 7% O_2 hypoxia for 2 hr (B and D, respectively). Positive cells were surrounded by brown particles. EPO-producing cells were peritubular interstitial cells in the inner cortex both in ICR and ICGN mice. (A, B, C and D; thick closed arrowheads <× 400> and inset <× 800>)
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hyperlipidemia, and systemic edema [9, 20–22, 29, 31–36]. Recently, we demonstrated that the ICGN mouse was an appropriate model for anemia associated with CRD [35]. Hematological and histopathological analyses revealed significant decreases in Ht, hemoglobin concentration and red blood cells, indicating that ICGN mice developed a normochromic and normocytic anemia with the deterioration of renal function. In our previous report [35], we showed that a wide range of serum EPO levels was observed in ICGN mice and the insufficient levels of serum EPO might account for the anemic conditions in ICGN mice. The improvement of anemia by rhEPO treatment without histopathological changes in the bone marrow and kidneys suggested that the EPO deficiency was directly related to the anemia in ICGN mice [36]. The EPO deficiency in ICGN mice was considered to be primarily the result of insufficient production in the kidneys, and we also detected urinary leakage of EPO due to proteinuria in the terminal stage of CRD. In addition to EPO deficiency, iron deficiency induced by hemolysis in the spleen and the leakage of transferrin into urine also contributed to anemic condition in ICGN mice [35].

EPO is a blood-circulating glycoprotein, and controls erythropoiesis in mammals [12, 14, 24]. The kidney is a principal organ of EPO production in adult animals [3, 14, 26]. The cloning of the murine EPO gene [28] enabled us to detect the EPO-producing cells in the kidney by *in situ* hybridization and transgenic technology. Even though some studies suggested that tubular and glomerular epithelial cells were the site of EPO production [16, 17], most recent research has indicated that peritubular epithelial cells or peritubular endothelial cells were plausible candidates of EPO-producing cells in the kidneys [2, 13, 15, 18]. Most studies utilized radioactive *in situ* hybridization methods on sections from anemic or hypoxic animals. Radioactive *in situ* hybridization methods have difficulty in identifying the cell types, especially in the interstitium, because of the high background compared to non-radioactive methods. To settle the controversy about EPO-producing cells, we used long non-radioactive DIG-labeled cRNA probes and *in situ* hybridization amplified by biotin-labeled tyramide and horseradish peroxidase in the present study. The amplified technique enabled us to detect the EPO-producing cells precisely without any anemic or hypoxic stimulation. We concluded that the EPO-producing cells were fibroblast-like interstitial cells adjacent to both tubules and blood capillaries in the mid-cortex. They were neither glomerular nor tubular epithelial cells. In the cortical interstitium, it is considered that interstitial cells are divided into two types morphologically: one is type 1 cortical interstitial cells that resemble fibroblasts, while the others are a less common type 2 cortical interstitial cells that resemble lymphocytes [30]. According to this division, the EPO-producing cells resembling fibroblasts seemed to be type 1 cortical intersti-

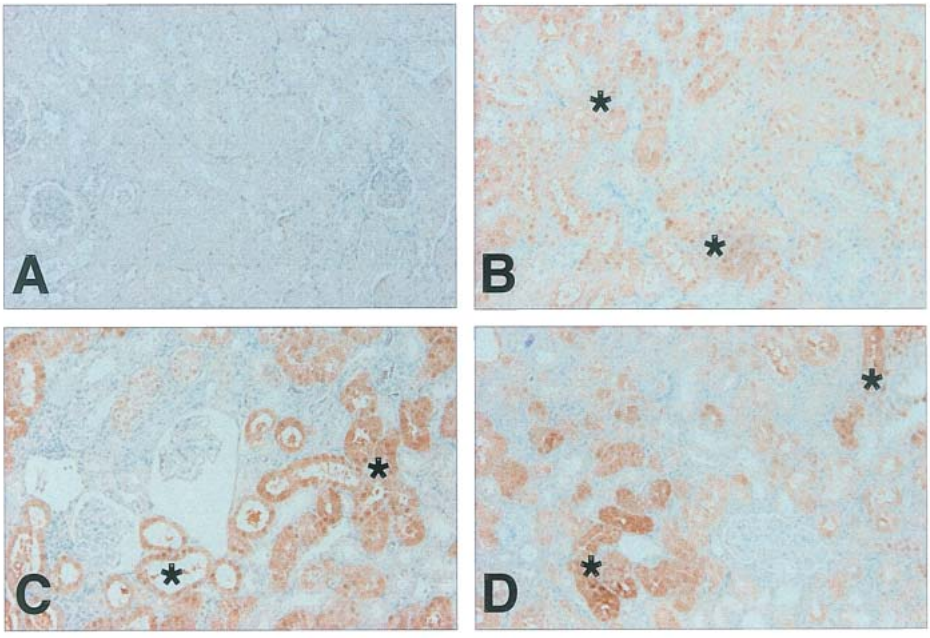

Fig. 4. Kidney sections prepared from normoxic ICR (A) and ICGN (C) mice, and hypoxic ICR (B) and ICGN (D) mice. Oxygen gradients in the kidneys were detected with the use of a horseradish peroxidase technique. Under hypoxia, numerous dense positive reactions were found in the proximal tubular epithelial cells in the renal cortex both in ICR and ICGN mice (× 200, asterisks in B and D, respectively). No interstitial cells in the cortex were positive. In contrast to the lack of positive cells in ICR mice, normoxic ICGN mice showed strong reactions in most of the proximal tubular epithelial cells of the renal cortex (× 200, A and asterisks in C, respectively).

tial cells. EPO-producing cells were rare in normoxic ICR mice, and even in hypoxia, only a minor portion of interstitial cells produced EPO.

Previous studies clarified that blood-letting and hypoxic stimulation induced exponential increases in the EPO mRNA level and the number of EPO-producing cells in the kidneys [13]. It is said that oxygen gradients exist in the renal cortex [1, 23], but it remains unclear that whether renal EPO-producing cells directly sense the change of oxygen tension. Our result using a Hypoxyprobe^{TM}-1 kit, which is able to detect cells undergoing hypoxia (pO_2 < 10mmHg), revealed that only the proximal tubule cells in inner cortex suffered from low oxygen tension. This result was contrary to our expectation that the interstitial region containing EPO-producing cells would be strongly detected.

Our present results indicate that the limited or specialized interstitial cells adjacent to both tubules and blood capillaries in the mid-cortical region have the potential to produce EPO. Although low oxygen tension is a strong inducer of EPO production in the whole kidney, renal EPO-producing cells seem not to sense the lowered oxygen tension directly. We hypothesize that oxygen sensors are present in proximal tubular epithelial cells which are rich in mitochondoria and present where oxygen is highly consumed. Under conditions of low oxygen tension, proximal tubular epithelial cells may release unknown signals to EPO-producing interstitial cells located outside of the tubules. After receiving the signal from the proximal tubular epithelial cells, EPO-producing cells begin transcription and translation of the urgently required EPO, and release EPO directly into adjacent blood vessels. The localization of EPO-producing cells in the interstitium, which is faced by numerous renal tubules and capillaries, is considered to be suitable for both monitoring the peripheral oxygen tension and quickly releasing the produced EPO.

In the present study to elucidate the cause of anemia associated with CRD, we compared the competence for and the site of EPO production between ICR and ICGN mice developing anemia associated with CRD. Both in normoxia and in hypoxia, EPO-producing cells were precisely detected by use of a highly sensitive technique, amplified *in situ* hybridization as well. The type and location of EPO-producing cells in ICGN mice were the same as those in ICR mice, and increases in the number of those cells and renal EPO mRNA were detected in ICGN mice. Serum EPO levels in ICGN mice did not increase despite adequate stimulation (hypoxia) of EPO mRNA synthesis, which was indicated by the increased level of EPO mRNA and the increased number of EPO-producing cells in Fig. 2. It seems that EPO-producing cells of ICGN mice were dysfunctional for EPO-synthesis with respect to the process of translation and /or the ability of the cells to export the protein. In both hypoxic

ICR and ICGN mice, low oxygen tension was not observed in the cortical interstitium or in the medulla. Although Masson's trichrome stain revealed marked accumulation of ECM and fewer red blood cells both in the glomeruli and in the tubulointerstitium of ICGN mice, there was no positive reaction for low oxygen tension in those regions. Interestingly, most of the proximal tubular epithelial cells suffered from low oxygen tension in ICGN mice without any hypoxic stimulation.

Urinary leakage of EPO was slight in the progressing-stage ICGN mice [35], and it is considered that the insufficient serum EPO level in ICGN mice is due to the suppression of EPO protein production in the EPO-producing cells. As mentioned before, ICGN mice develop severe proteinuria as the renal function deteriorates, and we surmise that some toxic substances that leak into the renal tubule fluid or serum may disturb the translation of EPO mRNA in EPO-producing cells of the interstitium, although the proximal tubular epithelial cells can monitor the lowered oxygen tension accurately in ICGN mice.

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