In our previous study, we performed a two-stage liver carcinogenesis experiment in transgenic mice carrying human prototype of c-Ha-*ras* gene (rasH2 mice) administered fenofibrate for 8 weeks after DEN initiation and reported that the numbers of CK8/18-positive foci were significantly increased in the livers of rasH2 mice [18]. In Experiment I of the present study, hepatocellular foci observed in HE-stained sections were immunohistochemically positive for CK8/18, but foci that could not be detected in HE-stained sections were also positive for CK8/18. The multiplicity of hepatocellular foci that were observed in HE-stained sections and positive for CK8/18 was 10.17 and 18.50, respectively. These findings indicate that more hepatocellular foci could be detected in CK8/18 immunohistochemistry in addition to those observed in HE-stained sections. In Experiment II, hepatocellular foci observed in HE-stained sections were positive for CK8/18, but foci that could not be detected in HE-stained sections were also positive for CK8/18. In addition, hepatocellular foci that are negative for CK8/18 were observed in 5 of 6 mice (83 %). The total multiplicity of hepatocellular foci which were observed in HE-stained sections and positive/negative for CK8/18 was 4.47 and 23.17, respectively. These findings indicate that more hepatocellular foci could be detected in CK8/18 immunohistochemistry in addition to those observed in HE-stained sections. In Experiment II, hepatocellular adenomas observed in HE-stained sections were positive for CK8/18, while hepatocellular adenomas that are

negative for CK8/18 were observed in 4 of 6 mice (67 %). The total multiplicity of hepatocellular adenomas from the treated mice which were observed in HE-stained sections and positive/negative for CK8/18 was 11.17 and 11.17, respectively. These findings indicate that hepatocellular adenomas observed in HE-stained sections could be also detected in CK8/18 immunohistochemistry. Similarly, 3 of 6 treated mice (50 %) had hepatocellular carcinomas that were also positive for CK8/18. There were no hepatocellular carcinomas that were negative for CK8/18 in these treated mice. The total multiplicity of hepatocellular carcinomas from the treated mice that were observed in HE-stained sections and positive for CK8/18 was 1.5 and 1.5, respectively. These findings indicate that hepatocellular carcinomas observed in HE-stained sections could be also detected in CK8/18 immunohistochemistry. In addition, in Experiment I, the multiplicity of hepatocellular foci in paraffin-embedded sections which were observed in HE-stained sections and positive for CK8/18 was 10.17 and 18.50, respectively, while that of hepatocellular foci in frozen sections which were observed in HE-stained sections and positive/negative for GGT was 6.17 and 8.17, respectively. In Experiment II, the total multiplicity of hepatocellular foci in paraffin-embedded sections which were observed in HE-stained sections and positive/negative for CK8/18 was 4.47 and 23.17, respectively, while that of hepatocellular foci in frozen sections which were observed in HE-stained sections and positive/negative for GGT was 2.50 and 3.50, respectively. These

findings strongly suggest that more hepatocellular proliferative lesions can be detected in CK8/18 immunohistochemistry in addition to those observed in HE-stained sections, as compared with the results of GGT histochemistry.

On the other hand, we observed several CK8/18-negative foci and adenomas in mice given PBO for 25 weeks after DEN initiation but not in mice subjected to 8-week tumor promoting treatment of PBO. These CK8/18-negative proliferative lesions could be also detected as hepatocellular foci or adenomas in HE-stained sections. However, it is uncertain whether these lesions are positive for GGT histochemistry, since GGT histochemistry can't be performed in the same paraffin section as HE-stained and CK8/18-immunostained sections. Therefore, we could not clarify the biological role of these CK8/18-negative proliferative lesions. It has been suggested that hepatocellular proliferative lesions that are positive for GST-P in rats are induced by the overexpression of CK8 and CK18 complex due to CK8 phosphorylation [16]. Ku (2003) reported that mutations of CK8 and CK18 are risk factors for developing human liver diseases [20]. CK8/18 mutations may trigger oxidative injury in hepatocytes [32], and such an overexpression of CK8/18 is maintained in human hepatocellular carcinomas [2]. Thus, CK8 phosphorylation and CK8/18 mutations are probably responsible for the induction of CK8/18-positive liver proliferative lesions. Therefore, CK8/18-negative lesions may be induced by the different mechanism other than

CK8 phosphorylation and CK8/18 mutations, but further studies are necessary to clarify the biological behavior of CK8/18-negative proliferative lesions in mice.

In conclusion, the results of our study suggest that CK8/18 immunohistochemistry can be used as a useful marker for detecting liver preneoplastic and neoplastic lesions in mice, although further studies using other hepatocarcinogens in mice are necessary to confirm the usefulness of CK8/18 immunohistochemistry for hepatocellular proliferative lesions in mice.

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LEGENDS TO FIGURES

Fig. 1. Microscopic photographs of HE-stained sections (A, D, G, J, L and O), CK8/18 immunohistochemistry (B, E, H, K, M and N) and GGT histochemistry (C, F and I) in the livers of male ICR mice given PBO for 8 or 25 weeks after DEN initiation. Hepatocellular focus in a HE-stained section obtained from a mouse given PBO for 8 weeks (A), that is positive for CK8/18 (B: serial section for Figs. 1A) and positive for GGT (C: different section from Figs. 1A). Hepatocellular adenoma in a HE-stained section obtained from a mouse given PBO for 25 weeks (D), that is positive for CK8/18 (E: serial section for Figs. 1D) and positive for GGT (F: different section from Figs. 1D). Hepatocellular carcinoma in a HE-stained section obtained from a mouse given PBO for 25 weeks (G), that is positive for CK8/18 (H: serial section for Figs. 1G) and positive for GGT (I: different section from Figs. 1g). Hepatocellular adenoma (clear cell type) in a HE-stained section obtained from a mouse given PBO for 25 weeks (J), that is negative for CK8/18 (K). Hepatocellular adenoma (basophilic cell type) in a HE-stained section obtained from a mouse given PBO for 25 weeks (L), that is negative for CK8/18 (M). CK8/18-positive foci in a mouse given PBO for 25 weeks (N), that could not be detected in a HE-stained section (O). Bar = 100 μm (A, B, C, G, H, N and O) Bar = 500 μm (D, E, I, J, K, L and M) Bar = 1 cm (F).

Fig.1

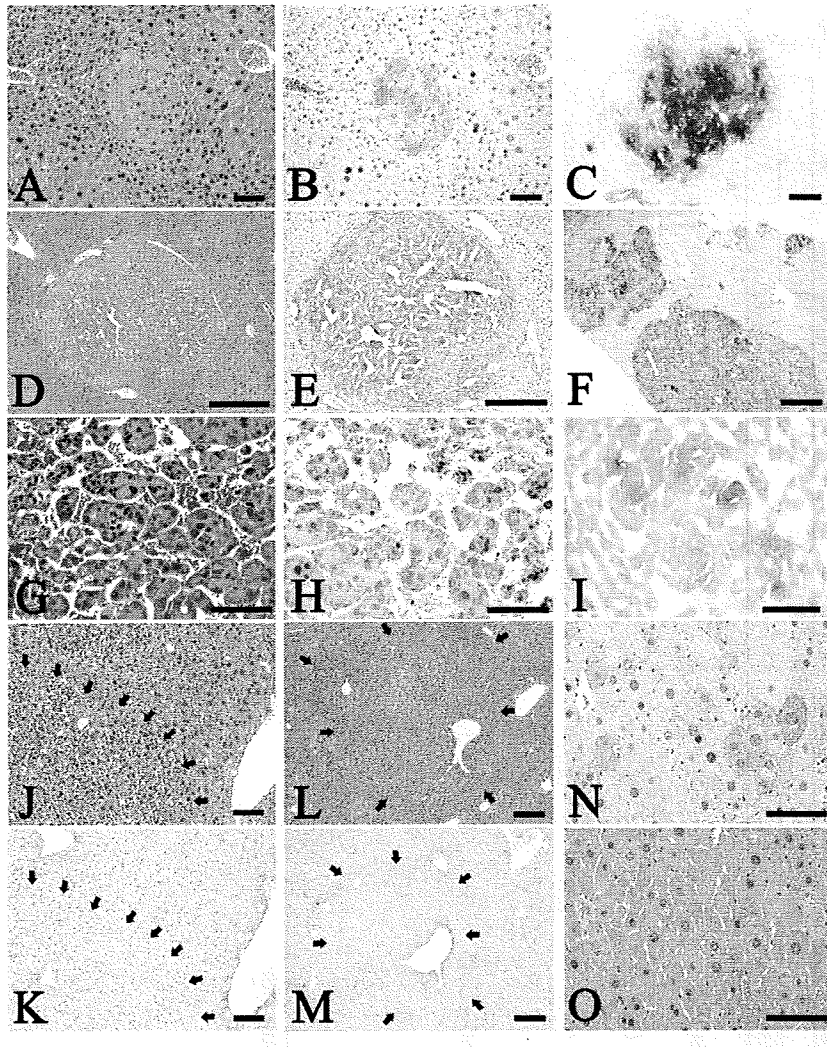

Table 1 Incidence and multiplicity of hepatocellular proliferative lesions in ICR mice given PBO for 8 weeks after DEN-initiation (Experiment I)

	Paraffin section				Frozen section			
	HE stain	CK8/18			HE stain	GGT		
		Positive	Negative	Total		Positive	Negative	Total
Incidence (%)								
Foci	6(100)	6(100)	0(0)	6(100)	6(100)	6(100)	6(100)	6(100)
Adenoma	0 (0)	0(0)	0(0)	0(0)	0 (0)	0(0)	0(0)	0(0)
Carcinoma	0 (0)	0(0)	0(0)	0(0)	0 (0)	0(0)	0(0)	0(0)
Multiplicity (No./2 sections)								
Foci	10.17 ± 7.83	18.50 ± 3.45	0	18.50 ± 3.45	6.17 ± 4.17	3.67 ± 2.34	4.50 ± 2.51	8.17 ± 4.07
Adenoma	0	0	0	0	0	0	0	0
Carcinoma	0	0	0	0	0	0	0	0

DEN: N-diethylnitrosamine, PBO: piperonyl butoxide.
The data represents mean ± S.D.

Table 2 Incidence and multiplicity of hepatocellular proliferative lesions in ICR mice given PBO for 25 weeks after DEN-initiation (Experimentll)

	Paraffin section				Frozen section			
	HE stain	CK8/18			HE stain	GGT		
		Positive	Negative	Total		Positive	Negative	Total
Incidence (%)								
Foci	6(100)	6(100)	5(83)	6(100)	6(100)	6(100)	3(50)	6(100)
Adenoma	6 (100)	6(100)	4(67)	6(100)	6 (100)	5(83)	5(83)	6(100)
Carcinoma	3(50)	3(50)	0(0)	3(50)	3(50)	1(17)	3(50)	3(50)
Multiplicity (No./2 sections)								
Foci	4.47 ± 2.31	19.67 ± 8.98	3.50 ± 3.45	23.17 ± 11.21	2.50 ± 1.52	2.00 ± 2.45	1.50 ± 1.97	3.50 ± 1.87
Adenoma	11.17 ± 3.66	9.83 ± 3.06	1.33 ± 1.51	11.17 ± 3.54	10.50 ± 4.09	3.83 ± 3.49	6.50 ± 5.09	10.33 ± 4.23
Carcinoma	1.50 ± 2.08	1.50 ± 1.97	0	1.50 ± 1.97	0.67 ± 0.82	0.17 ± 0.41	0.50 ± 0.55	0.67 ± 0.82

DEN: N-diethylnitrosamine, PBO: piperonyl butoxide.
The data represents mean ± S.D.

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Original article

Calculation of glomerular filtration rate in conscious rats by the use of a bolus injection of iodixanol and a single blood sample

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ABSTRACT

Introduction: To establish a simple and convenient procedure for the determination of glomerular filtration rate (GFR) in conscious rats, we developed a single-blood-sample method, in contrast to the conventional three-sample method, using a bolus injection of the nonionic contrast medium iodixanol. **Methods:** Iodixanol was intravenously administered at 1500 mg I/kg to healthy or renal-impaired rats, and blood was collected 60, 120, and 180 min later. Serum iodixanol concentrations were measured by HPLC, and serum urea nitrogen (UN) and creatinine concentrations were determined as renal function tests. **Results:** In rats subjected to 1/2 and 3/4 nephrectomies, GFR values decreased significantly without and with increases in serum UN and creatinine concentrations, respectively. In rats treated subcutaneously with gentamicin sulfate (GM) at 80 mg/kg/day or puromycin aminonucleoside (PAN) at 15 mg/kg/day for 10 consecutive days, the GFR values decreased or showed a tendency to decrease before increases in serum UN and creatinine concentrations. Accordingly, when the GFR decreased to more than 60% of the basal value, serum UN or creatinine concentrations became elevated. The GFR values obtained from the three-sample method were closely correlated ($r = 0.83$) with those calculated from the estimated distribution volume (V) and serum iodixanol concentration 120 min after iodixanol injection in the single-blood-sample method in which serum iodixanol concentrations ranged between 20 and 250 μg I/mL. **Discussion:** These results suggest that the single-blood-sample method with a bolus injection of iodixanol, allowing for the repeated use of the same animals, is an expedient procedure without ensuring accurate urine collection.

1. Introduction

In rats, although many reports have described the measurement of glomerular filtration rate (GFR) using inulin (Gabel, Ranaei, & Kivlighn, 1996; Onodera & Furuhama, 1983), contrast medium (Guesry et al., 1975), EDTA (Harvey, Jaffa, Loadholt, & Mayfield, 1988) and endogenous (Darling & Morris, 1991) or exogenous (Zager, 1987) creatinine, little information is available on a procedure that is easy, versatile and reliable, and required only a minimal number of animals without urine and/or repeated blood collections. In humans, the concentration of a tracer in a single plasma sample taken 180–240 min after injection is correlated with renal clearance (Groth & Aasted, 1981). Based on this information, Jacobsson (1983) devised a formula derived from a simple one-compartment model combined with the distribution (compartment) volume and optimum time for taking plasma using $^{99}Tc^{m}$-DTPA and accurately determined GFR. Later, based on this formula, it was reported that the clearance of iohexol, a nonionic monomeric X-ray contrast medium, was a simple, rapid and accurate alternative to that of inulin (Brown & O'reilly, 1991) or radiopharmaceuticals (Thomsen & Hvid-Jacobsen, 1991) for measuring GFR.

In an attempt to apply Jacobsson's formula to rats, we first measured GFR using a conventional three-sample method with iodixanol, an isotonic nonionic dimeric X-ray contrast medium, in experimental models with renal injuries such as partial nephrectomies and gentamicin sulfate (GM)- or puromycin aminonucleoside (PAN)-evoked nephropathy. By substituting these GFR values and serum iodixanol concentrations at 120 min into Jacobsson's formula, we sought the estimated distribution volume (V) in individuals. After confirming a negative relationship between the V values and serum iodixanol concentrations, we obtained a formula for calculating the V value. The GFR value in a single blood sample was obtained by substituting the V

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value and serum iodixanol concentration at 120 min in each animal into Jacobsson's formula once again. Because the *V* value is dependent on elimination kinetics of each substance (marker) and animal size, it is necessary to obtain it in the respective animals and species.

Iodixanol is rapidly excreted in urine without metabolic degradation and no or very little protein binding in rats (Heglund, Michelet, Blazak, Furuhama, & Holtz, 1995) or humans (Jacobsen, Blindheim, & Skotland, 1995). More recently, it has been reported that iodixanol is less nephrotoxic than iohexol in randomized, double-blind, prospective, multicenter studies using patients with chronic renal diseases (Aspelin et al., 2003; McCullough, Bertrand, Brinker, & Stacul, 2006), although iohexol has been used as a gold standard for measuring GFR so far (Brown & O'reilly, 1991). Additionally, the contrast effect of iodixanol was confirmed to be superior to that of iohexol because it possesses twice the amount of iodine in one molecule with a low osmolarity, when the equivalent iodine dose was administered to beagle dogs (Kishimoto et al., 2007, 2008).

Here, we selected iodixanol and Jacobsson's formula to establish a simple procedure for the determination of GFR in conscious rats.

2. Methods

2.1. Materials

Iodixanol (Visipaque 320®; 320 mg I/mL, 290 mOsm/kg H_2O) was purchased from Daiichi-Sankyo (Tokyo, Japan), gentamicin sulfate (GM) from Schering-Plough (Osaka, Japan), and puromycin aminonucleoside (PAN) from Wako Chemicals (Osaka, Japan). All other chemicals and reagents were of the highest grade available from commercial sources, unless otherwise stated.

2.2. Animals

Male Fischer 344 (F344) rats at 6–10 weeks of age were obtained from Japan SLC, Inc. (Shizuoka, Japan), and studies began after at least a 2-day acclimation period. Male F344 rats were chosen for the present investigations because they show a high susceptibility to nephrotoxicant (Tarloff, Goldstein, & Hook, 1989). The rats were housed in an air-conditioned facility (temperature, 22 ± 3 °C; relative humidity, 55 ± 25%; lighting, 8:00 AM to 8:00 PM with a 12-h light cycle) and fed commercial rodent chow (MEQ, Oriental Yeast Co., Ltd., Tokyo) and tap water *ad libitum*. All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science (1987) and also approved by the Animal Experimental Ethics Committee of Iwate University (Morioka, Japan).

2.3. Optimum iodixanol dose

To identify the optimum dose for GFR measurement, iodixanol was administered intravenously at a dose of 375, 750, or 1500 mg I/kg to healthy 6-week-old F344 rats, and blood was collected 60, 120, 180, and 240 min later under conscious conditions by a previously reported procedure (Hatanaka, Kondoh, Kawarabayashi, & Furuhama, 1994). Iodixanol was diluted with a 0.9% saline solution and administered at 5 mL/kg to each animal. Before the iodixanol injection, a constant volume (5 mL) of distilled water was given orally by gavage to all animals to avoid dehydration due to repeated blood collection. The blood was centrifuged and sera were stored at −30 °C until assayed.

2.4. Measurement of GFR in healthy and renal-impaired rats

To measure the GFR in healthy rats, iodixanol was administered intravenously at 1500 mg I/kg to F344 rats at 6–9 weeks of age, weighing from 150 to 210 g, and blood was withdrawn 60, 120, and 180 min later using the aforementioned procedure (three-sample method). The GFR values were represented as mL/min/kg.

For partial nephrectomy models, 10-week-old F344 rats were fasted overnight with free access to water before the operation. After anesthesia with ether, a surgical ablation was performed on the right kidney (1/2 nephrectomy) or the right kidney with both poles of the left kidney (3/4 nephrectomy) according to the previous report (Sampaio-Maia, Serrao, Guimaraes, Vieira-Coelho, & Pestana, 2005). In 3/4 nephrectomized rats, the mean percentage of remnant renal mass was 32 ± 5%, based on the removed kidney weight. Animals subjected to sham surgery under the same conditions served as the sham control. The day the operation was performed was regarded as day 1. On day 2, the GFR was measured immediately after collecting the blood specimen to measure serum urea nitrogen (UN) and creatinine concentrations, and then all animals were euthanized by exsanguination under ether anesthesia.

For renal nephropathy models, GM and PAN were administered subcutaneously at 80 mg/kg/day and 15 mg/kg/day, respectively, to 11-week-old F344 rats for 10 consecutive days (days 1–10). Control animals received 0.9% saline solution (saline, 10 mL/kg) in the same way. The GFR was measured on days 0 (pre-dose), 6, and 11 in conjunction with the determination of serum UN and creatinine concentrations in the same animals. The dosage level and administration period of GM and PAN were selected based on the results of previous studies (Furuhama & Onodera, 1986; Onodera & Furuhama, 1983). On day 11, all animals were euthanized by exsanguination under ether anesthesia.

2.5. Laboratory tests and renal pathology

Serum UN and creatinine concentrations were measured with an autoanalyzer (Hitachi, Tokyo). In rats subcutaneously given GM, PAN or saline, the kidney was excised, fixed in 10% formalin, embedded in paraffin wax, cut at 3-μm thickness, stained with hematoxylin and eosin (H–E), and histopathologically examined.

2.6. Analysis of serum iodixanol concentrations

Serum iodixanol concentration was measured with reversed-phase high-performance liquid chromatography (HPLC) according to a previously reported procedure (Jacobsen et al., 1995), with some modifications. Serum specimens (0.1 mL) were deproteinized by adding 20% trichloroacetic acid (TCA; Wako Chemicals) at a ratio of 1:1 and placed at 4 °C for 30 min to complete precipitation before removal of the proteins by centrifugation (14,000×g, 10 min, 4 °C). The supernatant was centrifuged again under the same conditions. The HPLC system consisted of separation equipment (Alliance™ Waters 2690 Separations Module; Waters, Milford, MA, USA), a UV detector (Waters 996 Photodiode Array Detector; Waters) and analytical software (Millennium^{32}; Waters) equipped with a 250×4.6-mm C-18 reverse-phase column (RP-18 GP, 5 μm; KANTO CHEMICAL Co., Inc., Tokyo). The stepwise mobile phase profile was composed of distilled water followed by 80% acetonitrile in distilled water, and the flow-rate was maintained at 1 mL/min. The detection wavelength was 244 nm, which is the approximate absorbance maximum for iodixanol. The standard was prepared at known concentrations of iodixanol, and the results from the standard were used to calculate the concentration in each sample. A linear relationship existed between the summation of exso- and endo-isomers of iodixanol and the logarithm of iodixanol concentration. The qualification limit of serum iodixanol concentration was 6.25 μg I/mL.

2.7. Iodixanol clearance

In the three-sample method, the clearance calculations are based on the one-compartment model (Bröchner-Mortensen, 1972). Briefly,

the area under the iodine concentration curve (AUC) was calculated by the linear trapezoidal rule with extrapolation using the final three serum samples, and a clearance value (Cl_1) was calculated from the following formula.

$$Cl_1 = Q_{tot} / AUC,$$

where Q_{tot} is the dose of iodixanol injected. However, this formula overestimates clearance because it does not consider the serum concentration during the distribution phase. Therefore, the clearance (Cl_2) was corrected by the following empirically determined formula (Bröchner-Mortensen, 1972; Thomsen & Hvid-Jacobsen, 1991).

$$Cl_2 = 0.991 \times Cl_1 - 0.00122 \times (Cl_1)^2$$

The distribution volume (V_1) in each animal was back-calculated by substituting Cl_2 values and serum iodixanol concentrations (C_{t_2}) at 120 min (t_2) obtained from the three-sample method into the following Jacobsson's formula using the "Goal-Seek" command of Microsoft Office Excel (Microsoft 2007, Microsoft Co., Tokyo). In this function, when we know the result we want to return a formula, but we do not know the input values the formula needs to reach that result, we can use the command.

$$Cl_2 = 1 / (t_2 / V_1 + 0.0016) \times \ln[Q_{tot} / (V_1 \times C_{t_2})]$$

To seek the estimated distribution volume (V) in each animal, after the correlation between the V_1 value and C_{t_2} was assessed with a scatter diagram, a formula for calculating V_1 was determined. In this case, therefore, the V_1 value sought by using C_{t_2} was regarded as the V value ($V_1 = V$).

Finally, the Cl value in a single-blood-sample method was determined by substituting the V value and C_t at 120 min (t) from each animal into the following Jacobsson's formula once again. The Cl term was the GFR in the present work.

$$Cl = 1 / (t / V + 0.0016) \times \ln[Q_{tot} / (V \times C_t)]$$

2.8. Statistical analysis

Quantitative data are expressed as the mean ± SEM of the group. Statistical evaluation was occasionally performed by a one-way ANOVA, and differences between treatment and control groups were analyzed by Dunnett's test (among three groups) or Student's t-test (between two groups). A probability level of $p<0.05$ indicates statistical significance. Correlations were determined by Pearson's correlation coefficient (r).

3. Results

3.1. Disappearance of iodixanol from serum

In 6-week-old healthy male rats ($n=2$) given 1500 mg I/kg of iodixanol (Fig. 1), mean concentrations of iodixanol in serum had a linear disappearance until 240 min. At doses of 375 and 750 mg I/kg, however, serum concentrations decreased almost to the qualification limit (6.25 μg I/mL) 120 and/or 180 min after injection. In a subsequent three-sample method, therefore, a 1500 mg I/kg of iodixanol with sample times of 60, 120, and 180 min was chosen.

3.2. GFR obtained by the three-sample method in healthy or renal-impaired rats

The background GFR value in healthy male F344 rats at 6–9 weeks of age was 9.6 ± 0.6 mL/min/kg ($n=18$).

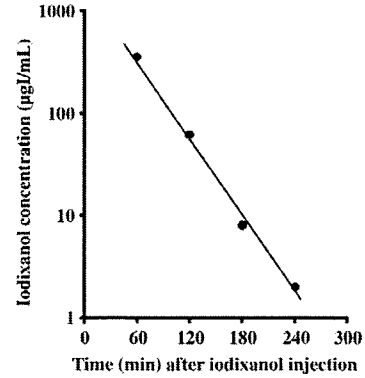

Fig. 1. Representative disappearance of iodixanol from serum of healthy 6-week-old male F344 rats given a bolus injection of iodixanol at 1500 mg I/kg. Blood was collected from the tail vein under conscious conditions 60, 120, 180, and 240 min after iodixanol injection, and serum iodixanol concentrations were measured by HPLC. Each plot shows the mean of two animals.

In rats subjected to partial nephrectomy (Fig. 2), the GFR values (mean: 4.5 mL/min/kg) decreased significantly without alterations in serum UN or creatinine concentrations in the 1/2 nephrectomy group, whereas significantly decreased GFR values (2.3 mL/min/kg) were observed with increases in serum UN (53.6 mg/dL) and creatinine (0.97 mg/dL) concentrations in the 2/3 nephrectomy group when compared to the sham control group (GFR, 6.8 mL/min/kg).

In rats subcutaneously administered GM or PAN for 10 days (Fig. 3), the GFR values showed a tendency to decrease in GM-treated rats (6.9 mL/min/kg) or statistically decreased in PAN-treated rats (5.6 mL/min/kg) without alterations in serum UN or creatinine concentrations on day 6. On day 11, however, markedly decreased GFR values (0.38–0.41 mL/min/kg) were noted with significant increases in serum UN and creatinine concentrations in both nephropathy groups. The GFR values in the corresponding saline control group ranged between 7.7 and 8.4 mL/min/kg throughout the experimental period. Renal histopathological examinations revealed that necrosis in the proximal epithelium and mitotic figures of epithelial cells with casts in the proximal tubule were observed in rats given GM, and that hydroponic vacuolated podocytes with hyaline casts were noted in rats receiving PAN.

3.3. Relationship between GFR values versus serum UN or creatinine concentrations

The relationship between the GFR values sought by the three-sample method versus serum UN or creatinine concentrations was assessed using a total of 48 animals (sample numbers, 125, where the number shows the sum total of the GFR values collected from the same animal on different days) from the preliminary and present (main) studies. When the GFR value decreased to more than 60% of the basal background level (9.5 mL/min/kg), serum UN or creatinine concentrations likely began to increase (Fig. 4A and B).

3.4. GFR by the single-blood-sample method

A formula for calculating the V value was determined from a scatter diagram (Fig. 5A) as follows:

$$y = -0.70x + 277 (n = 25;\ \text{sample numbers, } 47)$$

where y is the V value and x is serum iodixanol concentration at 120 min. This formula was valid only between 20 and 250 μg I/mL serum iodixanol concentrations, showing a close correlation ($r=0.55$; $p<0.01$). Within these concentration ranges, the GFR values were calculated as 2.9–13.7 mL/min/kg in a single-blood-sample method.

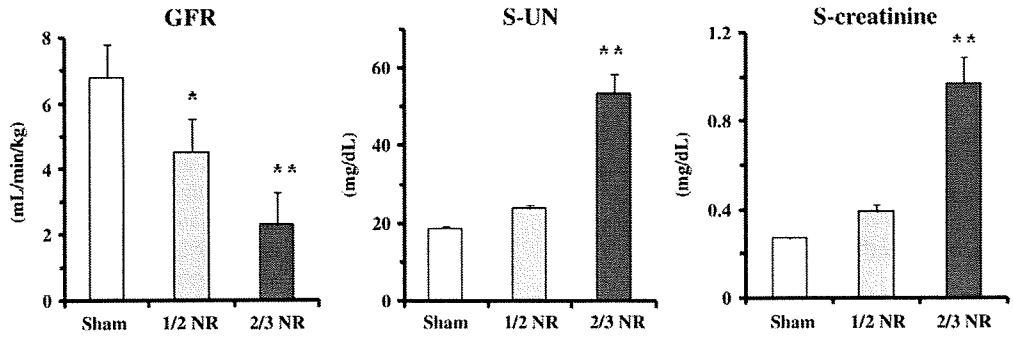

Fig. 2. Glomerular filtration rate (GFR) and serum urea nitrogen (UN) and creatinine concentrations 24 h after operation in male F344 rats subjected to 1/2 or 2/3 nephrectomy (NR). Animals that underwent a sham operation served as the sham control (sham). The GFR value was determined by the three-sample method. Each column and vertical bar represents the mean ± SEM of five animals. $^{*}p<0.05$ and $^{**}p<0.01$ versus the sham control group. S: serum.

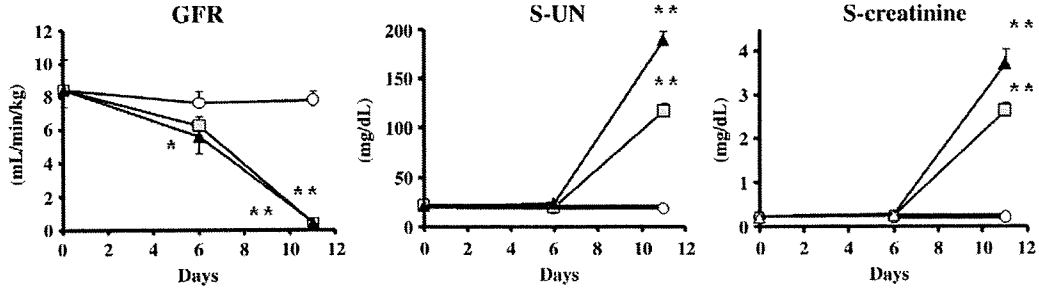

Fig. 3. Glomerular filtration rate (GFR) and serum urea nitrogen (UN) and creatinine concentrations on days 0 (pre-dose), 6, and 11 in male F344 rats treated subcutaneously with gentamicin sulfate (GM, gray squares) at 80 mg/kg/day or puromycin aminonucleoside (PAN, closed triangles) at 15 mg/kg/day for 10 consecutive days. Animals given 0.9% saline solution in the same way served as the control (open circles). The GFR value was determined by the three-sample method. Each column and vertical bar represents the mean ± SEM of 5–7 animals. $^{*}p<0.05$ and $^{**}p<0.01$ versus the saline group. S: serum.

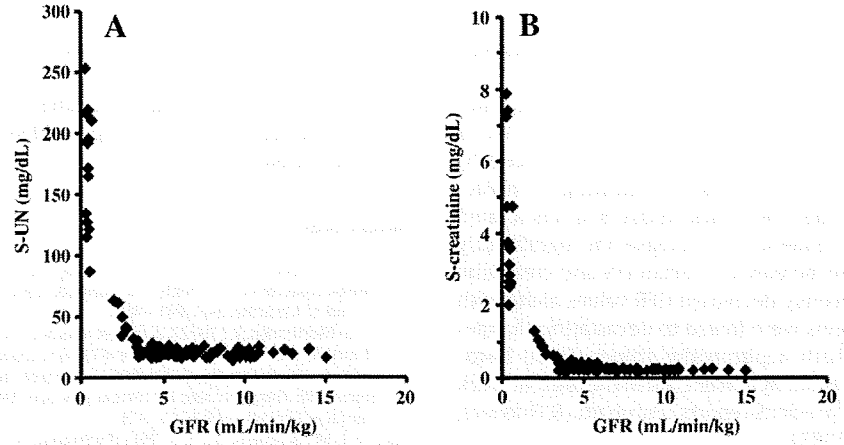

Fig. 4. Relationship between the Glomerular filtration rate (GFR) values versus serum urea nitrogen (UN, A) or creatinine (B) concentrations using data collected from healthy and renal-impaired rats ($n=48$; sample numbers, 125). The GFR value was determined by the three-sample method. S: serum.

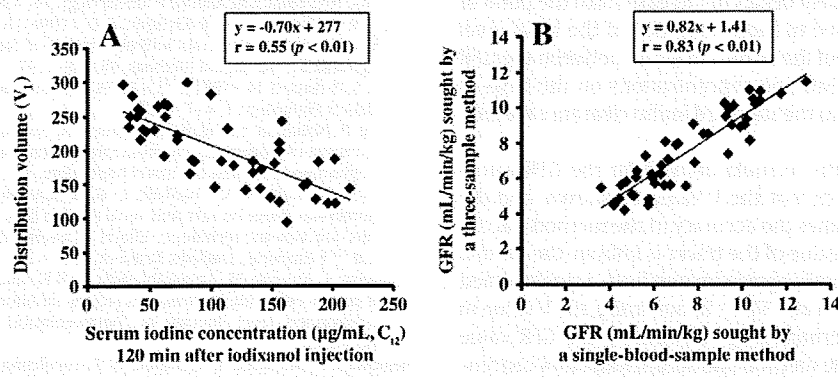

Fig. 5. Correlation between the distribution volumes (V_1) and serum iodixanol concentrations (C_{t_2}) 120 min (t_2) after iodixanol injection (A) and between the GFR values determined by the three-sample method and those determined by the single-blood-sample method (B). The V value in the single-blood-sample method was calculated from $y=-0.70x+277$ ($n=25$; sample numbers, 47).

No significant correlation with serum iodixanol concentrations was found at 20 μg I/mL or less, and 250 μg I/mL or more (data not shown).

A comparison of the GFR values obtained from the three-sample method with those of the single-blood-sample method yielded a correlation coefficient of 0.83 ($n = 28$; sample numbers, 47), indicating a close correlation ($p < 0.01$, Fig. 5B). The GFR value obtained by the single-blood-sample method in healthy male F344 rats at 6–9 weeks old were 10.1 ± 0.8 mL/min/kg ($n = 18$), indicating no significant difference from the three-sample method.

4. Discussion

According to previous iodixanol pharmacokinetic data (Heglund et al., 1995), an intravenous treatment of male Wistar rats with 200–300 mg I/kg of ^{125}I-iodixanol resulted in a rapid half-life of 25 min in plasma and a total clearance of 8.0 ± 1.9 mL/min/kg ($n = 10$) with less than 1% of the injected dose present in tissues after 24 h. In our work with 1500 mg I/kg of iodixanol, a linear semilogarithmic plot of serum iodixanol concentrations versus time demonstrated the suitability of using a one-compartment model for the clearance calculation (GFR). The GFR values (9.6 ± 0.6 mL/min/kg) in healthy male F344 rats determined by the three-sample method were nearly consistent with the above clearance values. Furthermore, these iodixanol GFR values were in good agreement with our historical data (8.6 ± 1.5 mL/min/kg in Sprague–Dawley rats, $n = 30$, Onodera & Furuhama, 1983) or previously reported data (0.798 ± 0.073 mL/min/100 g in Sprague–Dawley rats, $n = 34$, Gabel et al., 1996) using a standard ^{14}C-inulin clearance, although the experimental conditions were very different. When converting the GFR values from bodyweights (mL/min/kg) to body surface areas (mL/min/m^2, Holt, Rhode, & Kines, 1968) using healthy rat data, the fluctuations (44.2–88.9 mL/min/m^2) in the latter were almost similar to those (7.1–14.9 mL/min/kg) in the former, suggesting that the effect of gains in the bodyweight on GFR calculations can be ignored under the conditions of this study.

In partially nephrectomized rats, the GFR values decreased statistically without and with significant increases in serum UN or creatinine concentrations in 1/2 and 3/4 nephrectomies, respectively, demonstrating no alteration in these parameters following 1/2 reductions in renal mass. Likewise, the GFR values on day 6 in rats treated with GM and PAN showed a tendency to decrease and significantly decreased, respectively, before increases in serum UN and creatinine concentrations. On day 11, severely decreased GFR values along with marked increases in serum items were linked to devastating changes in the renal morphology of both nephropathy groups. Renal histopathological findings brought about by GM or PAM in the present work were well consistent with our previous reports (Furuhama & Onodera, 1986; Onodera & Furuhama, 1983).

Based on cumulative data collected from the preliminary and present studies including healthy and renal-impaired rats, serum UN or creatinine concentrations likely began to increase from the point at which the GFR values decreased to more than 60% of the basal level (9.6 mL/min/kg). The findings of the scatter diagram between the GFR values versus serum UN or creatinine concentrations on the basis of these data resembled those from the standard inulin clearance method (Onodera & Furuhama, 1983).

It has been reported that the formula derived for the GFR calculation with one sample requires that the V value be known, and the accuracy in the V value determines the accuracy in the method (Jacobsson, 1983). Similarly, if the V value of the tracer is known, the plasma disappearance curve can be closely approximated from a single, timed plasma measurement (Harvey et al., 1988). In our study, the V value in an individual animal was determined by substituting the GFR value obtained from the three-sample method and serum iodixanol concentrations at 120 min into Jacobsson's formula. Although the 120 min sample time for rats is shorter than the 300 min for humans (Jacobsson, 1983), this was based on the fact that iodixanol clearance value is 3- to 4-fold higher in rats (Heglund et al., 1995) than in humans (Svaland, Haider, Langseth-Manrique, Andrew, & Hals, 1992). Moreover, the GFR values calculated at 120 min were apparently stable in healthy rats compared to those at 60 or 180 min, because a relatively high concentration at 60 min or an extremely low concentration at 180 min was included. It was essential to have a close relationship between the V values and serum iodixanol concentrations as a prerequisite for a formula to calculate the V value. The formula obtained was valid between 20 and 250 μg I/mL in C_{t_2}, in which the GFR values were calculated as 2.9–13.7 mL/min/kg with the single-blood-sample method. These results implied a margin of error in the GFR values (2.8 mL/min/kg or less) for rats showing severe renal impairment. However, because serum UN or creatinine concentrations evidently increased under these diseased states (about 40 mg/dL and 1.0 mg/dL in Fig. 4A and B, respectively), the clinical significance of the GFR determination may be low. Although a close correlation ($r = 0.83$) existed between the GFR values obtained from the three-sample method and that from the single-blood-sample method, the actual values (10.1 ± 0.8 mL/min/kg) in healthy rats by the latter were somewhat higher than those by the former, as described above. Therefore, when this procedure is applied to pharmacological or toxicological studies in rats, a control group is necessary for each protocol. Further studies are required to collect cumulative background data including differences in strains, genders, and ages. The highlight of this investigation is to be more efficient in animal use.

These results suggest that the single-blood-sample method with a bolus injection of iodixanol, allowing for the repeated use in the same animals, is an expedient procedure without ensuring accurate urine collection, or using radioisotopes.

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