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マストミス精子の凍結保存法の検討
Development of a method for cryopreservation of mastomys spermatozoa

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Mastomys (*Mastomys coucha*) is a rodent which has been used as an laboratory animal in biomedical researches such as oncology, parasitology and epidemiology. In this study, we attempted to develop a suitable method for the cryopreservation of mastomys sperm. Four sugars (sucrose, lactose, trehalose and raffinose), egg yolk and surface-active agents (Equex Stem, SDS) were examined as cryopotectants for the mastomys sperm cryopreservation. Sparmatozoa from cauda epididymides of mastomys were transferred into different cryoprotectants containing solutions and the sperm suspension was loaded into plastic straw and frozen in LN_2 vapor for 5 mins before being plunged into NL_2. The frozen sperm suspension was thawed in 37℃ water and was diluted with incubation media at 37℃ to evaluate sperm motility. When the spermatozoa frozen in the solution of various

concentration of cryoprotectants alone or combined, the highest sperm motility (20%) after freezing/thawing was obtained using the solution containing 18% raffinose, 25% egg yolk, 0.7% Equex Stem. Fertilizability of the frozen/thawed mastomys spermatozoa is under investigation.

Key Words: マストミス、精子、凍結保存、精子運動率

緒言

アフリカ原産のマストミスは、マウスやラットと同類の齧歯目ネズミ科に属する動物である。この動物は、1940年代に実験動物化されてから[1)]、腫瘍学や寄生虫学、疫学といった様々な研究分野で用いられてきた。また、マストミスは多くの近交系が確立されているほか、毛色変異などの遺伝的多様性が認められるため、遺伝資源としても興味深い。現在、我々は数系統のマストミスを維持しており、繁殖生物学的応用を目指した研究を行っている。

実験動物の系統維持や供給体制の確立、繁殖学的見地などから配偶子の凍結保存は大変重要なことである。中でも、精子の凍結保存は 1 匹の雄から大量の精子サンプルを採取することが可能で、様々な手法により多数の卵母細胞と受精させることができるという利点を有している。さらに、配偶子の状態で凍結することは、それらの組み合わせ次第で遺伝的多様性をもたせることを可能とし、接合体の凍結保存に比べて優れている。

今回、マストミスの実験動物としての有用性向上および系統維持を目的として、精子凍結保存法開発の検討を行ったので報告する。

材料および方法

供試動物

供試動物には、国立感染症研究所で飼育、継代されている MCC（シャモア色）、MST（野生色）、RI-7（シャモア色）の近交系 3 系統の 4〜8 ヶ月齢成熟雄マストミスを用いた。飼育室は 22±2℃に維持し、明期 14 時間、暗期 10 時間（5:00 時点灯）とした。飼料（CMF, γ線照射飼料、オリエンタル酵母工業）と水は自由摂取させた。

精子採取

成熟雄マストミスを頚椎脱臼により安楽死させた後、精巣上体尾部を摘出した。精巣上体尾部は眼科用バサミを用いて切開し、切開部から精子を排出した。25G の注射針で精子塊を掬い取り、35mm プラスティックディッシュ

(FALCON) に用意した凍結保存液 50 μl に室温で浮遊させた (精子濃度 1-3 $\times 10^7$/ml)。

精子の凍結

凍結保存液に精子を 2 分浮遊させた後、その精子懸濁液 20～30 μl を 0.25ml プラスティックストローに封入した。ストローを液体窒素気相下で 5 分間保持して予備凍結を行い、液体窒素に投入した。凍結ストローは液体窒素中に 24 時間以上保存し、融解実験に用いた。

精子の融解

凍結精子の融解は、凍結ストローを 37℃の温水中に 10 秒維持することで行った。融解した精子ストローの精子懸濁液を 35mm プラスティックディッシュに回収し、精子懸濁液 1 μl を 200 μl の精子培養メディウムに添加した (精子濃度 0.5-1.5$\times 10^5$)。凍結-融解精子は、37℃の温度条件下で培養した。

精子の運動率

精子運動率の判定は、1 サンプルあたり総数 300 以上の精子をカウントし、頭部運動を示す精子を運動精子として算出した。

実験 1：耐凍保護剤としての糖の種類と濃度選択

凍結保存液として、スクロース水溶液、ラクトース水溶液、トレハロース水溶液 (それぞれ最終濃度 3, 6, 12, 24% w/v)、ラフィノース水溶液 (最終濃度 2.25, 4.5, 9, 18% w/v) を用い、糖の種類と濃度差に対する凍結-融解精子の運動率変化を検討した。凍結-融解精子は、M16 メディウムを用いて、37℃、5% CO_2 の大気下で培養した。

実験 2：凍結保存液への卵黄と界面活性剤添加の影響

凍結保存液は、18% (w/v) ラフィノース水溶液をベースに、さらに卵黄 {最終濃度：25% (v/v)} と界面活性剤 {SDS (0-4%), Equex Stem (宮崎化学薬品, 0-2.8%)} を添加して、凍結-融解精子の運動率の変化を調べた。凍結-融解精子は、予備実験において最も成績の良かった Whittingham メディウム を改変した rAIM (Table.1) を用いて、37℃、大気相下で培養した。

統計解析

凍結-融解精子運動率の経時的変化は、精子運動率に角度変換を施した値で Scheffe あるいは Fisher の PLSD による post-hoc test を用いて有意性を検討した。その他の条件は、観察時間ごとに Student's *t*-test によって有意性を判定した。

Table.1 Composition of a medium for rat artificial insemination (rAIM)

	mM
NaCl	114.0
KCl	2.70
HEPES ($C_8H_{18}N_2O_4S$)	20.0
$C_6H_{12}O_6$	5.50
$NaH_2PO_4 \cdot 2H_2O$	0.36
Sodium pyruvate	0.10
Streptomycin sulfate	0.1 mg/ml
Penicillin G potassium	100 unit/ml
$MgCl_2 \cdot 6H_2O$	0.49
$NaHCO_3$	25.0
$CaCl_2 \cdot 2H_2O$	1.80
BSA	3 mg/ml

結果

実験 1：耐凍保護剤としての糖の種類と濃度選択

凍結保存液中の糖の種類と濃度が凍結-融解精子の運動率に及ぼす影響を Table.2 に示した。検査した二糖類（スクロース、ラクトース、トレハロース）では、それぞれ 12%(w/v)の濃度で凍結-融解精子運動率が高かった。三糖類（ラフィノース）では、18%(w/v)の濃度で運動精子率が高く、これらは二糖類と比較しても最も成績が良かった。全ての実験群において、前進運動を示す精子は認められなかったが、最も高い運動率を示した 18%(w/v)ラフィノース水溶液を凍結保存液のベースとして採用した。

実験 2：凍結保存液への卵黄と界面活性剤添加の影響

凍結保存液中への卵黄｛最終濃度：25%(v/v)｝と界面活性剤（SDS あるいは Equex Stem）の添加が凍結-融解精子の運動率に及ぼす影響を Table.3 に示した。18%(w/v)ラフィノース水溶液に卵黄のみを添加したとき、精子運動率はほとんど変化しなかった。しかしながら、卵黄と共に界面活性剤の SDS や Equex Stem を添加することで、精子運動率は改善した。SDS は 2.0%(v/v)の濃度で最も高い精子運動率を示し、Equex Stem は 0.7%(v/v)と 1.4%(v/v)の濃度で高い運動率を示した。界面活性剤の濃度に関わらず、融解後 0～60 分の間では各実験群の精子運動率に有意差は認められなかった。しかし、融解後 90 分以降では 0.7%(v/v)Equex Stem 添加群の精子運動率が、全濃度の SDS 添加群や 0 および 0.35%(v/v)Equex Stem 添加群の精子運動率に比べて有意に高かった。また、精子の運動性も新鮮精子と同様の前進運動を示した。

Table.2　Effect of Saccharide as a cryoprotectant

Saccharides	Conc. (%, w/v)	Sperm motility (%) after thawing					
		0 min	15 min	30 min	60 min	90 min	120 min
Sucrose	3.0**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	6.00**	0.1±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	12.00	0.9±0.7	0.0±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	24.00**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Lactose	3.00**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	6.00*	0.4±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	12.00	1.8±1.3	0.3±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	24.00**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Trehalose	3.00**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	6.00	1.4±2.3	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0
	12.00	3.4±2.1	0.7±0.9	0.2±0.3	0.1±0.1	0.0±0.0	0.0±0.0
	24.00	1.8±1.1	0.2±0.2	0.0±0.1	0.0±0.0	0.0±0.0	0.0±0.0
Raffinose	2.25**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	4.50**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	9.00*	0.3±0.3	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	18.00	6.9±2.7	2.2±0.4	0.4±0.8	0.3±0.3	0.0±0.0	0.0±0.0

Means±S.D. Experiments were replicated three times.

* P<0.05, ** P<0.01 (vs 18.00% raffinose solution)

考察

マウスなど齧歯類の精子凍結保存液には耐凍保護剤として糖類が広く用いられている[2,3]。今回、耐凍保護剤としての糖類およびその濃度の検討を行った結果、検討した糖類の中では18%(w/v)ラフィノース水溶液で高い凍結-融解精子の運動率が得られた。精子の凍結保存の成功において、凍結保存液の浸透圧が重要な要因であることが良く知られており、糖類はその浸透圧調整に関与している。18%(w/v)ラフィノース溶液の浸透圧は約 400Osm/kg に相当し、これは報告されているマウスの精子凍結保存に最適な浸透圧と一致している[2]。

Table.3 Effect of addition of Egg Yolk and Detergents as a cryoprotectant.

Detergents	Conc.	Sperm motility (%) after thawing					
	(%,v/v)	0 min	15 min	30 min	60 min	90 min	120 min
18%(w/v) Raffinose†	0.00	6.9±1.9	3.8±2.4	3.2±1.4	4.9±0.9	2.6±1.3	1.8±1.3
SDS	0.00	5.2±6.5	3.6±4.4	4.5±4.9	5.0±4.0	2.3±0.9**	2.9±1.8**
	0.50	9.9±13.7	7.8±9.1	9.8±6.6	9.2±9.1	5.8±2.3**	5.5±2.0**
	1.00	11.8±13.5	6.7±9.0	8.9±6.8	9.0±7.6	5.2±1.0**	4.3±2.4**
	2.00	18.6±3.1	15.7±5.1	17.9±6.6	14.9±6.9	7.7±2.0*	6.4±5.0**
	4.00	10.1±9.1	8.7±9.8	10.1±8.8	7.4±6.7	4.5±3.3**	4.7±4.9**
Equex Stem	0.00	5.7±6.2	4.4±5.6	4.7±6.4	4.4±6.3	4.5±4.1**	5.7±5.5**
	0.35	6.2±3.7	3.5±3.3	7.3±6.4	5.9±4.3	7.3±2.1*	7.2±2.5**
	0.70	13.9±6.8	10.3±7.7	13.2±9.2	14.3±8.1	16.2±5.5	20.2±4.6
	1.40	12.1±2.2	10.0±3.2	15.0±8.2	15.2±5.2	17.0±4.7	19.2±4.2
	2.80	7.1±6.2	9.1±6.3	11.2±6.4	11.0±4.4	13.5±4.8	13.9±5.2

Means±S.D. Experiments were replicated three times for each treatment.

18%(w/v) raffinose + 25%(v/v) Egg Yolk solution used as a cryoprotectant.

† 18%(w/v) raffinose solution used as a cryoprotectant

* P<0.05, ** P<0.01 (vs 0.70% Equex Stem group)

マストミス精子の形態学的特徴は、ラット精子とよく似ている。そのため、ラット精子の凍結保存液[4]に用いられている卵黄と界面活性剤の添加によるマストミス凍結-融解精子の運動率への影響を検討した。その結果、18%(w/v)ラフィノース水溶液に卵黄〔最終濃度：25%(v/v)〕とEquex Stem〔最終濃度：0.7%(v/v)〕を添加した際に、顕著に凍結-融解精子の運動率が改善した。卵黄の添加によって凍結-融解精子の運動率が増加したことは卵黄の精子原形質膜保護作用によるものと考えられる[4]。また、界面活性剤Equex Stemの精子への直接作用については定かではないが、凍結保存液内での卵黄の懸濁作用を有しており、その結果として卵黄の精子原形質膜保護作用を促進しているものと推定される[4]。

以上のように、マストミス精子では、18%(w/v)ラフィノース、25%(v/v)卵黄、0.7%(v/v)Equex Stem水溶液を凍結保存液に用い、液体窒素保存前の予備凍結時間5分、融解温度37℃、融解時間10秒の条件で凍結-融解し、37℃、大気相下でrAIMを用いて培養することで、最大20%の凍結-融解精子運動率と良好な運動性を示す精子を得ることに成功

した。これらの凍結-融解精子は、マストミスの人工授精や体外受精に使用可能であることが期待される。

現在、この凍結-融解精子の受精能判定の研究を継続中である

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Birth of Offspring After Transfer of Mongolian Gerbil (*Meriones Unguiculatus*) Embryos Cryopreserved by Vitrification

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ABSTRACT The Mongolian gerbil (*Meriones unguiculatus*) has been used as a laboratory species in many fields of research, including neurology, oncology, and parasitology. Although the cryopreservation of embryos has become a useful means to protect valuable genetic resources, its application to the Mongolian gerbil has not yet been reported. In this study, we investigated the in vitro and in vivo developmental competence of Mongolian gerbil embryos cryopreserved by vitrification. In vivo-fertilized embryos were vitrified on the day of collection using the ethylene glycol (EG)-based solutions EFS20 and EFS40, which contained 20% and 40% EG, respectively, in PB1 containing 30% (w/v) Ficoll 70 and 0.5M sucrose. First, we compared one-step and two-step vitrification protocols. In the one-step method, the embryos were directly transferred into the vitrification solution (EFS40), whereas in the two-step method, the embryos were exposed serially to EFS20 and EFS40 and then vitrified. After liquefying (thawing), late two-cell embryos (collected on day 3) vitrified by the two-step method showed significantly better rates of in vitro development to the morula stage compared to those vitrified by the one-step method (65% vs. 5%, $P < 0.0001$). We then examined whether the same two-step method could be applied to early two-cell embryos (collected on day 2), four-cell embryos (day 4), morulae (day 5), and blastocysts (day 6). After liquefying, 87%–100% of the embryos were morphologically normal in all groups, and 23% and 96% developed to the compacted morula stage from early two- and four-cell embryos, respectively. After transfer into recipient females, 3% (4/123), 1% (1/102), 5% (4/73), and 10% (15/155) developed to full-term offspring from vitrified and liquefied early two-cell embryos, late two-cell embryos, morulae, and blastocysts, respectively. This demonstrates that Mongolian gerbil embryos can be safely cryopreserved using EG-based vitrification solutions. *Mol. Reprod. Dev. 70: 464–470, 2005.* © 2005 Wiley-Liss, Inc.

Key Words: cryopreservation; ethylene glycol; embryo transfer

INTRODUCTION

The Mongolian gerbil (*Meriones unguiculatus*), also called the "laboratory gerbil", is a myomorph rodent that is native to China and Mongolia. It has been widely used as a laboratory animal in biomedical research, including the study of epilepsy (Jobe et al., 1991), tumor (Meckley and Zwicker, 1979), hypercholesterolemia (Dictenberg et al., 1995), and cerebral ischemia (Levine and Payan, 1966). This species has also been used to develop good animal models for a variety of infectious diseases caused by bacteria, viruses, and parasites; for example, *Helicobacter pylori* (Yokota et al., 1991; Sugiyama et al., 1998), Borna disease virus (Nakamura et al., 1999), *Echinococcus multilocularis* (Williams and Oriol, 1976), *Cryptospordium muris* (Koudela et al., 1998), *Brugia-pahangi* (Klei et al., 1981), *Giardia duodenalis* (Buret et al., 1991), and *Entamoeba histolytica* (Chadee and Meerovitch, 1984). Although gerbils were randomly bred in closed laboratory colonies for the first decades of their use, selective breeding has recently been conducted to establish laboratory strains that are suited for each research purpose. The best-characterized strains include seizure-sensitive and -resistant strains (Loskota et al., 1974; Robbins, 1976; Seto-Ohshima et al., 1997) and mutant strains with different coat colors (Robinson, 1973; Shimizu et al., 1990).

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For many domestic and laboratory species, assisted reproductive technologies have been developed to enhance live animal production and safe cryopreservation of genetic materials. However, the major assisted reproductive technologies—including in vitro fertilization, embryo culture, embryo transfer, and embryo cryopreservation—have not yet been established for Mongolian gerbils. This is a major drawback of working with Mongolian gerbils and it impedes their broad exploitation in biomedical research. The present study was undertaken to develop a reliable experimental protocol for cryopreservation of gerbil embryos. For this purpose, we employed embryo vitrification methods, which have been successfully used for mice (Kasai et al., 1990), rabbits (Kasai et al., 1992), cattle (Tachikawa et al., 1993), horses (Hochi et al., 1994), humans (Mukaida et al., 1998), mastomys (Mochida et al., 2001), and rats (Han et al., 2003). As little information is available concerning techniques related to embryo manipulation in gerbils, we also examined whether the protocols for superovulation and embryo transfer conventionally used for mice and rats could be applied to gerbils.

MATERIALS AND METHODS

Animals

Mongolian gerbils (*Meriones unguiculatus*) from inbred strains MGS/Sea (agouti coat color, Seac Yoshitomi, Ltd., Fukuoka, Japan) and MGB (black coat color, from the Nippon Medical School, Tokyo, Japan; Shimizu et al., 1990) were maintained under specific-pathogen-free conditions at the National Institute of Infectious Diseases, Japan. They were kept under controlled lighting conditions (light: 05:00–19:00) and provided with water and commercial laboratory mouse chow *ad libitum*. All animals were maintained and handled in accordance with the guidelines of the National Institute of Infectious Diseases, Japan. As the results obtained from different strains of gerbil were not significantly different, they were combined in this study.

Collection of Embryos

Mature females (7–18 weeks of age) were induced to superovulate by intraperitoneal injection of 10 IU pregnant mare's serum gonadotrophin (PMSG) between 3 and 5 pm, followed by injection of 10 IU human chorionic gonadotrophin (hCG) 44–46 hr later. The superovulated females were mated with mature males in cages with wire net floors. The next morning (designated day 1 of pregnancy), the presence of a copulation plug was confirmed. The early two-cell embryos, late two-cell embryos, four-cell embryos, and morulae were collected by flushing the oviducts (at 48, 72, 96, 120 hr post-hCG) with modified phosphate buffered saline (PB1, Whittingham, 1971a); blastocysts were also collected by flushing the uteri (144 hr post-hCG). The collected embryos were placed in culture dishes containing droplets of M16 medium (Whittingham, 1971b) covered with paraffin oil and cultured at 37°C under 5% CO_2 in air until cryopreservation or embryo transfer.

Cryopreservation of Embryos

In the first series of experiments, we assessed the toxicity of different cryoprotectants to optimize the cryopreservation solution suitable for gerbil embryos. Freshly collected late two-cell (day 3) embryos were immediately suspended in 2M solutions of ethylene glycol (EG), glycerol, dimethyl sulfoxide (DMSO), propylene glycol (PG), or acetamide in PB1 at room temperature (22°C) for 10 min. After washing by serial transfers into three drops of PB1 at room temperature, the rate of in vitro development to the compacted morula stage was assessed using the culture conditions described above. Preliminary experiments showed that the morula stage was the most advanced stage to which fresh two-cell embryos developed under our in vitro culture conditions.

Vitrification was performed according to the method developed for mouse embryos by Kasai et al. (1990), with slight modifications. We prepared two vitrification solutions, EFS20 and EFS40, which consisted of 20% and 40% (v/v) EG, respectively, in PB1 solution containing 30% (w/v) Ficoll (average molecular weight 70,000), and 0.5M sucrose. In this study, we employed both one- and two-step vitrification protocols. For the one-step method, 13–20 embryos, together with a minimal amount of culture medium, were directly introduced, using a fine glass pipette, into approximately 40 µl EFS40 solution in a 0.25-ml plastic straw held horizontally. The plug ends were sealed with polyvinyl alcohol powder. After exposure of embryos to the EFS solution for 2 min at room temperature, the straw was immersed in liquid nitrogen. For the two-step method, 13–20 embryos were suspended in EFS20 solution for 2 min at room temperature. They were then directly transferred to approximately 40 µl EFS40 solution in a straw, as described above. After exposure of the embryos to EFS40 solution at room temperature for 30 sec, the sealed straw was immersed in liquid nitrogen.

To liquefy, (thaw; for terminology, see Shaw and Jones, 2003) the embryos for further evaluation, after storage in liquid nitrogen for at least 2 days, a straw was warmed rapidly in 22°C water for about 8 sec. Immediately after warming, the EFS solution containing the embryos was expelled from the straw onto a watch glass using a metal rod. The solution was diluted by addition of 0.8 ml PB1 medium containing 0.25M or 0.5M sucrose (S-PB1) and the embryos were retrieved into fresh S-PB1 medium. At 5 min after liquefying, the embryos were transferred to PB1 medium.

Embryo Transfer

Two types of pseudopregnant recipient females were prepared for embryo transfer: hormone-treated recipients and nontreated recipients. For the former, females were pre-treated with hormones for superovulation, as described above. Only virgin females were used for both groups. The vasectomized males for induction of pseudopregnancy were prepared at least 4 weeks before the experiments and infertility was confirmed by the

absence of sperm in the vagina of females after mating. Each recipient female was mated with a mature vasectomized male in a cage with a wire net floor. On the following morning (designated day 1 of pseudopregnancy), the females were examined for the presence of a vaginal plug and used for embryo transfer. Shortly before embryo transfer, the recipient females were anesthetized with an intraperitoneal injection of sodium pentobarbital (57.5 mg/kg).

Statistical Analysis

The results were evaluated using Fisher's exact probability test. Values of P less than 0.05 were considered statistically significant.

RESULTS

Effects of Cryoprotectants on the Development of Gerbil Embryos

Gerbil embryos were exposed to one of five different cryoprotectants and their subsequent development was assessed. As shown in Figure 1, embryos exposed to EG and PG showed rates of development to the compacted morula stage similar to those of nonexposed control embryos ($P > 0.05$). In contrast, those exposed to glycerol or acetamide had significantly decreased developmental competence (Fig. 1). Exposure to DMSO had an intermediate effect on embryo development. Therefore, we selected EG-based solutions for use in our cryopreservation experiments for gerbil embryos, as has been done for mouse embryos (Kasai et al., 1990).

Comparison of One- and Two-step Methods

Late two-cell (day 3) gerbil embryos that had been vitrified using the one- or two-step methods were liquefied and examined for subsequent development in vitro. More embryos developed to the compacted morula stage in vitro when they had been vitrified by the two-step method using EFS20 and EFS40, and liquefied in 0.25M sucrose solution (36/55, 69%) as compared to the other vitrification and liquefying procedures (Fig. 2). Only 5% of embryos (2/43) developed to the morula stage after the one-step vitrification method using EFS40 alone (Fig. 2).

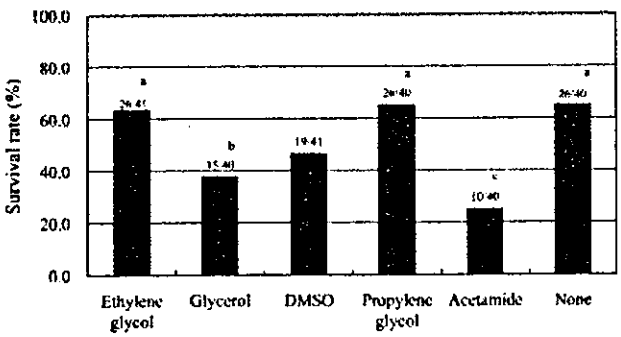

Fig. 1. Survival of late two-cell (day 3) gerbil embryos after exposure to cryoprotectants in PB1 for 10 min at 22°C. The numbers on the bars indicate surviving embryos per exposed embryos. The values with different letters (a, b, and c) are statistically different ($P < 0.05$).

In light of these results, we employed the two-step method for the subsequent vitrification experiments.

Effect of the Stage of Development Upon Cryopreservation on Subsequent In Vitro Embryo Development

Using the two-step method described above, we vitrified embryos at different stages of development and later liquefied and examined them to determine their survival and subsequent development in vitro and in vivo. When recovered into normal culture medium, most embryos (>87%) appeared to be morphologically normal, regardless of the stage at which the embryos were vitrified (Table 1). When cultured in vitro, embryos vitrified at the late two-cell (day 3) and four-cell (day 4) stages showed in vitro development comparable to that of nonvitrified control embryos at the same stage (Table 1). When embryos were vitrified at the early two-cell stage (day 2), in vitro developmental competence was significantly lower than that of controls (Table 1).

Development of Vitrified Gerbil Embryos After Embryo Transfer

We found that hormonal treatment of the recipient females made the efficiency of mating with males more consistent compared with natural mating, which occurs at random. However, as hormonal treatment may compromise the oviductal and uterine environments for transferred embryos, we first assessed whether hormonally treated recipient females conceived after embryo transfer. Early two-cell embryos (day 2) and blastocysts (day 6) transferred into the recipient oviducts and uteri, respectively, developed to term, regardless of whether the recipient females had been treated with hormones (Table 2). For further embryo transfer experiments, therefore, we used recipient females prepared by natural mating without hormone treatment, to optimize the conditions for efficient embryo transfer.

Early two-cell embryos (day 2), late two-cell embryos (day 3), morulae (day 5), and blastocysts (day 6) were vitrified using the two-step method, liquefied, and assessed for their developmental competence after transfer into recipient females. Four-cell embryos (day 4) were not transferred because day 3 oviduct (ampullar) is not an appropriate transfer site in gerbils owing to the distance from the position of the native oocytes, which at this stage have descended to a point near the uterine–oviductal junction.

Although the rates of development to full-term offspring were not high, normal pups were born from embryos vitrified at all stages, indicating that at least a portion of the embryos were completely viable and competent after vitrification and liquefying (Table 3; Fig. 3). The implantation rates were also low (Table 3), indicating that most embryos died before implantation, or simply failed to attach to the uterine epithelium. The weaning rates varied according to the experiment, but all of weaned pups developed into adults with normal appearance, to the extent examined.

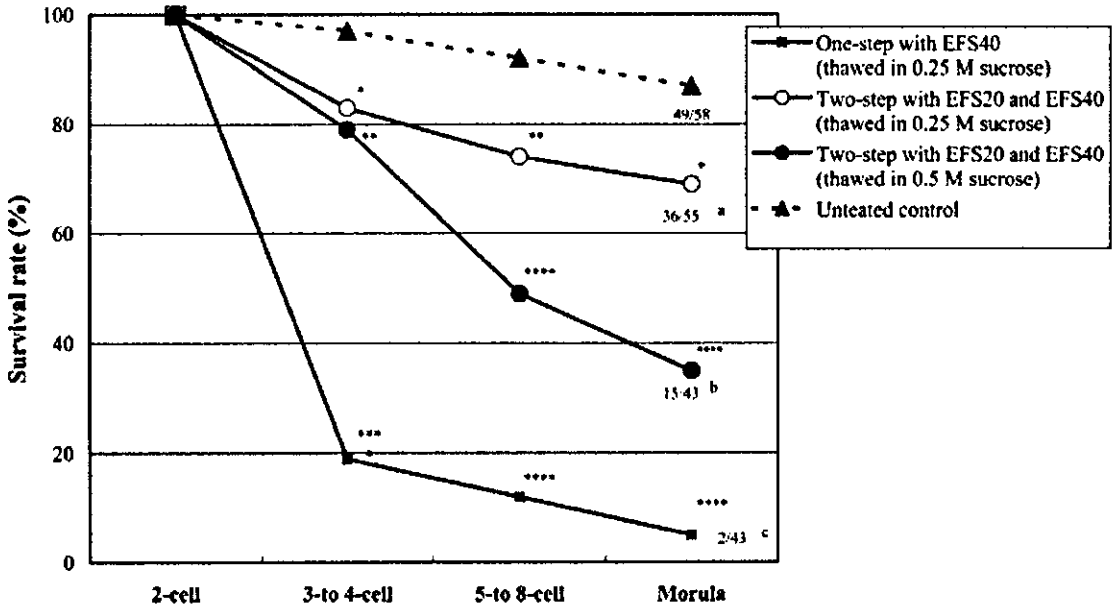

Fig. 2. In vitro development of late two-cell (day 3) gerbil embryos vitrified in EFS solution by the one-step or two-step method. Numbers indicate surviving embryos per cultured embryos. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.001$, as compared with the nonvitrified control at the same stage. $^{a,b}P < 0.001$, $^{b,c}P < 0.005$, and $^{a,c}P < 0.0001$.

DISCUSSION

The present study clearly demonstrated that Mongolian gerbil embryos could survive freezing and liquefying procedures at high rates and that some of them develop into normal full-term offspring. We employed a vitrification method using EG-based cryoprotectant solutions. Embryo cryopreservation by vitrification was first developed by Rall and Fahy (1985) for mouse embryos. It has potential advantages over conventional slow-freezing methods because of its very rapid cooling time and minimal cell injury caused by extracellular crystallization (Rall, 1987). However, the original vitrification solution consisted of four cryoprotectants, including acetamide, which is known to be very toxic to embryos; therefore, its application eventually was limited to embryos of certain strains of mice. Later, this complication was overcome by development of less toxic vitrification solutions using EG or glycerol (Kasai et al., 1990; Zhu et al., 1994; Rall et al., 2000). The use of EG also increased the flexibility of the conditions for vitrification protocols (e.g., exposure time to cryoprotectant) and thus increased the reproducibility of the vitrification experiments (Kuleshova et al., 1999; Nowshari and Brem, 2001). In this study, we confirmed the low toxicity of EG for gerbil embryos, but glycerol showed moderate toxic effects for gerbil embryos. To date, successful vitrification using EG has been reported for

TABLE 1. In Vitro Development of Gerbil Embryos After Vitrification by the Two-Step Method With EFS20 and EFS40

		No. (%) of embryos				
Stage of embryos (day)	Treatment	Vitrified	Recovered	Morphologically normal	Cultured	Developed to morula
Early two-cell (2)	Vitrified	40	39 (98)	39 (100)	39	9 (23)*
	Control	—	—	—	36	31 (86)*
Late two-cell (3)	Vitrified	176	169 (96)	147 (87)	55	38 (69)
	Control	—	—	—	49	39 (80)
Four-cell (4)	Vitrified	54	50 (93)	50 (100)	50	48 (96)
	Control	—	—	—	60	51 (85)
Morula (5)	Vitrified	94	92 (98)	87 (95)	—	—
Blastocyst (6)	Vitrified	50	46 (92)	46 (100)	—	—

*$P < 0.001$.

TABLE 2. In Vivo Development of Gerbil Embryos Transferred Into Pseudopregnant Recipients With or Without Treatment for Induction of Sterile Mating

Stage of embryos (day)	Treatment before sterile mating	No. (%) of recipients that became pregnant	No. of embryos transferred	No. (%)		
				Implanted	Live offspring delivered	Offspring weaned
Early two-cell (2)	No treatment	3/7 (43)	80	12 (15)	9 (11)	4 (5)
	Hormone treatment	4/8 (50)	124	23 (19)	10 (8)	7 (6)
Blastocyst (6)	No treatment	5/5 (100)	77	40 (52)	37 (48)*	31 (40)*
	Hormone treatment	5/7 (71)	111	34 (31)	20 (18)*	15 (14)*

Early two-cell and blastocyst were transferred into the oviducts (day 1) and uteri (day 5) of recipient females, respectively.
*$P < 0.001$.

many mammalian species (see Introduction). In mice, its applicability for embryos at each developmental stage has been assessed in detail (Miyake et al., 1993).

As vitrification solutions contain very high concentrations of cryoprotectants and sucrose to circumvent intracellular ice formation, the embryos are exposed to extremely high osmolality before they are vitrified. It had been reported that this osmotic shock may compromise the viability of the embryos, but can be reduced by the step-wise exposure of embryos to solutions with increasing osmolalities. We found that this was also the case with gerbil embryos. When late two-cell gerbil embryos were vitrified using the two-step method with EFS20 and EFS40, their developmental competence was significantly improved; as many as 65% of the frozen and liquefied embryos reached the morula stage in vitro. The osmolalities of EFS20 and EFS40 were about 4.5 Osmol/kg and 9 Osmol/kg, respectively, as measured by an automated osmometer. In mice, we found that better survival rates could be achieved for embryos from certain strains (e.g., DBA/2, ddY) using the two-step method, as compared to a one-step method with EFS30. Thus far, mouse embryos from 248 strains, including genetically modified mice, have been safely cryopreserved using the two-step method and their viability confirmed by full-term development after liquefying and embryo transfer (unpublished).

In the last series of experiments, we assessed the viability and competence of vitrified gerbil embryos by transfer into recipient females. Because there have been very few studies on embryo transfer in gerbils, the best combinations of the embryo stage and the day of pseudopregnancy of the recipient females are not known. In preliminary experiments, late two-cell (day 3) embryos did not survive when transferred into 1-day pseudopregnant oviducts. In this study, therefore, the recipient females were implanted with embryos 1 day older than the female's pseudopregnancy. The oviducts and uteri of gerbils accepted embryos at different stages of development as long as a 1-day difference existed between the recipients and embryos. Embryos at every developmental stage developed into normal offspring, regardless of whether the embryos had been vitrified. However, the rates of normal birth per transfer were very low, being less than 10% in most cases. We also found that the implantation rates of both vitrified and fresh embryos were low, except in the case of fresh 6-day embryos. This indicates that embryo transfer techniques for gerbils can still be improved, probably by optimizing the transfer timing. The in vitro culture medium for gerbil embryos should also be improved, because no two-cell or four-cell embryos reached the blastocyst stage in M16 medium, which was originally developed for mouse embryos. We have previously reported that embryos of

TABLE 3. In Vivo Development of Vitrified Gerbil Embryos After Transferred Into Pseudopregnant Females

Stage of embryos (day)	Recipient female			No. of embryos transferred	No. (%) of		
	Stage (day)	Transfer site	No. (%) that became pregnant		Implanted	Live offspring delivered	Offspring weaned
Early two-cell (2)	1	Oviduct	2/8 (25)	123	6 (5)*	4 (3)**	2 (2)**
Late two-cell (3)	2	Oviduct	1/6 (17)	102	6 (6)*	1 (1)*	1 (1)**
Morula (5)	4	Uterus	2/6 (33)	73	9 (12)	4 (5)	2 (3)
Blastocyst (6)	5	Uterus	3/10 (33)	155	30 (19)*	15 (10)*,**	13 (8)**
Blastocyst (6)	5[a]	Uterus	3/7 (43)	110	25 (23)	17 (15)	15 (14)

Embryos were transferred into recipient females on the day of thawing.
[a]Without hormone treatment (natural cycle).
*$P < 0.005$.
**$P < 0.05$.

Fig. 3. Gerbil pups born after transfer of vitrified blastocysts (black). They looked normal and showed active movement.

mastomys, a laboratory rodent native to Africa, developed into blastocysts in glutamine-containing medium, but not in a medium lacking glutamine (Ogura et al., 1997). Future experiments should examine whether this is also the case with gerbil embryos.

No conventional methods yet exist for embryo transfer in the Mongolian gerbil, in part because, unlike other rodents such as mice and rats, induction of pseudopregnancy in female gerbils is difficult due to the unique character of the reproductive biology of this species. First, females and males caged together after reaching sexual maturity often show very aggressive behavior because of their monogamous nature. It has been reported that the incidence of fighting and mortality can be decreased to some extent by combining an elder male and a virgin female (Norris and Adams, 1972). We employed this combination for our embryo transfer experiments. Second, unlike in mice and rats, it is difficult to identify the estrous cycle in gerbils by vaginal smears due to their irregular patterns. The estrous cycle of the Mongolian gerbil generally lasts for 4 to 7 days, varying by individual animal (Marston and Chang, 1965). In a preliminary experiment, we examined whether the cycle could be synchronized by the hormone treatment used for superovulation. On the day following hCG administration and mating, 61% (n = 41) of females had a copulation plug. As these hormone-treated females were proven to conceive after embryo transfer, we conventionally employed this method to conduct transfer experiments using vitrified embryos. Successful embryo transfer in Mongolian gerbils was first reported by Norris and Rall (1983), who used lactating pregnant females as recipients after ligating their single oviduct during early pregnancy. This method gave excellent results by exploiting lactation-induced delayed implantation for embryo transfer.

In conclusion, Mongolian gerbil embryos can be cryopreserved safely using a two-step vitrification method with EG-based cryoprotectant solutions. The offspring derived from vitrified embryos appeared normal and grew into fertile adults. This strategy will enable efficient maintenance of gerbil breeding colonies and avoid microbiological and genetic contamination that may occur during natural breeding.

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FULL PAPER *Anatomy*

Decreased Expression of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinase in the Kidneys of Hereditary Nephrotic (ICGN) Mice

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ABSTRACT. Matrix metalloporoteinases (MMPs), which are dominantly regulated by tissue inhibitors of metalloproteinase (TIMPs), play important roles in extracellular matrix (ECM) degradation and are involved in the progression of kidney diseases. In glomeruli and tubulointerstitum of hereditary nephrotic (ICR-derived glomerulonephritis: ICGN) mouse kidneys, hyper-accumulation of ECM components occurred, and MMP activity decreased. In the present study, because lower levels of MMP activity may contribute to the progression of renal fibrosis in ICGN mice, Western blotting analysis and immunohistochemical staining for MMPs and TIMPs were performed to verify the expression levels of these proteins. Levels of MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 in the kidneys were decreased in ICGN mice in comparison with normal ICR mice. These results indicate that small amounts and low levels of activity of MMPs cause the progression of renal fibrosis in ICGN mice.

KEY WORDS: extracellular matrix (ECM), ICR-derived glomerulonephritis (ICGN) mouse, matrix metalloproteinase (MMP), renal fibrosis, tissue inhibitor of metalloproteinase (TIMP).

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The quality and quantity of extracellular matrix (ECM) components are tightly regulated in normal tissue, cell migration, proliferation, apoptotic cell death and so on. A balance between the production and degradation of the ECM is maintained to achieve tissue homeostasis, but is disrupted under pathological conditions [1]. Hyper-production and/or hypo-degradation of the ECM can cause fibrosis in many organs, ex. kidneys, liver, lungs and so on. ECMs are dominantly degraded by serine proteinase, plasmin, and matrix metalloproteinases (MMPs) [14, 15, 20]. Based on substrate specificity, MMPs are classified as follows: (1) MMP-1 (interstitial collagenase), primarily responsible for the degradation of type I collagen; (2) MMP-2 and MMP-9 (gelatinases), dominantly degrade type IV collagen; (3) MMP-3 (stromelysin), has a broad substrate specificity and degrades type IV and V collagens, proteoglycans and laminin; and (4) membrane type MMP (MT-MMP: membrane associated MMP), degrades not only various ECM components, such as type I collagen, but also processes the precursor MMP. Each MMP is secreted as precursor enzyme (pro-MMP; non-active form) into the extracellular space and binds with tissue inhibitor of metalloproteinase (TIMP), which is a dominant regulator of MMP activation. When the degradation of ECM is required, the appropriate TIMP is removed from the MMP via digestion by a proteolytic enzyme (ex. Plasmin, MT-MMP and so on), and pro-MMP is activated and the against ECM.

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ICR-derived glomerulonephritis (ICGN) mice develop severe proteinuria at an early age which progresses to nephrosis [7, 8]. This strain suffers from hypoalbuminemia, hypercholesterolemia, and anemia. Histological studies show a thickened glomerular basement membrane (GBM) and effacement of podocyte foot processes [6–11]. We previously showed that components of the ECM accumulate in glomeruli and tubulointerstitum of ICGN kidneys [11], and that the accumulation was due to hyper-production and less degradation of the ECM [17, 18]. We biochemically measured the activity of MMP-1, MMP-2 and MMP-9 in ICGN kidneys, and found decreased levels in comparison with normal ICR mice [17]. However, the reason for the decrease was not clear. In the present study, to verify whether lower expression levels of these proteins provoke lower levels of activity or not, we compared the expression levels of MMPs (MMP-2, MMP-9 and MT1-MMP) and TIMPs (TIMP-1 and TIMP-2) in the kidneys of ICGN mice and in of normal ICR mice by Western blotting and immunohistochemical staining.

MATERIALS AND METHODS

Animals and tissue preparation: ICGN mice were prepared by mating homozygous males (*nep/nep*) with heterozygous females (*nep/–*) at the laboratory of the National Institute of Infectious Diseases (NIID) [8–11]. Early stage (8-week-old; slightly renal fibrosis) and terminal stage (15-week-old: progressed stage of renal fibrosis) male homozygous ICGN mice from a specific-pathogen-free colony in NIID [16–18] and age- and sex-matched ICR mice pur-

chased from Clea Japan (Tokyo, Japan) were used (n=6 in each group). All animals were housed in autoclaved metal cages and were given a standard diet (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 #86–23; revised 2000).

For biochemical analyses, urine samples were collected during the 24 hr before sacrifice (24-hr-urine samples), and then blood samples were obtained from the cervical vein under mild ether anesthesia. The animals were sacrificed under deep ether anesthesia, and the kidneys were rapidly removed. One kidney was immediately fixed in 10% neutral-buffered formalin (pH 7.4) for conventional histopathological evaluation and immunohistochemical study. The other kidney was frozen in liquid nitrogen and used for biochemical study.

Clinical biochemistry: To evaluate the nephrotic state and loss of renal function, blood and 24-hr urine samples were examined on the basis of the following biochemical parameters. Serum and urinary albumin (sAlb and uAlb, respectively), serum creatinine (sCr), blood urea nitrogen (BUN) and serum total cholesterol (sTC) levels were enzyme-biochemically measured with an automatic analyzer (Dri-Chem 3500U, Fuji Film, Tokyo, Japan). All procedures were performed according to the manufacturers' protocols.

Renal histopathology and immunohistochemistry: Formalin-fixed kidney samples were dehydrated through a graded ethanol series and embedded in Histosec (Merck, Darmstadt, Germany). Sections 3 μm thick were mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Sigma Aldrich Chemical, St. Louis, MO, U.S.A.), deparaffinized with xylene and rehydrated through a graded ethanol series. For conventional histopathological evaluation, kidney sections were stained with hematoxylin and eosin. As previously reported [16–19], the extent of glomerulosclerosis was expressed as the degree of ECM deposition, which was assessed on sections stained with Sirius-red solution (saturated picric acid in distilled water containing 0.1% Sirius-red F3B; BDH Chemicals, Poole, U.K.). Sirius red staining detects interstitial collagen. All slides were mounted with Entellan (Merck), and examined under a light microscope (BX51; Olympus, Tokyo, Japan) at least three sections/mouse. In each kidney specimen, approximately 100 glomeruli and 100 tubular fields were selected at random and histopathologically evaluated. Briefly, the mesangial expansion in the glomeruli scored according to the extent of the sclerotic lesion in the glomerulus, the morphological changes in the glomeruli (capillary aneurysm: thickened GBM and foot process effacement of podocytes), and tubular (cystic tubular dilation, epithelial cellular atrophy and intraluminal cast formation) and tubulointerstitial (tubulointerstitial expansion and mononuclear cell filtration around arterioles) lesions were evaluated [16–18].

Serial sections were immunohistochemically stained for MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 proteins. Briefly, the sections were preincubated with 0.3% H_2O_2 in methanol for 30 min to inhibit endogenous peroxidase, rinsed with 0.05% Triton-X100 in Tris-HCl buffered saline (TBS, pH 7.6) for 30 min at room temperature (22–25°C), and then incubated with each primary antibody for 60 min at 20°C. Primary antibodies used were as follows: mouse monoclonal anti-MMP-2 IgG1, mouse monoclonal anti-MMP-9 IgG1, mouse monoclonal anti-MT1-MMP IgG1, mouse monoclonal anti-TIMP-1 IgG1, and mouse monoclonal anti-TIMP-2 IgG1 (Daiichi Fine Chemicals, Takaoka, Japan). Each primary antibody was prepared as follows: each primary antibody diluted at 1/20 with 50% TBS and 50% Envision-horseradish peroxidase mouse solution (Dako, Glostrup, Denmark) was preincubated for 1 hr at room temperature, added to a 1/20 volume of mouse normal serum (Dako), and then incubated for 1 hr at room temperature. After incubation with the primary antibody, the sections were treated with 1 mg/m*l* of 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals, Osaka, Japan) and 0.1% H_2O_2 in 50 mM Tris-HCl (pH 7.6) for 1 min at room temperature. After a wash with distilled water, they were counterstained with hematoxylin, mounted with Entellan, and examined under a light microscope.

Western blotting analysis: Frozen kidney samples were homogenized with lysis buffer [20 mM Tris-HCl (pH 7.5), 138 mM NaCl, 10% Glycerol, 1% IGEPAL (Sigma), 2 mM EDTA, 2.1 mg/m*l* of Aprotinin (Sigma), 1 μg/m*l* of Leupeptin (Sigma), 1 μg/m*l* of Pepstatin A (Sigma), 0.25% sodium deoxycholate (Sigma) and 1 mM Pefabloc-SC (Roche Diagnostics, Mannheim, Germany)]. Then, an equal volume of sample buffer [120 mM Tris-HCl (pH 6.8), 5% sodium dodecyl sulfate (SDS), 1440 mM β-mercaptoethanol and 3% bromophenol blue] was added, mixed well, and boiled for 5 min. After centrifugation at 3,000 g, an aliquot of protein (15 μg) from each tissue sample was electrophoresed on 10% SDS-polyacrylamide gel, and separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). The membranes were preincubated with blocking buffer [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2.5% bovine serum albumin (BSA: Sigma) and 0.1% Tween 20] for 60 min at room temperature, and then incubated with each primary antibody (as above described) diluted at 1/20 with blocking buffer. Then, the membranes were washed with washing buffer [10 mM Tris-HCl (pH 7.6), 100 mM NaCl and 0.1% Tween 20], and incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Dako) diluted at 1/2,000 with 5% skim-milk and 1% BSA in washing buffer for 60 min at room temperature. After a wash with washing buffer, chemiluminescence was visualized using an ECL-system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) according to the manufacturer's protocol. The chemiluminescence was recorded with a digital recorder (LAS-1000; Fuji Film), and then protein expression levels were quantified using ImageGauge (Fuji Film) on a Macintosh computer.

Statistical methods: ANOVA analyses with Fisher's least

significant differences test comparison for biochemical data, and Wilcoxon's signed rank test for histopathological estimation were carried out with the StatView IV program (Abacus Concepts, Berkely, CA, U.S.A.) using a Macintosh computer. Differences at a probability of $P<0.05$ were considered significant. All data are expressed as mean values ± SD (n=6 in each group).

RESULTS

Clinical biochemistry and kidney histopathology: Clinical biochemical data showed that terminal stage ICGN mice (15 week-old) used in the present study developed progressed nephrosis (high serum creatinine and blood urea nitrogen levels, hypercholesterolemia and hypoalbuminemia) (Table 1). Progressed fibrosis in the glomeruli and tubulointerstitum, enlargement of the mesangial region without cellular proliferation, tubulointerstitial expansion, appearance of cysts, expansion of renal tubules, and infiltration of lymphocytes in the tubulointerstitum were observed in the kidneys of terminal (15-week-old)-ICGN mice, and compared with those of early (8-week-old)-ICGN mice and age-matched control ICR mice (Table 2 and Fig. 1). Histopathological findings in the kidneys of terminal-ICGN mice were the same as in previous reports [16–19].

Western blotting analysis for MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2: To assess the expression levels of MMP-2, MMP-9 and MT1-MMP proteins in the kidneys, Western Blotting was performed. With the progression of renal disease in the kidneys of ICGN mice, MMP-2, MMP-9 and MT1-MMP protein levels decreased (Fig. 2). When compared with the kidneys of control ICR mice, a 39 and 58% decrease in the level of MMP-2, a 30 and 26% decrease in the level of MMP-9, and a 16 and 91% decrease in the level of MT1-MMP were found in the kidneys of early-ICGN mice and terminal-ICGN mice, respectively. Furthermore, the expression levels of TIMP-1 and TIMP-2 proteins also decreased with the progression of renal disease in the kidneys of ICGN mice (Fig. 3). When compared with ICR mouse kidneys, a 26 and 68% decrease in the level of TIMP-1, and a 25 and 27% decrease in the level of TIMP-2, were noted in early-ICGN mouse kidneys and terminal-ICGN mouse kidneys, respectively. The decrease in the levels of MT1-MMP and TIMP-1 in the kidneys of terminal-ICGN mice was prominent.

Immunohistochemistry for MMP-2, MMP-9, TIMP-1 and TIMP-2: Changes in the localization of MMP and TIMP proteins in the kidneys of ICGN mice with the progression of renal disease were immunohistochemically demonstrated. Slight or weak staining for MMP-2 was demonstrated in proximal and distal tubules in control ICR mice (Fig. 4A), whereas that trace or slight staining in proximal and distal tubules, and weak staining in mesangium cells of glomeruli were observed in the kidneys of terminal-ICGN mice (Fig. 4B). Immunostaining for MMP-9 was observed in proximal and distal tubules of ICR and early-ICGN mice (Fig. 4C and D, respectively), while a slight decrease in staining intensity was seen in those of terminal-ICGN mice. Strong staining for TIMP-1 was demonstrated in the basal area of distal tubules in ICR mice (Fig. 5A), whereas decreased staining intensity in the basal area of distal tubules and slight staining in apical and lateral areas of distal tubules were seen in the kidneys of terminal-ICGN mice (Fig. 5B). Immunostaining for TIMP-2 was seen in proximal and distal tubules of ICR and ICGN mice (Fig. 5C and D, respectively). Decreased staining for TIMP-2 was observed in those of terminal- ICGN mice (Fig. 5D).

DISCUSSION

The ECM binds to receptors, integrins, which link with cytoskeletal components and signal transducing compo-

Table 1. Serum and urine biochemical features in ICR and ICGN mice

Strains	ICR mice		ICGN mice	
Age (week-old)	8	15	8	15
Serum albumin (g/dl)	3.43 ± 0.24	3.42 ± 0.13	2.82 ± 0.13*	2.13 ± 0.16***
Serum creatinine (mg/dl)	0.29 ± 0.03	0.30 ± 0.03	0.41 ± 0.04*	0.90 ± 0.10***
Blood urea nitrogen (mg/dl)	32.0 ± 1.4	31.8 ± 1.9	48.0 ± 4.7***	70.7 ± 5.4***
Serum total cholesterol (mg/dl)	98.0 ± 3.9	120.2 ± 5.1	139.2 ± 11.4***	204.5 ± 15.1***

* and ***: $p < 0.05$ and 0.001 vs each ICR group, respectively.

Table 2. Histopathological changes of glomeruli in the renal cortex area of control ICR and ICGN mice

	ICR mice	Early-ICGN mice (8 week-old)	Terminal-ICGN mice (15 week-old)
Normal[a)] (%)	97.8 ± 1.2	78.7 ± 13.1*	35.8 ± 6.8***
Expansion of mesangial areas[a)] (%)	2.2 ± 1.2	8.3 ± 2.4*	54.8 ± 4.9***
Capillary aneurysm[a)] (%)	Not observed	4.4 ± 0.3	13.6 ± 4.0

a) Histopathological changes are described in detail in the Materials and Methods.
* and ***: $P < 0.05$ and 0.001 versus control ICR mice, respectively.

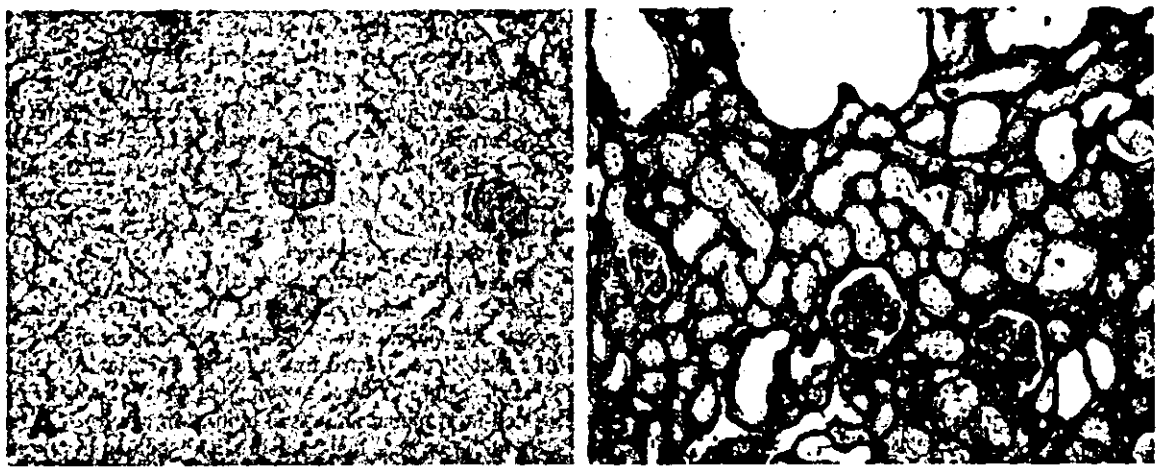

Fig. 1. Sirius-red staining demonstrating interstitial collagen accumulation. As compared with the kidney of ICR mouse (A), marked collagen accumulation was observed in the kidney of ICGN mouse (B). (× 200)

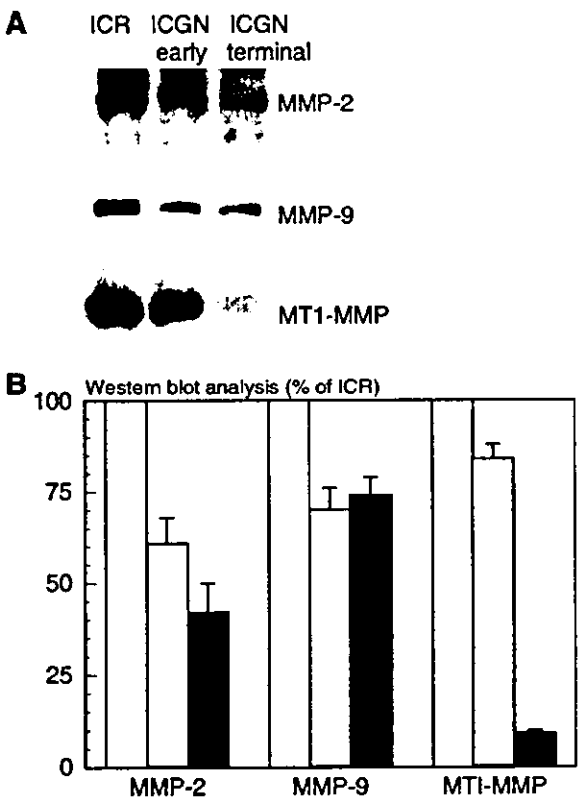

Fig. 2. Western blotting analyses for MMP-2, MMP-9 and MT1-MMP proteins. Representative photographs are shown in A, and quantitative data are summarized in B. Open, dotted and closed columns in B are ICR, early-ICGN and terminal ICGN mice, respectively. The expression levels of MMP-2, MMP-9 and MT1-MMP proteins were decreased in the kidneys of ICGN mice as compared with those of ICR mice.

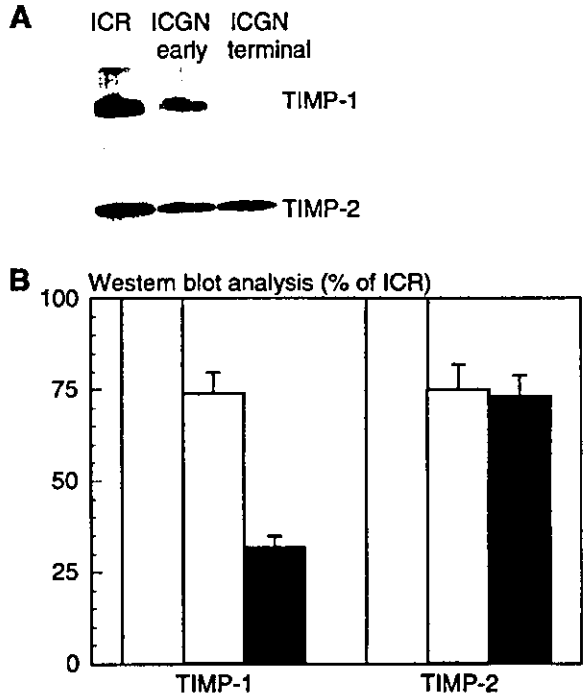

Fig. 3. Western Blotting analyses for TIMP-1 and TIMP-2 proteins. Representative photographs are shown in A, and quantitative data are summarized in B. Open, dotted and closed columns in B are ICR, early-ICGN and terminal ICGN mice, respectively. The expression levels of TIMP-1 and TIMP-2 proteins were decreased in the kidneys of ICGN mice in comparison with those of ICR mice.

nents, and influence cell morphology and functions, such as cell migration, proliferation, apoptosis and so on. The production and degradation of the ECM are accurately regulated in quantity and quality in healthy mammals, but under

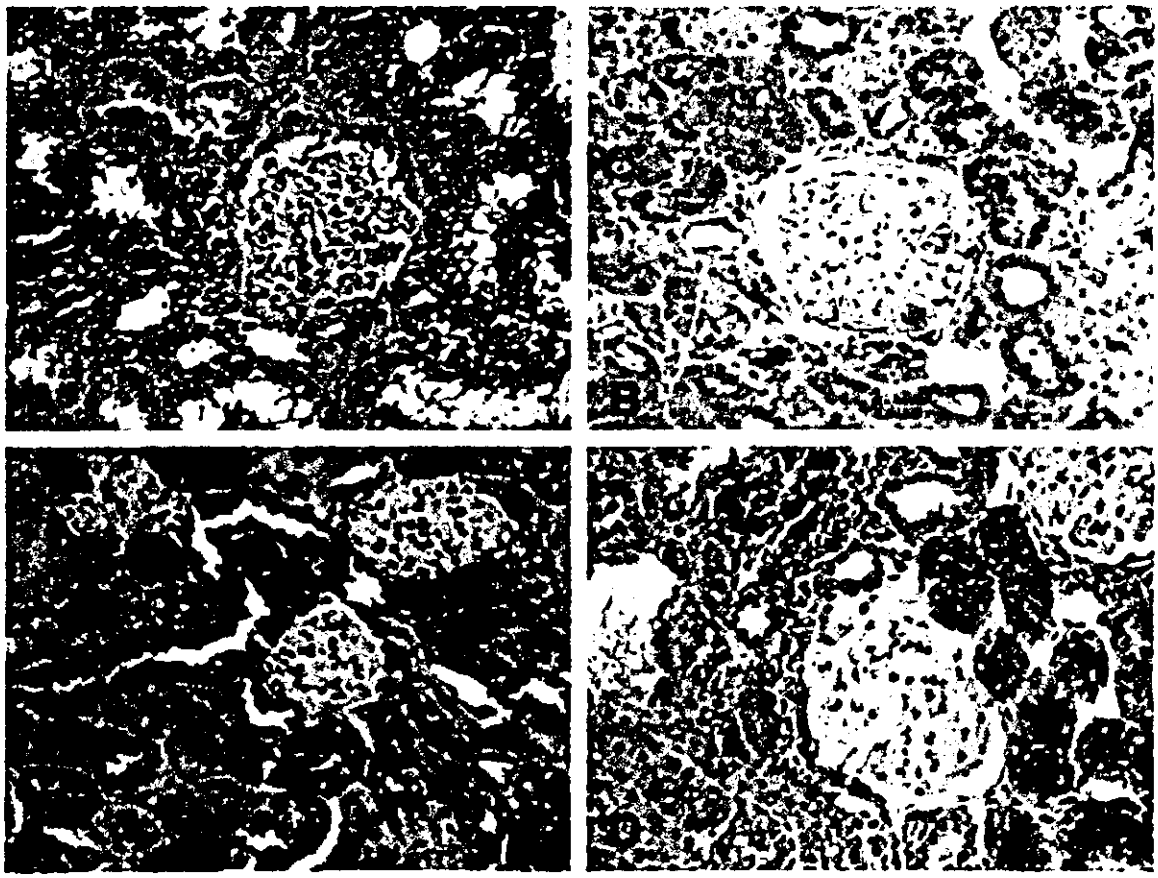

Fig. 4. Immunohistochemical staining for MMP-2 (A and B) and MMP-9 proteins (C and D). MMP-2 protein was detected in proximal and distal tubules in ICR (A) and terminal-stage ICGN mice (B). In the kidneys of ICGN mice, MMP-2 levels were slightly decreased in some tubules, and weak signals were observed in glomeruli. MMP-9 protein was detected in proximal and distal tubules of ICR (C) and terminal-stage ICGN mice (D). In ICGN mice, a slight decrease in the level of MMP-9 was observed. (× 200)

pathological conditions this balance is upset, and the homeostatic regulation of cell morphology and function can not be maintained [1, 14]. Consequently, abnormal regulation of ECM metabolism causes the progression of fibrosis.

ICGN mice, a spontaneous glomerulonephritis model, show a hyper-accumulation of ECM components in glomerular and tubulointerstitial regions. Notably, basement membrane components in glomeruli are accumulated at the earliest stage of renal fibrosis in the kidneys of ICGN mice [11]. Previously, we showed that hyper-production and hypo-degradation of ECMs occurred in ICGN kidneys, and such an abnormal metabolism may induce renal fibrosis [16–18]. Briefly, *in situ* hybridization data showed ECM production in glomeruli and tubulointerstitum of ICGN mice to be up-regulated [18]. Decreased levels of activity by MMP-1, MMP-2 and MMP-9, estimated biochemically, in the kidneys of ICGN mice were found [17]. The hyper-accumulation of ECM components is due to hyper-production and hypo-degradation. Moreover, an abnormal localization of integrins was demonstrated in kidneys of ICGN mice in comparison with healthy controls [19].

In the present study, we found decreased levels of MMP and TIMP proteins in the kidneys of ICGN mice. Particularly low levels of MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 were noted. These catabolism-regulating molecules are involved in the degradation of basement membrane components. In the kidneys of ICGN mice, thickened basement membranes of glomeruli and renal tubules were confirmed [11]. However, our previous study revealed that the production of basement membrane components, such as type IV collagen and laminin, was not markedly increased in the kidneys of ICGN mice [16, 18]. Moreover, the expression levels of TIMPs, which inhibit the activation of MMPs and degradation of ECM components, were also decreased in the kidneys of ICGN mice. Down-regulated MMPs are responsible for the lower levels of ECM degradation, in spite of the down-regulation of TIMPs. Generally, down-regulated TIMPs augment ECM components, but down-regulated TIMPs, lower activities of MMPs and augumented ECM components were detected in the kidneys of ICGN

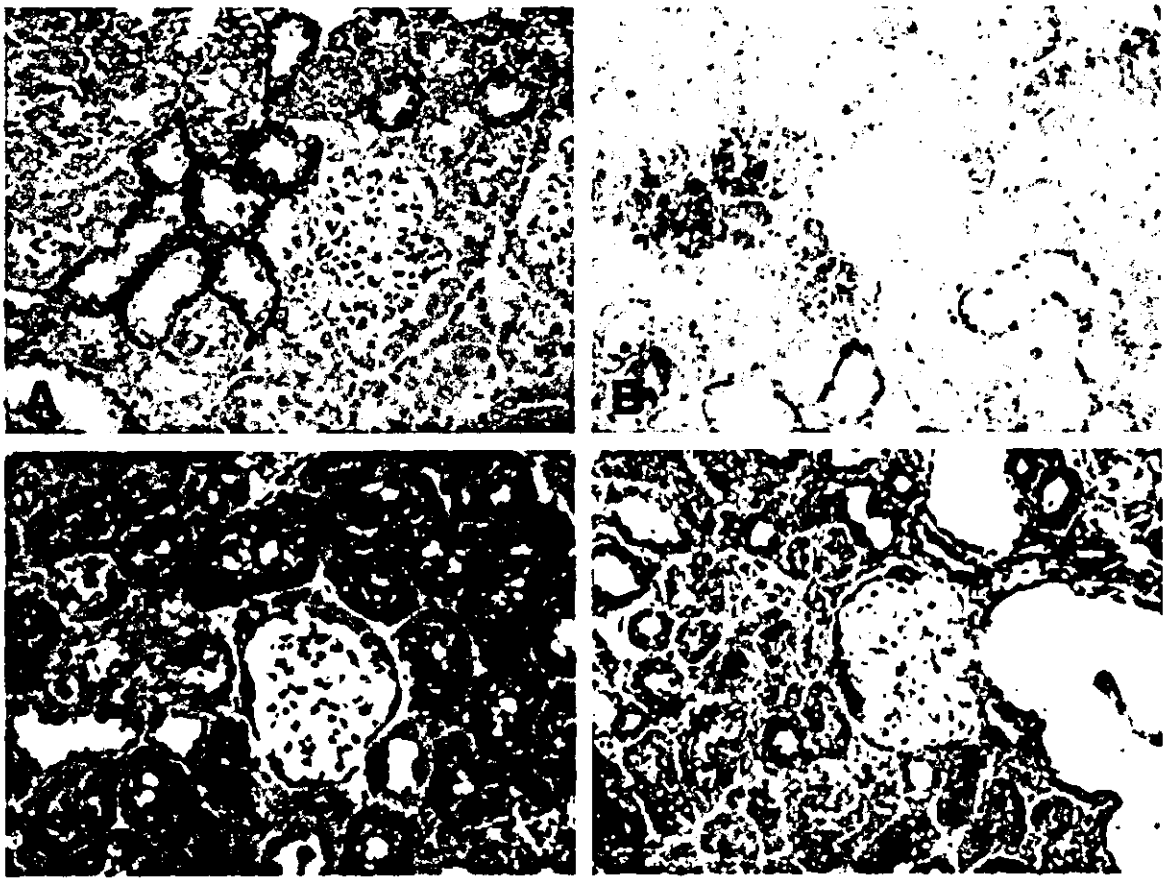

Fig. 5. Immunohistochemical staining for TIMP-1 (A and B) and TIMP-2 (C and D) proteins. TIMP-1 protein was detected in basal area of distal tubules in the kidneys of ICR mice (A). On the other hand, it was detected in the apical and lateral areas of distal tubules in the kidneys of terminal-stage ICGN mice (B). TIMP-2 protein was detected in proximal and distal tubules of ICR (C) and terminal-stage ICGN mice (D). TIMP-2 levels were decreased in ICGN mice. (× 200)

mice. We suspect extreme decreases of MMPs account for extremely low MMPs activities. Furthermore, MT1-MMP is related to the activation of MMP-2. In the kidneys of ICGN mice, MT1-MMP levels were strikingly decreased, and such a decrease may bring about down-regulation of MMP-2 activation. In other nephrotic animal models, such as the Heymann nephritis model, anti-GBM model and anti-Thy.1.1 model, the up-regulation of MMPs and TIMPs is observed [2–4, 12, 13]. However, in the kidneys of ICGN mice, a characteristic down-regulation of both MMP and TIMP expression was demonstrated. Thus, we conclude that the decreased level of ECM degradation by MMPs results in thickened basement membranes in the kidneys of ICGN mice. We emphasize that ICGN mice are an unique model for studying the molecular mechanism of ECM catabolism and for the pathogenic mechanism of renal fibrosis, and that the present study would contribute to our understanding of the processes of renal fibrosis.

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