strain, C57BL/6 (Abe et al. 2004). Considering that the genomic difference between humans and chimpanzees is 1.23% (Fujiyama et al. 2002), this nucleotide difference makes the MSM/Ms strain a useful model for functional genomic studies. Corresponding to these genomic sequence differences, many aspects of the phenotype of MSM/Ms mice are also distinct from those of commonly used laboratory strains; for example, they have smaller body size, resistance to carcinogenesis (Miyashita and Moriwaki 1987; Nakanishi et al. 2007; Okumoto et al. 1995), high locomotive activity (Koide et al. 2000), and resistance to high-fat-diet-induced diabetes (Kobayashi et al. 2004). It is therefore expected that genetic studies using MSM/Ms will be able to provide new clues to gene function that cannot be obtained from studies with common laboratory strains. However, genetic manipulation of the MSM/Ms genome has been difficult due to their poor response to superovulation and the absence of an embryonic stem (ES) cell line for this strain.

In this study, we have established three ES cell lines, Mol/MSM-1, -2, and -3, from MSM/Ms embryos and have devised an efficient way to produce germline chimeras by injecting ES cells into blastocysts obtained from mating C57BL/6J female and BDF1 male mice (B6 × BDF1).

Materials and methods

Culture medium

Mouse embryos were cultured using KSOM medium (Lawitts and Biggers 1993) (ARK-Recource Co. Ltd., Kumamoto, Japan). ES cells were cultured in KSR-GMEM medium consisting of Glasgow Minimum Essential Medium (GMEM) (Sigma, St Louis, MO) with 1 × MEM nonessential amino acids (Gibco Invitrogen, Grand Island, NY), 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1% fetal bovine serum (FBS) (HyClone, Thermo Fisher Scientific Inc., Waltham, MA), 14% Knockout^{TM} Serum Replacement (KSR) (Gibco Invitrogen), and 1100 U/ml leukemia inhibitory factor (LIF) (ESGRO, Chemicon, Temecula, CA). For neutralization of trypsin, FBS-GMEM was used in which the KSR in KSR-GMEM was replaced with FBS (final 15% FBS).

Mice and embryos

MSM/Ms morula-stage embryos were collected from the oviducts of naturally mated MSM/Ms females, cryopreserved using the vitrification method (Nakao et al. 1997) at RIKEN BRC, and sent to Kumamoto University. They were quickly thawed according to the method reported by Nakao et al. (1997) and cultured in KSOM (Lawitts and Biggers 1993) for 1 day until they reached blastocyst stage.

F1 hybrid BDF1 (C57BL/6 × DBA2) male mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). C57BL/6J and CD-1 female mice were purchased from CLEA Japan (Tokyo, Japan) and Charles River Laboratories, Inc. (Wilmington, MA), respectively.

To obtain morulae and blastocysts, C57BL/6J and CD-1 females were induced to superovulate by injection of 5 IU PMSG (pregnant mare's serum gonadotropin) (ASKA Pharmaceutical Co. Ltd., Tokyo, Japan), followed 48 h later by injection of 5 IU of hCG (human chorionic gonadotropin) (ASKA Pharmaceutical Co. Ltd.). They were then mated with BDF1 and CD-1 males, respectively. Two-cell-stage embryos were collected from the oviducts of females 42 h after hCG injection and cultured in KSOM until the morula (for 24 h) or blastocyst stages (for 48 h).

Establishment of ES cell line

ES cells were cultured at 37°C in a humidified atmosphere of 6.5% CO_2 in air. MSM/Ms blastocysts were plated individually on a 48-well plate coated with 0.15% gelatin in KSR-GMEM medium. The blastocysts were allowed to hatch and attach to the dish and were refed every 3 days with KSR-GMEM medium. After 10 days in culture, the inner cell mass (ICM) outgrowth was dissociated in threefold-diluted 0.25% trypsin/1 mM EDTA (Sigma), and then plated onto a 24-well plate with a feeder layer of mitomycin C-treated primary mouse embryo fibroblasts. After this first passage, the ES cells were gradually plated onto larger culture plates with feeder layers. ES cells were routinely passaged and diluted five- to sixfold every 2 days, and the medium was changed on alternate days.

Characterization of ES cell lines

ES cells were stained for alkaline phosphatase activity using an Alkaline Phosphatase Staining kit (Sigma) according to the protocol supplied by the manufacturer. Karyotype analysis was performed as described previously (Robertson 1987).

Two polymorphic markers, *D18Mit145* and *D14Mit196*, which distinguish between C57BL/6 and MSM/Ms [Mouse Microsatellite Data Base of Japan (MMDBJ), http://www.shigen.nig.ac.jp/mouse/mmdbj/top.jsp], were used to examine genomic DNA from the established ES cells, a C57BL/6J mouse, TT2 ES cells, E14Tg2a ES cells, and an MSM/Ms mouse, according to the protocol obtained from MMDBJ. The sequences of the primer pairs and the product sizes in C57BL/6, 129 Sv/J, and MSM/Ms were as follows: *D18Mit145* (primers: TTCAGGTGCACCACCAAGTT, CTCCGTCAAAGAAAATGTTAAATC; product size: C57BL/6, 201 bp; 129 Sv/J, 202 bp, MSM/Ms, 269 bp) and *D14Mit196* (primers: CAGGCACAAACAAGTGCTGT, GTGAGTTCTA

GGACATCCCAGG; product size: C57BL/6, 230 bp; 129SV/J, 232 bp; MSM/Ms, 319 bp).

To determine the sex of established ES cells, PCR detecting the *Sry* gene was performed with the primer pair, Sry-F: TGACTGGGATGCAGTAGTTC and Sry-R: TGTGCTAGAGAGAAACCCTG. The size of PCR product was 0.23 kb.

Electroporation

The details of the trap vector pU-21T will be described elsewhere. The main elements of these vectors are the splice acceptor site of the mouse *En-2* gene, and the *βgeo* gene (Taniwaki et al. 2005). Cells transfected with these vectors become G418-resistant. For electroporation, 20–30 μg of *Spe*I-digested plasmid DNA was used. ES cells ($2–3 \times 10^6$ cells) were suspended in 0.8 ml phosphate-buffered saline, electroporated using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) set at 800 V and 3 μF, and then fed with KSR-GMEM medium supplemented with 200 μg/ml G418 after 48 h. Selection was maintained for 7 days, after which the colonies were counted, picked, and placed in 24-well plates.

Production of chimeric mice and confirmation of germline transmission

Chimeric mice were produced by aggregation of ES cells with 8-cell embryos of ICR or B6 × BDF1 mice, and also by injection of ES cells into blastocysts of ICR or B6 × BDF1 mice, as described elsewhere (Nagy et al. 2003). Germline transmission was confirmed by the coat color of F1 offspring. Male chimeras produced from ICR and B6 × BDF1 embryos were mated with ICR and with C57BL/6J or JF1/Ms females, respectively. If no pregnancy was observed within 2 months of mating, in vitro fertilization was performed. For in vitro fertilization, unfertilized eggs were collected from superovulated ICR or C57BL/6J females, and insemination and transfer of fertilized embryos were performed as described previously (Nagy et al. 2003). Because MSM/Ms mice are wild-type with respect to the agouti and albino loci (Aw/Aw, Tyr+/Tyr+), only the F1 progeny derived from MSM/Ms ES cells should exhibit agouti coat color, both in pairings of ICR chimeras with ICR (Tyrc/Tyrc) mice and B6 × BDF1 chimeras with either C57BL/6J (a/a, Tyr+/Tyr+) or JF1/Ms (a/a, Tyr+/Tyr+) mice.

Results

Derivation of ES cells from MSM/Ms blastocysts

Fifteen blastocysts were cultured in KSR-GMEM medium for 10 days, and three ICM-derived colonies were successfully passaged into 10-cm dishes, stocked, and designated Mol/MSM-1, -2, and -3. These first stocks corresponded to passage number (p) 5. The morphologies of the three established ES lines at p7 are shown in Fig. 1a. The appearances of the ES cells were not uniform; they consisted of a mixture of tight, thick colonies and separate, flat colonies. Both populations stained positively for alkaline phosphatase, indicating that

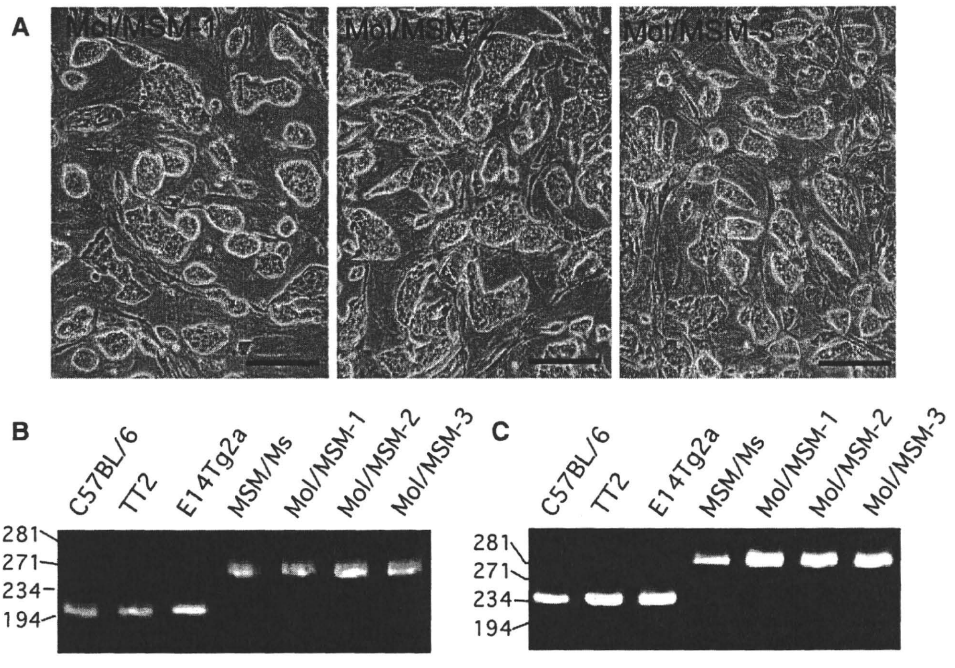

Fig. 1 Generation of Mol/MSM ES lines. **a** Phase contrast photographs of ES lines (p7) stained for alkaline phosphatase activity. Scale bar = 250 μm. **b, c** The genomic DNAs of established ES lines and control genomic DNAs from a C57BL/6J mouse, TT2 ES cells, E14Tg2a ES cells, and an MSM/Ms mouse were examined using two microsatellite markers, *D18Mit145* (**b**) and *D14Mit196* (**c**). Mol/MSM ES cells gave a 269-bp band in (**b**) and a 319-bp band in (**c**) which is characteristic of MSM/Ms. Positions of size marker bands are indicated on the left side

they were undifferentiated. These two types of morphology were still observed after several times of passage and also colony isolation, although the percentage of the two populations varied among subclones. The genomes of the established ES lines were examined using two microsatellite markers, *D18Mit145* and *D14Mit196*, and band sizes characteristic of MSM/Ms were demonstrated, as shown in Fig. 1b, c. The sex of the established ES lines was also examined by genomic PCR detecting the *Sry* gene on the Y chromosome, and all lines were *Sry*-positive, meaning that they were male ES lines (data not shown). For further characterization, metaphase spreads were prepared from the three ES lines and chromosome numbers were counted. As shown in Fig. 2a, over 90% of cells from the Mol/MSM-1 and -3 lines showed normal 40 chromosome numbers, whereas 18% of cells in the Mol/MSM-2 line were tetraploid.

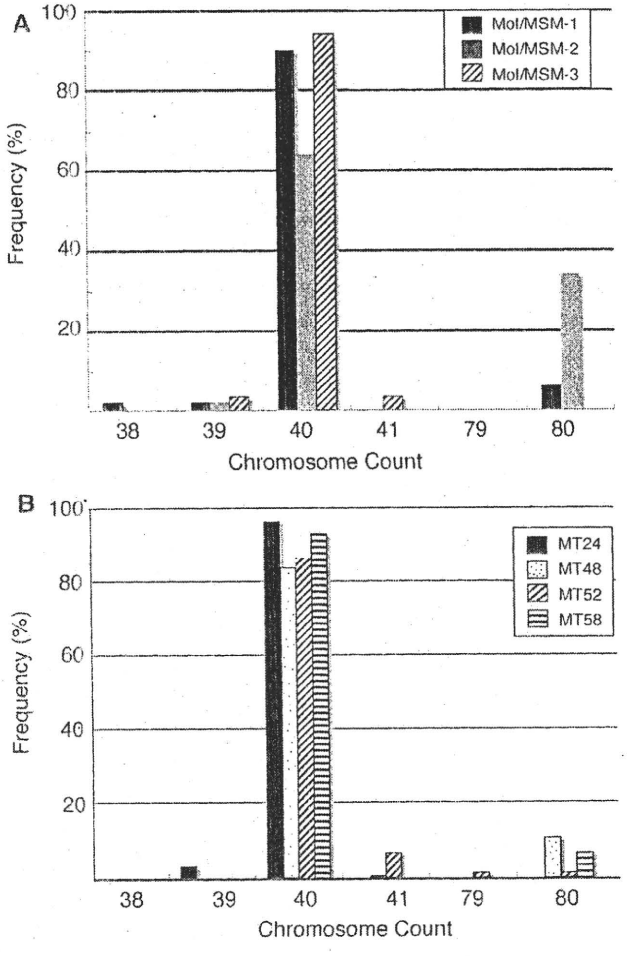

Fig. 2 Chromosome counts of Mol/MSM cell lines and subclones. **a** Chromosome counts of Mol/MSM-1, -2, and -3 lines at p8. **b** Chromosome counts of gene trap clones obtained from Mol/MSM-1. Metaphase spreads of ES cells were prepared, and 50 metaphase spreads were counted for each cell line

Chimeric mouse production and germline transmission

To compare chimera production efficiency, the three ES lines were aggregated with ICR morulae (Table 1). All ES lines resulted in production of male chimeric mice with 100% contribution of ES cells, as shown by coat color. All of the 100% chimeras were able to pass the ES cell genome onto the next generation. The Mol/MSM-1 line showed the highest efficiency of chimera production among the three lines, therefore this line was chosen for subsequent experiments.

To test the stability of the pluripotency, Mol/MSM-1 ES cells were passaged seven times from the first stock without feeder layers (corresponding to p12) and then subjected to aggregation with ICR morulae. As shown in Table 1, the rate of chimera production from these cells was similar to that of earlier passage cells, indicating that Mol/MSM-1 ES cells could be maintained stably.

Injection into blastocysts is a common and orthodox method for the production of chimeras from most ES cell lines. The Mol/MSM-1 line was therefore tested for chimera production by injection into blastocysts derived from ICR or B6 × BDF1 mice. Because mating with BDF1 males could produce higher fertilization rates than mating with C57BL/6 males, and the number of embryos obtained from C57BL/6 females was much larger after mating with BDF1 males than with C57BL/6 males, B6 × BDF1 embryos were used for chimera production. As shown in Table 1, injection into blastocysts increased the efficiency of chimera production (26.7% with ICR and 34.3% with B6 × BDF1) compared with aggregation using ICR morulae (19.2%). However, chimeras obtained by ICR blastocyst injection showed a lower degree of chimerism, determined by coat color (30–70%, data not shown), even in germline chimeras. In contrast, most of the chimeras obtained by B6 × BDF1 blastocyst injection demonstrated 90–100% chimerism. The ratio of germline chimeras to total chimeras produced by B6 × BDF1 blastocyst injection was also higher than that produced by ICR blastocyst injection (Table 1). Thus, B6 × BDF1 blastocyst injection was an efficient method for the production of chimeras using Mol/MSM-1 ES cells.

Germline transmission from subclones after electroporation

To evaluate the ability of the Mol/MSM-1 line to maintain germline competency after genetic manipulation, it was subjected to electroporation and subcloning, and the subclones were then tested for chimera production. A gene trap vector carrying a splice acceptor sequence and the *βgeo* gene was electroporated to Mol/MSM-1 cells. In two experiments, 400 colonies were formed and 142 trap clones

Table 1 Germline transmission of Mol/MSM ES lines

ES cell line (passage no.)	Method of chimeric mouse production	No. of transferred embryos	No. of mice born (% of transferred embryos)	No. of weaned chimeras (% of transferred embryos)	No. of germline chimeras/tested chimeras
Mol/MSM-1 (p6)	Aggregation with ICR morulae	125	43 (34.4%)	24 (19.2%)	9/11
Mol/MSM-2 (p6)	Aggregation with ICR morulae	100	15 (15%)	6 (6%)	1/3
Mol/MSM-3 (p6)	Aggregation with ICR morulae	100	16 (16%)	8 (8%)	2/3
Mol/MSM-1 (p12)	Aggregation with ICR morulae	50	13 (26%)	7 (14%)	2/3
Mol/MSM-1 (p7)	Injection into ICR blastocysts	75	36 (48%)	20 (26.7%)	8/14
Mol/MSM-1 (p7)	Injection into B6 × BDF1 blastocysts	70	28 (40%)	24 (34.3%)	15/18

were stocked. The colony-forming efficiency and growth rate of the G418-resistant subclones after colony isolation were similar to those of the conventional ES cell line, TT2 (Araki et al. 1999). Four trap clones were analyzed for chromosome counts to examine whether euploidy was maintained during subcloning. As shown in Fig.2b, all clones harbored over 80% euploid metaphases, suggesting stability of Mol/MSM-1 line.

For the production of chimeras with trap clones, the aggregation method was initially used. However, as shown in Table 2, only 23% (5 of 22) of trap lines could produce germline chimeras by aggregation with ICR morulae. Although the use of B6 × BDF1 morulae improved the ratio of germline-competent trap lines (31%), this method resulted in a very low production rate of germline chimeras (0.28% with ICR and 0.79% with B6 × BDF1). This meant that 357 ICR morulae or 127 B6 × BDF1 morulae were necessary to obtain one germline chimera.

To further analyze the best combination of methods to produce the most efficient germline transmission, we selected two trap lines, MT6 and MT23, that failed to produce germline chimeras by aggregation with either ICR or B6 × BDF1 morulae (Table 3). Injection into ICR or B6 × BDF1 blastocysts was performed using these two trap lines. As shown in Table 3, ICR blastocyst injection failed to produce any germline chimeras, but 2 of 8 or 11 of 19 chimeras obtained from injection into B6 × BDF1 blastocysts showed germline transmission. These results suggested that B6 × BDF1 blastocyst injection was suitable for chimera production, and we therefore selected a further three trap clones (MT13, MT54, and MT58), which produced a small number of newborn pups but no weaned chimeras after aggregation with B6 × BDF1 morulae (Table 4). MT54 failed to produce any live newborn pups, even using 350 morulae. As shown in Table 4, B6 × BDF1 blastocyst injection using all three clones resulted in efficient germline chimera production, with 7 of 8, 9 of 16, and 10 of 16 germline chimeras being obtained from injection with MT13, MT54, and MT58 trap clones, respectively. Overall, the five trap clones tested for B6 × BDF1 blastocyst injection could produce germline chimeras at an average production rate of 11.2%, meaning that only nine blastocysts were required to obtain one germline chimera. These results clearly suggested that injection of trap clones into blastocysts obtained from B6 × BDF1 mice was the most efficient method of producing germline chimeras.

Discussion

In this study we have demonstrated the establishment of germline-competent ES cells from a wild-derived inbred strain of mice, MSM/Ms. In addition, their subclones, obtained through electroporation and drug selection, could also efficiently produce germline chimeras by injection into B6 × BDF1 blastocysts, suggesting the feasibility of using the Mol/MSM-1 line for genetic manipulation. This ES cell line should provide a powerful tool for the functional analysis of genes in *M. m. molossinus*.

Table 2 Chimera production by aggregation using trap ES lines isolated through electroporation and drug selection

Embryo	No. of trap lines tested	No. of lines producing live pups	No. of lines producing male chimeras	No. of lines producing germline chimeras	Average percentage of germline chimeras to transferred embryos (%)
ICR	22	20 (91%)	18 (82%)	5 (23%)	0.28
B6 × BDF1	39	25 (64%)	13 (33%)	12 (31%)	0.79

Trap clones passaged 4–5 times from G418-resistant colonies were used for chimeric production

Table 3 Chimera production with two trap clones by aggregation or injection method using embryos from ICR or B6 × BDF1 mice

Trap lines	Method for production of chimeras	Recipient embryo	No. of transferred embryos	No. of newborn pups (% of transferred embryos)	No. of weaned male chimeras (% of transferred embryos)	No. of germline chimeras (% of transferred embryos)
MT6	Agg	ICR	150	24 (16)	2 (1.3)	0 (0)
	Inj	ICR	70	19 (27)	4 (5.7)	0 (0)
	Agg	B6 × BDF1	150	6 (4)	1 (0.7)	0(0)
	Inj	B6 × BDF1	70	10 (14)	8 (11)	2 (2.9)
MT23	Agg	ICR	75	7 (9.3)	2 (2.7)	0 (0)
	Inj	ICR	70	52 (74)	19 (27)	0 (0)
	Agg	B6 × BDF1	75	2 (2.7)	0 (0)	0 (0)
	Inj	B6 × BDF1	70	34 (49)	19 (27)	11 (16)

Trap clones passaged 4-5 times from G418-resistant colonies were used for chimeric production. All weaned male chimeras were tested for germline transmission

Agg aggregation with morulae, *Inj* injection into blastocysts

Table 4 Chimera production with three trap clones by aggregation or injection method using embryos from B6 × BDF1 mice

Trap lines	Method for production of chimeras	No. of transferred embryos	No. of new born pups (% of transferred embryos)	No. of weaned male chimeras (% of transferred embryos)	No. of germline chimeras (% of transferred embryos)
MT13	Agg	125	2 (1.6)	0 (0)	0 (0)
	Inj	70	10 (14)	8 (11)	7 (7)
MT54	Agg	350	0 (0)	0 (0)	0 (0)
	Inj	70	20 (29)	16 (23)	9 (13)
MT58	Agg	225	1 (0.4)	0 (0)	0 (0)
	Inj	70	21 (30)	16 (23)	10 (14)

Trap clones passaged 4–5 times from G418-resistant colonies were used for chimeric production. All weaned male chimeras were tested for germline transmission

Agg aggregation with morulae, *Inj* injection into blastocysts

We used KSR to establish and maintain Mol/MSM ES cells. KSR has previously been used for the culture of ES cells from C57BL/6 mice by two other groups, who reported an efficiency of about 20% (Cheng et al. 2004; Shimizukawa et al. 2005). The efficiency of ES cell establishment with MSM/Ms blastocysts in our study was also 20% (three lines from 15 blastocysts), indicating that the MSM/Ms strain is permissive for ES cell derivation. However, this value was almost half that of the ES establishment efficiency (45%) found with F1 blastocysts obtained by injecting MSM/Ms spermatozoa into C57BL/6 oocytes (Shinmen et al. 2007). The higher efficiency in this previous study might have been due to hybrid vigor.

We could obtain germline chimeras from the untreated Mol/MSM-1 line using both the aggregation and the injection methods. However, after electroporation and subcloning, only injection into B6 × BDF1 blastocysts was efficient and was therefore considered to be the more promising method for producing germline chimeras. The decreased efficiency of chimera production using the aggregation method could be due to a reduction in the developmental potential of Mol/MSM-1 ES cells after subcloning. It is known that chimeric mice produced using 8-cell-stage embryos showed higher ES cell contributions than chimeras produced by blastocyst injection (Peli et al. 1996; Tokunaga and Tsunoda 1992). The Mol/MSM-1 ES cell line also showed a similar tendency in that most aggregation chimeras demonstrated 100% chimerism in coat color, while in injection chimeras using B6 × BDF1 blastocysts, 100% chimeras were scarce, and most chimeras showed only 60–90% chimerism. If a subclone with reduced developmental potential was used for aggregation and 100% chimeras were generated with such cells, the chimeras could be lost during prenatal or perinatal development, similar to the instances of perinatal death observed in tetraploid aggregation chimeras (Nagy et al. 1990). The effect of multiple electroporations on germline transmission is currently being investigated. Another reason for the low frequency of germline chimeras produced by the aggregation method is that B6 × BDF1 morulae seem to be more sensitive to treatment with acidic Tyrode's solution, which was used to remove the zona pellucida.

Injection of Mol/MSM-1 cells into B6 × BDF1 blastocysts was able to produce germline chimeras, whereas injection into ICR blastocysts resulted in only a low percentage of chimerism. When the ES cell line derived from the 129 strain was used for gene targeting, the efficiency of germline chimera production was believed to be higher when injected into C57BL/6 blastocysts compared with those from ICR mice (Nagy et al. 2003). This was the case for the ES cell line derived from the MSM/Ms strain, although we used B6 × BDF1 recipient embryos, instead of C57BL/6 embryos, in order to obtain a larger number of embryos. The difference in body size may also be related to the low level of chimera production with ICR blastocyst injection; MSM/Ms mice have a small body size (about 10 g at 8 weeks of age), which is almost one-third that of ICR mice. The different growth rate between MSM/Ms cells and ICR cells might prevent normal development of chimeras.

The use of genetically engineered mice is now indispensable for functional analysis of the mammalian genome. Phenotypes of knockout or knockin mice are profoundly influenced by their genetic backgrounds. Most laboratory mouse strains are derived from a few suppliers and have been selected for special traits, such as susceptibility to cancer. Therefore, the phenotypes observed in laboratory mouse strains might not reflect those of wild animals, including humans. Because wild-derived mouse strains, such as MSM/Ms, have not been selected for any particular traits, mutations in MSM/Ms mice could result in different phenotypes from those caused by the same mutations in common laboratory mouse strains, thus providing new insights into gene function. Thus, the Mol/MSM-1 ES line should provide an excellent new tool for the study of functional genomics.

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—Review—
Review Series: Animal Bioresource in Japan

Current Activities of CARD as an International Core Center for Mouse Resources

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Abstract: The Center for Animal Resources and Development (CARD), Institute of Resource Development and Analysis, Kumamoto University was established in 1998 based on recommendations published in the report "Preservation, Supply and Development of Genetically Engineered Animals" by the Ministry of Education, Culture, Sports, Science and Technology. We provide a comprehensive and integrated set of research services designed for the mouse-based biological research community. All services are conducted in accordance with the highest standards of animal health and genetic quality and are delivered to meet researcher's research goals. To promote biological sciences worldwide, we produce genetically engineered mice and exchangeable gene trap ES clones, cryopreserve mouse embryos and sperm, supply these resources, organize training courses to educate people, and form a hub of the domestic and international networks of both mutagenesis and resource centers. Up to now, we have produced more than 600 genetically engineered mouse strains and have more than 1,100 strains and stocks of mice for supply to the scientific community. More than 150 studies using genetically engineered mice produced or supplied by CARD have been published so far. As a founding member of the Federation of International Mouse Resources, the Asian Mouse Mutagenesis and Resource Association, and the International Gene Trap Consortium, we are contributing to the promotion of biological sciences in the world.

Key words: cryopreservation, knockout, mouse, resource, transgenic

Introduction

The Center for Animal Resources and Development (CARD), Kumamoto University was established in 1998 based on recommendations published in the report "Preservation, Supply and Development of Genetically Engineered Mice" by the Subdivision of Resource Study, Council for Science and Technology, Ministry of Education, Culture and Science on July 10, 1997. That report recommended the establishment of at least two centers which could preserve, supply, and develop genetically engineered mice in Japan. At the Institute of Molecular Embryology and Genetics, Kumamoto University, we established the Laboratory of Transgenic Technology in 1992 and started to produce transgenic mice in response to requests from scientists. This was the first trial for

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such a service in Japan. The first transgenic mice were produced by Gordon *et al.* in 1980 [4]. Since then, transgenic mice have been used in many biological studies and have been shown to be a rich resource for such studies. In 1989, the first knockout mice were produced using a homologous recombination technique in embryonic stem (ES) cells [8]. With this procedure we aimed to promote scientific activities through the production of genetically engineered mice. In the mid 1990s, we realized that one transgenic mouse or knockout mouse strain can be used in many different areas of biological science, and that there was a strong demand for the preservation and supply of genetically engineered mouse strains. At the same time, the technology for the cryopreservation of mouse embryos had developed enough to be able to respond to such demand. In 2000, a new facility for embryo cryopreservation was completed and we started to cryopreserve, supply, and develop genetically engineered mice.

Goals of CARD

Our goals are as follows: 1) the establishment of mouse resources unique in Asia by integration of advanced technologies in production, cryopreservation, and the supply of genetically engineered mice; 2) the development of technologies relevant to these areas to keep our techniques advanced; 3) the establishment of a supply system between our center and other institutions for cryopreserved embryos or gametes to protect against infection which may arise through the transfer of live mice; 4) the training and education of workers and researchers in this field, especially those involved in reproductive engineering; and 5) the formation of domestic and international networks to promote biological sciences globally. To accomplish these goals, we became a founding member of the Federation of International Mouse Resources (FIMRe) [3]. FIMRe is a collaborating group of Mouse Repository and Resource Centers worldwide whose collective goal is to archive and provide strains of mice as cryopreserved embryos and gametes, ES cell lines, and live breeding stock to the research community. Goals of the FIMRe are (1) Coordinate repositories and resource centers to archive valuable genetically defined mice and ES cell lines being created worldwide and meet research demand for these genetically defined mice and ES cell lines, (2) Establish consistent animal health standards of the highest quality in all resource centers, (3) Provide genetic verification and quality control for genetic background and mutations, (4) Provide resource training to enhance user ability in utilizing cryopreserved resources.

In addition, we organized the Asian Mouse Mutagenesis and Resource Association (AMMRA) in 2006. The AMMRA is a collaborative group of Mouse Mutagenesis and Resource Centers in Asia. Its mission is "To promote mouse mutagenesis projects and to facilitate access to mouse resources in Asia". Its goals are "The use of mouse models for understanding genome function and the improvement of human health". The founding members of AMMRA are (1) Biological Resource Center, Singapore, (2) National Laboratory Animal Center, Taipei, (3) National Resource Center for Mutant Mice, Nanjing University, Nanjing, (4) Shanghai Institute of Biological Sciences, Shanghai, (5) Nanfang Center for Model Organisms, Shanghai, (6) Peking University-BLARC, Beijing, (7) Beijing Institute of Laboratory Animal Science, CAMS, PUC, Beijing, (8) Bio-Evaluation Center, KRIBB, Daejeon, (9) Riken BioResource Center, Tsukuba, (10) Center for Animal Resources and Development, Kumamoto.

Characteristics of the Mouse

Development of the laboratory mouse as a research model really began with genetic experiments in the early 1900s. Today, a large number of inbred strains of mice or various strains of mice with spontaneous or induced mutations (i.e., transgenics, targeted mutations, chemically induced mutations) have been produced in a variety of laboratories worldwide. They are prized for many qualities, including their small size, short generation time, and ease of breeding within the laboratory. The fact that they are genetically the best characterized of all mammals increases their value for all fields of study.

Technical Services Available at CARD

According to our aims, we provide a comprehensive,

Table 1. Production, cryopreservation, supply, and cleaning of genetically engineered mice at CARD since 1998

	Tg	Chimera	Total	No. of strains cryopreserved	No. of strains supplied	No. of strains cleaned
1998	67	6	73			3
1999	22	1	23			53
2000	66	11	77	144	4	62
2001	60	15	75	97	10	73
2002	59	21	80	67	34	47
2003	61	23	84	89	33	20
2004	44	14	58	116	58	65
2005	43	18	61	111	39	83
2006	23	13	36	153	42	83
2007	25	7	32	207	35	48
2008	37	6	43	142	53	106
Total	507	135	642	1,126	308	643

integrated, and highly customizable set of research services designed for the mouse-based biomedical research community. All services are conducted in accordance with the highest standards of animal health and genetic quality and are delivered to meet user's schedule and research goals. The intellectual property right of each resource belongs to the depositor, not to our center. Users should consider this issue before submitting a request for resources, and undertake signed agreements to any conditions attached to a resource.

(1) Production of genetically engineered mice

Upon requests by researchers, we produce either transgenic mice or chimeric mice. So far, we have produced 507 transgenic mouse lines and 135 chimeric mouse lines (Table 1). Researchers should prepare and send DNA constructs for microinjection into fertilized eggs. We usually use fertilized eggs obtained from C57BL/6 mice. We microinject DNA into fertilized mouse eggs and transfer them into oviducts of foster mothers. We rear new born mice up to 4 weeks of age and then send these mice to requesters. So, researchers need to carry out screening for transgenic mice at their facilities. For chimeric mouse production, researchers should prepare knockout ES clones at their facilities. We make chimeric mice by injecting targeted ES cells into blastocysts, which are then transferred to the uteri of foster mothers. We send all chimeric mice to requesters at 4 weeks of age. It should be noted that researchers should have permission for recombinant DNA experiments at their institutions.

(2) Cryopreservation of embryos and sperm

For deposit, researchers should send their mice to CARD. The numbers of mice that should be shipped to CARD for embryo freezing is as follows. For genetically engineered mouse strains, researchers should send at least 3 homozygous or 3 heterozygous male mice. Alternatively, researchers can send at least 5 homozygous males and 20 homozygous females if they desire cryopreservation of homozygous embryos. For other mouse strains, we need at least 5 males and 20 females for cryopreservation.

We generally produce embryos from these mouse strains using *in vitro* fertilization. Ten straws containing spermatozoa and 8 tubes containing 40 two-cell embryos are cryopreserved for each strain. We use the simple vitrification method for embryo freezing [6] and the Nakagata method for sperm freezing [5]. For quality control, some of the frozen embryos are thawed to check if the frozen embryos develop into viable young and whether or not the developed mice are microbiologically clean. For genetically engineered mouse strains, transgene transmission is confirmed by PCR analysis of genomic DNA from tail tissue of the produced mice.

Currently our center has 1,126 strains and stocks of mice. These include inbred mouse strains, spontaneous

mutants, transgenic mice, and knockout mice. CARD R-BASE (Resource Database) provides resource-related strain, gene and reference information (http://cardb.cc.kumamoto-u.ac.jp/transgenic/index.jsp).

(3) Supply of mouse strains and frozen embryos/sperm

Cryopreserved embryos or recovered populations from cryopreserved embryos are supplied to the scientific community. The cost is141,750 JY or 109,200 JY for supply of recovered mice or frozen embryos, respectively. The application procedure for the supply of laboratory mice is shown on our web page (http://card.medic.kumamoto-u.ac.jp/card/english/sigen/gyoumu/ebank/index.htm). We have already supplied 90 strains of cryopreserved embryos and 218 strains of mice worldwide.

(4) Exchangeable gene trap clones (EGTC)

Our center has more than 600 exchangeable gene trap ES clones. In general, gene trapping is a high-throughput approach that is used to introduce insertional mutations across the genome in mouse embryonic stem (ES) cells. In addition to generating standard loss-of-function alleles, newer gene trap vectors offer a variety of post-insertional modification strategies for the generation of other experimental alleles. We are using the exchangeable gene trap method for isolation of gene trap clones [1, 2, 9]. This method has a great advantage. The exchangeable gene trap vector is usually inserted around the exon containing the ATG codon in a mouse endogenous gene, resulting in the null mutation. As the β-geo gene is flanked by lox71 and loxP, the β-geo gene can be replaced with the gene of interest by electroporating a new vector containing the gene of interest flanked by lox66 and loxP together with a Cre expression vector. The newly introduced gene of interest can be expected to be expressed under the control of the trapped gene. Using this system, a mouse gene can be replaced with a homologous human gene, leading to the production of a humanized mouse at the gene level. These clones can be accessed through the EGTC database (http://egtc.jp/view/index). As we are also the founding member of the international gene trap consortium (IGTC) [7], the EGTC database is connected to the integrated database of the IGTC. The IGTC database contains all publicly available gene trap cell lines, which are available on a non-collaborative basis for nominal handling fees. Researchers can search and browse the IGTC database for cell lines of interest using accession numbers or IDs, keywords, sequence data, tissue expression profiles, and biological pathways. At the moment, the IGTC database has 380,863 cell lines. IGTC members are BayGenomics (USA), Centre for Modelling Human Disease (Toronto, Canada), Embryonic Stem Cell Database (University of Manitoba, Canada), German Gene Trap Consortium (Germany), Sanger Institute Gene Trap Resource (Cambridge, UK), Soriano Lab Gene Trap Database (Mount Sinai School of Medicine, New York, USA), Texas Institute for Genomic Medicine—TIGM (USA), TIGEM-IRBM Gene Trap (Naples, Italy), and our institution, Exchangeable Gene Trap Clones (Kumamoto University, Japan).

(5) Other resources

In the Gene Technology Center, which also belongs to the Institute of Resource Development and Analysis, we started the GTC P-stock service in April 2004. This is not a plasmid bank; however, we do store valuable transgenes or targeting constructs for homologous recombination and supply them upon request (http://gtc.egtc.jp/view/pstock/index).

(6) Courses and education

CARD sponsors stimulating courses and conferences on our campus at Honjo in Kumamoto and at other locations inside and outside Japan. CARD training courses for reproductive engineering techniques were introduced in November 2000. Spring and autumn courses are staged each year at CARD. The courses teach the following techniques:1) Preparing and Assembling Pipettes for Embryo Handling, 2) *In Vitro* Fertilization, 3) Simple Vitrification of Mouse Embryos, 4) Vitrification and Transplantation of Mouse Ovaries, 5) Cryopreservation of Mouse Spermatozoa, 6) *In Vitro* Fertilization using Cryopreserved Spermatozoa, 7) Collecting Two-Cell-Stage Embryos, 8) Vasectomy for the Creation of Sterile Males, 9) Embryo Transfer into the Oviduct, 10) Production of Chimeric Mice by 8-Cell Aggregation, 11) Embryo Transfer into the Uterus, and 12) Caesarean Section and Fostering. We sometimes hold the training course at other venues inside and outside Japan.

Popular Strains: Characteristic Features and Their Use for Research

Table 2 shows the mouse strains which are requested by many researchers. A recent trend is that requests for Cre mice are increasing substantially due to the performance of its conditional knockout.

Among Cre mice, P0-Cre is one of the most frequently requested strains. Although this strain of mouse has been described in a published manuscript [10], we would like to briefly introduce the characteristics of this mouse. Neural crest cells are embryonic, multipotent stem cells that give rise to various cell/tissue types and thus serve as a good model system for the study of cell specification and mechanisms of cell differentiation. For analysis of neural crest cell lineage, transgenic mice harboring a Cre gene driven by a promoter of protein 0 (P0) were generated. To detect and visualize the Cre-mediated DNA recombination in neural crest cells and derivatives, we utilized another transgenic (Tg) line with the CAG-CAT-Z indicator construct [10]. This Tg line carries a *lacZ* reporter gene downstream of a chicken β-actin promoter and a "stuffer" fragment flanked by two *loxP* sequences. The *lacZ* is expressed only when the stuffer is removed by the action of Cre recombinase. Using these transgenic mice, we demonstrated that a subset of migrating neural crest cells and a wide variety of cells in the neural crest cell lineage could be marked by *lacZ* expression. Thus, the use of this system may facilitate many interesting experiments, including lineage analysis, purification, and manipulation of the mammalian neural crest cells. Also, this cell-type-specific transgenesis system should facilitate functional analysis of genes of interest in the neural crest cell lineage. Up to now, this strain of mouse has been used in many studies and is now one of the most useful Cre-driver mice.

Deposition and Request for Mouse Strains and EGTC

An outline of the deposition and request procedures for mouse strains is illustrated in Fig. 1. Information on the application procedure is available on the web page (Fig. 2) (http://card.medic.kumamoto-u.ac.jp/card/english/index.html). As we are one of the founding members of FIMRe (http://www.fimre.org/), we also offer our mouse data to the integrated database IMSR (International Mouse Strain Resources) (http://www.informatics.jax.org/imsr/index.jsp). Thus, researchers also can find mouse strains using these databases.

Table 2. Frequently requested mouse strains

ID: 148	C57BL/6J-Tg(PO-Cre)94Imeg
ID: 250	Tg(K5-Cre)
ID: 709	B6;C3H-Tg(K19-Wnt1/K19-Ptgs2/K19-Ptges)
ID: 428	C57BL/6N-Tg(CAG-AURKA(WT)Card
ID: 196	B6;129-Synd4^{tm1}
ID: 189	Tg(E/nestin:EGFP-50)
ID: 175	Tg(E/nestin:EGFP-25)
ID: 91	Tg(CAG-Cre)
ID: 89	Tg(lck-Cre)
ID: 509	B6;D2-Tg(CAG-CAT-EGFP)39Miya
ID: 355	C57BL/6-CD9^{tm1};Tg(ZP3-EGFPCD9)
ID: 312	B6;CB-Dtr$^{tm2(lox)}$
ID: 290	C57BL/6 -Tg(Act-EGFP)C14-Y01-FM131Osb
ID: 88	C57BL/6-Rag1$^{tm1(GFP)Imku}$

Major Achievement: Publication

More than 150 studies using genetically engineered mice produced by CARD, have been published so far. Among these some representative manuscripts are listed in Table 3. This list clearly suggests that technical services in mouse embryo manipulation and reproductive engineering contribute greatly to the promotion of science.

Related Information

JMSR (http://www.shigen.nig.ac.jp/mouse/jmsr/top.jsp): The Japan Mouse/Rat Strain Resources Database (JMSR) is a searchable online database of mouse/rat strains and stocks available in Japan. The JMSR was developed in 2001 through discussions with the Mouse Genetic Resources Subcommittee (http://www.shigen.nig.ac.jp/shigen/grc/grc_mouse.jsp). The objective of this database is to provide a portal site which will help users to locate and obtain mouse/rat resources. This site is maintained by the Genetic Informatics Laboratory, National Institute of Genetics in close cooperation with data providers.

IGTC (http://www.genetrap.org/): The International

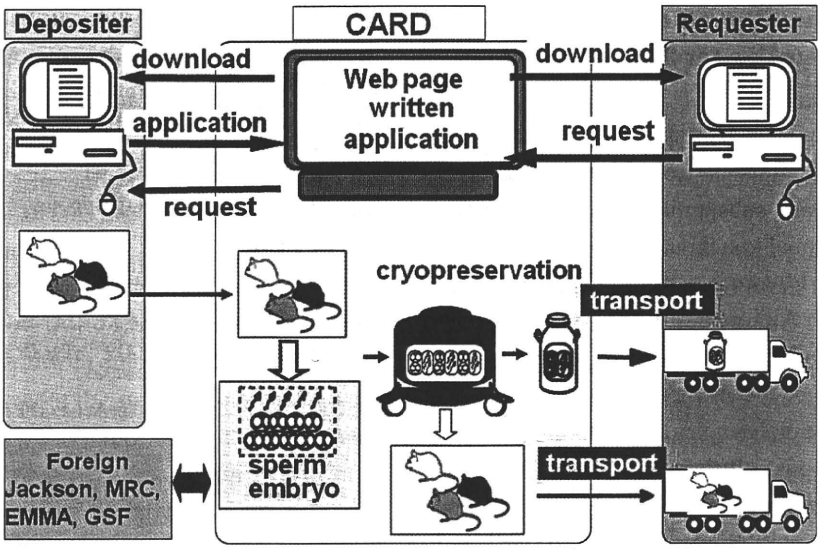

Fig. 1. Outline of deposition and request procedures for mouse strains. Information on the application procedure and necessary documents are available on the web page. Mouse embryos or sperm obtained from mice sent from depositors are cryopreserved and stored in liquid nitrogen tanks. Frozen embryos or live mice obtained by transfer of thawed embryos into foster mothers can be sent to requesters.

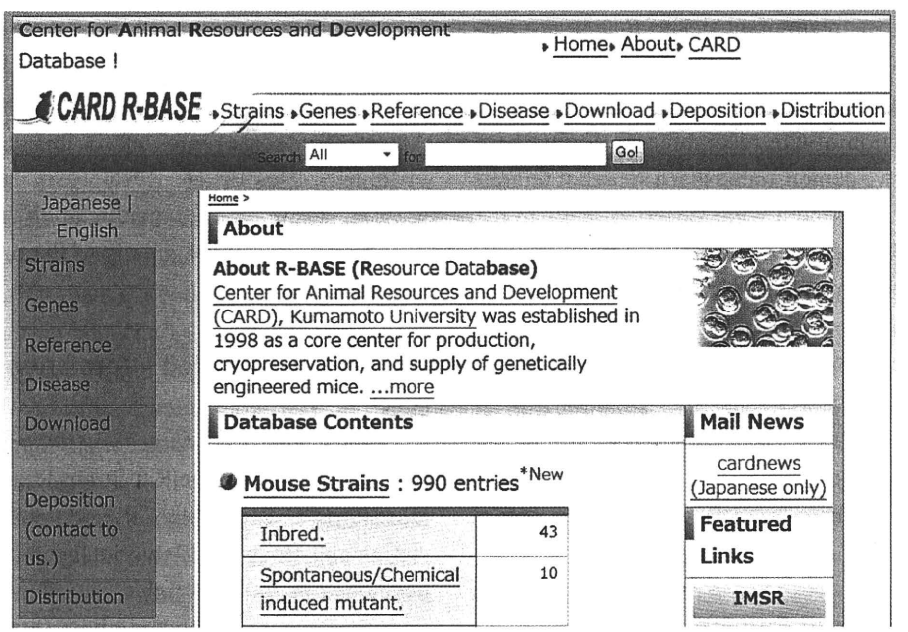

Fig. 2. Web page of CARD R-BASE. Only part of the web page is shown in this figure. Both Japanese and English versions are available.

Gene Trap Consortium (IGTC) consists of laboratories around the world working together to generate a public library of mutated murine ES cell lines. Such cell lines can be obtained on a non-collaborative basis by scientists interested in generating reporter-tagged, loss-of-function mutations in mice. In addition to loss of function, new-

Table 3. Selected publications

Ohnishi, N. *et al. Proc. Natl. Acad. Sci. U.S.A.* 105: 1003–1008, 2008.
Sato, T. *et al. Nature* 448: 366–369, 2007.
Imai, T. *et al. Science* 314: 657–661, 2006.
Serizawa, S. *et al. Cell* 127: 1057–1069, 2006.
Shim, J.H. *et al. Genes Dev.* 19: 2668–2681, 2005.
Terada, K. *et al. EMBO J.* 24: 611–622, 2005.
Tachibana, M. *et al. Genes Dev.* 19: 815–826, 2005.
Ohmuraya, M. *et al. Gastroenterology* 129: 696–705, 2005.
Itoh, H. *et al. Gastroenterology* 127: 1423–1435, 2004.
Yamazaki, S. *et al. J. Cell Biol.* 163: 469–475, 2003.
Ishida, D. *et al. Cancer Cell* 4: 55–65, 2003.
Serizawa, S. *et al. Science* 302: 2088–2094, 2003.
Wakabayashi, Y. *et al. Nat. Immunol.* 4: 533–539, 2003.
Yokosuka, T. *et al. J. Exp. Med.* 195: 991–1001, 2002.
Pepys, M.B. *et al. Nature* 417: 254–259, 2002.
Tachibana, M. *et al. Genes Dev.* 16: 1779–1791, 2002.
Yamauchi, T. *et al. Nat. Genet.* 30: 221–226, 2002.
Hasegawa, S. *et al. Proc. Natl. Acad. Sci. U.S.A.* 99: 297–302, 2002.
Yamaguchi, S. *et al. Nature* 409: 684, 2001.
Nishimura, H. *et al. J. Exp. Med.* 191: 157–169, 2000.
Hisahara, S. *et al. EMBO J.* 19: 341–348, 2000.
Nakai, A. *et al. EMBO J.* 19: 1545–1554, 2000.
Mcllroy, D. *et al. Gene. Dev.* 14: 549–558, 2000.
Serizawa, S. *et al. Nat. Neurosci.* 3: 687–692, 2000.
Ishiguro, K. *et al. J. Clin. Invest.* 106: 873–878, 2000.
Shinkura, R. *et al. Nat. Genet.* 22: 74–77, 1999.
Terauchi, Y. *et al. Nat. Genet.* 21: 230–235, 1999.
Watanabe, N. *et al. J. Exp. Med.* 190: 461–469, 1999.
Ihara, Y. *et al. Proc. Natl. Acad. Sci. U.S.A.* 95: 2526–2530, 1998.
Fujii, H. *et al. EMBO J.* 17: 6551–6557, 1998
Watanabe, D. *et al. Cell* 95: 17–27, 1998.
Sawada, S. *et al. J. Exp. Med.* 187: 1439–1449, 1998.

er gene trap vectors offer a variety of post-insertional modification strategies to allow for the generation of other experimental alleles. The cooperative goal of the IGTC is to generate an international resource representing all or most genes in the mouse genome, and to provide the bioinformatics and logistical support to make the resource valuable and available to scientists.

IMSR (http://www.informatics.jax.org/imsr/index.jsp): The International Mouse Strain Resources (IMSR) is a searchable online database of mouse strains and stocks available worldwide, including inbred, mutant, and genetically engineered mice. The goal of the IMSR is to assist the international scientific community in locating and obtaining mouse resources for research. The data content found in the IMSR is as it was supplied by data provider sites.

EUCOMM (http://www.eucomm.org/): The European Conditional Mouse Mutagenesis Integrated Project (EUCOMM) is funded by the European Union Framework 6 programme. The goal of EUCOMM is to generate a collection of up to 13,000 mutated genes in mouse C57BL/6N embryonic stem (ES) cells using conditional gene trapping and gene targeting approaches. This library will enable mouse mutants to be established worldwide in a standardized and cost-effective manner, making mouse mutants available to a much wider biomedical research community than has been possible previously.

NorCOMM (http://norcomm.phenogenomics.ca/): NorCOMM (North American Conditional Mouse Mutagenesis project) is a large-scale research initiative focused on developing and distributing a library of mouse embryonic stem (ES) cell lines carrying single conditional knockout mutations across the mouse genome.

KOMP (http://www.nih.gov/science/models/mouse/knockout/): The Knockout Mouse Project is a trans-NIH initiative that aims to generate a comprehensive and public resource comprised of mouse embryonic stem (ES) cells containing a null mutation in every gene in the mouse genome.

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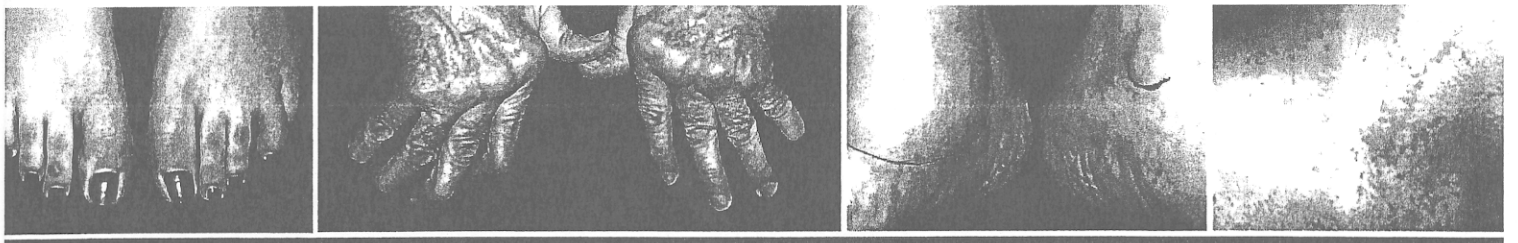

RHEUMATOID ARTHRITIS

MARC C. HOCHBERG • ALAN J. SILMAN • JOSEF S. SMOLEN
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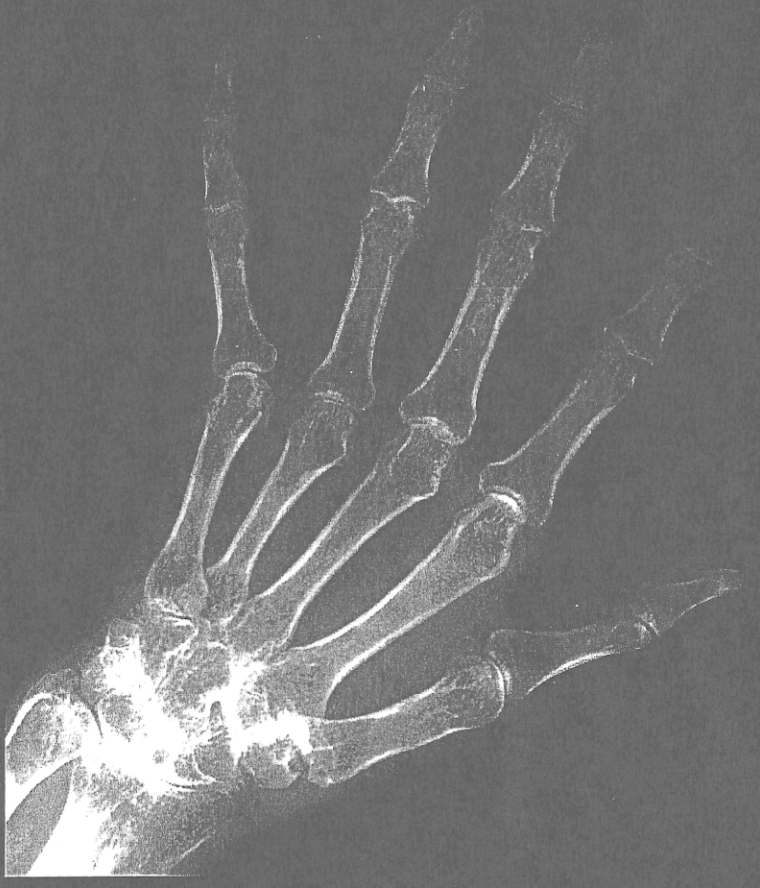

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Tocilizumab

Norihiro Nishimoto and Toru Mima

CHAPTER 10J

Interleukin (IL)-6 was originally identified as B-cell stimulatory factor-2 (BSF-2), a T-cell–derived factor that induces B-cell differentiation.[1] After the cDNA for BSF-2 was found to be identical to that of interferon-β2, a hybridoma/plasmacytoma growth factor, a 26-kDa protein, and a hepatocyte stimulating factor, these entities were unified under the name IL-6.[2] IL-6 is produced not only by T cells but by B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, and some tumor cells.[3] Although IL-6 plays important physiologic roles in the regulation of immune response, inflammatory reaction, and hematopoiesis, constitutive overproduction of IL-6 has been implicated in the development of immune-mediated and inflammatory diseases.[4]

In patients with rheumatoid arthritis (RA), IL-6 levels are elevated in both serum and synovial fluids.[5–8] IL-6 augments autoimmune responses through the activation of both B cells, resulting in hyper-γ-globulinemia and increased autoantibody titers, and autoreactive T cells. In animal models, it has been shown that in the presence of IL-6 transforming growth factor-β (TGF-β) induces differentiation of Th17 cells—which in turn produce IL-17, IL-6, and TNF.[9–13] In contrast, TGF-β in the absence of IL-6 induces $CD4^+CD25^+$Forkhead box P3 $(FOXP3)^+$ T regulatory cells—which inhibit autoimmune reactions. It appears that IL-6 may therefore be a key cytokine in the pathogenesis of effector Th17 cells and autoimmune responses, although further confirmatory studies will be needed in humans.

IL-6 acts synergistically with IL-1β and TNF to induce production of vascular endothelial growth factor (VEGF), which plays a role in stimulating angiogenesis in the hyperplastic synovial tissues of RA patients.[14] In the presence of soluble IL-6 receptors, IL-6 also induces osteoclast differentiation[15]—which mediates the joint destruction and osteoporosis associated with RA. As a proinflammatory mediator, IL-6 causes systemic symptoms of fever and fatigue,[16] as well as increased production of acute-phase proteins[17] such as C-reactive protein (CRP), fibrinogen, α1-antitrypsin, and serum amyloid A (SAA). Overexpression of IL-6, as a megakaryocyte-activating factor, induces thrombocytosis.[18] Finally, IL-6 induces production of the iron regulatory peptide hormone hepcidin.[19,20] By suppressing iron absorption and increasing hepatic iron storage, hepcidin promotes a hypoferremic anemia of chronic inflammation.

Recognition that IL-6 is involved in numerous inflammatory responses led to the idea that inhibition of IL-6 might be a plausible therapy for RA. Tocilizumab, a humanized antihuman IL-6 receptor (IL-6R) monoclonal antibody specifically targeting IL-6,[21] has been the subject of recent safety and efficacy trials in patients with RA.

Pharmacologic Features of Tocilizumab

Mode of Action

Tocilizumab was engineered by grafting complementarity-determining regions (CDRs) from mouse antihuman IL-6R antibody to human IgG1 to create a human IL-6R binding site on a human antibody.[21] This strategy proved effective for minimizing immunogenicity, as fewer neutralizing antibodies have been detected with repetitive tocilizumab administration than with treatments using mouse antibodies or mouse and human chimeric antibodies. This results in a prolonged half-life for tocilizumab.

IL-6 signal transduction is mediated by a ligand-binding IL-6R and a non ligand-binding but signal-transducing chain, gp130, on the surface of target cells. Soluble forms of IL-6R, found in blood and synovial fluids, are also capable of signal transduction through "trans-signaling"[22]—a signaling mechanism unique to the IL-6R system. Tocilizumab recognizes IL-6 binding sites on both the membranous and soluble forms of IL-6R, and competitively inhibits IL-6 binding to IL-6R (Figure 10J-1). Using the standard tocilizumab dosing regimen, neither antibody-dependent nor complement-dependent cellular cytotoxicity has been observed in cells that express IL-6R.

Pharmacokinetics

Serum tocilizumab concentrations showed nonlinear pharmacokinetics in the dose range of 2 to 8 mg/kg when intravenously administered by drop infusion for 2 hours.[23] The half-life of tocilizumab (t1/2) was dose dependent, and approximated the half-life of human IgG1 (241.8 ± 71.4 hours) by the third dose of 8 mg/kg. The mean area under the curve (AUC) for serum tocilizumab concentration peaked at approximately 10.66 ± 4.07 mg*hour/ml.[23]

Interestingly, CRP and SAA levels were undetectable in RA patients with serum concentrations of free tocilizumab greater than 1 μg/ml, suggesting that IL-6 is essential for CRP and SAA production *in vivo*. In fact, CRP levels have been shown to function as a surrogate marker for the level of tocilizumab activity.[23]

Clinical Studies of Tocilizumab in RA Patients

Clinical studies of tocilizumab in patients with immune-inflammatory diseases were initially performed in the Osaka University Hospital of Japan. The pilot studies, which were performed in patients with refractory disease, demonstrated the therapeutic potential of tocilizumab.[14,24,25] Tocilizumab

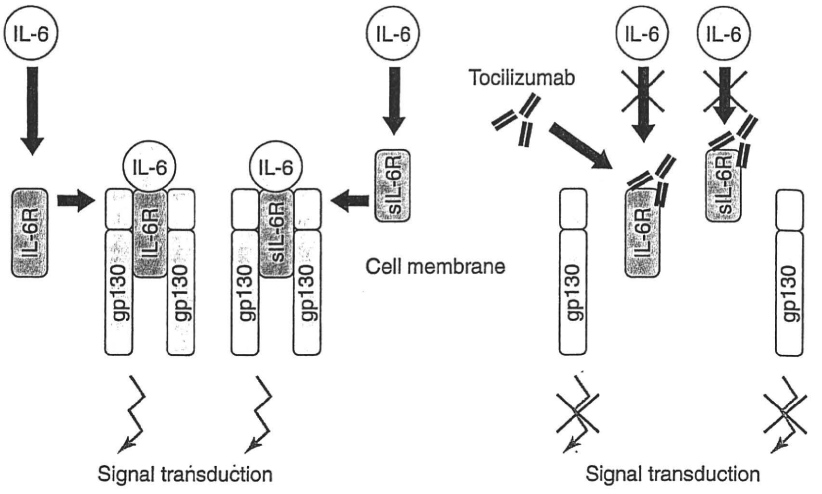

Figure 10J-1. Inhibitory action of tocilizumab in IL-6 signaling. IL-6 signal transduction is mediated by a ligand-binding IL-6R and a non–ligand-binding but signal-transducing chain, gp130, on the cell surface. Soluble IL-6R (sIL-6R) is also capable of signal transduction. Tocilizumab recognizes IL-6 binding sites on both the membranous IL-6R and sIL-6R and inhibits IL-6 signal transduction.

treatment dramatically improved both inflammatory symptoms and laboratory abnormalities, including serum levels of acute-phase proteins, albumin, hemoglobin, and VEGF. On the basis of these findings, clinical trials were designed and conducted.

Phase I/II Studies in the United Kingdom and Japan

The phase I/II study in the United Kingdom was a double-blind placebo-controlled trial testing the pharmacokinetics, safety, and efficacy of single-dose intravenous administration of tocilizumab at the following doses: 0.1, 1, 5, and 10 mg/kg.[26] The treatment was well tolerated, and normalization of CRP was achieved in the 5- and 10-mg/kg groups. In the 5-mg/kg group, 55.6% of the patients met the American College of Rheumatology (ACR) response criteria ACR 20, compared to 0% of the patients in the placebo group.

A Japanese phase I/II study then tested the pharmacokinetics, safety, and efficacy of repeated intravenous tocilizumab doses at 2, 4, and 8 mg/kg every 2 weeks in an open-label trial.[23] At 6 months, 86% and 33% of the patients had met ACR 20 and ACR 50 criteria, respectively. Treatments were again well tolerated.

Phase II Studies for RA

In 2001, the safety and efficacy of tocilizumab monotherapy for RA was evaluated in a multicenter double-blind randomized placebo-controlled phase II trial in Japan. In this study, 164 patients with refractory RA were randomized to receive intravenous treatment with placebo, tocilizumab treatment at 4 mg/kg, or tocilizumab treatment at 8 mg/kg. Treatment was administered at three sessions spaced 4 weeks apart, and the clinical responses were evaluated at 12 weeks using the ACR criteria.[27] Tocilizumab treatment significantly improved all measures of disease activity, and a dose-response relationship was observed across the 4- and 8-mg/kg groups. Patients had a mean disease duration of 8 years, and had used an average of four to five antirheumatic drugs before beginning tocilizumab treatment. Despite their advanced disease, 78% of the patients in the 8-mg/kg group met ACR 20 criteria—compared to 57% in the 4-mg/kg group ($p = 0.02$) and only 11% in the placebo group ($p < 0.001$). ACR 50 and ACR 70 responses in the 8-mg/kg group were 40% and 16%, respectively, and both were superior to those in the placebo group ($p < 0.001$, $p = 0.002$). The efficacy of tocilizumab monotherapy was also confirmed by the disease activity score in 28 joints (DAS28): 91% of patients in the 8-mg/kg group reported "good or moderate" joint scores, as opposed to 19% in the placebo group ($p < 0.001$). Complete normalization of CRP was observed in 76% of the patients in the 8-mg groups, but only 1.9% of patients in the placebo group. Significant improvement was also noted in lab values for hemoglobin, platelet count, fibrinogen, SAA, RF, albumin, bone formation markers, and bone resorption markers.

Safety data did not show a dose-dependent relationship. The overall incidence for adverse events was 56%, 59%, and 51% in the placebo, 4-, and 8-mg/kg groups, respectively. Serious adverse events were reported in three patients receiving tocilizumab (2.8%) and two patients receiving placebo (3.7%). One patient died from hemophagocytosis syndrome associated with reactivation of chronic active Epstein-Barr virus (EBV) infection after receiving a single dose of 8 mg of tocilizumab. This patient had increased serum EBV DNA levels before study enrollment, and it was later determined that she had developed Hodgkin's disease prior to tocilizumab treatment.[28] Although the mechanism of EBV reactivation is currently unclear, a careful pretreatment examination is necessary to assess whether the patient has a concurrent infectious disease. Other serious adverse events included allergic pneumonitis and super-infection of a burn, both of which were adequately treated with no long-term sequelae.

Abnormalities in laboratory values were also reported, notably including increased total cholesterol (TC) in 44% of patients receiving tocilizumab. High-density lipoprotein (HDL) cholesterol also increased, however, leaving the atherogenic index (total cholesterol – HDL cholesterol/HDL cholesterol) unchanged throughout the study period. The TC values tended to stabilize in the upper normal range, and no cardiovascular complications were reported. Similar increases in TC concentrations were also reported with TNF inhibitor treatments,[29] suggesting that the dyslipidemia may be secondary to the improvement in disease activity. Mild to moderate increases in liver function tests were observed in 14 of 109 patients (12.8%) receiving tocilizumab. They were all transient and normalized with the repeated administration of tocilizumab. There were no increases in antinuclear antibodies or anti-DNA antibodies, as is occasionally reported with TNF inhibitor treatments. These results indicate that treatment with tocilizumab was generally well tolerated.

A European phase II trial later tested the safety and efficacy of tocilizumab in combination with methotrexate (MTX) among patients with inadequate responses to MTX monotherapy.[30] This multi-arm trial randomized 359 patients into seven parallel arms. Patients received MTX (10–25 mg/week) with tocilizumab placebo; tocilizumab at 2, 4, or 8 mg/kg with MTX; or tocilizumab at 2, 4, or 8 mg/kg with MTX placebo. Tocilizumab or tocilizumab placebo was infused every 4 weeks for a total of 16 weeks.

ACR 20 criteria were met by 61% and 63% of patients, respectively, receiving 4 mg/kg and 8 mg/kg of tocilizumab as monotherapy, and by 63% and 74% of patients, respectively, receiving 4 mg/kg and 8 mg/kg of tocilizumab plus MTX. In contrast, only 41% of patients receiving placebo plus MTX satisfied the ACR 20 criteria. However, no statistical difference was observed in the ACR 20 response rate between the groups receiving monotherapy with 8 mg/kg tocilizumab and those receiving combination therapy with 8 mg/kg tocilizumab and MTX. ACR 50 and ACR 70 responses, as well as the European League Against Rheumatism (EULAR) remission rates, were significantly higher in patients receiving a combination therapy than in patients receiving MTX alone. EULAR remission was achieved in 34% of patients receiving tocilizumab 8 mg/kg plus MTX, compared to 17% in the group receiving 8 mg/kg tocilizumab monotherapy and 8% in the group receiving only MTX. The clinical benefits of tocilizumab, however, appeared to be similar with and without concomitant MTX therapy—although concomitant use of MTX increases the efficacy of TNF inhibitors.

In this study, tocilizumab was also well tolerated—with approximately 50% of patients experiencing adverse events, the majority of which were mild or moderate. No clear dose-dependent pattern was observed. Thirty-five serious adverse events were reported, with higher rates in the 2-mg/kg tocilizumab monotherapy group. Serious anaphylactic reactions were observed only in the groups receiving monotherapy with either 2 or 4 mg/kg of tocilizumab, and may be related to the development of anti-tocilizumab antibodies. Two cases of sepsis developed in patients receiving combination therapy with 8 mg/kg tocilizumab and MTX. Clinically significant laboratory abnormalities included increased transaminase levels, which seemed to be exacerbated by MTX. It is possible that tocilizumab treatment diminishes the protective effect of IL-6 on hepatocytes against MTX-induced toxicity. As in earlier trials, increased TC and decreased neutrophil counts were noted.

Phase III Studies for RA

The Japanese phase III trials consisted of two studies: The SATORI study evaluated the safety and efficacy of tocilizumab monotherapy for RA patients with inadequate responses to MTX treatment,[31] and the SAMURAI trial investigated the efficacy of tocilizumab monotherapy in slowing joint damage.[32]

In the SATORI study, 127 RA patients with inadequate responses to MTX (8 mg/week) were randomized to receive tocilizumab 8 mg/kg every 4 weeks in addition to a MTX placebo (tocilizumab group) or tocilizumab placebo in addition to MTX 8 mg/week (MTX group) for 24 weeks. ACR 20 improvement rates at 24 weeks served as the primary endpoint, with additional outcome measures including ACR 50 and ACR 70 improvement rates, DAS28, EULAR response, and ACR-N AUC.[31] At 24 weeks, ACR 20 response rates were significantly higher in the tocilizumab group than in the MTX group (80.3% vs. 25.0%, $p < 0.001$). Patients in the tocilizumab group also reported significantly higher ACR 50 and ACR 70 rates than patients treated by MTX [49.2% vs. 10.9% ($p < 0.001$) and 29.5% vs. 6.3% ($p < 0.001$), respectively]. Similarly, tocilizumab therapy was associated with improvements in both the EULAR response rates and the ACR-N AUC. Tocilizumab was very well tolerated, and withdrawals due to adverse events (tocilizumab, $n = 2$; MTX, $n = 3$) as well as the occurrence of serious adverse events (tocilizumab, $n = 4$; MTX, $n = 3$) were similar between tocilizumab and MTX groups. The frequency of liver function test abnormalities was higher in the MTX group (mean alanine transaminase [ALT] increase of 10.9%), although most cases were grade 1.

The SAMURAI study was designed to assess the change in total Sharp score (TSS), a quantitative radiographic evaluation of bone erosion and joint space narrowing in hand and foot joints of RA patients. This x-ray reader-blinded open-label randomized 1-year controlled trial enrolled RA patients who had been diagnosed less than 5 years prior to the study start date.[32] A total of 306 patients with active RA were randomly allocated to receive tocilizumab 8 mg/kg every 4 weeks or conventional disease-modifying antirheumatic drugs (DMARDs) for 52 weeks. Patients in the tocilizumab group showed significantly less radiographic progression, as measured by the change in TSS, than those receiving DMARDs (2.3 ± 5.6 versus 6.1 ± 11.4, $p = 0.001$). Tocilizumab was also superior to DMARDs in preventing both erosion and joint space narrowing ($p < 0.001$ and $p = 0.018$, respectively). The overall incidence of adverse events (including laboratory abnormalities) was 89% and 82% in the tocilizumab and DMARDs groups, respectively (serious adverse events: 18% and 13%, respectively; serious infections: 7.6% and 4.1%, respectively).

The results of two tocilizumab global phase III studies, OPTION and TOWARD, were reported in 2007.[33,34] In the OPTION study, 623 patients with moderate to severe RA received maintenance pre-study doses of MTX therapy in addition to placebo, 4 mg/kg of tocilizumab, or 8 mg/kg of tocilizumab. A significantly higher proportion of tocilizumab-treated patients achieved the primary endpoint of meeting ACR 20 criteria at 24 weeks (59% in the tocilizumab 8-mg/kg group; 48% in the tocilizumab 4-mg/kg group; 27% in the placebo group; $p < 0.0001$). ACR 50 endpoints were achieved in 44%, 32%, and 11% (respectively)—and ACR 70 endpoints were achieved in 22%, 12%, and 2% of patients in the tocilizumab 8-mg/kg group, the tocilizumab 4-mg/kg, and the placebo group, respectively.[33]

Safety profiles revealed a similar frequency of adverse events for tocilizumab and placebo groups, although serious infections were observed more frequently in the tocilizumab group (2.9% in the tocilizumab 8-mg/kg group; 1.4% in the tocilizumab 4-mg/kg group; 1% in the placebo group). Tocilizumab treatment significantly improved quality of life in patients with RA, as assessed by the health assessment questionnaire (HAQ), functional assessment of chronic illness therapy-fatigue scale (FACIT-fatigue), and short form-36 health survey (SF-36).[35]

Similarly, the TOWARD study evaluated the efficacy and safety of tocilizumab in a larger population of 1,216 RA patients with inadequate responses to a range of DMARDs.[34] Patients continued maintenance DMARD treatment while receiving intravenous tocilizumab 8 mg/kg or placebo every 4 weeks for 24 weeks. ACR 20, ACR 50, and ACR 70 responses were significantly higher in the tocilizumab group than in the placebo group (61% vs. 25%, 38% vs. 9%, and 21% vs. 3%, respectively; $p < 0.0001$). This study also

confirmed that tocilizumab treatment improved physical function, fatigue, and physical and mental health scores as assessed by HAQ, the FACIT-fatigue scale, and SF-36.[36]

Tocilizumab Safety Concerns

Because IL-6 plays a crucial role in immunity regulation, IL-6 inhibition may result in increased susceptibility to infectious diseases. In addition, suppression of inflammatory symptoms and laboratory value abnormalities may delay detection of infections. Clinical trials have demonstrated that the incidence of infections is similar to that of control groups, and similar to that reported for TNF inhibitors. It is noteworthy that tuberculosis was observed in only 2 of more than 4000 patients treated with tocilizumab, without pretrial screening or prophylactic use of anti-tuberculosis drugs. In comparison, tuberculosis frequently emerges during treatment with TNF inhibitors. This may be explained by the fact that TNF plays an important role in the formation of granulomas, whereas IL-6 is largely uninvolved in this immune response.

Several reports have claimed that TNF blockade may increase the risk of malignancy in RA patients, and it is possible that suppression of IL-6 action may also be carcinogenic. To investigate the risk of malignancy in RA patients treated with tocilizumab, outcomes from patients treated with long-term tocilizumab were compared to those of a Japanese cohort of RA patients (IORRA cohort) and a Japanese population database.[37] The incidence of malignancy, adjusted for age and gender, was calculated by direct and indirect methods.

In the tocilizumab cohort (mean age 51.5, female 79.8%, mean disease duration 6.21 years), 11 malignancies (male 3, female 8) were identified among 618 patients (1,459 person-years). In the IORRA cohort (mean age 55.9, female 81.8%, mean disease duration 8.73 years), 173 malignancies (male 56, female 115) were identified among 7,656 patients (25.567 person-years). The crude incidence of malignancies was 753.7 (male 1171.3, female 664.8) per 100,000 in the tocilizumab cohort and 676.7 (male 1331.5, female 542.2) per 100,000 in the IORRA cohort. Compared to the IORRA cohort, the standardized incidence ratio (SIR) of malignancies in the tocilizumab cohort was overall 1.33 (95% CI: 0.74–2.40). The SIR for males in this study was 1.18 (95% CI: 0.38–3.66), and that for females was 1.39 (95% CI: 0.70–2.79). Compared to the Japanese population database, the SIR was overall 1.66 (95% CI: 0.92–3.00)—and for males was 1.58 (95% CI: 0.51–4.88) and for females 1.70 (95% CI: 0.85–3.39). The incidence of malignancy in the tocilizumab cohort, therefore, did not exceed those of the Japanese RA cohort nor the Japanese population database.

Conclusion

Clinical studies have demonstrated that IL-6R inhibition by tocilizumab is a promising therapeutic approach for RA. The mechanisms by which IL-6 inhibition mediates improvements in RA, however, are not yet fully understood. Because regulation of inflammatory cytokines, including IL-6, is involved in the pathogenesis of RA future research will be needed to identify compounds that may act synergistically with tocilizumab. The long-term safety of tocilizumab treatment—particularly involving infection, malignancy, and cardiovascular risk—will also require investigation.

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