Establishment and Characterization of the Komeda Diabetes-prone Rat as a Segregating Inbred Strain

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Abstract: The Komeda diabetes-prone (KDP) rat is a spontaneous animal model of human autoimmune type 1 diabetes. By positional cloning of the non-MHC major susceptibility locus \textit{Iddm/kdp1}, we recently identified a nonsense mutation in \textit{Cblb} and also found that lymphocytes of KDP rats infiltrate into various tissues, indicating autoimmunity. The maintenance and production of KDP rats has been a critical problem owing to the poor reproductive ability of diabetic animals. To solve the problem, we here established the KDP rat as a segregating inbred strain. We first identified animals that were heterozygous at the \textit{Iddm/kdp1} region in a breeding colony of KDP rats. The heterozygous region spans at least from D11Yok1 to Cblb on rat chromosome 11. By mating between the heterozygous rats, we obtained homozygotes, heterozygotes and wild-types with the expected ratio of 1 : 2 : 1 and found that only the homozygotes developed diabetes, suggesting that these genotypes represent those of \textit{Iddm/kdp1}. We then tried to maintain KDP rats by mating between the heterozygotes, which resulted in a segregating inbred strain. Within 210 d of age, about 80\% of \textit{Iddm/kdp1} homozygotes developed diabetes with severe insulitis, while neither heterozygotes nor wild-types developed diabetes. The phenotypic characteristics of the homozygotes are the same as those of progeny of diabetic parents in the original KDP rats. The segregating inbred KDP rat strain described here would serve as a useful animal model for autoimmune diseases, including type 1 diabetes.

Key words: autoimmune disease, heterozygosity, Komeda diabetes-prone rat, segregating inbred strain, type 1 diabetes

Introduction

The Komeda diabetes-prone (KDP) rat is one of the best animal models of human type 1 diabetes \cite{5}. The phenotypes of KDP rats are characterized by autoimmune destruction of pancreatic $\beta$-cells, rapid onset of overt diabetes with no sex difference, and no significant T-cell lymphopenia. Most of the animals show
moderate to severe lymphocyte infiltration into pancreatic islets (insulitis), and about 80% of them develop diabetes within 220 d of age.

We previously performed genetic analysis of type 1 diabetes in KDP rats and found that most of the genetic predisposition to diabetes was explained by two major susceptibility loci, MHC on chromosome 20 and Iddm/kdp1 on chromosome 11 [11]. We recently identified a nonsense mutation in Cblb (Casitas B-lineage lymphoma b) as Iddm/kdp1 and proved it by transgenic complementation experiments with wild-type Cblb [12]. Cblb controls CD28 dependence of T-cell activation. Co-stimulation of the T-cell antigen receptor and CD28 is required for activation of T cells, and co-stimulatory signaling through CD28 seems to be essential for tolerance induction [3, 9]. If Cblb is disrupted, signaling through CD28 is not required for T-cell proliferation, interleukin-2 production [1, 2], or lipid raft aggregation [6], and this may contribute to autoimmunity. We found that lymphocytes of KDP rats infiltrate into pancreatic islets and several tissues, indicating autoimmunity [12]. Accordingly, we concluded that Cblb functions as a negative regulator of autoimmunity and that Cblb is a major susceptibility gene for type 1 diabetes in KDP rats.

Although the KDP rat is quite a unique model and should be used widely, there has been a critical problem of maintenance and production of the strain owing to poor reproductive ability in diabetic animals. Diabetic female rats have minimum levels of reproductive ability, while most of diabetic male rats have extremely poor reproductive ability. To make the KDP rat a more useful model, the problem of maintenance and production has to be solved.

In this study, we identified Iddm/kdp1 heterozygous animals in a colony of KDP rats, which allowed us to maintain and establish the strain as a segregating inbred strain. We also describe the genetic and phenotypic characteristics of the segregating inbred KDP rats.

Materials and Methods

Animals

We maintained KDP and Tester Moriyama (TM) rats under specific pathogen free conditions with a 12-h light-dark cycle, a commercial diet FR-1 (Funabashi Farm Co. Ltd., Chiba, Japan) and water ad libitum at the Animal Research Center, Tokyo Medical University. We used diabetic male KDP rats to generate backcross progeny with TM rats: (TM × KDP) F1 × KDP [11, 12]. We phenotyped and genotyped the progeny as described previously [11]. Diabetes was defined as glycosuria positivity with blood glucose levels more than 200 mg/dl under ad libitum dietary conditions. Animal care and procedures were approved by the Steering Committee of Research-Related Laboratory Animals of Chiba University and Tokyo Medical University.

Genetic markers

Most of simple sequence length polymorphism (SSLP) markers used in this study have been described elsewhere (Mouse Genome Database [7], RATMAP [8] and Yokoi et al. [11, 12]). Primer sequences that have not been described previously [12] are as follows: D11M16Mit14, 5'-TTTACACTGATTTTCACTG-3' and 5'-AGAATAATTCCAGACATCAA-3'; D11M16Mit85, 5'-TTTGTAGCAAATATTTATCA-3' and 5'-TGAGCAAGTTTTAAAAACTA-3'; and D11Yok1, 5'-ATATCCTTTAGCTTTGGTGA-3' and 5'-TTACTTGCCACTTCAATG-3'. For Cblb, genotyping was performed by PCR-RFLP analysis using primers, 5'-TGCCCCTTCTGTCGCTGTGA-3' and 5'-CCTCGGTTTTGAATCAACAG-3', and restriction enzyme TaqI [12]. For Alcam, genotyping was performed by PCR-RFLP analysis using primers, 5'-AAAGTGCTACAGCCTGTTGA-3' and 5'-TACTCCAAGGAGGAAGTCATG-3', and restriction enzyme AluI.

Histological analysis

We performed histological analysis as described previously [5, 11]. We graded the degree of insulitis of each animal from 1 (slight insulitis) to 4 (severe insulitis) based mainly on the percentages of moderately and severely infiltrated islets.

Results

Genetic linkage mapping of Iddm/kdp1 locus on rat chromosome 11

We recently reported a genetic linkage map of rat chromosome 11 in the vicinity of Iddm/kdp1 [12]. Using (TM × KDP) F1 × KDP backcross progeny, we here provide an up-graded version of the map including a new marker, Alcam. The backcross panel consisting of
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65 diabetic animals placed Iddm/kdp1 in a genomic segment of 3.1 cM between D11Yok1 and D11M16Mit14 ([12] and Fig. 1). Iddm/kdp1 co-segregated with Alcam and Cblb genes (Fig. 1). All the diabetic animals showed homozygosity for the KDP allele at Iddm/kdp1 (Fig. 1A), strongly implying that Iddm/kdp1 acts in a completely recessive manner. We also constructed a comparative map of the Iddm/kdp1 region on the rat, mouse and human chromosomes (Fig. 1B). These results indicate that the genomic segment including Iddm/kdp1 is highly conserved in these species.

Iddm/kdp1 heterozygosity in the KDP colony

During the positional cloning study of Iddm/kdp1 [12], newly developed markers in and around the Iddm/kdp1 region were examined for polymorphism among rat strains including KDP rats. When the marker D11Yok1 was examined, we noticed rats with two PCR products in the KDP colony, suggesting heterozygosity. Similar results were obtained with polymorphic markers developed from the Alcam and Cblb genes. By mating between the putative heterozygous animals, we obtained homozygous, heterozygous and wild-type animals with the expected ratio of 1 : 2 : 1, and also found that only the homozygous animals developed diabetes, indicating that these genotypes represent those of Iddm/kdp1. Since D11M16Mit46 and D11M16Mit14 markers are monomorphic in the KDP colony (Fig. 2) and its original Long-Evans Tokushima Lean (LETL) rats [4] (data not shown), it is not clear whether the regions in the vicinity of these markers have been fixed. The heterozygous region spans at least from D11Yok1 to Cblb (Fig. 2).

Establishment and characterization of the segregating inbred KDP rats

Since Iddm/kdp1 homozygous animals (most of them are diabetic) in the KDP colony have poor reproductive ability, we tried to maintain the colony by mating between Iddm/kdp1 heterozygous animals (all of them are non-diabetic). We successfully maintained the colony with this mating method through eight generations, and established the KDP rat as a segregating inbred strain. It is noteworthy that the problem of maintenance and production was solved by this breeding method.

To characterize the reproductive ability of animals of each genotype in the segregating inbred strain, we performed several types of mating among animals of the three genotypes (Table 1). Iddm/kdp1 homozygous male rats produced no offspring, whereas homozygous female rats produced but showed relatively low ability as compared with heterozygotes. Iddm/kdp1 heterozygous rats of both sexes have reproductive ability
comparable to that of wild-types. These data generally support the previous observation that diabetic rats (Iddm/kdp1 homozygotes) have poor reproductive ability, except for the fact that some diabetic male rats were previously reported to produce offspring.

The incidence of diabetes in Iddm/kdp1 homozygous through the first six inbreeding generations ranged from 70 to 100% (Fig. 3). The onset of diabetes averaged 95.9 d in 39 males and 100.6 d in 25 females after birth. The cumulative incidence of diabetes in Iddm/kdp1 homozygotes was 82.1% at 210 d of age (Fig. 4). In contrast, neither heterozygous nor wild-type animals developed diabetes (Fig. 4). In Iddm/kdp1 homozygous animals, all the diabetic rats showed severe insulitis, and most of the remainder showed mild to moderate insulitis (Table 2). Consistent with the non-diabetic status, most of the heterozygous and wild-type animals showed only slight insulitis, and none of them developed moderate to severe insulitis (Table 2).

**Discussion**

In this study, we have described the establishment and characterization of the segregating inbred KDP rat

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**Table 1.** Reproductive ability in the segregating inbred KDP rats

<table>
<thead>
<tr>
<th>Mating (female × male)</th>
<th>No. of females</th>
<th>No. of pregnant animals</th>
<th>No. of offspring</th>
<th>Litter size</th>
<th>No. of weanlings</th>
<th>Weaning rate a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo × Homo b)</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hetero × Homo</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Homo × Hetero</td>
<td>22</td>
<td>18 (82%)</td>
<td>101</td>
<td>5.6</td>
<td>91</td>
<td>90%</td>
</tr>
<tr>
<td>Hetero × Hetero</td>
<td>69</td>
<td>63 (91%)</td>
<td>473</td>
<td>7.5</td>
<td>454</td>
<td>96%</td>
</tr>
<tr>
<td>Wild × Hetero</td>
<td>5</td>
<td>5 (100%)</td>
<td>36</td>
<td>7.2</td>
<td>33</td>
<td>92%</td>
</tr>
<tr>
<td>Hetero × Wild</td>
<td>3</td>
<td>3 (100%)</td>
<td>22</td>
<td>7.3</td>
<td>22</td>
<td>100%</td>
</tr>
<tr>
<td>Wild × Wild</td>
<td>7</td>
<td>7 (100%)</td>
<td>52</td>
<td>7.4</td>
<td>52</td>
<td>100%</td>
</tr>
</tbody>
</table>

a) No. of weanlings/No. of offspring × 100. b) Genotypes at Iddm/kdp1 were determined by PCR-RFLP analysis of the Cblb mutation: Homo, homozygote; Hetero, heterozygote; Wild, wild-type.

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**Fig. 2.** Iddm/kdp1 heterozygosity in the KDP colony. PCR-SSLP (D11M16Mit46, D11Yok1 and D11M16Mit14) or PCR-RFLP (Alcam and Cblb) analyses were performed on Iddm/kdp1 homozygous (Homo), heterozygous (Hetero) and wild-type (Wild) animals in the KDP colony. Heterozygosity was observed for D11Yok1, Alcam and Cblb. See Fig. 5 for detailed information on the Alcam marker.
strain. At first, we identified putative heterozygosity at the \textit{Iddm/kdp1} region in the KDP colony. Secondly, we obtained homozygous, heterozygous and wild-type animals by mating between the heterozygous animals, and found that only the homozygous animals developed diabetes, indicating that these genotypes represent those of \textit{Iddm/kdp1}. Finally, we successfully maintained the KDP colony by mating between the heterozygous animals, resulting in the establishment of a segregating inbred strain. Within 210 d of age, about 80\% of \textit{Iddm/kdp1} homozygous animals developed diabetes with severe insulitis, while neither heterozygous nor wild-type animals developed diabetes.

In the original KDP rats, the cumulative incidence of diabetes in progeny of diabetic parents was 81.8\% at 220 d of age [5]. When one or both of the parents was non-diabetic, the cumulative incidence of diabetes was 64.8\% [5]. It is now clear that the former animals correspond to \textit{Iddm/kdp1} homozygotes and the latter to a mixture of the homozygotes and heterozygotes. Therefore, the phenotypic characteristics of \textit{Iddm/kdp1} homozygotes in the segregating inbred strain described here are the same as those of progeny of diabetic parents in the original KDP rats.

We previously established the Komeda non-diabetic (KND) rats [5] as well as the KDP from inbred LETL

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Fig. 3. Incidence of type 1 diabetes in \textit{Iddm/kdp1} homozygous animals in the segregating inbred KDP rats. Inbreeding generations from separation from the original KDP rats are indicated. Animals were examined for onset of diabetes until 210 d of age. Genotypes at \textit{Iddm/kdp1} were determined by PCR-RFLP analysis of the \textit{Cblb} mutation.

**Table 2.** Association of \textit{Iddm/kdp1} genotype with degree of insulitis and onset of diabetes in the segregating inbred KDP rats

<table>
<thead>
<tr>
<th>\textit{Iddm/kdp1} genotype(^a)</th>
<th>Degree of insulitis(^b)</th>
<th>Slight</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe, Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (n=6)</td>
<td></td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Heterozygote (n=29)</td>
<td></td>
<td>24</td>
<td>5</td>
<td>0</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Homozygote (n=78)</td>
<td></td>
<td>3</td>
<td>8</td>
<td>3</td>
<td>64 (82.1%)</td>
</tr>
</tbody>
</table>

\(^a\) Genotypes at \textit{Iddm/kdp1} were determined by PCR-RFLP analysis of the \textit{Cblb} mutation. \(^b\) The degree of insulitis for each animal was determined shortly after the onset of diabetes or at 210 d of age for non-diabetic animals. All the diabetic animals showed severe insulitis, while non-diabetic animals showed slight to moderate insulitis. Data were obtained from animals in the first six inbreeding generations.

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Fig. 4. Cumulative incidence of type 1 diabetes in the segregating inbred KDP rats. Animals were examined for onset of diabetes until 210 d of age. Genotypes at \textit{Iddm/kdp1} were determined by PCR-RFLP analysis of the \textit{Cblb} mutation (homozygote, n=78; heterozygote, n=29; wild-type, n=6). Data were obtained from animals in the first six inbreeding generations.
In KND rats, the Iddm/kdp1 region is fixed in the wild-type allele (Fig. 5 and data not shown). Except for the Iddm/kdp1 region, we could not detect any genetic differences between KDP and KND rats ([5] and data not shown), strongly indicating that only the Iddm/kdp1 region is different in the two strains. KND rats are therefore indistinguishable from the wild-type animals in the segregating inbred KDP rats.

The LETL rat has been established by selective (mostly nondiabetic) brother-sister mating for more than 20 generations to obtain less than 30% incidence of diabetes [4], since high incidence of diabetes resulted in poor reproductive performance. The major diabetes susceptibility locus Iddm/kdp1 would, therefore, remain heterozygous in this inbred strain. In KDP rats, the Iddm/kdp1 region also has not been fixed despite selective (diabetic or nondiabetic with insulitis) brother-sister mating for eight generations [5]. This indicates that KDP rats have been maintained by mating in which at least one of the mating partners was heterozygous for Iddm/kdp1.

We here developed a PCR-RFLP marker for Alcam based on a single nucleotide variation (G to C) at codon 184 leading to an amino acid substitution (Val184Leu). The LETL rats have the same variation as that of the KDP, while the KDP-related non-diabetic strains, KND and Long-Evans Tokushima Otsuka (LETO) [4], have only the wild-type alleles (Fig. 5). However, this variation is not specific to KDP and LETL rats, since several rat strains, including DA and exogenous hypercholesterolemic (ExHC) rats, have the same variation (Fig. 5). It was also suggested that this variation is not involved in the pathogenesis of diabetes in KDP rats, because transgenic complementation with wild-type Cblb gene rescued the KDP phenotype [12].

As described previously [12], in addition to the pancreatic islets, KDP rats showed severe lymphocyte infiltration into the thyroid gland and mild to moderate localized infiltration into various tissues, including the submandibular gland, kidney, adrenal, and pituitary. These data indicate that the KDP rat could be an animal model for other autoimmune diseases, especially for the autoimmune thyroid disease.

In conclusion, we have established the KDP rat as a segregating inbred strain. The segregating inbred KDP rat strain should contribute to understanding of human autoimmune diseases, including type 1 diabetes.

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