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Transgenic mouse expressing human CCR5 as a model for in vivo assessments of human selective CCR5 antagonists

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Abstract

The species selectivity of receptor antagonists often hinders their preclinical assessment in vivo. In order to evaluate human selective CC chemokine receptor type 5 (CCR5) antagonists in vivo, we generated human CCR5 transgenic mice that expressed the transgene on both peripheral blood leukocytes as well as thymocytes. The selective CCR5 ligand CC chemokine ligand 4 (CCL4)/macrophage inflammatory protein (MIP)-1β induced the chemotaxis of thymocytes that had been derived from the transgenic mice, but not from littermate mice, suggesting that the human CCR5 expressed in the transgenic mice were functional. The binding of the human CCR5 specific antibody 45531 to peripheral blood granulocytes from the transgenic mice was inhibited by human selective CCR5 antagonist SCH-351125. Using this antibody, we developed an ex vivo assay system that is suitable for the evaluation of a test compound's ability to occupy the human CCR5 receptor on mouse peripheral blood leukocytes. This transgenic mouse model is useful for estimating the pharmacodynamics of human selective CCR5 antagonists in vivo.

Keywords: Chemokine CCR5 receptor; Human CCR5 transgenic mouse; Receptor occupancy; Pharmacodynamics

1. Introduction

CCR5 is a G protein-coupled receptor for CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) that functions as a coreceptor for human immunodeficiency virus (HIV) type 1 (Alkhatib et al., 1996) (Deng et al., 1996) (Choe et al., 1996). CCR5 has been shown to be essential for HIV pathogenesis, as individuals homozygous for the CCR5 delta32 mutation, which lacks cell surface expression of CCR5, are highly resistant to HIV infection (Liu et al., 1996) (Samson et al., 1996). Several small molecule CCR5 antagonists, such as SCH-351125 ((Z)-(4-bromophenyl){1′-[(2,4-dimethyl-1-oxido-3-pyridinyl)carbonyl]-4′-methyl-1,4′-bipiperidin-4-yl}methanone O-ethyloxime), TAK-779, and E-913 have been described as having potent antiviral activity against macrophage-tropic R5 viruses in vitro (Strizki et al., 2001) (Baba et al., 1999) (Maeda et al., 2001). Epidemiologic studies on CCR5 delta32 homozygous individuals also suggest the involvement of CCR5 in the pathogenesis of many inflammatory diseases, such as

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transplant rejection (Fischereder et al., 2001), rheumatoid arthritis (Zapico et al., 2000), asthma (Srivastava et al., 2003), multiple sclerosis (Sellebjerg et al., 2000), myocardial infarction (Gonzalez et al., 2001), and Sjögren's syndrome (Petrek et al., 2002). Therefore, CCR5 antagonists are expected to become potential treatments for a wide variety of diseases.

Human CCR5 is less similar to mouse CCR5 with 81% of the amino acid sequences being identical (Boring et al., 1996). In fact, most of CCR5 antagonists, including SCH-351125, TAK-779, and E-913, showed obvious species selectivity (unpublished observations). In this paper, in order to evaluate human CCR5 selective antagonists in vivo, we constructed transgenic mouse model expressing human CCR5 under the transcriptional control of human CD11a promoter. We observed functional human CCR5 expression on the thymocytes in transgenic mice. We also developed an ex vivo assay system that is suitable for the evaluation of a test compound's ability to occupy the human CCR5 receptor on mouse peripheral blood leukocytes.

2. Materials and methods

2.1. Human and mouse CCR5-expressing B300-19 cells

Mouse pre-B cell line B300-19 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 50 μM 2-mercaptethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. The expression vector, pEF-BOS-Neo (Mizushima and Nagata, 1990) carrying either full-length human CCR5 (GenBank accession no. X91492) or mouse CCR5 cDNA (GenBank accession no. U47036), was transfected into B300-19 cells using electroporation. Then, G418-resistant stable transformants were isolated.

2.2. Binding studies

Cell membranes (5 μg/ml protein) were incubated at 25 °C for 3 h with 50 pM [^{125}I]-CCL3 (PerkinElmer Life Sciences, Boston, MA, USA) and 5 mg/ml wheat germ agglutinin SPA beads (Amersham, Bucks, UK) in the presence of various concentrations of SCH-351125 in 50 mM HEPES (pH 7.4) buffer containing 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% (W/V) bovine serum albumin (BSA). Radioactivity was counted using a TopCount (Packard, Meriden, CT, USA). Nonspecific binding was determined in the presence of 50 nM CCL3 (PeproTech, Rocky Hill, NJ, USA).

2.3. Intracellular calcium concentration ($[Ca^{2+}]_i$) elevation assay

Either human or mouse CCR5-expressing cells were incubated with HEPES-buffered salt solution [20 mM HEPES (pH 7.4), 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 0.05% BSA] containing 5 μM Fura-2 acetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan) at 37 °C for 45 min. SCH-351125 was added 1 min prior to the addition of 1 nM CCL3. $[Ca^{2+}]_i$ was analyzed with CAF-110 (JASCO, Tokyo, Japan), and the values of $[Ca^{2+}]_i$ were calculated from the ratio of fluorescence intensities ($F_{340/380}$) (Grynkiewicz et al., 1985).

2.4. Generation of human CCR5 transgenic mice

To generate human CCR5 transgenic mice, the human CCR5 coding sequence was placed under the transcriptional control of human CD11a promoter, which has been shown to produce high levels of transgene expression in peripheral blood leukocytes (Ritchie et al., 1995) (Fig. 1). The 1.7 kb DNA containing the human CD11a promoter carrying a 5′ *Hind*III site and a 3′ *Not*I site, was obtained through polymerase chain reaction (PCR) using two primers (CCAAGCTTTTGCACAGCAGTCACGC-CATTTTACATTTC, and GCGCGGCCGCTTGCTGGCAACC-TAGACAGGGTGAAAGAGG) and human genome DNA. A 1.1 kb DNA fragment of human CCR5 cDNA carrying a 5′ *Xho*I site and a 3′ *Xba*I site, was obtained through reverse transcription-PCR (RT-PCR) using two primers (GG*CTCGAG*ACCATGGATTAT-CAAGTGTCA, and GC*TCTAGA*CACTTGAGTCCGTGTCA-CAAG) and human leukocyte poly(A)$^+$mRNA as a template. The RT-PCR products were digested with *Xho*I and *Xba*I, and then inserted into the *Xho*I and *Xba*I site of the plasmid pME18S (Takebe et al., 1988) which contained SV40 late 16S intron, a gene expression booster. Using PCR and the resulting plasmid as a template, a 5′ *Not*I site and a 3′ *Apa*I site were added to the 1.5 kb DNA fragment that encompasses the SV40 late 16S intron, human CCR5 cDNA, and SV40 early poly(A)$^+$ signal. Then the 1.7 kbp *Hind*III-*Not*I DNA fragment containing human CD11a promoter and the 1.5 kb *Not*I-*Apa*I DNA fragment containing human CCR5 cDNA were ligated and subcloned into the *Hind*III and *Apa*I site of the pBluscript II KS(+) plasmid.

Transgenic mice were generated by injecting the 3.2 kb *Hind*III-*Apa*I DNA fragment containing human CD11a promoter and human CCR5 cDNA into fertilized (C57BL/6 × C57BL/6) mouse oocytes. Five positive founders were identified using polymerase chain reaction on the specific transgene sequence. In this study, all experiments were done using 6 to 30-week-old heterozygous transgenic mice along with normal littermates (Japan SLC, Shizuoka, Japan).

The animal experiments were performed in compliance with the regulations of the Institutional Animal Ethical Committee of Yamanouchi Pharmaceutical Co., Ltd.

2.5. Flow cytometry analysis

Blood was collected from the eye ground of the transgenic mouse, heparinized and incubated with anti-CD16/CD32 (Fc Block, BD PharMingen, San Diego, CA, USA) for 5 min. Either fluorescein isothiocyanate (FITC)-conjugated anti-human CCR5 monoclonal antibody (mAb), 2D7 (BD Pharmingen) diluted at 1:40, or FITC-conjugated anti-human CCR5 mAb, 45531 (R and D systems, Minneapolis, MN, USA) at 2.5 μg/ml was added and

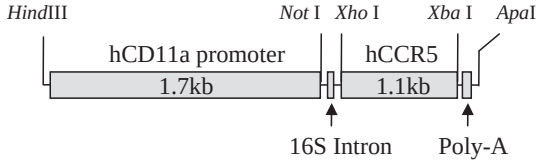

Fig. 1. Diagram of the human CD11a–CCR5 transgene. The human CCR5 coding sequence (hCCR5) was placed under the transcriptional control of the human CD11a (hCD11a) promoter.

Table 1
Inhibitory activities of SCH-351125 to human and mouse CCR5 receptors

Human CCR5 binding	$[Ca^{2+}]_i$	Mouse CCR5 binding	$[Ca^{2+}]_i$
13 ± 1.5	81 ± 6.4	>10,000[a]	>10,000[a]

IC_{50} values (nM) obtained from the binding and $[Ca^{2+}]_i$ assays are shown. $n = 10$. [a] $n = 2$.

further incubated for 60 min. An isotype-matched antibody was used as a negative control. The red blood cells were lysed with FACS™ Lysing Solution (BD Biosciences, San Jose, CA, USA) and analyzed using a FACScan flow cytometer (BD Biosciences).

2.6. Thymocyte chemotaxis assay

Chemotaxis assays were performed using 6.5-mm Transwell tissue culture inserts with a 5-μm pore size (Costar, Cambridge, MA, USA). Thymocytes were suspended at 1×10^7 cells/ml in RPMI 1640 plus 0.5% BSA, and 100 μl of cell suspension was added to an insert in a well with 600 μl of medium containing CCL4 or CCL25 (PeproTech). Assays were run for 2.5 h at 37 °C in a humidified 95% O_2/5% CO_2 incubator. The numbers of cells migrating to the bottom were counted using a hemocytometer.

2.7. Receptor occupancy study using flow cytometry

SCH-351125 was synthesized by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). For in vitro assessments, the heparinized blood from transgenic mice was preincubated for 30 min with increasing concentrations of SCH-351125 before the addition of the human CCR5-specific antibody, 45531. After a 60-min incubation with 45531, the erythrocytes were lysed and analyzed by FACScan. For in vivo assessments, SCH-351125 dissolved in sterile water was administered orally 1 h prior to blood sample collection. The collected blood was incubated with 45531 as described above. Receptor occupancy (%) was calculated as $100 \times$ (mean channel fluorescence [SCH-351125] − mean channel fluorescence [negative control])/(mean channel fluorescence [medium] − mean channel fluorescence [negative control]). The value obtained in the presence of excess amounts of SCH-351125 (10^{-5} M) was defined as 100% occupancy.

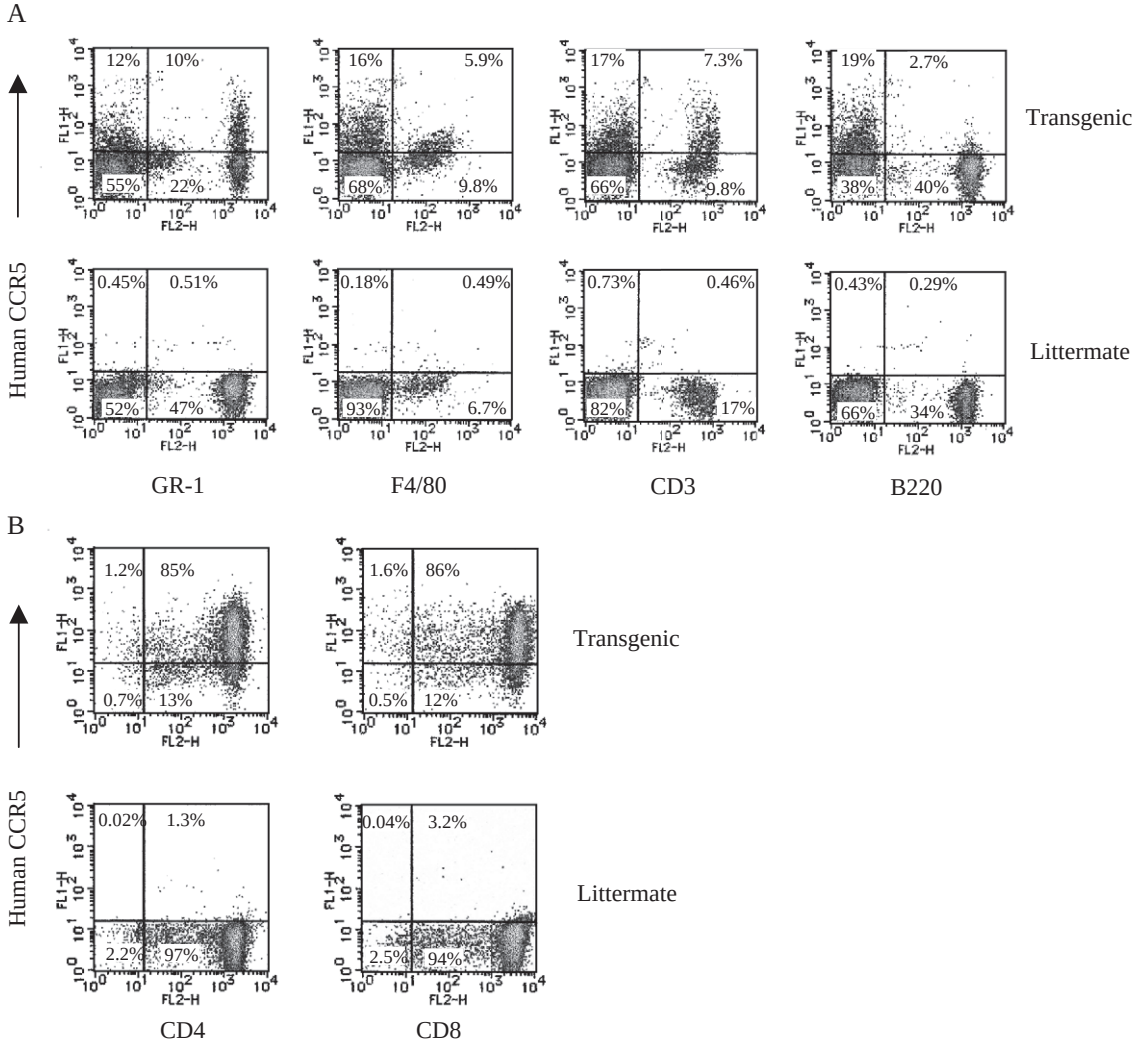

Fig. 2. Detection of human CCR5 with 2D7 antibody. (A) Human CCR5 expression on peripheral blood leukocytes in transgenic and littermate mice. (B) Human CCR5 expression on thymocytes. Similar results were obtained in 4 separate experiments.

2.8. Analysis of data

Results are expressed as the mean ± S.E. Statistical significance was assessed by a one-way analysis of variance followed by Dunnet's multiple comparison test. Probabilities of less than 5% ($P < 0.05$) were considered significant.

3. Results

3.1. Activity of SCH-351125 with regard to human and mouse CCR5

SCH-351125 inhibited the binding of [^{125}I]-CCL3 to human CCR5 with an IC_{50} of13 ± 1.5 nM. It also inhibited the $[Ca^{2+}]_i$ increase induced by CCL3 with an IC_{50} of 81 ± 6.4 nM. In contrast, it did not show any inhibitory activities with regard to mouse CCR5 up to a concentration of 10,000 nM (Table 1). These results indicate that SCH-351125 is highly selective for human CCR5.

3.2. Expression of human CCR5 on peripheral blood leukocytes and thymocytes

Expression of human CCR5 on peripheral blood leukocytes and thymocytes were analyzed by flow cytometry using 2D7, the FITC-conjugated anti-human CCR5. In order to define a leukocyte cell population, we also used phycoerythrin-conjugated antibodies against several cell surface markers: GR-1 on granulocytes; F4/80 on monocytes and macrophages; CD3, CD4, and CD8 on T cells; and B220 on B cells (Fig. 2). The percentage of positive and negative cells and the fluorescent intensity for each of these markers is roughly the same for the transgenic and littermate mice, suggesting that the transgene did not affect endogenous lymphocyte differentiation nor regulation of these lineage markers. In the peripheral blood of transgenic mice, a significant proportion of GR-1$^+$ cells, F4/80$^+$ cells, and CD3$^+$ cells expressed human CCR5, but B220$^+$ cells hardly expressed human CCR5. Within the GR-1$^+$, F4/80$^+$, CD3$^+$, and B220$^+$ gate, 31%, 38%, 43%, and 6.3% cells were positive to human CCR5, respectively. The human CCR5 expression level indicated by the fluorescent intensity was higher

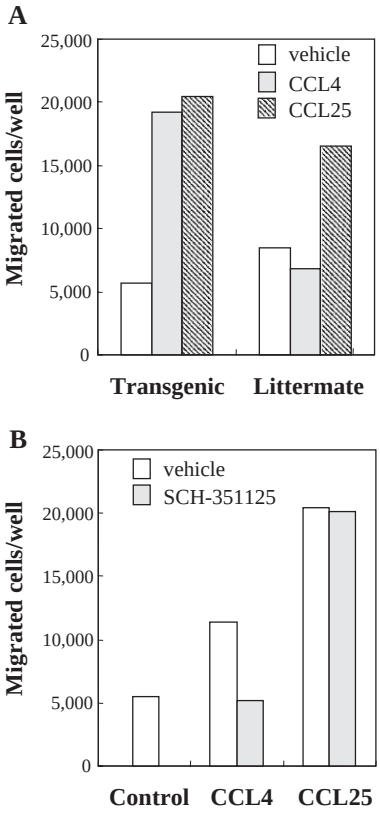

Fig. 3. Chemotactic responses of thymocytes from transgenic and littermate mice. (A) Chemotaxis by 20 nM CCL4 and 200 nM CCL25. (B) Effects of the human CCR5 selective antagonist, SCH-351125 (1 μM), on CCL4 and CCL25-induced chemotaxis.

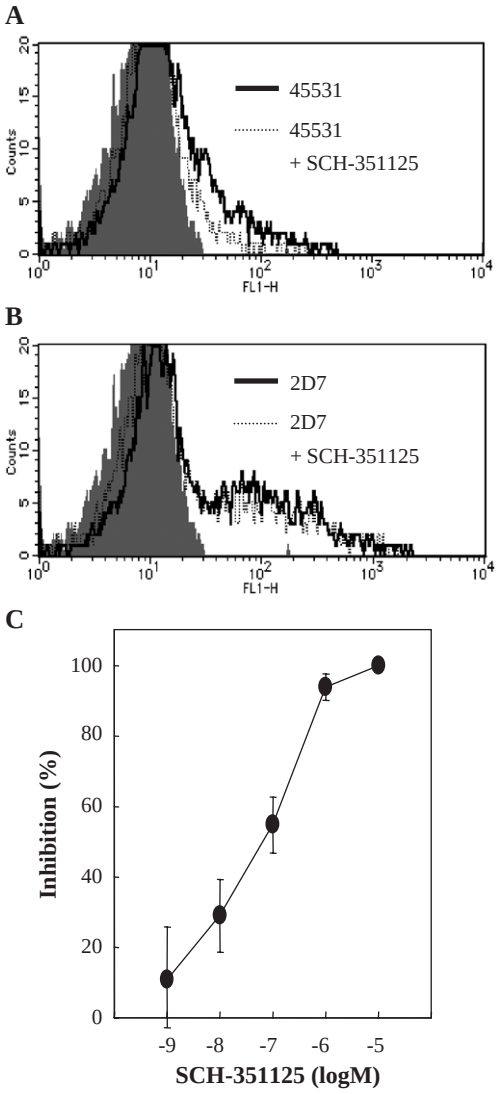

Fig. 4. Effects of SCH-351125 on human CCR5 antibody binding in vitro. (A) and (B): Transgenic mouse whole blood leukocytes were stained with human CCR5 antibody, 45531 (A) and 2D7 (B). The granulocytes indicated by the side and forward scatter properties were analyzed for the presence of CCR5 (thick line). The effects of 10^{-5} M SCH-351125 on 45531 or 2D7 binding (dotted line), and an isotype-matched mAb as the negative control (gray area), are represented. (C): The concentration-dependent inhibition of 45531 binding to human CCR5 by SCH-351125. The results shown are the mean values ± S.E.M. of four separate experiments.

both on GR-1$^+$ and CD3$^+$ cells, compared with that on F4/80$^+$ cells. In the thymocytes of transgenic mice, most CD4$^+$ and CD8$^+$ cells strongly expressed human CCR5. In contrast, human CCR5 was hardly detectable in the peripheral blood and thymocytes of the littermate mice.

3.3. Functional activity of the transgene in thymocytes

Next we determined whether the human CCR5 expressed in the transgenic mice was functional or not. CCL25/TECK, which is a ligand for CCR9, induced the chemotaxis of thymocytes in both the transgenic and the littermate mice. On the other hand, CCR5 selective agonist, CCL4/MIP-1β induced the chemotaxis only of the thymocytes from the transgenic mice (Fig. 3A), and this chemotaxis was suppressed to background levels in the presence of the human CCR5 selective antagonist, SCH-351125 (Fig. 3B). These results indicated that the human CCR5 expressed in the transgenic mice is functional.

3.4. Receptor occupancy study in vitro and in vivo

We examined the inhibitory effects of SCH-351125 on the binding of the two different antibodies, 2D7 and 45531, to whole blood granulocytes from transgenic mice. Pretreatment with 10^{-5} M SCH-351125 inhibited 45531 binding, but not 2D7 binding (Fig. 4A and B). SCH-351125 concentration-dependently inhibited 45531 binding with an IC_{50} of 50 nM (Fig. 4C). Since the inhibitory effects indicated that SCH-351125 was occupying the human CCR5 receptor, we conclude that the antibody 45531 is a useful tool for estimating the pharmacological effects of CCR5 antagonists.

We applied this whole blood antibody binding assay to the evaluation of the receptor occupancy by SCH-351125 in vivo. SCH-351125 was orally administered to the transgenic mice, and the blood was collected for the occupancy assay. One milligram per kilogram of SCH-351125 almost fully occupied the human CCR5 receptors (Fig. 5).

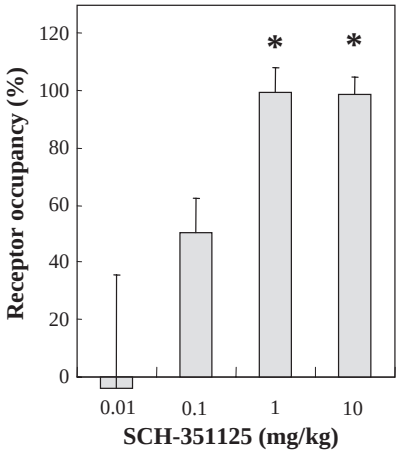

Fig. 5. Receptor occupancy assay by SCH-351125 in vivo. SCH-351125 was administered orally 1 h prior to the collection of blood from the transgenic mice. The antibody, 45531, was added to the collected blood and the granulocytes indicated by the side and forward scatter properties were analyzed for the presence of CCR5. The results shown are the mean values ± S.E.M. ($n=5$). *$P<0.05$ compared with data in the absence of SCH-351125.

4. Discussion

Low-molecular-weight antagonists against chemokine receptors such as CCR1, CCR5, and CXC chemokine receptor type 4 (CXCR4), that can inhibit the infiltration of inflammatory leukocytes into inflammatory sites, were effective on many animal disease models for diseases such as, rheumatoid arthritis (Yang et al., 2002) (Matthys et al., 2001), nephritis (Anders et al., 2004), multiple sclerosis (Liang et al., 2000a), and heart transplant rejection (Horuk et al., 2001). Among them, CCR1 antagonist was effective in clinical trials on patients with rheumatoid arthritis (Haringman et al., 2003). These facts suggest that chemokine receptor blockade in peripheral blood leukocytes is a valid therapy for various inflammatory diseases. However, the candidate compounds for clinical development sometimes show strong species preferences, that is, strong activity with regard to human targets, but poor or no activity with regard to the rodent counterparts, as shown by several antagonists for CCR1 (Liang et al., 2000b), adenosine A_3 (Gao et al., 2003), and tachykinin NK_3 receptor (Chung et al., 1995). Although CCR5 antagonists are expected to become drugs for the treatment of HIV infection and several other inflammatory diseases, they possess species-specific properties. To overcome the difficulties of evaluating human selective CCR5 antagonists in small animals, we developed human CCR5-expressing transgenic mice. The mice also express the natural mouse CCR5 receptor, and the activity of chemokines is expected to be mediated both by the human and the mouse receptors. This means that the transgenic mice model cannot be used for assaying the efficiency of human selective CCR5 antagonists in mouse disease models. However, the existence of mouse CCR5 can be ignored when estimating the pharmacodynamics of human selective CCR5 antagonists by 45531, because the antibody is highly specific to human CCR5. The homologous gene knock-in mice model is an alternative method for the evaluation of human-selective compounds, as has been reported in the case of CCR2 (Prosser et al., 2002). However, we did not choose the CCR5 knock-in mice, since the expression level of CCR5 in granulocytes, monocytes and T/B cells in mice were very low (Mack et al., 2001).

We demonstrated that human CCR5 was expressed on transgenic thymocytes (Fig. 2B), and the expressed receptor was functional (Fig. 3). SCH-351125 inhibited 45531 binding to human CCR5 with an IC_{50} of 50 nM, but did not inhibit 2D7 binding (Fig. 4). The IC_{50} value was comparable to that obtained in the binding assay and $[Ca^{2+}]_i$ assay (Table 1). It has been reported that 2D7 binds to the N-terminal half of the second extracellular loop 2 of CCR5, and 45531 binds to the C-terminal half of the second extracellular loop 2 domain of CCR5 (Lee et al., 1999). Since the binding site of SCH-351125 was reported to be in the transmembrane domain, but not in second extracellular loop 2 (Tsamis et al., 2003), it is unlikely that SCH-351125 directly interfered with the bind-

ing of 45531 to human CCR5. The binding of SCH-351125 to the transmembrane domain seems to induce a structural change around the C-terminal half of second extracellular loop 2. It was reported that another CCR5 antagonist, E-913, also interfered with the binding of 45531, but not the binding of 2D7 (Maeda et al., 2001).

SCH-351125 dose-dependently occupied the human CCR5 receptor on peripheral blood granulocytes from the transgenic mice with the ED_{50} value of 0.10 mg/kg (Fig. 5). The antiviral effects of SCH-351125 seen in clinical trials have recently been reported. Oral administration of SC-351125 (25 mg/person) reduced the viral load in HIV-1-infected patients (Este, 2002). The extrapolation of the dose to a 70-kg human is 0.36 mg/kg. This value well agrees with the ED_{50} value obtained in our transgenic mice model.

In this report, we have generated human CCR5 transgenic mice and developed an ex vivo human CCR5 occupancy assay. This assay system is useful for the in vivo screening of human-selective CCR5 antagonists. Our approach using transgenic mice expressing the human receptor and the antibody specific to the receptor will be applicable to the estimation of the pharmacodynamics of any other human-selective antagonist in vivo.

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