ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2007, p. 707–715
0066-4804/07/$08.00+0 doi:10.1128/AAC.01079-06

Vol. 51, No. 2

Isolation and Characterization of Human Immunodeficiency Virus Type 1 Resistant to the Small-Molecule CCR5 Antagonist TAK-652[▽]

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Received 27 August 2006/Returned for modification 20 October 2006/Accepted 9 November 2006

TAK-652, a novel small-molecule chemokine receptor antagonist, is a highly potent and selective inhibitor of CCR5-using (R5) human immunodeficiency virus type 1 (HIV-1) replication in vitro. Since TAK-652 is orally bioavailable and has favorable pharmacokinetic profiles in humans, it is considered a promising candidate for an entry inhibitor of HIV-1. To investigate the resistance to TAK-652, peripheral blood mononuclear cells were infected with the R5 HIV-1 primary isolate KK and passaged in the presence of escalating concentrations of the compound for more than 1 year. After 67 weeks of cultivation, the escape virus emerged even in the presence of a high concentration of TAK-652. This virus displayed more than 200,000-fold resistance to TAK-652 compared with the wild type. The escape virus appeared to have cross-resistance to the structurally related compound TAK-779 but retained full susceptibility to TAK-220, which is from a different class of CCR5 antagonists. Furthermore, the escape virus was unable to use CXCR4 as a coreceptor. Analysis for Env amino acid sequences of escape viruses at certain points of passage revealed that amino acid changes accumulated with an increasing number of passages. Several amino acid changes not only in the V3 region but also in other Env regions seemed to be required for R5 HIV-1 to acquire complete resistance to TAK-652.

The introduction of highly active antiretroviral therapy with reverse transcriptase inhibitors and protease inhibitors has achieved significant progress in the treatment of human immunodeficiency virus type 1 (HIV-1) infection (31). In addition, novel inhibitors targeting other essential molecules for viral replication, such as CCR5 and integrase, are now in human clinical trials (8, 22, 25). The chemokine receptors CCR5 and CXCR4 act as major coreceptors of HIV-1 in consort with the primary receptor CD4 (4, 16, 17). It has been reported that HIV-1 using CCR5 as a coreceptor (R5 HIV-1) is isolated predominantly during the asymptomatic stage (5). R5 HIV-1 is also responsible for virus transmission between individuals. On the other hand, HIV-1 using CXCR4 as a coreceptor (X4 HIV-1) generally emerges at the advanced stage of the disease and is related to acceleration of its progression (5, 20). However, several lines of evidence suggest that R5 HIV-1 still plays a major role even in the advanced stage (11, 30). Therefore, suppression of R5 HIV-1 in infected individuals may be more important than that of X4 HIV-1 in terms of blocking viral transmission and delaying disease progression. This hypothesis has been supported by the finding that individuals having homozygous CCR5-Δ32, a truncated and nonfunctional form of CCR5, display profound resistance to HIV-1 infection without obvious health problems (6, 12, 21). These findings have given us the idea that CCR5 antagonists may be effective as anti-HIV-1 agents without serious side effects, even though CCR5 is a host cellular factor.

The first small-molecule CCR5 antagonist, TAK-779, has been reported to be a potent and selective inhibitor of HIV-1 replication by our group (3). This compound inhibits R5 HIV-1 replication at nanomolar concentrations in cell cultures. However, TAK-779 is an anilide derivative with a quaternary ammonium moiety and could not be further developed as an antiretroviral agent because of its poor oral bioavailability. In the meantime, several groups have identified different classes of small-molecule and orally bioavailable CCR5 antagonists, most of which appeared to be promising candidates for further development (8, 13, 25). TAK-220 and TAK-652, novel orally bioavailable CCR5 antagonists, are successors of TAK-779. TAK-220 is one of a novel series of compounds with chemical structures totally different from that of TAK-779 (27). TAK-220 is orally bioavailable and highly inhibitory to HIV-1 replication in vitro. The other compound, TAK-652, is a derivative of TAK-779 with high oral bioavailability and favorable pharmacokinetic profiles in humans (2). This compound is also a highly potent inhibitor of R5 HIV-1 replication in vitro. Thus, both compounds are considered promising candidates for clinical development.

There may be no exceptions that drug-resistant HIV-1 will emerge under the selective pressure of any single antiretroviral agent. In the case of CCR5 antagonists, there is a serious concern that their long-term use could induce the evolution of X4 HIV-1 in patients (17, 19). In fact, drug-resistant viruses were isolated in long-term cultures of R5 HIV-1-infected cells by the selection

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▽ Published ahead of print on 20 November 2006.

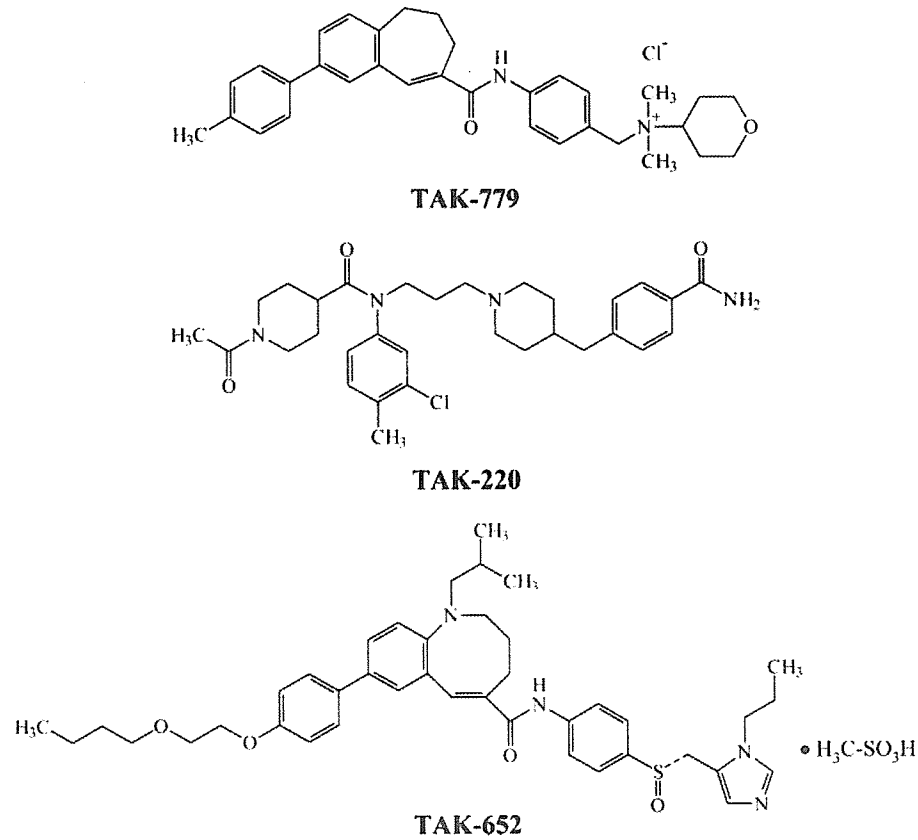

FIG. 1. Structures of TAK-779, TAK-220, and TAK-652.

pressure of some CCR5 antagonists, such as AD101 and vicriviroc (10, 15, 29). However, the escape viruses were found to retain the R5 phenotype. Therefore, in vitro isolation and analyses of drug-resistant viruses may be able to provide useful information for future clinical development of CCR5 antagonists. In this study, we conducted a long-term culture experiment with R5 HIV-1-infected peripheral blood mononuclear cells (PBMCs) with escalating concentrations of TAK-652. After serial passages of the infected cells for more than 1 year, an escape virus was obtained which displayed complete resistance to TAK-652 but retained full susceptibility to TAK-220.

MATERIALS AND METHODS

Compounds. The small-molecule CCR5 antagonists TAK-779 (3), TAK-220 (27), and TAK-652 (2) and the CXCR4 antagonist AMD3100 (23) were synthesized by Takeda Pharmaceutical Company, Osaka, Japan. The chemical structures of the CCR5 antagonists are shown in Fig. 1.

Cells and virus. PBMCs were obtained from healthy volunteers after obtaining their informed consent. The cells were isolated with Ficoll-Hypaque gradient density centrifugation and stimulated with 5 μg/ml phytohemagglutinin (PHA) in RPMI 1640 medium supplemented with 20% fetal bovine serum, 100 U/ml recombinant human interleukin-2 (Takeda Pharmaceutical Company), 100 U/ml penicillin G, and 100 μg/ml streptomycin for 3 days. The above medium without PHA was used in viral replication assays. U87 astroglioma cells expressing human CD4 and either CCR5 or CXCR4 (U87.CD4.CCR5 cells or U87.CD4.CXCR4 cells, respectively) were obtained from D. Littman (New York University School of Medicine, New York, NY) and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 300 μg/ml Geneticin, 1 μg/ml puromycin, and antibiotics. The above medium without Geneticin and puromycin was used in viral replication assays. The KK strain of R5 HIV-1 was used for infection of PBMCs. This strain was isolated from a patient in Kagoshima University Hospital who had no treatment history with any antiretrovirals until virus isolation. Its coreceptor usage was previously determined by a replication assay in U87.CD4.CCR5 cells and U87.CD4.CXCR4 cells, as described below.

Long-term culture of infected PBMCs with TAK-652. The KK strain of HIV-1 (100 ng of p24) was added to 6 ml of PHA-stimulated PBMCs (5×10^6 cells) and incubated at 37°C. To achieve sufficient infection of PBMCs with the clinical isolate, the cells were incubated for 2 days in the absence of compounds. After virus adsorption, the cells were washed three times with culture medium to remove unadsorbed virus particles. The cells were resuspended with culture medium (10 ml) in the presence of 0.2 nM TAK-652. On day 4 after virus infection, the infected cells were subcultured at a ratio of 1:4 with fresh culture medium containing the same concentration of the compound. On day 7, the number of viable cells was counted and adjusted to 1×10^6 cells/ml. Then, 1 ml of the infected cells and 4 ml of freshly prepared and uninfected PBMCs (4×10^6 cells) were suspended with culture medium (10 ml) in the presence of an appropriate concentration of TAK-652 and incubated at 37°C. As control cultures, exactly identical passages of the infected PBMCs in the absence of the compound were carried out in parallel with the cultures exposed to TAK-652. At each passage, p24 antigen levels of culture supernatants were monitored by using an enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix Corp., Buffalo, NY) to confirm virus replication. The concentration of TAK-652 was escalated when the p24 level in the TAK-652-treated culture exceeded 50% of that in the control culture at two consecutive passages. To exclude the effect of different PBMC donors, PBMCs from the same (one) donor were used for passages throughout the experiment. The escape viruses as well as the control viruses were propagated once in PBMCs to remove the compound, their infectivity was determined, and they were used for further experiments.

Susceptibility assay of escape viruses to CCR5 antagonists. PHA-stimulated PBMCs (4×10^6 cells) were infected with 1,400 50% cell culture infective doses of the virus and incubated at 37°C. After a 4-h incubation, the cells were washed with culture medium to remove unadsorbed viral particles and seeded into a 96-well plate (2×10^5 cells/well) with culture medium containing various concentrations (0.1 to 10,000 nM) of test compounds. On day 4 after virus infection,

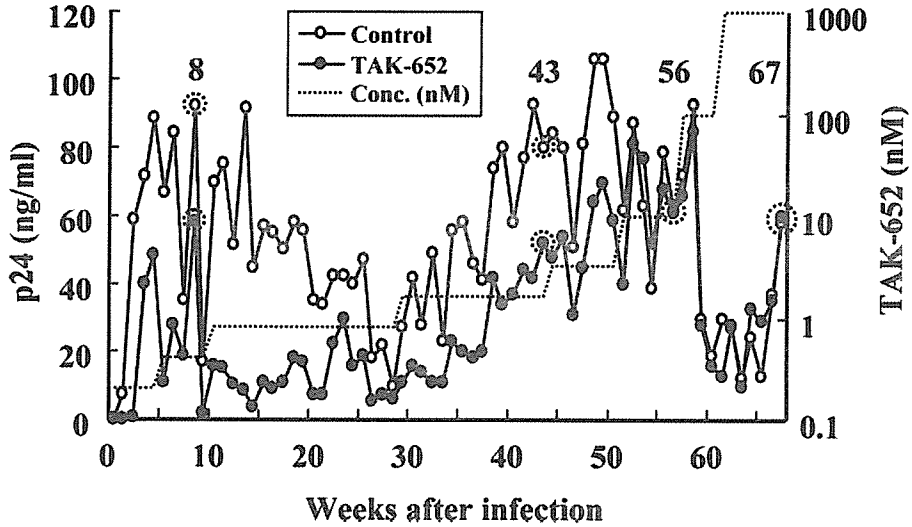

FIG. 2. Long-term culture of infected PBMCs with escalating concentrations of TAK-652. PHA-stimulated PBMCs were infected with an R5 HIV-1 clinical isolate (KK strain) and were passaged weekly by replenishing with fresh PBMCs in the presence or absence of TAK-652 at the indicated concentrations. The solid and dotted lines indicate the p24 levels and the concentrations of TAK-652 in culture supernatants, respectively. PBMCs obtained from one healthy volunteer were used throughout the experiment. At passages 8, 43, 56, and 67, viruses were isolated from the supernatants and subjected to phenotypic and genotypic analyses.

the cells were subcultured at a ratio of 1:2 with fresh culture medium containing the same concentration of the test compounds. On day 7 after infection, the culture supernatants were collected and p24 antigen levels were determined by using an ELISA kit (ZeptoMetrix Corp.).

Determination of coreceptor usage. U87.CD4.CCR5 or U87.CD4.CXCR4 cells were seeded into a 48-well plate (1×10^4 cells/well) and incubated overnight at 37°C. The culture supernatants were removed, and the cells were inoculated with the culture supernatant of each passage in a total volume of 400 μl. After an overnight incubation, the cells were washed thoroughly with culture medium to remove unadsorbed viral particles and further incubated. On day 4 after infection, the culture supernatants were removed and incubation continued with fresh culture medium. On day 6, the culture supernatants were collected and p24 antigen levels were determined by using an ELISA kit (ZeptoMetrix Corp.).

Sequence analysis of *env* genes. Genomic DNA was extracted from the infected PBMCs with a DNA extraction kit (Wako, Tokyo, Japan). The extracted DNA was subjected to PCR. The PCR consisted of 30 cycles (95°C for 15 s, 55°C for 30 s, and 68°C for 150 s) with the forward and reverse primers EnvS (5′-GAGCAGAAGACAGTGGCAATGAGAGTGAAG-3′) and EnvA (5′-TTTTGACCACTTGCCACCCATCTTATAGCA-3′), respectively, which generated a fragment including nucleotides −18 through 2566 of the *env* gene corresponding to the JR-FL strain of HIV-1 (GenBank accession number U63632). The amplified products were isolated by gel electrophoresis and purified with a PCR DNA and gel band purification kit (Amersham Pharmacia Biotech, Piscataway, NJ). The purified DNA was sequenced directly with a cycle sequence kit (BigDye Terminator version 3.1; Applied Biosystems Inc., Foster City, CA), using both forward and reverse primers on an automated DNA analyzer (model 3730; Applied Biosystems Inc.). Depending on the sequence result obtained by the analysis, the primer for the next sequence analysis was designed.

Data analysis. The 50% inhibitory concentrations (IC_{50}) of test compounds were calculated using the SAS system procedure NLIN, which produces least-squares estimates of the parameters of a nonlinear model (logistic model).

RESULTS

Isolation of escape viruses. To successfully isolate TAK-652-resistant viruses, it seemed important to start an experiment with a genetically heterogeneous primary R5 HIV-1 isolate. In addition, an isolate from a treatment-naïve patient would be preferable. Therefore, we chose the KK strain as the source of R5 HIV-1. In our previous study, TAK-652 was found to inhibit replication of the KK strain with an IC_{50} and IC_{90} of 0.043 nM and 0.19 nM, respectively (2). Therefore, PBMC cultures were started in the absence or presence of TAK-652 at a concentration of 0.2 nM (almost identical to its IC_{90}). After three passages, the p24 level of the TAK-652-treated culture rapidly increased and reached more than 50% of the control culture level (Fig. 2). Therefore, the compound concentration was elevated to 0.4 nM at passage 5. At passage 8, viruses were isolated from the TAK-652-treated and control cultures and designated as $KK_{652\text{-}8}$ and $KK_{C\text{-}8}$, respectively (Fig. 2).

Further passages of the infected PBMCs were carried out weekly with an increasing concentration of TAK-652 from 0.8 to 1.6 nM. During this period, HIV-1 replication in the TAK-652-treated culture appeared to be suppressed well, compared to that in the control culture. However, the p24 levels gradually increased after 37 passages, and viruses from the TAK-652-treated and control cultures were isolated at passage 43 ($KK_{652\text{-}43}$ and $KK_{C\text{-}43}$, respectively) (Fig. 2). At passage 52, the p24 levels of the TAK-652-treated and control cultures were comparable. After this point, suppression of HIV-1 replication was not observed for the TAK-652-treated culture, even when its concentration was elevated to 100 nM. Viruses were obtained from the TAK-652-treated and control cultures at passage 56 ($KK_{652\text{-}56}$ and $KK_{C\text{-}56}$, respectively) and passage 67 ($KK_{652\text{-}67}$ and $KK_{C\text{-}67}$, respectively) (Fig. 2). The concentrations of TAK-652 were 10 and 1,000 nM at passages 56 and 67, respectively.

Susceptibility of escape viruses to CCR5 antagonists. When TAK-652 was examined for its inhibitory effect on $KK_{652\text{-}67}$ replication in PBMCs obtained from two different donors, it did not show any significant inhibition at concentrations up to 10,000 nM (Fig. 3). TAK-779 exhibited a dose-dependent but only partial antiviral activity against $KK_{652\text{-}67}$. Interestingly, TAK-220, from a different class of CCR5 antagonists, was highly inhibitory to the replication of $KK_{652\text{-}67}$, irrespective of

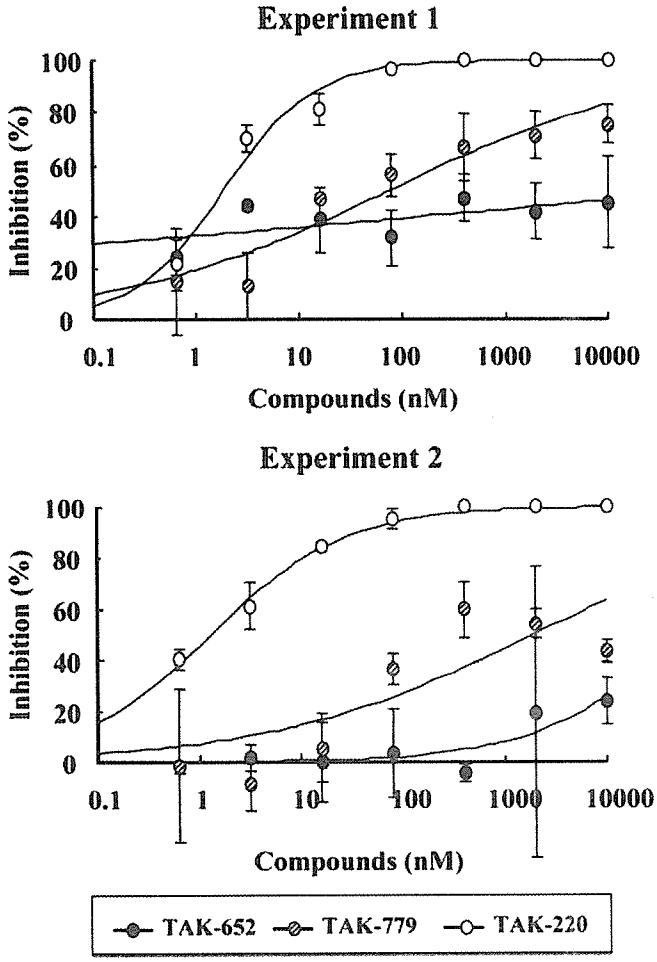

FIG. 3. Antiviral activities of TAK-652, TAK-220, and TAK-779 against the escape virus at passage 67 (KK_{652-67}). PHA-stimulated PBMCs were infected with the virus and incubated for 4 h. The cells were washed to remove unadsorbed viral particles and seeded into a 96-well plate with culture medium containing various concentrations of test compounds. On day 4 after virus infection, the cells were subcultured at a ratio of 1:2 with fresh culture medium containing the same concentration of the test compounds. On day 7 after infection, the culture supernatants were collected and p24 antigen levels were determined by ELISA. The results of two different experiments are shown. In each experiment, different donors were used for the antiviral assays of the compounds.

PBMC donor. As shown in Table 1, the IC_{50} of TAK-652 for KK_{652-67} was more than 10,000 nM. Considering that the IC_{50} of TAK-652 for the wild-type virus (KK_{WT}) was 0.043 nM in PBMCs, KK_{652-67} had strong (more than 200,000-fold) resistance to this compound. The IC_{50}s of TAK-779 for KK_{652-67} were 77 and 2,000 nM in experiments 1 and 2, respectively. In another experiment under identical assay conditions, an IC_{50} of 2.1 nM was obtained with TAK-779 for the wild type (Table 1), suggesting that KK_{652-67} displayed cross-resistance to TAK-779. In contrast, KK_{652-67} appeared to retain complete susceptibility to TAK-220 because little, if any, increase in its IC_{50} was observed in comparison with those for KK_{WT} (Table 1). Surprisingly, TAK-652 did not inhibit the replication of the control virus isolated at passage 67 (KK_{C-67}) at concentrations up to 10,000 nM (data not shown), suggesting that the virus is an X4

TABLE 1. Anti-HIV-1 activities of TAK-652, -220, and -779 against KK_{652-67} in PBMCs[a]

Compound	KK_{WT}[b]	IC_{50} (nM) (fold increase)	
		KK_{652-67}	
		Expt 1	Expt 2
TAK-652	0.043	>10,000 (>230,000)	>10,000 (>230,000)
TAK-220	1.2	1.9 (1.6)	1.3 (1.1)
TAK-779	2.1	77 (37)	2,000 (940)

[a] Cells from different donors were used in each experiment. The IC_{50} is the 50% inhibitory concentration based on the reduction of p24 antigen levels in culture supernatants on day 7 after virus infection. Assays were performed in triplicate wells. Values in parentheses represent the fold increase (ratio of the IC_{50} for the wild type to the IC_{50} for KK_{652-67}).

[b] The IC_{50}s of TAK-652 and TAK-220 for the wild type were taken from references 2 and 27, respectively. The IC_{50} of TAK-779 for the wild type is based on an experiment performed under identical assay conditions.

HIV-1, a dual-tropic (R5X4) HIV-1, or a mixture of R5 HIV-1 and X4 HIV-1.

It was important to examine the susceptibility of the viruses isolated at certain points during the long-term passage of infected PBMCs. For accurate evaluation and comparison of these viruses, all escape and control viruses as well as the wild type needed to be examined simultaneously. In the first experiment, the IC_{50} of TAK-652 for the wild type was 0.14 nM (Table 2). This value was 3.3-fold higher than that obtained previously (2). When the IC_{50} for the escape virus at passage 8 (KK_{652-8}) was compared to that for its control virus (KK_{C-8}), no reduction in its susceptibility to TAK-652 was observed in either experiment 1 or 2. However, slight (2.0- to 3.3-fold) and considerable (110- to 380-fold) increases in the IC_{50}s were identified for KK_{652-43} and KK_{652-56}, respectively, compared with those of the corresponding control viruses. Thus, viruses with a different degree (low, middle, or high) of resistance to TAK-652, namely, KK_{652-43}, KK_{652-56}, and KK_{652-67}, were obtained in the long-term culture experiment.

Coreceptor usage of escape viruses. To determine whether the escape viruses and their control viruses acquired the ability to use CXCR4 as an alternative coreceptor, the replication of these viruses were examined in U87.CD4.CCR5 or U87.CD4.CXCR4 cells. All viruses except for KK_{C-67} replicated well in

TABLE 2. Anti-HIV-1 activity of TAK-652 against escape and control viruses obtained at passages 8, 43, and 56 in PBMCs[a]

Virus	IC_{50} (nM) (fold increase)	
	Expt 1	Expt 2
KK_{WT}	0.14	ND[b]
KK_{C-8}	0.24 (1)	0.27 (1)
KK_{652-8}	0.13 (0.55)	0.26 (0.97)
KK_{C-43}	0.20 (1)	0.34 (1)
KK_{652-43}	0.66 (3.3)	0.69 (2.0)
KK_{C-56}	0.63 (1)	0.64 (1)
KK_{652-56}	240 (380)	67 (110)

[a] Cells from different donors were used in each experiment. The IC_{50} is the 50% inhibitory concentration based on the reduction of p24 antigen levels in culture supernatants on day 7 after virus infection. Assays were performed in triplicate wells. Values in parentheses represent the fold increase (ratio of the IC_{50} for virus from the control passage to the IC_{50} for virus from the corresponding TAK-652-treated passage).

[b] ND, not determined.

TABLE 3. Replication of escape and control viruses obtained at passages 8, 43, 56, and 67 in U87.CD4.CCR5 and U87.CD4.CXCR4 cells[a]

Virus	p24 (ng/ml)[b]	
	U87.CD4.CCR5	U87.CD4.CXCR4
KK_{C-8}	+	−
KK_{C-43}	+	−
KK_{C-56}	+	−
KK_{C-67}	+	+
KK_{652-8}	+	−
KK_{652-43}	+	−
KK_{652-56}	+	−
KK_{652-67}	+	−

[a] Assay procedures are described in Materials and Methods.
[b] +, the p24 level in the culture supernatant was above 1 ng/ml or the virus replicated in the indicated cells; −, the p24 level was below 0.1 ng/ml or the virus did not replicate in the indicated cells.

U87.CD4.CCR5 cells but not in U87.CD4.CXCR4 cells (Table 3), indicating that they could not use CXCR4 as a coreceptor for infection. Although we did not examine their replication in U87 cells expressing other chemokine receptors, it would be unlikely that these viruses could use a chemokine receptor other than CCR5 and CXCR4. KK_{652-67} was found to be highly susceptible to TAK-220 (Fig. 2 and Table 1), which is an antagonist highly specific to CCR5 (27).

KK_{C-67} could replicate in both U87.CD4.CCR5 and U87.CD4.CXCR4 cells (Table 3). Therefore, to determine whether the virus is a dual-tropic (R5X4) HIV-1 or a heterogeneous mixture of R5 HIV-1 and X4 HIV-1, PBMCs were infected with KK_{C-67} and cultured in the presence of either 5 μM TAK-652, 5 μM AMD3100 (a specific inhibitor of CXCR4), or both. These concentrations of TAK-652 and AMD3100 are enough to suppress the replication of R5 HIV-1 and X4 HIV-1, respectively. After a 4-day incubation, the cells were washed thoroughly and further incubated for 3 days in the absence of any compounds. Active replication of KK_{C-67} was observed for the cells initially exposed to TAK-652 alone or AMD3100 alone. However, no replication was observed for the cells initially exposed to both compounds (data not shown). Then, PBMCs were infected with the virus derived from the TAK-652-exposed culture and incubated in the presence of 5 μM AMD3100, and no virus replication was identified (data not shown). An identical result was obtained when PBMCs were infected with the virus derived from the AMD3100-exposed culture and incubated in the presence of 5 μM TAK-652 (data not shown). These results indicate that KK_{C-67} is likely to be a heterogeneous mixture of R5 HIV-1 and X4 HIV-1 rather than R5X4 HIV-1.

Amino acid changes of escape viruses. To determine what amino acid changes are associated with resistance to TAK-652, full-length *env* genes of four escape and four control viruses as well as the wild type were sequenced. Several amino acid changes were observed not only in the gp120 subunit but also in the gp41 subunit (Fig. 4). Each amino acid change could be classified into one of three categories. The first category includes changes that were identified for both escape and control viruses. The second one is changes that were identified only for the control viruses. The last one, which is the most important, includes the changes that were identified only for the escape viruses under the selection pressure of TAK-652. The amino acid changes in the last category have been summarized in Table 4. KK_{652-8} had no amino acid changes in this category, which corresponds to the observation that the susceptibility of KK_{652-8} to TAK-652 was comparable that of KK_{C-8} and KK_{WT} (Table 2). Six amino acid changes, including one heterogeneous change, were observed for the modestly resistant virus KK_{652-43}. The number of amino acid changes increased with increasing passage number and resistance. The highly resistant virus KK_{652-67} had 12 amino acid changes: one in C2, two in V3, two in V4, two in C4, and five in gp41 (Table 4).

DISCUSSION

In this study, we isolated an R5 HIV-1 virus highly resistant to the novel CCR5 antagonist TAK-652 through long-term culture of infected PBMCs. The phenotypic analysis revealed that the virus was highly resistant to TAK-652 and had partial cross-resistance to TAK-779, probably due to their structural similarities (Fig. 1 and Table 1). In contrast to TAK-779, TAK-220, a structurally different CCR5 antagonist, was equally inhibitory to the replication of the TAK-652-resistant virus and the wild type. A similar finding has been reported for SCH-C and vicriviroc (SCH-D) (25, 26). Although both compounds are structurally related, the subtype G Russian clinical isolate RU570, which was weakly susceptible to inhibition by SCH-C (IC_{50} > 1 μM), retained high susceptibility to vicriviroc. Furthermore, four amino acid changes in the V3 region of gp120 were necessary and sufficient to confer resistance to SCH-C (10), whereas vicriviroc-resistant viruses had no amino acid changes in the V3 region (1). Thus, Env amino acid changes responsible for resistance to CCR5 antagonists differ from one compound to another. It would be of special interest to determine whether TAK-652 has sufficient antiviral activity against TAK-220-resistant R5 HIV-1.

We have also conducted a long-term culture experiment with R5 HIV-1-infected PBMCs under the selection pressure of TAK-220. However, no escape virus could be obtained with escalating concentrations of TAK-220, even after 132 passages (data not shown). At present, the reason for such difficulty in inducing TAK-220-resistant viruses is unclear. Nishikawa and colleagues recently analyzed the binding sites for TAK-220 on human CCR5 and found that TAK-220 shares some interacting amino acid residues with TAK-779 but also requires distinct amino acid residues for its inhibitory effect on HIV-1 (18). It is possible that the conformation of CCR5 might be more extensively altered by binding of TAK-220 to CCR5 than by binding of TAK-652. Nevertheless, TAK-652 has unique properties with which TAK-220 and other CCR5 antagonists are not endowed. TAK-652 has good oral bioavailability and a long plasma half-life in humans (2). Therefore, it is assumed that TAK-652 is able to retain a plasma concentration sufficiently higher than that required for virus inhibition by once-daily administration at a reasonable dose. TAK-652 is a potent inhibitor of ligand binding not only to CCR5 but also to CCR2b, which has been observed for neither TAK-220, maraviroc, vicriviroc, nor aplaviroc (8, 13, 25, 27).

Amino acid changes in the Env region accumulated with an increasing period of cultivation (Fig. 4). Among the amino acid

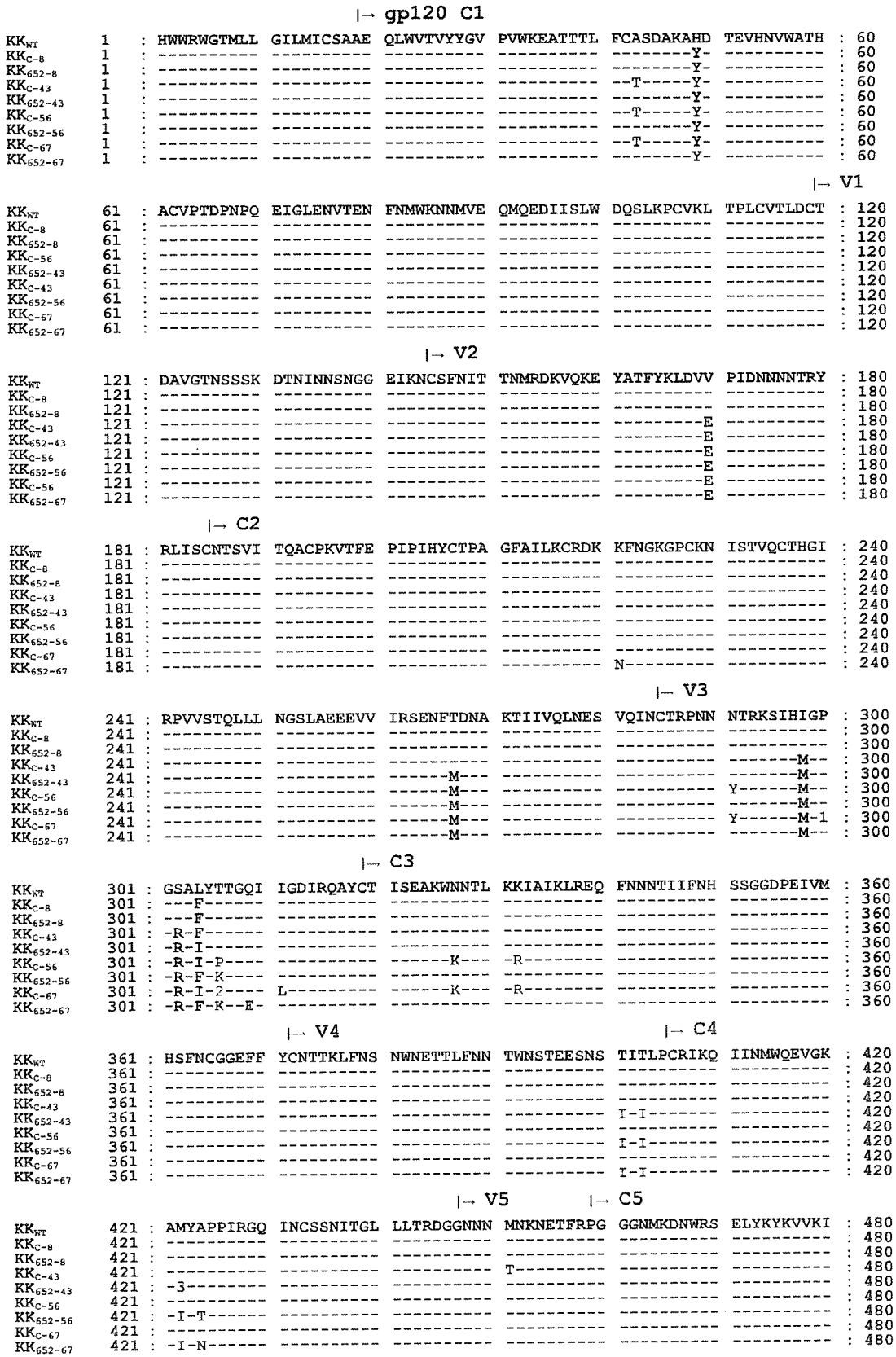

FIG. 4. Env amino acid sequences of isolated viruses. Blue letters indicate the amino acid changes identified for both drug escape and control viruses. Green letters indicate the amino acid changes identified only for the control viruses but not for the escape viruses. Red letters indicate the amino acid changes identified only for the escape viruses but not for the control viruses. There was heterogeneity for four amino acids, which are indicated numerically (1, P or A; 2, P or T; 3, M or I; 4, S or L).

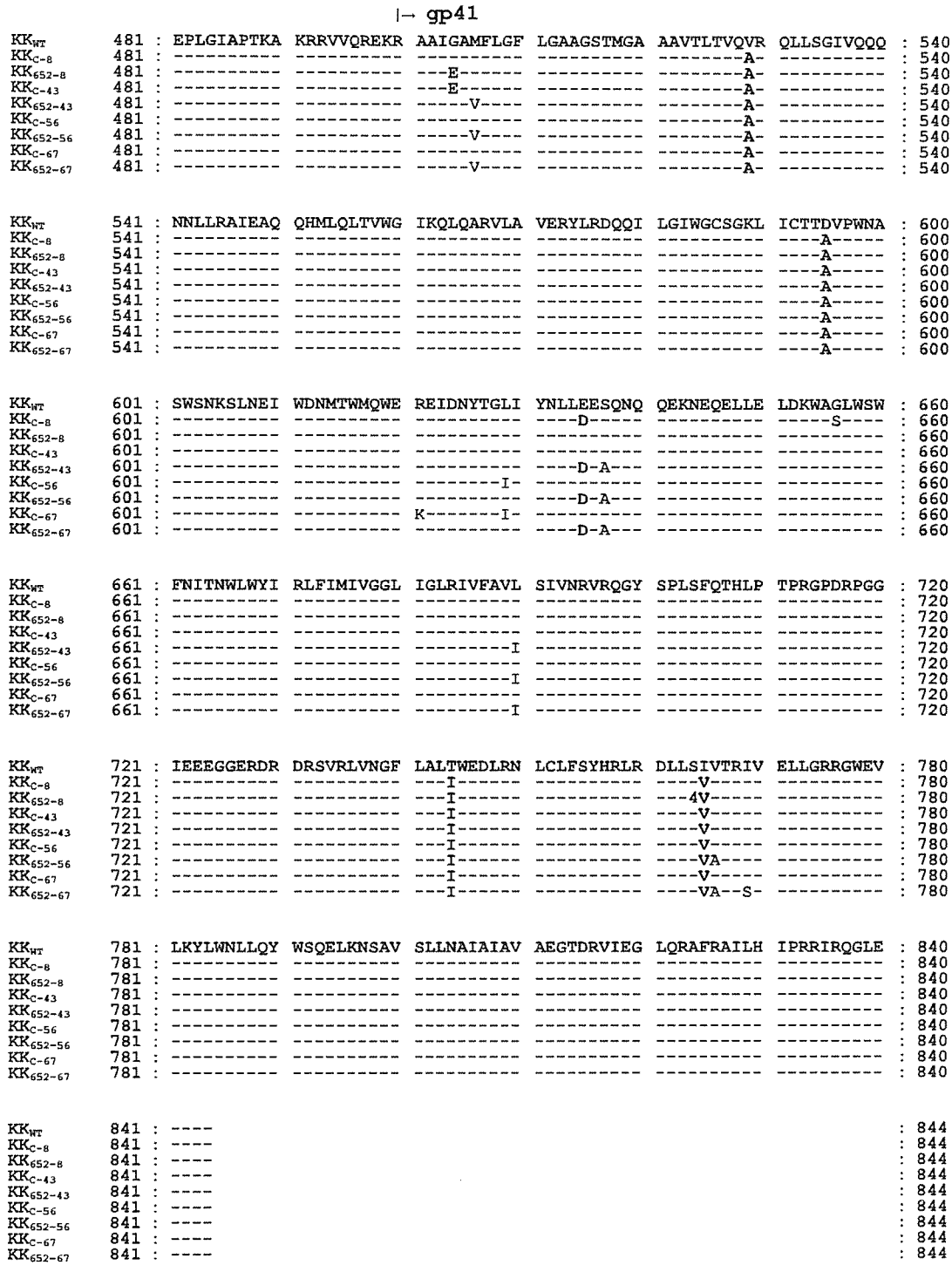

FIG. 4—*Continued.*

changes, several changes did not seem to be attributable to the selection pressure by TAK-652 but were the consequences of in vitro passage of infected cells, since these changes could be identified not only for the escape viruses but also for the corresponding control viruses. In addition, there were some amino acid changes that were found only for the control viruses. Selection of viruses with certain amino acid changes despite the absence of compounds has been commonly observed after in vitro passages of primary isolates and may be attributed to better replication fitness of these viruses. Apart from the amino acid changes unrelated to TAK-652 resistance, there were amino acid changes identified only for the escape viruses, and they accumulated with an increased period of cultivation (Table 4). In particular, a considerable gap in drug

TABLE 4. Env amino acid changes considered to be involved in TAK-652 resistance[a]

Virus	Amino acid at position:											
	221 (C2)	306 (V3)	309 (V3)	401 (V4)	403 (V4)	422 (C4)	424 (C4)	506 (gp41)	637 (gp41)	690 (gp41)	766 (gp41)	769 (gp41)
KK_{WT}	K	T	Q	T	T	M	A	M	S	L	V	I
$KK_{652\text{-}8}$	—[b]	—	—	—	—	—	—	—	—	—	—	—
$KK_{652\text{-}43}$	—	—	—	I	I	M/I	—	V	A	I	—	—
$KK_{652\text{-}56}$	—	K	—	I	I	I	T	V	A	I	A	—
$KK_{652\text{-}67}$	N	K	E	I	I	I	N	V	A	I	A	S

[a] Based on the amino acid sequence data shown in Fig. 4.
[b] —, identical to the amino acid of the wild type.

susceptibility was found between $KK_{652\text{-}43}$ and $KK_{652\text{-}56}$ (Table 2). In addition to the amino acid changes observed for $KK_{652\text{-}43}$, the three changes T306K (V3), M424T (C4), and V766A (gp41) were identified for $KK_{652\text{-}56}$. Furthermore, three amino acid changes, K221N (C2), Q309E (V3), and I769S (gp41), occurred in the Env region of the highly resistant virus $KK_{652\text{-}67}$. It has been reported that, unlike the resistance to reverse transcriptase and protease inhibitors, one amino acid change of the Env region does not bring about the resistance to CCR5 antagonists (10, 14). Trkola and colleagues have proposed two possible mechanisms that confer resistance to CCR5 antagonists on HIV-1 (29). The first one is the increase of gp120 binding affinity to CCR5. In this case, the virus can compete more strongly with a CCR5 antagonist and infect target cells. The second one is the creation of a substantially different binding site on gp120 for CCR5. In this case, the virus is still able to infect the target cells, even when the binding site of gp120 is already occupied with a CCR5 antagonist. Since the two mechanisms may not be mutually exclusive and can act sequentially, further studies, including the introduction of site-directed mutations, are required to elucidate the amino acids responsible for the resistance to TAK-652.

In accordance with previous experiments by others (29), no HIV-1 coreceptor switch from CCR5 occurred in the escape viruses in this study (Table 3). Instead, the control virus, $KK_{C\text{-}67}$, could use both CCR5 and CXCR4 for infection. It is known that only a few amino acid changes, especially in the V3 region of gp120, can convert R5 HIV-1 to X4 HIV-1 (7, 9, 24). Indeed, seven amino acid changes, including two heterogeneous changes, were found in the V3 region of $KK_{C\text{-}67}$, suggesting that the virus might be a heterogeneous mixture of R5 HIV-1 and X4 HIV-1 rather than a dual-tropic (R5X4) virus. This assumption was confirmed by the drug-swapping experiment with TAK-652 and AMD3100 (see Results for details). Since the original strain, KK_{WT}, is a clinical isolate from a treatment-naïve patient, it is not surprising that a small population of X4 HIV-1 existed in the infected cells and expanded during their long-term culture.

In conclusion, this study provides important information on TAK-652-resistant viruses, such as no cross-resistance to TAK-220 and no coreceptor switch to X4 HIV-1. While our experiments using a clinical isolate and a single PBMC donor may reflect the in vivo scenario of drug resistance better than those using a laboratory strain and multiple donors, it is possible that different mutants will be selected in individual experiments. Furthermore, in a clinical setting, CCR5 antagonists must be used in combination with existing antiretrovirals (28), which may alter the pattern for TAK-652 resistance. Thus, the emergence of drug resistance should be further investigated and confirmed in clinical trials.

ACKNOWLEDGMENT

This work was supported in part by a research grant from the Ministry of Health Labor and Welfare, Japan.

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Immunogenetics (2006) 57: 944–952
DOI 10.1007/s00251-005-0075-0

ORIGINAL PAPER

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Genetic variability in the extracellular matrix protein as a determinant of risk for developing HTLV-I-associated neurological disease

Received: 12 October 2005 / Accepted: 4 December 2005 / Published online: 10 January 2006

Abstract Aggrecan, which is a well-known proteoglycan in joint cartilage, also exists in the spinal cord and plays an important role in maintaining water content in the extracellular matrix structure. In this study, we first examined the variable number of tandem repeat (VNTR) polymorphism of the *aggrecan* gene in 227 HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients, in 217 HTLV-I-infected healthy carriers (HCs), and in 85 normal controls. The VNTR allele 28 (1,630 bp) was more frequently observed in HAM/TSP patients than in HCs (χ^2=12.02, p=0.0005, odds ratio 1.79, 95% C.I. 1.29–2.50) and in controls (χ^2=13.43, p=0.0002, odds ratio 2.54, 95% C.I. 1.52–4.25), although this allele was not related to disease progression or to HTLV-I provirus load. We also found that the aggrecan concentration in cerebrospinal fluid (CSF) from rapidly progressive HAM/TSP patients was significantly higher than in slowly progressive patients (corrected p=0.0145) but not in infected non-inflammatory neurological other disease controls (OND) (corrected p=0.078). We then analyzed this aggrecan VNTR polymorphism in the different set of patients with HAM/TSP (n=58) and healthy carriers (n=70). This analysis, again, revealed that allele 28 was detected more frequently in HAM/TSP group than in HCs (χ^2=11.03, p=0.0009, odd ratio 3.04, 95% C.I. 1.55–5.97). The reproducibility of our study was regarded as a second- or third-class association by comparing combined p values and the Better Associations for Disease and GEnes (BADGE) system. Our results suggest that aggrecan polymorphism can be a novel genetic risk factor for developing HAM/TSP.

Keywords Aggrecan · Extracellular matrix · HTLV-I · VNTR · HAM/TSP

Financial support: Grant in Aid for Research on Brain Science of the Ministry of Health, Labor and Welfare, Japan.

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Introduction

HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive inflammatory disease of the spinal cord, which occurs in only 1–2% of HTLV-I-infected individuals (Gessain et al. 1985; Osame et al. 1986). As we have previously reported, that the main HTLV-I-harboring cells in the spinal cord of HAM/TSP patients are not neuronal cells but $CD4^+$ T cells (Moritoyo et al. 1996), a T-cell-mediated immunologic process initiated by HTLV-I infection can be a possible pathological process of HAM/TSP. Although the factors that cause different manifestations of HTLV-I infection are not fully understood, our recent population association studies of more than 200 cases each of HAM/TSP and HTLV-I-infected healthy carriers (HCs) in Kagoshima, an endemic area of HTLV-I infection in Japan, have revealed several important risk factors (Jeffery et al. 1999, 2000; Nagai et al. 1998; Vine et al. 2002). One of the major risk factors for developing HAM/TSP is the provirus load. The median provirus load was approximately 16 times higher in HAM/TSP patients than in HCs, and a high provirus load is also associated with an increased risk for

progression to HAM/TSP (Nagai et al. 1998). We have also reported that *HLA-A*02* and *Cw*08* genes were associated with a lower HTLV-I provirus load and protection from HAM/TSP, whereas *HLA-DRB1*0101* and *B*5401* were associated with susceptibility to HAM/TSP (Jeffery et al. 1999, 2000). Moreover, we have revealed non-HLA genetic risk factors such as TNF−α, SDF-1, and IL-15 (Vine et al. 2002), as well as the association between *HTLV-I Tax* gene variation and the risk for HAM/TSP (Furukawa et al. 2000). From these observations, we now can identify approximately 88% of cases of HAM/TSP in the Kagoshima cohort.

Our detailed clinical analysis of 213 patients with HAM/TSP has revealed that 17% showed arthropathy (Nishioka et al. 1989) characterized by erythema, swelling, and severe pain on moving which mainly occur in large joints (Nakagawa et al. 1995). As the recent study by Levin et al. identified an autoantibody against heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1) which cross-reacts with HTLV-I Tax protein in IgG isolated from HAM/TSP patients (Levin et al. 2002), it is possible that host recognition of 'self' molecules that mimic HTLV-I contributes to the tissue damage seen in HAM/TSP and its accompanying arthropathy. If this is the case, an immune reaction against a protein that exists in both the spinal cord and joint may be a good candidate for autoantigen.

Human aggrecan is a major extracellular matrix protein expressed in both joint cartilage and the spinal cord, and consists of a core protein and attached glycosaminoglycan (GAG) side chains (Asher et al. 1995; Doege et al. 1991; Milev et al. 1998; Moon et al. 2003; Takahashi-Iwanaga et al. 1998; Watanabe et al. 1998). The reported functions of aggrecan are, first, to maintain the high water content in the extracellular matrix, and second, to act as a barrier against cell migration and a guide for axonal growth in the central nervous system (CNS) along with other chondroitin sulfate (CS) proteoglycans such as phosphocan, neurocan, and versican (Adams et al. 1993; Ang et al. 1999; Asher et al. 2000; Grumet et al. 1993; Moon et al. 2003; Oohira et al. 2000; Perris and Perissinotto 2000). Some reports provide evidence that aggrecan is produced by astrocytes in the perineurial region of the CNS (Matthews et al. 2002; Takahashi-Iwanaga et al. 1998).

Interest in aggrecan function has been increasing as a result of recent research on autoimmune and inflammatory arthritis (Glant et al. 1998; Poole 1998; Zhang et al. 1998b). There are reports showing that aggrecan may act as an immunogenic epitope of T and B cells both in vivo and in vitro. Once the G1 domain has been removed from the core protein of aggrecan by the enzyme aggrecanase (Feng et al. 1998; Zhang et al. 1998a), the molecule discloses a T-cell epitope. It has also been reported that a decrease of CS content elicits a T-cell immune response, whereas a decrease of keratan sulfate (KS) content elicits a B-cell response (Glant et al. 1998).

Based on these findings, we wished to consider the possibility that genetically determined characteristics of extracellular matrix proteins and their degradation are related to the pathogenesis of HAM/TSP. To test this possibility, we analyzed the variable number of tandem repeat (VNTR) polymorphism that was recently identified in the second exon of the *aggrecan* gene, and which encodes a CS attachment site (Doege et al. 1997), in 227 HAM/TSP patients, 217 HCs, and 85 normal controls, and in 58 HAM/TSP patients and 70 HCs. We also examined the protein level of *aggrecan* in both serum and CSF from HTLV-I-infected individuals.

Finally, we have employed a special criterion proposed as the Better Association for Disease and GEnes (BADGE) system (Manly 2005) to assure the reproducibility of our genetic association study. This is because some genetic association studies have problems on reproducibility. In fact, several studies have shown poor reproducibility (Becker et al. 2004; Cardon and Bell 2001; Colhoun et al. 2003; Hirschhorn et al. 2002; Ioannidis et al. 2001; Redden and Allison 2003). This novel system is simple to use and is useful when one needs to estimate reproducibility in the absence of direct experimental replication.

Materials and methods

Study population

The genomic DNA sequences of the *aggrecan* gene were compared among 227 HAM/TSP patients, randomly selected 217 HCs, and 85 normal controls. All cases, HCs, and controls were Japanese and resided in Kagoshima Prefecture, which is an endemic area of HTLV-I infection in Japan. All HCs and normal controls were blood donors and were not related to the patients. The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria (Osame 1990). Sex and ages of subjects were as follows: HAM/TSP group, 69 males and 158 females, 23–76 (mean 57) years old; HC group, 101 males and 116 females, 20–74 (mean 50) years old; control group, 35 males and 50 females, 35–55 (mean 48) years old. The second set of DNA samples were derived from 58 patients with HAM/TSP and 70 HCs from our area. Sex and ages of subjects of this second group were as follows: HAM/TSP group, 20 males and 38 females, 40–65 (mean 50) years old; HC group, 30 males and 40 females, 35–50 (mean 42) years old.

To measure the level of aggrecan in serum and CSF, we used serum samples from 33 HAM/TSP patients and from 11 HCs and CSF samples from 52 HAM/TSP patients, CSF samples from 18 HTLV-I-infected non-inflammatory other disease controls (OND) (five motor neuron disease, four spinocereberal degenaration, two Parkinson's disease, two quadriceps myopathy, one thyroid dysfunction, one essential tremor, one hemifacial spasm, one arrhythmia, and one leg fracture). There was no paired sample of serum and CSF.

We defined rapidly progressive HAM/TSP patients as those who became unable to walk within 3 years after onset of the disease. Sex and ages of rapidly progressive HAM/TSP patients were seven males and 11 females, 48–65 (mean 55) years old, and those of chronic HAM/TSP patients were 11 males and 23 females, 40–64 (mean 54) years old. All samples were taken under written informed consent. The

Ethical Committee of Kagoshima University Faculty of Medicine approved this study.

Serum, CSF, and genomic DNA preparation

Fresh peripheral blood mononuclear cells (PBMCs) were obtained by Histopaque-1077 (Sigma, Tokyo, Japan) density gradient centrifugation, and washed three times with phosphate buffered saline (PBS) with 1% fetal calf serum (FCS). Isolated PBMCs were cryopreserved in liquid nitrogen until use. Genomic DNA was extracted from PBMCs using a QIAamp blood kit (Qiagen Ltd, Tokyo, Japan) according to the manufacturer's instructions. The CSF and serum samples were also collected, and stored at −70°C until use.

Determination of polymorphism and provirus load measurement

The *aggrecan* gene contains a large exon (exon 12) of 3.5 kb, which encodes the entire glycosaminoglycan (GAG) attachment regions of its core protein (Doege et al. 1991). This region consists of numerous repeated sequences, including a particularly highly conserved set of repeats in the CS attachment site. The VNTR polymorphism of the *aggrecan* gene in exon 10 has already been reported (Doege et al. 1997) This VNTR can be detected by PCR as different lengths of PCR products. A genomic PCR was performed with 20 ng of genomic DNA as template, 50 pmol of each primer (forward: 5′-TAG AGG GCT CTG CCT CTG GAG TTG-3′ and reverse: 5′-AGG TCC CCT ACC GCA GAG GTA GAA-3′), 20-mM deoxynucleotide triphosphates (dNTPs), 15-mM $MgCl_2$, reaction buffer provided by the manufacturer, and one unit of Takara-Taq DNA polymerase (Takara, Tokyo, Japan) in a final volume of 10 μl. PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 60 s, and elongation at 72°C for 60 s with a final extension at 72°C for 10 min. PCR products were separated on 1% agarose gels, visualized by ethidium bromide staining, after which the products were determined. Several of the alleles differ by only one repeat in size (59 bp), and care was taken to identify these alleles using appropriate gels and size markers. Two independent readers scored the alleles.

Provirus load of the samples was measured by a quantitative PCR method using an ABI Prism 7700 (PE-Applied Biosystems) (Nagai et al. 1998).

Quantification of aggrecan in serum and CSF

Serum, as well as CSF aggrecan concentration, was measured in duplicate using a commercial ELISA kit (BiSource Europe S.A., Nivelles, Belgium). According to the manufacturer's instruction, the kit detects aggrecan and aggrecan fragments, and the assay system used is sensitive to detect 0.9 ng/ml of aggrecan in samples. Serum aggrecan levels in normal adults ranged between 1 and 4.4 μg/ml, whereas no information was available regarding CSF levels. When we needed to separate CSF aggrecan amounts into two groups, we selected 0.9 ng/ml as the cut-off level, as this value was the lowest value of the cut-off range and there was no previous report measuring CSF aggrecan concentration. Optical density at 450 nm was measured on the ImmunoMini NJ-2300 (Nippon Inter Med, Tokyo, Japan) and aggrecan concentration was determined by linear regression from a standard curve using the aggrecan supplied with the kit as standard.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows release 7.0, run on an IBM-compatible computer (Analytical software, Version 7, Tallahassee, FL, USA). Comparison of whole-allele distribution between patients with HAM/TSP and HCs was performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. The distribution of each allele and genotype of the VNTR polymorphism of the *aggrecan* gene in patients with HAM/TSP patients was compared with those in HCs using a chi-square test for a 2×92 and 2×3 contingency table. Bonferroni multiple adjustments (Motulsky 1995) were made to the level of significance because of the multiple comparisons for VNTR allele frequencies. This level was set at $p<0.0057$ ($p=1-0.95^{(1/9)}$).

To assure reproducibility of our study, we have combined p values from the analysis on two sets of populations and have compared the combined p values to the BADGE (Manly 2005).

Serum and CSF aggrecan levels in patients and controls in three different groups were compared using either ANOVA or Kruskal–Wallis test. A p value less than 0.05 was considered statistically significant. When aggrecan levels in three groups were different, multiple comparisons were done by Scheffe's test. We also performed multiple-hypotheses testing when it was needed, and the level was set at $p<0.017$ ($p=1-0.95^{(1/3)}$).

Results

Frequency of aggrecan VNTR allele 28 was significantly higher in HAM/TSP than HCs and normal control

We applied two-step analysis on our cohort. We first typed 100 samples from each group observing nine aggrecan VNTR alleles, and found the difference between the groups [$\chi^2=18.18$ ($df=8$), $p=0.019$]. We then proceeded to analyze whole samples in this study (227 HAM/TSP patients and 217 HCs) (Table 1).

Comparison of whole allele distribution among patients with HAM/TSP, in HCs and normal controls was performed using a chi-square test for 3×9 contingency table with a significance level $p<0.05$. This analysis has revealed

Table 1 Distribution of aggrecan *VNTR* polymorphism in *HAM/TSP* patients, in healthy carriers (*HCs*) and in normal control subjects

		Allele*						Genotype**					
		HAM/TSP		*HCs*		Control		*HAM/TSP*		*HCs*		Control	
Allele	Length	Obs	Freq (%)	Obs	Freq (%)	Obs	Freq (5)	Homozygote	Heterozygote	Homozygote	Heterozygote	Homozygote	Heterozygote
32	1858	2	0.4	2	0.4	1	0.6	0	2	0	2	0	1
29	1687	18	3.9	12	2.7	6	3.5	2	14	3	6	0	6
28	1630	115	25.3	69	15.9	20	11.8	23	69	12	45	5	10
27	1573	141	31	155	35.7	57	33.5	32	77	37	81	13	31
26	1516	90	19.8	102	23.5	44	25.9	15	60	22	58	8	28
25	1459	62	13.6	53	12.2	25	14.7	4	54	9	35	1	23
22	1288	23	5	37	8.5	15	8.8	6	11	11	15	3	9
21	1231	2	0.4	3	0.6	1	0.6	1	0	1	1	0	1
18	1060	1	0.2	1	0.2	1	0.6	0	1	0	1	0	1
		454		434		170							

* Comparison of whole allele distribution among patients with *HAM/TSP*, *HCs* and normal controls was performed using a chi-square test for 3×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=27.33(df=16)$, $p=0.038$.
Comparisons of whole allele distribution between each two groups were performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=17.84(df=8)$, $p=0.02$ (*HAM/TSP* vs *HCs*), $\chi^2=16.53(df=8)$, $p=0.035$ (HAM/TSP vs normal controls), and $\chi^2=3.24(df=8)$, $p=0.918$ (*HCs* vs normal controls). The distribution of each allele of the *VNTR* polymorphism of the *aggrecan* gene in patients with *HAM/TSP* patients was compared with those in *HCs* using a chi-square test for a 2×2 contingency table. Allele 28 has been detected more frequently in patients group than HCs ($\chi^2=12.02$, $p=0.0005$, odds ratio 1.79, 95% C.I. 1.29–2.50)
**: The p value of genotype among three groups was calculated by χ^2 test with a 3×3 contingency table. This analysis revealed that genotype of the 28 repeat was frequently observed in *HAM/TSP* than *HCs* ($\chi^2=19.68$, $p=0.003$, $df=6$). Then p values of genotype in each two groups were calculated by χ^2 test with a 2×3 contingency table. This analysis revealed that genotype of the 28 repeat was frequently observed in *HAM/TSP* than in *HCs* ($\chi^2=10.41$, $p=0.005$, $df=2$) and in *HAM/TSP* than in normal controls ($\chi^2=14.65$, $p=0.0007$, $df=2$), but not in *HCs* and in normal controls ($\chi^2=3.31$, p=0.19, $df=2$)

$\chi^2=27.33$ ($df=16$), $p=0.038$. Comparisons of whole allele distribution between each two groups were performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=17.84$ ($df=8$), $p=0.02$ (HAM/TSP vs HCs), $\chi^2=16.53$ ($df=8$), $p=0.035$ (HAM/TSP vs normal controls), and $\chi^2=3.24$ ($df=8$), $p=0.918$ (HCs vs normal controls).

Allele 28 was observed in 25.3% of HAM/TSP patients, whereas, only 15.9% of HCs and 11.8% of normal controls carried this allele. We, therefore, compared the distribution of allele 28 in patients with HAM/TSP and in HCs, and that in normal controls using a chi-square test for a 2×2 contingency table. As nine alleles appeared in our analysis, we set $p<0.0057$ ($p=1-0.95^{(1/9)}$) using the Bonferroni adjustment for multiple comparisons. Allele 28 has been detected more frequently in patients group than in HCs ($\chi^2=12.02$, $p=0.0005$, odd ratio 1.79, 95% C.I. 1.29–2.50) and than in normal controls ($\chi^2=13.43$, $p=0.0002$, odd ratio 2.54, 95% C.I. 1.52–4.25).

The p value of genotype in three groups was calculated first by χ^2 test with a 3×3 contingency table. This analysis revealed that genotype of the 28 repeat was frequently observed in HAM/TSP than in HCs in and normal controls ($\chi^2=19.68$, $p=0.003$, $df=6$). We, then, calculated the p values of genotype between two groups by χ^2 test with a 2×3 contingency table. This analysis revealed that genotype of the 28 repeat was frequently observed in HAM/TSP than in HCs ($\chi^2=10.41$, $p=0.005$, $df=2$) and in HAM/TSP than in normal controls ($\chi^2=14.65$, $p=0.0007$, $df=2$), but not in HCs and in normal controls ($\chi^2=3.31$, $p=0.19$, $df=2$). The observed frequency of alleles other than allele 28 was very similar to the frequency reported in a European population (Doege et al. 1997).

Table 2 Distribution of aggrecan 1630-bp allele in HAM/TSP patients at different provirus load

Provirus load	Total number of Patients	Patients with allele 1630	Frequency (%)
<100	28	10	35.7
<300	33	12	36.3
<600	48	18	37.5
<1000	34	14	41.2
<2000	46	18	39.1
>2000	16	8	50.0

Provirus load is presented as number of the cells in 10^4 PBMC
Mann–Whitney's U test has revealed that the distribution of allele 1630 positive patients is not different at different provirus load ($p=0.402$)

We assessed the reproducibility of our study by comparing combined p values and the BADGE system (Manly 2005). We first multiplied the p value for the 3×9 χ^2-square test from the first population (0.038) and that of the 2×9 χ^2-square test from the second population (0.0001). This yielded the combined p value of 0.0000038. This estimate suggested that the association of our study should be regarded as a second-class association in the BADGE system. We also tried to assess the reproducibility on the test applied to allele 28. We multiplied the p value from the first population (0.0005) with that from the second population (0.0009) and applied Bonferroni correction by multiplying 9 on each p value. This produced the combined p value of 0.00004 as a third-class association.

The possession of allele 28 was not related to disease progression or HTLV-I provirus load

Of 52 HAM patients with CSF aggrecan analyzed, eight patients with allele 1630 showed rapid progression while ten were without this allele. A chi-square test for 2×2 contingency table revealed that disease progression was not correlated with allele possession (χ^2=0.188, p=0.66, odds ratio 1.29, 95% C.I. 0.41–4.12).

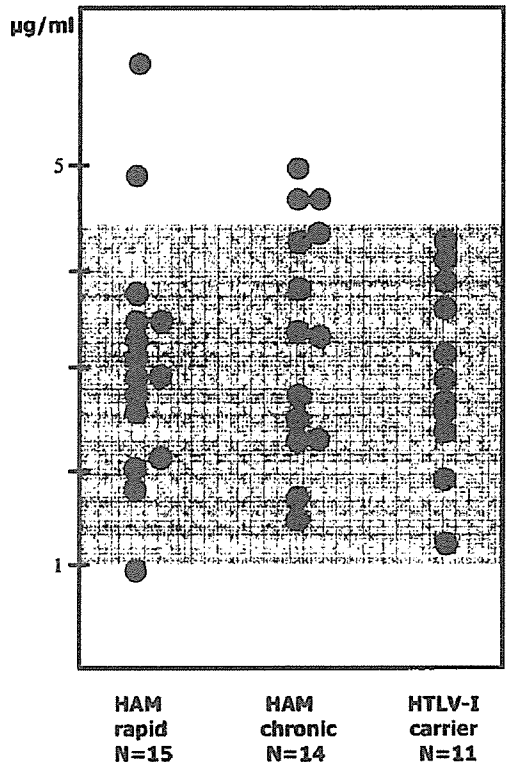

Fig. 1 The amount of aggrecan in serum (normal range 1–4.4 μg/ml, *shadowed area*) showed similar level among *HAM/TSP* patients with rapid progression, with slow progression and *HTLV-I* carriers (*HCs*)

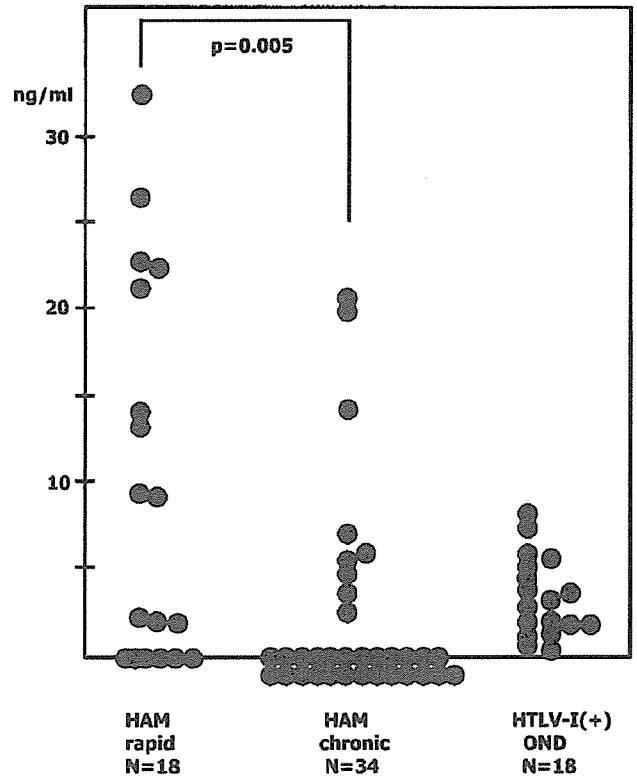

Fig. 2 The amount of aggrecan in *CSF* of patients with *HAM/TSP* showing rapid or slow progression, and other non-inflammatory disease (*OND*)

The distribution of allele 1630 positive patients in the entire sample of 205 HAM patients

In these samples, the provirus load was measured and was not correlated with the provirus load (Table 2) (Mann–Whitney's U test, p=0.402). We measured the provirus load of samples when we had an approval to measure the provirus load.

The CSF level of aggrecan was significantly higher in rapidly progressive HAM/TSP than in chronic HAM/TSP

We, next, compared the serum aggrecan level in rapidly progressive and chronically progressive HAM/TSP patients and HCs (Fig. 1). However, there was no significant difference among these three groups (F=0.78, p=0.47). We then compared CSF aggrecan levels among rapidly and chronically progressive HAM/TSP patients as well as OND (Fig. 2). The results showed that aggrecan levels in CSF in the three different groups were different by Kruskal–Wallis test (H=13.45, df=2, p=0.0006, corrected p=0.0018) and the level in the rapidly progressive HAM/TSP patients was significantly higher than that in the chronically progressive HAM/TSP (p=0.0049, corrected p=0.0145) but not in that of OND (p=0.026, corrected p=0.078) (Scheffe's test).

Table 3 Distribution of aggrecan *VNTR* polymorphism in the second group of *HAM/TSP* patients compared with the second healthy carrier (*HCs*) group

		Allele*				Genotype**			
		HAM/TSP		*HCs*		*HAM/TSP*		*HCs*	
Allele	Length	Obs	Freq (%)	Obs	Freq (%)	Homozygote	Heterozygote	Homozygote	Heterozygote
32	1858	0	0	2	1.5	0	0	0	2
29	1687	3	2.6	7	5	1	1	2	3
28	1630	31	26.8	15	10.8	7	17	3	9
27	1573	42	36.3	49	35	5	32	8	33
26	1516	17	14.7	34	24.3	0	17	3	28
25	1459	13	11.3	26	18.6	0	13	1	24
22	1288	10	8.4	1	0.8	0	10	0	1
18	1060	0	0	2	1.5	0	0	0	2

* Comparison of whole allele distribution among patients with *HAM/TSP* and *HCs* in the second group was performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=31.09(df=8)$, $p=0.0001$. The distribution of each allele of the *VNTR* polymorphism of the *aggrecan* gene in patients with *HAM/TSP* patients was compared with those in *HCs* using a chi-square test for a 2×2 contingency table. Allele 28 has been detected more frequently in patients group than HCs ($\chi^2=11.03$, $p=0.0009$, odds ratio 3.04, 95% C.I. 1.55–5.97)

** The p value of genotype was calculated by χ^2 test with a 2×3 contingency table. This analysis revealed that the genotype of 28 repeat was frequently observed in *HAM/TSP* than *HCs* ($\chi^2=9.28$, $df=2$, $p=0.009$)

Aggrecan VNTR analysis in the different set of patients with HAM/TSP and HCs

We, then, analyzed this aggrecan VNTR polymorphism in the different set of patients with HAM/TSP ($n=58$) and healthy carriers ($n=70$) (Table 3). We performed this second analysis to ensure our first observation. Comparisons of whole allele distribution between two groups were performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. This analysis has revealed $\chi^2=31.09(df=8)$, $p=0.0001$. Allele 28 was observed in 26.8% of HAM/TSP patients and 10.8% of HCs in this second analysis. We compared the distribution of allele 28 in patients with HAM/TSP and that in HCs using a chi-square test for a 2×2 contingency table. The allele 28 was detected more frequently in HAM/TSP group than in HCs ($\chi^2=11.03$, $p=0.0009$, odds ratio 3.04, 95% C.I. 1.55–5.97). The p value of genotype was calculated by χ^2 test with a 2×3 contingency table. This analysis revealed that the genotype of 28 repeat was frequently observed in HAM/TSP than HCs ($\chi^2=9.28$, $df=2$, $p=0.009$).

Discussion

In this study, we report three findings. First, allele 28 (1630 bp) of the *aggrecan* gene was more frequently observed in HAM/TSP patients than in HCs and in normal controls. This frequent distribution of allele 28 was observed also in the different set of HAM/TSP patients and HCs. The reproducibility of our study was assessed by comparing combined p values and the BADGE system (Manly 2005) and was regarded as a second- or third-class association. Second, possession of allele 28 was not related to the disease progression or HTLV-I provirus load. Finally, the rapidly progressive HAM/TSP patients showed a higher aggrecan concentration in the CSF than the chronically progressive HAM/TSP patients.

Recent genetic analysis of the *aggrecan* gene has shown that it has 18 exons and that there is a polymorphic region in the 12th exon, which is the CS attachment site (Doege et al. 1991). This site has a VNTR of 57 bp. Using this VNTR, several reports have analyzed whether there is a correlation between osteoarthritis (OA) of the hand and a particular allele of the *aggrecan* gene (Horton et al. 1998). Another study of *aggrecan* gene VNTR polymorphism has shown that individuals with shorter VNTR tend to develop multilevel disc degeneration at an earlier age (Kawaguchi et al. 1999). Even though no disease association of aggrecan VNTR has been shown in chronic inflammatory or immunological disease of the nervous system, the reported nature and function of aggrecan and these association studies prompted us to investigate its relation to HTLV-I-related neurological diseases. Ours is the second report of aggrecan VNTR allele distribution in the Asian population, but the first study to examine the association between aggrecan polymorphism and a neurological disease. Regarding allele 28, Kawaguchi et al. (Kawaguchi et al. 1999) reported that allele frequency was 9.4% in their studied population, whereas we have observed 25.3% in patients with HAM/TSP, 15.9% in HCs and 11.8% in normal controls from our area (Table 1). We have, again, shown that the allele frequency of this allele 28 was 26.8% in HAM/TSP patients and 10.8% in HCs (Table 3). This has shown that the allele 28 is indeed increased in our patient population and there was no possibility for a population stratification artifact. To estimate the reproducibility of our study, we have employed the BADGE system to describe genetic association (Manly 2005). As shown in the results, the association of whole allele distribution of *aggrecan* gene to HAM/TSP has reached second-class and the association of allele 28 has reached a third-class association. We have, therefore,

assumed that our study suggests reproducibility under conservative assumptions for traits previously mapped to a chromosome or a small region.

Aggrecan was recently reported to be produced by astrocytes and to exist in the perineurial region of the CNS (Matthews et al. 2002). In general, aggrecan degenerates with age and is cleaved between the G1 domain and the KS binding domain by proteolysis with the enzyme aggrecanase (Lark et al. 1997). Fragments of aggrecan are produced by aging, mechanical processes and/or activation of cleaving enzymes. Once these fragments activate T cells, these T cells can infiltrate into the CNS through the blood–brain barrier and initiate inflammatory CNS diseases (Buzas et al. 1995; Lemons et al. 1999; Mikecz et al. 1988; Zhang et al. 1998b). The reported lower concentration of CS and lack of KS in brain aggrecan (Buzas et al. 1995;Glant et al. 1998; Koppe et al. 1997) may be related to this elicited immune response in the CNS, as decrease of CS or KS content are reported to generate T- or B-cell immune response (Glant et al. 1998). Previous studies on the pathological mechanism of HAM/TSP have revealed that the main disease process is T-cell-mediated inflammation of the thoracic spinal cord (Izumo et al. 2000; Umehara et al. 1993). Taking these findings together, it is of interest to know that the length of the CS attachment site determined by VNTR may have a correlation with HAM/TSP.

Next, we were not able to show the correlation between possession of allele 28 and disease progression or HTLV-I provirus load. This may be because the genetic background we have found in this study can be one independent factor in causing HAM/TSP. Our previous studies have revealed that higher provirus load correlates with strong inflammation of the spinal cord and that the load is related to the deterioration of motor disability in 64 HAM/TSP patients followed up for 10 years (Matsuzaki et al. 2001). We also reported that there were HAM/TSP patients with lower provirus load (Nakagawa et al. 1995). From these observations, we speculated that tissue damage during immune inflammation might not only be controlled only by the strength of the inflammation itself but by the strength of the tissue structure as well. Weak inflammation is sufficient when inflammation occurs in a genetically determined weak tissue. In this regard, our present study may open a novel approach in finding the cause of HTLV-I-related neurological diseases.

To investigate whether aggrecan leakage correlates with disease progression, we measured aggrecan concentration in sera of HAM/TSP patients and HCs, and in CSF of HAM/TSP patients and OND. We found higher CSF aggrecan concentration in rapidly progressive HAM/TSP patients than in chronically progressive patients. As our previous clinical analysis of HAM/TSP patients showed that the patients with later disease onset and knee-joint arthritis showed faster progression of the disease (Nakagawa et al. 1995), we speculated that aggrecan that leaked into the CSF was caused by the degradation of spinal cord tissue secondary to inflammation induced by HTLV-I infection. We also showed that the degree of aggrecan degradation was higher in rapidly progressive patients in this study, and would, therefore, like to propose that the concentration of aggrecan in CSF may be a marker for denaturing in the spinal cord. Although HAM/TSP is reported to occur more frequently in female and we have observed slightly more male cases in rapid progressive group than expected by the reported ratio, age of onset is the only factor, so far, that has been shown to correlate with the disease progression rate (Nakagawa et al. 1995). To find a correlation between sex and disease progression, we may need to measure aggrecan concentration in more cases. To our knowledge, this is the first study to show the presence of aggrecan in CSF. Analysis of CSF aggrecan in other neurological diseases may clarify the significance of this molecule.

As aggrecan and other proteoglycan family molecules play a role in neuronal regeneration and tissue repair after CNS injury (Davies et al. 1997; Gates et al. 1996; Koppe et al. 1997; Lemons et al. 1999), our present observation suggests the possibility that the genetically determined nature of aggrecan determines the efficiency of tissue damage of the spinal cord. This may explain the axonal damage of the spinal cord observed in HAM/TSP patients (Umehara et al. 2000). Profound spinal tissue damage after acute inflammation caused by HTLV-I infected T cells may lead to an acute course of the disease, and insufficient or excessive repair of spinal tissue due to the genetic background may accumulate in a chronic course of the disease. Further studies are necessary to clarify these points.

Acknowledgements We thank Ms Tomoko Muramoto and Yoko Nishino of Kagoshima University for their excellent technical assistance.

Part of this study has been presented at the 11th International Conference on Human Retrovirology: HTLV and Related Viruses in San Francisco from June 9 to 12, 2003. All subjects gave written informed consent. The Ethical Committee of Kagoshima University Faculty of Medicine approved this study. The authors do not have commercial or other associations that might pose a conflict of interest.

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