(21) Okimoto, N.; Kitayama, K.; Hata, M.; Hoshino, T.; Tsuda, M. *THEOCHEM* **2001**, *543*, 53.
(22) Okimoto, N.; Tsukui, T.; Kitayama, K.; Hata, M.; Hoshino, T.; Tsuda, M. *J. Am. Chem. Soc.* **2000**, *122*, 5613.
(23) Park, H.; Suh, J.; Lee, S. *J. Am. Chem. Soc.* **2000**, *122*, 3901.
(24) Okimoto, N.; Tsukui, T.; Hata, M.; Hoshino, T.; Tsuda, M. *J. Am. Chem. Soc.* **1999**, *121*, 7349.
(25) Ventrini, A.; López-Ortiz, F.; Alvarez, J. M.; Gonzalez, J. *J. Am. Chem. Soc.* **1998**, *120*, 1110.
(26) Liu, H.; Müller-Plathe, F.; Van Gusteren, W. F. *J. Mol. Biol.* **1996**, *118*, 3946.
(27) Weber, I. T.; Harrison, R. W. *Protein Eng.* **1996**, *9*, 679.
(28) Harrison, R. W.; Weber, I. T. *Protein Eng.* **1994**, *7*, 1353.
(29) Beveridge, A. J.; Heywood, G. C. *Biochemistry* **1993**, *32*, 3325.
(30) Goldblum, A. *Biochemistry* **1988**, *27*, 1653.
(31) Perryman, A. L.; Lin, J.-H.; McCammon, J. A. *Protein Sci.* **2003**, *13*, 1108.
(32) Shenderovich, M. D.; Kagan, R. M.; Heseltine, P. N. R.; Ramnarayan, K. *Protein Sci.* **2003**, *12*, 1706.
(33) Clemente, J. C.; Hemmrajani, R.; Blum, L. E.; Goodenow, M. M.; Dunn, B. M. *Biochemistry* **2003**, *42*, 15029.
(34) Piana, S.; Carloni, P.; Rothlisberger, U. *Protein Sci.* **2002**, *11*, 2393.
(35) Rick, S. W.; Topol, I. A.; Erickson, J. W.; Burt, S. K. *Protein Sci.* **1998**, *8*, 1750.
(36) Harte, W. E., Jr.; Beveridge, D. L. *J. Am. Chem. Soc.* **1993**, *115*, 1231.
(37) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B. G.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *Gaussian 98*; Gaussian, Inc.: Pittsburgh, PA, 1998.
(38) Cieplak, P.; Cornell, W. D.; Bayly, C.; Kollman, P. A. *J. Comput. Chem.* **1995**, *16*, 1357.
(39) Case, D. A.; Pearlman, D. A.; Caldwell, J. W.; Cheatham, T. E., III; Wang, J.; Ross, W. S.; Simmerling, C. L.; Darden, T. A.; Merz, K. M.; Stanton, R. V.; Cheng, A. L.; Vincent, J. J.; Crowley, M.; Tsui, V.; Gohlke, H.; Radmer, R. J.; Duan, Y.; Pitera, J.; Massova, I.; Seibel, G. L.; Singh, U. C.; Weiner, P. K.; Kollman, P. A. *AMBER 7*; University of California: San Francisco, 2002.
(40) Wang, J.; Cieplak, P.; Kollman, P. A. *J. Comput. Chem.* **2000**, *21*, 1049.
(41) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. *J. Chem. Phys.* **1983**, *79*, 926.
(42) Ryckaert, J.-P.; Ciccotti, G.; Berendsen, H. J. C. *J. Comput. Phys.* **1977**, *23*, 327.
(43) Zoete, V.; Michielin, O.; Karplus, M. *J. Mol. Biol.* **2002**, *315*, 21.
(44) Schneider, T. R. *Acta Crystallogr., Sect. D* **2002**, *58*, 195.
(45) Schneider, T. R. *Acta Crystallogr., Sect. D* **2000**, *56*, 714.
(46) Williams, T.; Kelley, C. *GNUPLOT*, 1998 (contact for further information http://www.gnuplot.info/).
(47) Lee, B.; Richards, F. M. *J. Mol. Biol.* **1971**, *55*, 379.
(48) Connolly, M. L. *J. Appl. Crystallogr.* **1983**, *16*, 548.
(49) Prabu-Jeyabalen, M.; Nalivaika, E. A.; King, N. M.; Schiffer, C. A. *J. Virol.* **2003**, *77*, 1306.
(50) Kollman, P. *Chem. Rev.* **1993**, *93*, 2395.
(51) Honig, B.; Nicholls, A. *Science* **1995**, *268*, 1144.
(52) Sitkoff, D.; Sharp, K. A.; Honig, B. *J. Phys. Chem.* **1994**, *98*, 1978.
(53) Karplus, M.; Petsko, G. A. *Nature* **1990**, *347*, 631.
(54) Kuhn, L. A.; Siani, M. A.; Pique, M. E.; Fisher, C. L.; Getzoff, E. D.; Tainer, J. A. *J. Mol. Biol.* **1992**, *228*, 13.
(55) Chothia, C. *J. Mol. Biol.* **1976**, *105*, 1.
(56) Nozaki, Y.; Tanford, C. *J. Biol. Chem.* **1971**, *7*, 2211.
(57) Hong, L.; Zhang, X. C.; Hartsuck, J. A.; Tang, J. *Protein Sci.* **2000**, *9*, 1898.
(58) Jenwitheesuk, E.; Samudrala, R. *BMC Struct. Biol.* **2002**, *3*, 2.
(59) Wang, W.; Kollman, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14937.
(60) Xie, D.; Gulnik, S.; Gustchina, E.; Yu, B.; Shao, W.; Qoronfleh, W.; Nathan, A.; Erickson, J. W. *Protein Sci.* **1999**, *8*, 1702.

J. Antibiot. 58(1): 65–68, 2005

NOTE

THE JOURNAL OF ANTIBIOTICS

Fungal Phenalenones Inhibit HIV-1 Integrase

Kazuro Shiomi, Ryosuke Matsui, Miki Isozaki, Harumi Chiba, Takahiro Sugai, Yuichi Yamaguchi, Rokuro Masuma, Hiroshi Tomoda, Tomoko Chiba, Hua Yan, Yoshihiro Kitamura, Wataru Sugiura, Satoshi Ōmura, Haruo Tanaka

Received: September 7, 2004 / Accepted: November 27, 2004

Abstract A phenalenone compound, atrovenetinone methyl acetal, was isolated from a culture broth of *Penicillium* sp. FKI-1463 as an HIV-1 integrase inhibitor, and it showed anti-HIV activity *in vitro*. HIV-1 integrase inhibition and anti-HIV activity of two other natural phenalenones were also studied. Among the tested compounds, funalenone inhibited HIV-1 integrase with an IC_{50} value of 10 μM and showed the best selectivity (anti-HIV, IC_{50}=1.7 μM; cytotoxicity, IC_{50}=87 μM).

Keywords: enzyme inhibitor, HIV interase, AIDS, phenalenone

Combined therapeutic regimens with reverse transcriptase inhibitors and protease inhibitors lead to a suppression of human immunodeficiency virus-1 (HIV-1) replication, reduction of viral load, and decline in morbidity and mortality [1, 2]. However, the therapy sometimes fails due to the emergence of mutant viruses that are resistant to these drugs [3]. Thus, it is critical to discover more effective and less toxic anti-HIV agents with different molecular targets in the viral replication cycle. We have previously screened microbial metabolites for new anti-HIV antibiotics that inhibit entry of HIV-1 into the susceptive cells, and found isochromophilones and chloropeptins by a gp120-sCD4 binding assay [4, 5] and actinohivin by a syncytium formation assay [6]. There are three viral enzymes essential for HIV-1 replication, reverse transcriptase, protease, and integrase. Of these, only integrase has not been the target of a clinically used inhibitor. HIV DNA is inserted into the host genome by a specialized DNA recombination reaction in which the viral integrase is the key player [7, 8]. The integration reaction is composed of three steps, 3′-processing, strand transfer, and gap filling, and integrase catalyses the first and second steps. The third step is thought to be catalyzed by cellular enzymes. Many natural and synthetic integrase inhibitors have been reported [8~12] but only a few compounds show high selectivity. Therefore, we screened microbial metabolites for HIV-1 integrase inhibitors, and found that a culture broth of *Penicillium* sp. FKI-1463 has the inhibitory

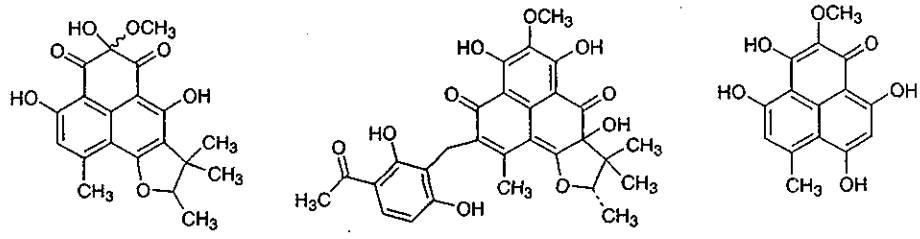
H. Tanaka (Corresponding author), **K. Shiomi, R. Matsui, M. Isozaki, H. Chiba, T. Sugai:** School of Pharmaceutical Sciences Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan, E-mail: tanakah@pharm.kitasato-u.ac.jp

Y. Yamaguchi, R. Masuma, H. Tomoda, S. Ōmura: The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

R. Masuma, H. Tomoda, S. Ōmura: Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

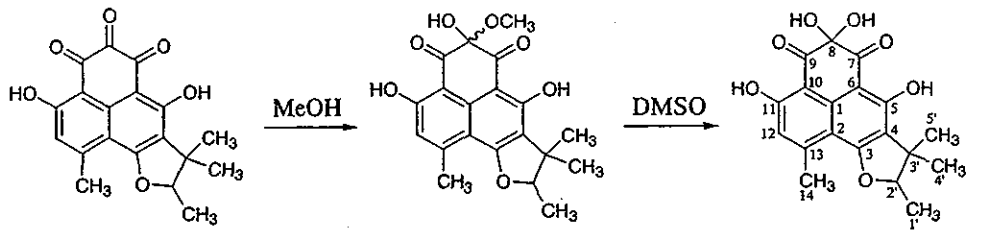
T. Chiba, H. Yan, W. Sugiura: AIDS Research Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan

Y. Kitamura: Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan



Atrovenetinone methyl acetal (**1**) Erabulenol B (**4**) Funalenone (**5**)

Fig. 1 Natural phenalenones.



2 **1** **3**

Fig. 2 Conversion of atrovenetinone.

activity. The active compound was identified as a phenalenone compound, atrovenetinone methyl acetal (**1**, Fig. 1) [13]. This paper presents integrase-inhibiting and anti-HIV activities of **1** and other natural phenalenones.

A slant culture of the strain FKI-1463 grown on YpSs agar was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 2.0%, Polypepton (Nihon Pharmaceutical Co.) 0.5%, yeast extract 0.2% (Oriental Yeast Co.), KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, and agar 0.1%, pH 6.0. It was cultured on a reciprocal shaker at 27°C for 3 days. One milliliter of the seed culture was transferred into each of twenty 500-ml Erlenmeyer flasks containing 100 ml of a production medium consisting of glycerol 3.0%, oatmeal (Nihon Shokuhin Seizo Co.) 2.0%, dry yeast (Gist-brocades) 1.0%, KH_2PO_4 1.0%, Na_2HPO_4 1.0%, $MgSO_4 \cdot 7H_2O$ 0.5%, pH not adjusted. The fermentation was carried out on a reciprocal shaker at 27°C for 7 days. The cultured broth (2.0 liters) was centrifuged and the mycelia were extracted with methanol, which was then removed from the extract by evaporation. The aqueous extract was partitioned with ethyl acetate at pH 3.0, and the organic layer was concentrated to dryness *in vacuo* to afford brown oil (644 mg). This was chromatographed over a silica gel column. Active fractions, eluted with $CHCl_3$-methanol (100 : 1) and $CHCl_3$-methanol (20 : 1), were concentrated to yield a crude material (284 mg). It was applied on a ODS silica gel column and eluted with aqueous CH_3CN. The 50% CH_3CN eluates were concentrated (95.5 mg) and chromatographed over Sephadex LH-20 to yield green oil (86.8 mg). It was further purified by reverse phase (Pegasil ODS, Senshu Scientific Co.) and normal phase (Pegasil Silica, Senshu Scientific Co.) HPLC to yield 50.5 mg of green oil.

The purified compound was implicated as **1** by comparison of the NMR data in $CDCl_3$ with the reported data by Nakanishi *et al.* [13]. Atrovenetinone (**2**) is easily converted into an acetal in alcohol (Fig. 2) [14], and the acetal is a mixture of diastereomers [13]. So, the NMR spectra of **1** are complicated. Since **2** exists as the hydrate (**3**) in DMSO [14], we observed the NMR spectra of the isolated compound in DMSO-d_6. The spectra were simplified, and each signal was assigned as follows: ^{1}H NMR (600 MHz) δ 13.67 (1H, s, 5-OH), 12.92 (1H, s, 11-OH), 6.86 (1H, s, 12-H), 4.70 (1H, q, J=6.5 Hz, 2′-H), 4.04 (1H, br s, 8-OH), 2.72 (3H, s, 14-H_3), 1.45 (3H, s, 5′-H_3), 1.22 (3H, s, 4′-H_3), 1.41 (3H, d, J=6.5 Hz, 1′-H_3); ^{13}C NMR (150 MHz) δ 197.7 (C-7), 196.2 (C-9), 165.1 (C-11), 164.8 (C-3), 164.5 (C-5), 147.9 (C-13), 136.7 (C-1), 118.1 (C-4), 117.6 (C-12), 109.0 (C-2), 104.9 (C-10), 101.9 (C-6), 91.1 (C-2′), 88.0 (C-8), 42.8 (C-3′), 25.2 (C-5′), 23.5 (C-14), 20.4 (C-4′), 14.3 (C-1′). The NMR data suggested that **1** was converted into **3** in DMSO solution (Fig. 2), and released methanol signals (δ_H 3.15 and δ_C 48.6) were also

Table 1 Biological activities of phenalenones

	IC_{50} (μM)			Selectivity (B/A)
	HIV-1 integrase inhibition	Anti-HIV activity (A)	Cytotoxicity (HPB-M(a)[a]) (B)	
Atrovenetinone methyl acetal (**1**)	19	6.7	13	1.9
Erabulenol B (**4**)	7.9	17	230	14
Funalenone (**5**)	10	1.7	87	51

[a]HPB-M(a) cells are human peripheral blood cells transformed by murine leukemia virus. Anti-HIV activity was measured using HPB-M(a) cells with LTR driven luciferase.

observed. Thus, the isolated compound was identified as **1**. It has been reported as a myosin light chain kinase inhibitor isolated from a culture broth of *Penicillium* sp. It may be derived from **2** during purification. Compound **2** is a phenalenone compound originally obtained by the oxidation of atrovenetin produced by *Penicillium* sp., and **2** was lately isolated from a culture broth of *Gremmeniella abietina* [14, 15].

We have previously isolated the other fungal phenalenones, erabulenol B (**4**) which inhibits cholesteryl ester transfer protein and funalenone (**5**) which inhibits collagenase [16, 17]. Funalenone was also reported to inhibit bacterial cell wall synthesis enzymes MraY and MurG [18]. We evaluated integrase inhibition and anti-HIV activity of **1** together with those phenalenones. HIV-1 integrase activity was measured by strand transfer assay according to Craigie *et al.* [7]. *In vitro* anti-HIV activities of the test compounds were measured by originally established reporter human T cell line with LTR driven luciferase. The cells were infected with wild type HIV-1, and the compounds were added at different concentrations ranging from 0.0016 to 125 μg/ml. Luciferase activities of the cells, which appeared to correlate with the level of HIV-1 replication, were measured at day 7, and anti-HIV IC_{50}s of the compounds were evaluated. The IC_{50} value of **1** against integrase was 19 μM, and it also showed anti-HIV activity at 6.7 μM (Table 1). However, its cytotoxicity was relatively high. Compounds **4** and **5** showed more potent inhibition against integrase than **1**, and also exhibited anti-HIV activity. The anti-HIV activity of **5** was the most potent (1.7 μM), and its cytotoxicity (87 μM) was lower than **1**. Though **5** was reported to inhibit collagenase and bacterial cell wall synthesis enzymes [17, 18], those inhibitions were less potent than the integrase inhibition and anti-HIV activity. Therefore, **5** may be a good candidate lead compound for anti-HIV agent. Inhibition of DNA polymerases by the other phenalenones have been reported, but they did not inhibit HIV reverse transcriptase [19]. A plant metabolite, hypericin [20], is the only *ortho*- and *peri*-fused aromatic compound reported to show integrase inhibition [21].

Acknowledgements We are grateful to Dr. Junji Inokoshi, School of Pharmaceutical Sciences, Kitasato University for providing funalenone. This work was supported in part by the Grant of Research for the Development of Anti-AIDS Pharmaceutical Products (KA12505), Japan Health Sciences Foundation, and the 21st Century COE Program, Ministry of Education, Culture, Sports, Science, and Technology.

References

1. Hogg RS, Rhone SA, Yip B, Sherlock C, Conway B, Schechter MT, O'Shaughnessy MV, Montaner JSG. Antiviral effect of double and triple drug combinations amongst HIV-infected adults: lessons from the implementation of viral load-driven antiretroviral therapy. AIDS 12: 279–284 (1998)
2. Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. N Engl J Med 338: 853–860 (1998)
3. Deeks SG. Treatment of antiretroviral-drug-resistant HIV-1 infection. Lancet 362: 2002–2011 (2003)
4. Matsuzaki K, Ikeda H, Masuma R, Tanaka H, Ōmura S. Isochromophilones I and II, novel inhibitors against gp120-CD4 binding produced by *Penicillium multicolor* FO-2338. I. Screening, taxonomy, fermentation, isolation and biological activity. J Antibiot 48: 703–707 (1995)
5. Tanaka H, Matsuzaki K, Nakashima H, Ogino T, Matsumoto

A, Ikeda H, Woodruff HB, Ōmura S. Chloropeptins, new anti-HIV antibiotics inhibiting gp120-CD4 binding from *Streptomyces* sp. 1. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. J Antibiot 50: 58–65 (1997)

6. Chiba H, Inokoshi J, Okamoto M, Asanuma S, Matsuzaki K, Iwama M, Mizumoto K, Tanaka H, Oheda M, Fujita K, Nakashima H, Shinose M, Takahashi Y, Ōmura S. Actinohivin, a novel anti-HIV protein from an actinomycete that inhibits syncytium formation: isolation, characterization, and biological activities. Biochem Biophys Res Commun 282: 595–601 (2001)
7. Craigie R, Hickman AB, Engelman A. Integrase. *In* HIV. Volume 2. *Ed.*, Karn J, pp. 53–71, IRL Press, Oxford (1995)
8. Pommier Y, Neamati N. Inhibitors of human immunodeficiency virus integrase. *In* Advances in Virus Research. Volume 52. *Ed.*, Maramorosch K *et al.*, pp. 427–458, Academic Press, San Diego (1999)
9. Cos P, Maes L, Vanden Berghe D, Hermans N, Pieters L, Vlietinck A. Plant substances as anti-HIV agents selected according to their putative mechanism of action. J Nat Prod 67: 284–293 (2004)
10. Hazuda D, Blau CU, Felock P, Hastings J, Pramanik B, Wolfe A, Bushman F, Farnet C, Goetz M, Williams M, Silverman K, Lingham R, Singh S. Isolation and characterization of novel human immunodeficiency virus integrase inhibitors from fungal metabolites. Antivir Chem Chemother 10: 63–70 (1999)
11. Singh SB, Jayasuriya H, Dewey R, Polishook JD, Dombrowski AW, Zink DL, Guan Z, Collado J, Platas G, Pelaez F, Felock PJ, Hazuda DJ. Isolation, structure, and HIV-1 integrase inhibitory activity of structurally diverse fungal metabolites. J Ind Microbiol Biotechnol 30: 721–731 (2003)
12. Ondeyka JG, Zink DL, Dombrowski AW, Polishook JD, Felock PJ, Hazuda DJ, Singh SB. Isolation, structure and HIV-1 integrase inhibitory activity of exophillic acid, a novel fungal metabolite from *Exophiala pisciphila*. J Antibiot 56: 1018–1023 (2003)
13. Nakanishi S, Toki S, Saitoh Y, Tsukuda E, Kawahara K, Ando K, Matsuda Y. Isolation of myosin light chain kinase inhibitors from microorganisms: dehydroaltenusin, altenusin, atrovenetinone, and cyclooctasulfur. Biosci Biotechnol Biochem 59: 1333–1335 (1995)
14. Ayer WA, Hoyano Y, Pedras MS, van Altena I. Metabolites produced by the Scleroderris canker fungus, *Gremmeniella abietina*. Part 1. Can Chem 64: 1585–1589 (1986)
15. Narasimhachari N, Vining LC. Studies on the pigments of *Penicillium herquei*. Can J Chem 41: 641–648 (1963)
16. Tomoda H, Tabata N, Masuma R, Si SY, Ōmura S. Erabulenols, inhibitors of cholesteryl ester transfer protein produced by *Penicillium* sp. FO-5637. I. Production, isolation and biological properties. J Antibiot 51: 618–623 (1998)
17. Inokoshi J, Shiomi K, Masuma R, Tanaka H, Yamada H, Ōmura S. Funalenone, a novel collagenase inhibitor produced by *Aspergillus niger*. J Antibiot 52: 1095–1100 (1999)
18. Zawadzke LE, Wu P, Cook L, Fan L, Casperson M, Kishnani M, Calambur D, Hofstead SJ, Padmanabha R. Targeting the MraY and MurG bacterial enzymes for antimicrobial therapeutic intervention. Anal Biochem 314: 243–252 (2003)
19. Perpelescu M, Kobayashi J, Furuta M, Ito Y, Izuta S, Takemura M, Suzuki M, Yoshida S. Novel phenalenone derivatives from a marine-derived fungus exhibit distinct inhibition spectra against eukaryotic DNA polymerases. Biochemistry 41: 7610–7616 (2002)
20. Pace N, Mackinney G. Hypericin, the photodynamic pigment from St. John'swort. J Am Chem Soc 63: 2570–2574 (1941)
21. Farnet CM, Wang B, Hansen M, Lipford JR, Zalkow L, Robinson WE, Jr, Siegel J, Bushman F. Human immunodeficiency virus type 1 cDNA integration: new aromatic hydroxylated inhibitors and studies of the inhibition mechanism. Antimicrob Agents Chemother 42: 2245–2253 (1998)

Antiviral Therapy 9:929-935

Influence of single-nucleotide polymorphisms in the multidrug resistance-1 gene on the cellular export of nelfinavir and its clinical implication for highly active antiretroviral therapy

*Dayong Zhu[1], Hitomi Taguchi-Nakamura[1], Mieko Goto[1], Takashi Odawara[2], Tetsuya Nakamura[2], Harumi Yamada[3], Hajime Kotaki[3], Wataru Sugiura[4], Aikichi Iwamoto[1,2] and Yoshihiro Kitamura[1]**

[1]Division of Infectious Diseases, Advanced Clinical Research Centre, Institute of Medical Science, University of Tokyo, Tokyo, Japan
[2]Department of Infectious Diseases and Applied Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan
[3]Department of Pharmacy, Research Hospital, Institute of Medical Science, University of Tokyo, Tokyo, Japan
[4]National Institute of Infectious Diseases, Tokyo, Japan

*Corresponding author: +81 3 5449 5336; Fax: +81 3 5449 5427; E-mail: yochan@ims.u-tokyo.ac.jp

Protease inhibitors (PIs) such as nelfinavir (NFV) suppress HIV replication. PIs are substrates of P-glycoprotein (P-gp), the product of the multidrug-resistance-1 (*MDR1*) gene. Three single-nucleotide polymorphisms (SNPs) are present in exons of the *MDR1* gene: *MDR1* 1236, *MDR1* 2677 and *MDR1* 3435. We speculated that these genetic polymorphisms affected PI concentration in the cell. To verify this hypothesis, we first genotyped these SNPs in 79 Japanese patients by the SNaPshot method and found incomplete linkage disequilibrium between the SNPs. Because the SNP at *MDR1* 3435 has been reported to be associated with P-gp expression, we evaluated the effect of that SNP on the export of NFV from HIV-positive patients' lymphoblastoid cell lines by measuring time-dependent decrease in the amount of intracellular NFV by high-performance liquid chromatography. We found the intracellular concentration of NFV in lymphoblastoid cell lines (LCLs) with the homozygous T/T genotype at *MDR1* 3435 were higher than that with C/C genotype with statistical significance. This suggests that the activity of P-gp in patients' LCL cells with the *MDR1* 3435 T/T genotype was lower. In a retrospective study we evaluated the effect of the SNPs on CD4 cell count recovery in response to antiretroviral treatment with PIs, and obtained statistically significant evidence that suggested marginal association of the SNP at *MDR1* 1236 but not at *MDR1* 2677 or *MDR1* 3435. As *in vitro* results were not consistent with the clinical evaluation, clinical importance of *MDR1* genotyping for antiretroviral therapy remains to be investigated in a larger, case-controlled study.

Introduction

Antiretroviral therapy with HIV protease inhibitors (PIs) in combination with reverse transcriptase inhibitors dramatically improved the prognosis of patients infected with HIV-1. However, some patients fail to achieve the maximal virological suppression. We speculate that such failure is partly because PIs do not accumulate in lymphocytes in their active free forms in a concentration high enough to inhibit viral replication [1,2], although the intracellular active PI levels have, to the best of our knowledge, not yet been determined. The activity of P-glycoprotein (P-gp), the product of the multidrug resistance-1 (*MDR1*) gene, appears to affect intracellular PI concentration, because PIs such as nelfinavir (NFV) are substrates of P-gp [2]. P-gp is a glycosylated membrane protein belonging to the ATP-binding cassette superfamily of membrane transporters. P-gp is expressed in many tissues and cell types including intestinal epithelial cells and lymphocytes, where it acts as an energy-dependent exporter [3–9]. The *MDR1* is polymorphic and at least three single-nucleotide polymorphisms (SNPs) have been identified in the exons in a healthy Japanese population [10] as well as in other ethnic groups [6]. *MDR1* 1236 and *MDR1* 3435 are silent mutations in exons 12 and 26 [3,11], respectively, whereas *MDR1* 2677 is a substitution mutation in exon 21 [11]. Reportedly, the SNP at *MDR1* 3435 is associated with the amount and activity of P-gp protein both *in vitro* and *in vivo* [3,12]. In addition, individuals with the T/T genotype at *MDR1* 3435 were found to express less P-gp in lymphocytes and in intestinal epithelial cells [3,13] and showed lower efflux of rhodamine from natural killer (NK)

cells than those with the C/C genotype [13]. According to these observations, *MDR1* polymorphisms seem to affect the intracellular PI concentration and the outcome of antiretroviral treatment. However, the role of *MDR1* 3435 SNP in the response to antiretroviral therapy is still controversial [12,14].

The objective of this study was to evaluate the effect of three *MDR1* SNPs on the intracellular concentrations of NFV and to evaluate the impact of those SNPs on virological and immunological response to antiretroviral treatment, including NFV and PIs. We genotyped the SNPs in 79 Japanese patients and compared the velocity of NFV efflux among selected patients' lymphoblastoid cell lines (LCLs) with different *MDR1 3435 genotypes*. We also analysed the viral loads and CD4 cell counts after initiation of antiretroviral treatment with prescriptions with PIs including NFV in 21 patients.

Materials and methods

Patients

A total of 79 HIV-positive Japanese patients were enrolled in this study. These patients attended a hospital AIDS clinic at the Institute of Medical Science, University of Tokyo (IMSUT). The patients provided their written informed consent to participate in the study and to supply blood samples for DNA analysis and cell culture. Of the 79 patients, 21 receiving highly active antiretroviral therapy (HAART) including PIs were divided into three groups: 11 patients receiving HAART with NFV, four patients receiving HAART with indinavir (IDV) and six patients receiving HAART with saquinavir (SQV) or lopinavir/ritonavir (LPV/RTV). CD4 cell counts and HIV-RNA of plasma were analysed for 9 months after the initiation of the antiretroviral treatment. The study has been approved by the ethics committee of IMSUT.

Single-nucleotide polymorphisms

We typed three single-nucleotide polymorphisms (SNPs) at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) by polymerase chain reaction (PCR) followed by ABI PRISM SNaPshot Multiplex Kit (PE Biosystems, Foster City, Calif., USA) [15]. Information on primers and conditions for PCR was obtained at http://snp.ims.u-tokyo.ac.jp [10].

Cells and determination of uptake and efflux of NFV

Peripheral blood mononuclear cells (PBMCs) were separated from patients' whole blood with Ficoll-Conray gradient centrifugation. LCLs were obtained by transforming PBMCs with Epstein-Barr virus (EBV), which was obtained from cell-free supernatants of EBV-producing B95-8 cell lines [16]. LCLs were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Mo., USA) supplemented with 10% heat-inactivated fetal calf serum.

To determine the time course of NFV uptake into LCL cells, LCL cells (1×10^6/10 ml, counted with a haematocytometer) were incubated at 37°C in a medium containing 10 μM NFV. Cells were harvested by centrifugation at 1500 ×*g* for 5 min at 4°C and immediately frozen at −80°C until high-performance liquid chromatography (HPLC) analysis. To determine the velocity of NFV efflux from LCL cells, these patients' LCL cells were incubated at 37°C in a medium containing 10 μM NFV for 3 h. The cells were then quickly washed twice with 10 ml ice-cold phosphate-buffered saline and cultured in 10 ml NFV-free medium for up to 3 h. After an interval, aliquot cells were harvested by centrifugation at 1500 ×*g* for 5 min at 4°C and immediately frozen at −80°C until HPLC analysis.

Reverse transcription-PCR (RT-PCR)

For quantification of *MDR1* transcript, RNA from 1×10^7 LCL cells was isolated using Trizol reagents (Invitrogen Corp, Carlsbad, Calif., USA). First strand cDNA was obtained by using ReverTra Ace (Toyobo, Osaka, Japan) with 1 μg of total RNA. cDNA was subjected to PCR. Information on primers and conditions for PCR was obtained as previously described [17]. We used human glyceraldehyde 3-phosphate dehydrogenase mRNA as a positive control.

Determination of intracellular concentration of NFV by HPLC

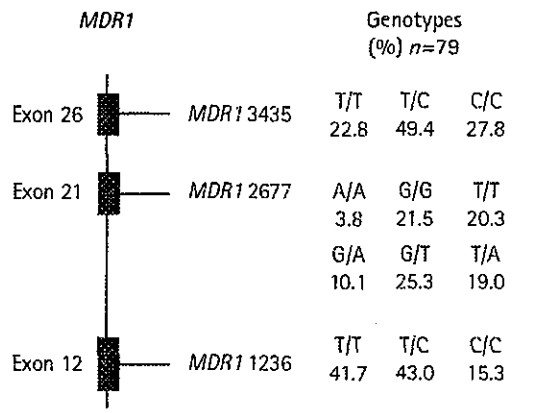
The patients' frozen LCL cells were extracted with 1.5 ml of ethanol. The extracts were then clarified by centrifugation at 2050 ×*g* for 10 min at 4°C. The ethanol extracts were evaporated at 30°C and dissolved in 180 μl of mobile phase, which was a mixture of phosphate buffer (containing 50 mM KH_2PO_4 and 50 mM Na_2HPO_4; pH 5.63) and acetonitrile (1:1, v:v) [18]. The amounts of NFV were measured using a Sensyu Pack ODS C_{18} column (5 μm particle size; 150×4.6 mm, Sensyu Scientific Co, Tokyo, Japan) at a flow rate of 1.5 ml/min by HPLC (Shimadzu Co, Tokyo, Japan). The UV detection wave length was 220 nm and efavirenz (EFV) was used as an internal standard. The lower limits of detection and quantification were 20 ng (30.1 pmole)/10^6 cells, and the calibration range was 20–2000 ng (30.1–3010 pmole/10^6 cells).

Results

We typed the three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) in DNA samples from 79 HIV-positive Japanese patients

(Figure 1). We found that it was consistent with the Hardy–Weinberg principle (Tables 1 and 2). Furthermore, in all possible two-way comparisons of the three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21, excluding the genotypes containing G) and *MDR1* 3435 (exon 26), we found significant linkage disequilibrium between *MDR1* 2677 A (T) and *MDR1* 1236 C (T), *MDR1* 2677 A (T) and *MDR1* 3435 C (T), and *MDR1* 1236 C (T) and *MDR1* 3435 C (T), respectively.

Figure 1. Frequency of SNPs in *MDR1*



The SNPs at *MDR1* 1236, *MDR1* 2677 and *MDR1* 3435 were typed by the SNaPshot method. Genotype frequencies at each site are shown as percentage among 79 HIV-infected Japanese patients. The thin vertical line at left represents the *MDR1* gene on human chromosome 7. The closed boxes represent exons 12, 21 and 26.

Table 1. Hardy–Weinberg principle at *MDR1* 1236 (n=79)

	T/T	T/C	C/C
Observed number of patients	33	34	12
Expected number of patients	31.7*	36.7†	10.6‡

p: Frequency for the T allele $\frac{33\times2+34}{2\times79}=0.633$

q: Frequency for the C allele 1–p=0.367

*$79\times p^2=31.7$

†$79\times2pq=36.7$

‡$79\times q^2=10.6$

Table 2. Hardy–Weinberg principle at *MDR1* 3435 (n=79)

	T/T	T/C	C/C
Observed number of patients	18	39	22
Expected number of patients	17.8*	39.4†	21.8‡

p: Frequency for the T allele $\frac{18\times2+39}{2\times79}=0.475$

q: Frequency for the C allele 1–p=0.525

*$79\times p^2=17.8$

†$79\times2pq=39.4$

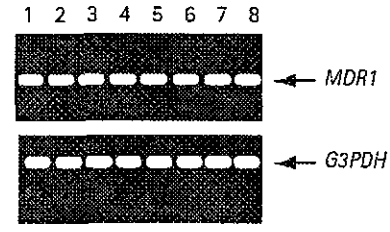
‡$79\times q^2=21.8$

Reportedly, *MDR1* 3435 T/T genotype was associated with lower expression of P-gp in leukocytes [13] so we hypothesized that the genotype was also associated with slower cellular export of NFV in patients' lymphocytes. To investigate this, we first established LCLs by immobilizing selected patients' PBMCs with EBV. We selected eight patients' LCLs with *MDR1* 3435 C/C (n=4) and T/T (n=4) and verified similar levels of *MDR1* in these LCLs by RT-PCR (Figure 2). We observed little variation in *MDR1* transcripts.

We found that uptake of NFV was rapid into LCLs reaching a steady-state within 5 min (Figure 3). We studied eight patients' LCLs with *MDR1* 3435 T/T and *MDR1* 3435 C/C to compare the steady-state intracellular concentration of NFV after 3 h incubation in a medium containing 10 μM NFV. The intracellular concentrations of NFV in LCLs with *MDR1* 3435 T/T and C/C genotypes were 2593 μM and 2411 μM, respectively (n=4), with no statistical difference. We calculated these values by hypothesizing that the LCLs are ideal spheres (10 μm diameter) and that NFV distributes uniformly in the cell.

We then compared NFV efflux from those LCLs with different genotypes at *MDR1* 3435. Before measuring export of NFV, LCLs were cultured with NFV to a saturated level. These NFV-loaded cells were transferred to NFV-free medium and cultured for 3 h with intermittent sampling of cell aliquots. We compared the efflux of NFV from the eight patients' LCLs with *MDR1* 3435 T/T and C/C (n=4 each), which had been verified to express *MDR1* mRNA by

Figure 2. *MDR1* mRNA expression in LCLs

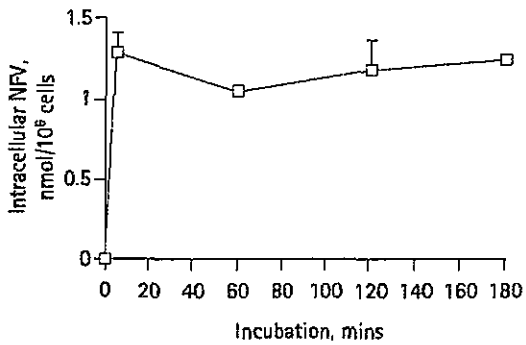


We selected eight patients' LCLs with *MDR1* 3435 C/C (lanes 1–4) and T/T (lanes 5–8) and measured the expression of *MDR1* mRNA. Total cellular RNA from LCLs was subjected to RT-PCR with primer sets for *MDR1* and *G3PDH* transcripts. Aliquots were subjected to agarose gel electrophoresis. The genotypes at *MDR1* 1236, 2677 and 3435: lanes 1 and 2, (T/T, G/G, C/C); lane 3, (T/C, G/A, C/C); lane 4 (C/C G/A C/C); lane 5 (T/T, G/T, T/T); and lanes 6–8 (T/T, T/T, T/T).

RT-PCR (Figure 2). The concentration of intracellular NFV in LCLs with the homozygous T/T genotype at *MDR1* 3435 was higher than in those with C/C genotype at 120 min and 180 min. This difference was statistically significant (P=0.04 and 0.02, respectively, Mann–Whitney U-test, Figure 4). This meant the NFV efflux in patients' LCL cells with the *MDR1* 3435 T/T genotype was slower than that with C/C genotype. Thus, we suspect the activity of P-gp in patients' LCLs with the *MDR1* 3435 T/T genotype is lower than that with the C/C genotype.

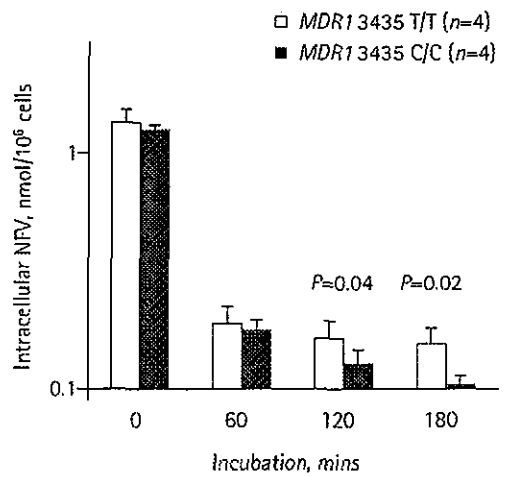
To examine the influence of *MDR1* 3435 genotypes on the response to treatment, we assessed increase in CD4 cell counts and viral suppression in 21 patients after initiation of HAART. At first, we hoped to analyse data obtained from a group of patients receiving NFV alone as a PI, but could not, due to the small number of NFV-receiving patients. Thus, we carried out the analysis in those patients receiving PIs including NFV (n=11), indinavir (n=4) and saquinavir/lopinavir/ritonavir (n=6). CD4 cell counts before treatment were similar among patients with various genotypes. Patients with various genotypes at *MDR1* 3435 showed similar changes in CD4 cell counts (Figure 5A) and viral suppression (Figure 6A) during 9 months of HAART. We found patients with the *MDR1* 1236 T/T showed higher increase in CD4 cell counts at 1 month (148 cells/μl) and 9 months (264 cells/μl) after initiation of therapy than those with *MDR1* 1236 C/C (20 cells/μl and 34 cells/μl, respectively) (Figure 5C). We suspected that *MDR1* 1236 T/T was associated with a higher rate of recovery of CD4 cell counts for patients receiving HAART with PI. We did not find differences in rates of viral suppression among the patients with various *MDR1* 1236 genotypes (Figure 6C). We did not observe a statistical difference in CD4 cell counts or viral loads among patients with different *MDR1* 2677 genotypes (Figures 5B and 6B).

Figure 3. A typical time course of NFV uptake



LCL cells (1 ×10⁶/10 ml) were incubated in medium containing 10 μM of NFV. Cells were harvested at 0, 5, 60, 120 and 180 min and assayed for intracellular NFV by HPLC. The horizontal axis shows the incubation time in min. The vertical axis shows the intracellular amount of NFV per 10^6 cells. The error bars represent the standard deviations.

Figure 4. NFV efflux from patients' LCLs

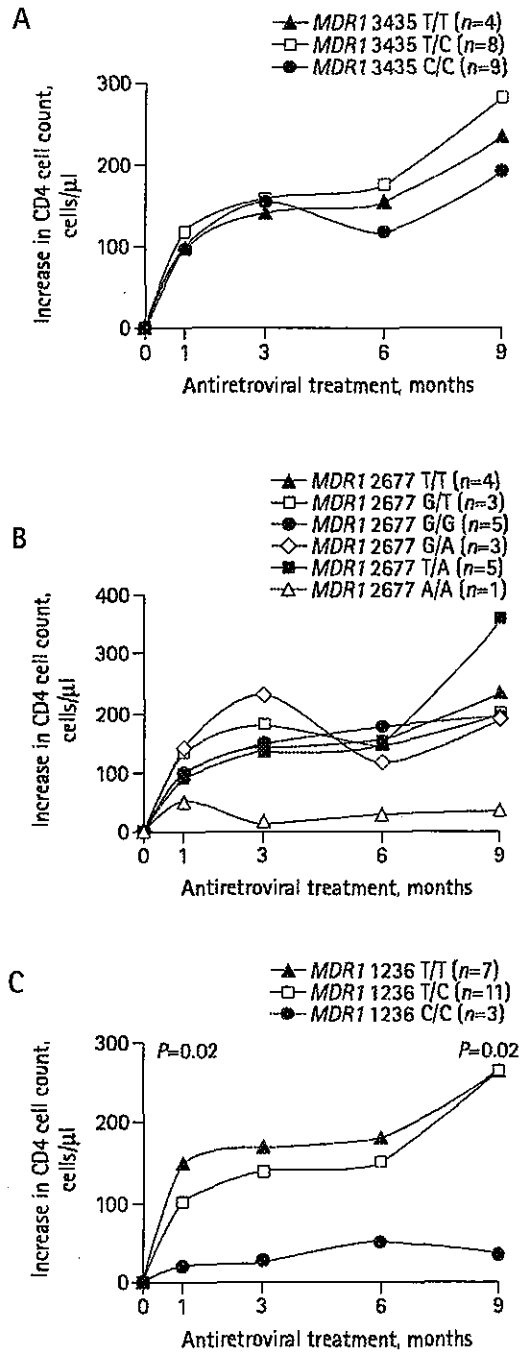


LCL cells were incubated in medium containing 10 μM of NFV for 3 h. Cells were then washed and cultured in NFV-free medium. Intracellular concentration of NFV was determined at 0, 60, 120 and 180 min by HPLC. The horizontal axis shows the incubation time in min. The vertical axis shows the intracellular amount of NFV per 10^6 cells. We selected eight patients (described in the legend to Figure 2) and examined the velocity of NFV efflux from those cells. The intracellular concentration of NFV was measured several times in all patients' LCLs, and data were similar in every test. The error bars represent the standard deviations.

Discussion

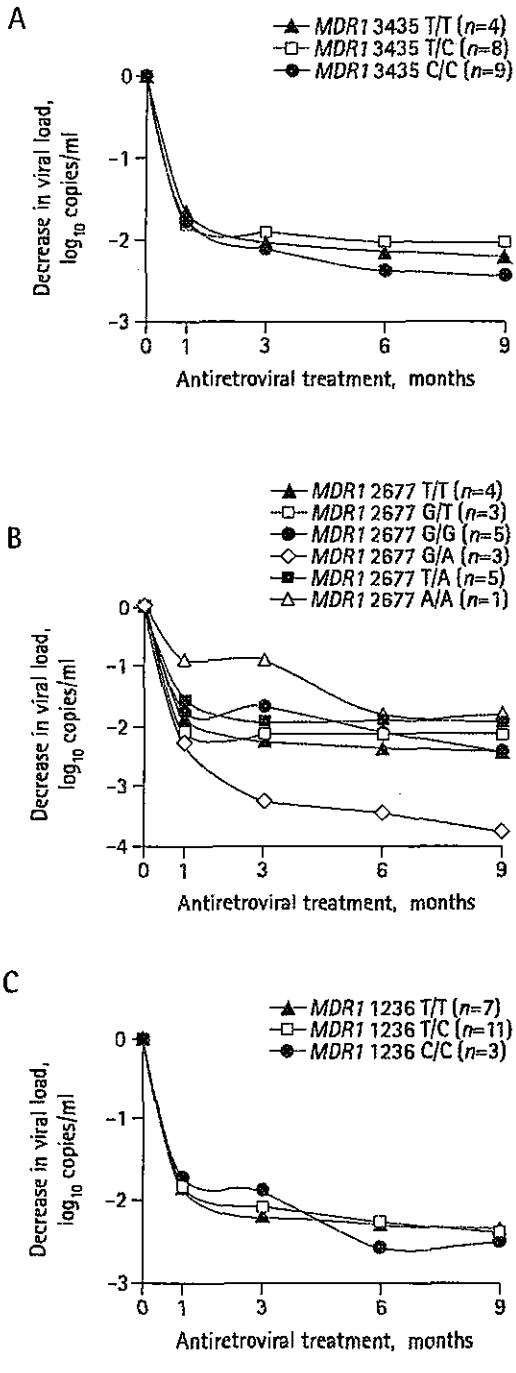
In this study, we genotyped three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) (Figure 1) in 79 HIV-positive Japanese patients and found incomplete linkage disequilibrium – as has also been reported in other ethnic groups [6]. We found that genotype frequencies of the SNPs at *MDR1* 1236 (exon 12) and *MDR1* 3435 (exon 26) in this population were in Hardy–Weinberg equilibrium. This suggested that the studied population was precisely genotyped and unbiased in terms of the *MDR1* gene. We compared the activity of P-gp among patients' LCLs with different *MDR1* 3435 genotypes by measuring NFV efflux from the cultured LCL cells by HPLC. We found that the intracellular concentration of NFV in LCLs with the homozygous T/T genotype at *MDR1* 3435 was higher than in those with the C/C genotype at 120 min and 180 min. This difference was statistically significant (P=0.04 and 0.02, respectively; Mann–Whitney U-test; Figure 4). In contrast, in the retrospective evaluation of 21 HIV-positive patients

Figure 5. Increase in CD4 cell count among patients with the various genotypes of *MDR1* during antiretroviral treatment



We assessed increase in CD4 cell counts among 21 patients. Every subject had CD4 cell counts and viral loads at months 0, 1, 3, 6 and 9. (A) *MDR1* 3435: T/T (▲); C/C (●); T/C (□). (B) *MDR1* 2677: T/T (▲); G/G (●); G/T (□); G/A (◇); T/A (■); A/A (△). (C) *MDR1* 1236: T/T (▲); C/C (●); T/C (□). The vertical axis shows the increase in CD4 cell count during treatment. *P* values were calculated by the Mann–Whitney U-test.

Figure 6. Suppression of viraemia among patients with various genotypes of MDR1 after antiretroviral treatment



We assessed suppression of viraemia among the same 21 patients as described in the legend to Figure 5. (A) *MDR1* 3435: T/T (▲); C/C (●); T/C (□). (B) *MDR1* 2677: T/T (▲); G/G (●); G/T (□); G/A (◇); T/A (■); A/A (△). (C) *MDR1* 1236: T/T (▲); C/C (●); T/C (□). The vertical axis shows decrease in viral load. Values are shown as $\log_{10}$ copies/ml plasma.

receiving PIs, we failed to observe a statistical difference in CD4 cell counts and viral suppression among patients with different *MDR1* 3435 SNPs (Figures 5A and 6A). Furthermore, we found that patients with the *MDR1* 1236 T/T genotype showed a greater increase in the CD4 cell counts during HAART therapy with PI at months 1 and 9 than patients with the *MDR1* 1236 C/C genotype (Figure 5C). The contribution of genetic variations in the *MDR1* gene to the patients' clinical characteristics, if any, seems very complicated and thus is difficult to evaluate in a straightforward manner.

As the steady-state intracellular concentration of NFV was about 250 times higher than that in the medium (10 μM), the uptake of NFV seems active rather than passive. However, these *in vitro* data depart from what has been observed in *in vivo* measurements of NFV in patients [19,20] presumably due to the presence of alpha(1)-acid glycoprotein to which NFV binds in plasma [21]. Furthermore, this discrepancy may also be due to the differential distribution of NFV among tissues rather than in free artificial medium. Therefore, our *in vitro* data should be considered as such, that is, *in vivo* lymphocytes may be unlikely to have this high intracellular to extracellular concentration ratio (250:1).

We observed an association of slower efflux of NFV *in vitro* with the T/T genotype at *MDR1* 3435. In fact, P-gp has been found to export PIs from lymphocytes and reduce their anti-HIV activity *in vitro*, and its low activity has been found to be associated with the T/T genotype at *MDR1* 3435 [13]. As the SNP at *MDR1* 3435 is a silent mutation, one possible explanation for this association is that the T/T genotype at *MDR1* 3435 renders *MDR1* mRNA unstable in the cell. Another possible explanation for the association is that *MDR1* 3435 SNP is in linkage disequilibrium with the SNPs at *MDR1* 1236 (exon 12) and *MDR1* 2677 (exon 21), the latter of which is a substitution mutation. This amino acid substitution from the *MDR1* 2677 SNP may be responsible for the observed difference (Figure 4) [11]. Another possible explanation is that *MDR1* 3435 SNPs are in linkage disequilibrium with a polymorphism(s) elsewhere in the genome that modifies *MDR1* expression or function [3,12].

Although an *in vitro* study showed that the velocity of NFV efflux in patients' LCLs with the *MDR1* 3435 T/T genotype was slower than that with the C/C genotype, we failed to observe a statistical difference in CD4 cell counts and viral suppression among patients with different *MDR1* 3435 genotypes (Figures 5A and 6A). Four equally possible accounts seem to explain this discrepancy. Firstly, since the C/C genotype at *MDR1* 3435 is also correlated with higher expression of P-gp in intestinal epithelial cells that adsorb PIs, the *MDR1* 3435 C/C is likely to be associated with higher absorption of PIs and higher PI concentration in plasma [12,22]. The higher plasma levels of NFV in 3435 C/C patients in one study [12] is puzzling and as yet not fully understood. Secondly, the sample size (n=21) in this study may be too small to evaluate CD4 cell counts or viral suppression in a statistical way. Thirdly, since the enrolled patients received different treatment combinations of PIs and reverse transcription inhibitors during antiretroviral therapy, the clinical evaluation was not normalized. Finally, because LCLs – immobilized B cells – but not CD4+ T cells were used in this study, the function of P-gp in a setting of HIV-1 infection may not have been accurately tested. In contrast to the *MDR1* 3435, we observed a marginal but statistically significant association of the *MDR1* 1236 SNP with the CD4 cell count increase although this SNP is a silent mutation. To our knowledge, this clinical association of *MDR1* 1236 with statistical significance is unprecedented, although its clinical significance remains to be investigated. In conclusion, a large-scale and case-controlled study would be required to test whether SNPs of *MDR1* affect the clinical course during antiretroviral therapy with PIs and the prognosis of infected patients.

Acknowledgements

This work was partly supported by grants for AIDS research from the Ministry of Health, Labor and Welfare of Japan, Grant-in-Aid for Scientific Research (A) from the Japan Society of the Promotion of Science (JSPS) and the Japan Health Sciences Foundation. We thank Dr Yusuke Nakamura (IMSUT) for Japanese SNP information.

References

1. Chaillou S, Durant J, Garraffo R, Georgenthum E, Roptin C, Dunais B, Mondain V, Roger PM & Dellamonica P. Intracellular concentration of protease inhibitors in HIV-1-infected patients: correlation with MDR-1 gene expression and low dose of ritonavir. HIV *Clinical Trials* 2002; 3:493–501.
2. Lee CG, Gottesman MM, Cardarelli CO, Ramachandra M, Jeang KT, Ambudkar SV, Pastan I & Dey S. HIV-1 protease inhibitors are substrates for the *MDR1* multidrug transporter. *Biochemistry* 1998; 37:3594–3601.
3. Hoffmeyer S, Burk O, von Richter O, Arnold HP, Brockmoller J, Johne A, Cascorbi I, Gerloff T, Roots I, Eichelbaum M & Brinkmann U. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity *in vivo*. *Proceedings of the National Academy of Sciences, USA* 2000; 97:3473–3478.
4. Chaudhary PM, Mechetner EB & Roninson IB. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. *Blood* 1992; 80:2735–2739.
5. Drach D, Zhao S, Drach J, Mahadevia R, Gattringer C, Huber H & Andreeff M. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. *Blood* 1992; 80:2729–2734.

6. Sakaeda T, Nakamura T & Okumura K. MDR1 genotype-related pharmacokinetics and pharmacodynamics. *Biological & Pharmaceutical Bulletin* 2002; 25:1391–1400.
7. Yacyshyn B, Maksymowych W & Bowen-Yacyshyn MB. Differences in P-glycoprotein-170 expression and activity between Crohn's disease and ulcerative colitis. *Human Immunology* 1999; 60:677–687.
8. Bellamy WT. P-glycoproteins and multidrug resistance. *Annual Review of Pharmacology & Toxicology* 1996; 36:161–183.
9. Mickley LA, Lee JS, Weng Z, Zhan Z, Alvarez M, Wilson W, Bates SE & Fojo T. Genetic polymorphism in MDR-1: a tool for examining allelic expression in normal cells, unselected and drug-selected cell lines, and human tumors. *Blood* 1998; 91:1749–1756.
10. Saito S, Iida A, Sekine A, Miura Y, Ogawa C, Kawauchi S, Higuchi S & Nakamura Y. Three hundred twenty-six genetic variations in genes encoding nine members of ATP-binding cassette, subfamily B (ABCB/MDR/TAP), in the Japanese population. *Journal of Human Genetics* 2002; 47:38–50.
11. Tanabe M, Ieiri I, Nagata N, Inoue K, Ito S, Kanamori Y, Takahashi M, Kurata Y, Kigawa J, Higuchi S, Terakawa N & Otsubo K. Expression of P-glycoprotein in human placenta: relation to genetic polymorphism of the multidrug resistance (MDR)-1 gene. *Pharmacology & Experimental Therapeutics* 2001; 297:1137–1143.
12. Fellay J, Marzolini C, Meaden ER, Back DJ, Buclin T, Chave JP, Decosterd LA, Furrer H, Opravil M, Pantaleo G, Retelska D, Ruiz L, Schinkel AH, Vernazza P, Eap CB & Telenti A. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. *Lancet* 2002; 359:30–36.
13. Hitzl M, Drescher S, van der Kuip H, Schaffeler E, Fischer J, Schwab M, Eichelbaum M & Fromm MF. The C3435T mutation in the human *MDR1* gene is associated with altered efflux of the P-glycoprotein substrate rhodamine 123 from CD56+ natural killer cells. *Pharmacogenetics* 2001; 11:293–298.
14. Nasi M, Borghi V, Pinti M, Bellodi C, Lugli E, Maffei S, Troiano L, Richeldi L, Mussini C, Esposito R & Cossarizza A. *MDR1* C3435T genetic polymorphism does not influence the response to antiretroviral therapy in drug-naive HIV-positive patients. *AIDS* 2003; 17:1696–1698.
15. Kobayashi N, Nakamura HT, Goto M, Nakamura T, Nakamura K, Sugiura W, Iwamoto A & Kitamura Y. Polymorphisms and haplotypes of the CD209L gene and their association with the clinical courses of HIV-positive Japanese patients. *Japanese Journal of Infectious Diseases* 2002; 55:131–133.
16. Miller G & Lipman M. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proceedings of the National Academy of Sciences, USA* 1973; 70:190–194.
17. Taipalensuu J, Tornblom H, Lindberg G, Einarsson C, Sjoqvist F, Melhus H, Garberg P, Sjostrom B, Lundgren B & Artursson P. Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. *Pharmacology & Experimental Therapeutics* 2001; 299:164–170.
18. Yamada H, Kotaki H, Nakamura T & Iwamoto A. Simultaneous determination of the HIV protease inhibitors indinavir, amprenavir, saquinavir, ritonavir and nelfinavir in human plasma by high-performance liquid chromatography. *Journal of Chromatography. B, Biomedical Sciences & Applications* 2001; 755:85–89.
19. Hennessy M, Clarke S, Spiers JP, Kelleher D, Mulcahy F, Hoggard P, Back D & Barry M. Intracellular accumulation of nelfinavir and its relationship to P-glycoprotein expression and function in HIV-infected patients. *Antiviral Therapy* 2004; 9:115–122.
20. Ford J, Cornforth D, Hoggard PG, Cuthbertson Z, Meaden ER, Williams I, Johnson M, Daniels E, Hsyu P, Back DJ & Khoo SH. Intracellular and plasma pharmacokinetics of nelfinavir and M8 in HIV-infected patients: relationship with P-glycoprotein expression. *Antiviral Therapy* 2004; 9:77–84.
21. Schon A, del Mar Ingaramo M & Freire E. The binding of HIV-1 protease inhibitors to human serum proteins. *Biophysical Chemistry* 2003; 105:221–230.
22. Kim RB, Leake BF, Choo EF, Dresser GK, Kubba SV, Schwarz UI, Taylor A, Xie HG, McKinsey J, Zhou S, Lan LB, Schuetz JD, Schuetz EG & Wilkinson GR. Identification of functionally variant MDR1 alleles among European Americans and African Americans. *Clinical Pharmacology & Therapeutics* 2001; 70:189–199.

Received 26 March 2004, accepted 11 August 2004

ABSTRACT 98

Antiviral Therapy 2004; 9:S109.

Changes in prevalence and patterns of drug resistant mutations in Japan – summary of nationwide HIV-1 drug resistance surveillance study (1996 to 2003) in Japan

W Sugiura[1], M Matsuda[1], T Chiba[1], J Kakizawa[1], M Nishizawa[1], H Miura[1], M Hamatake[1], T Ueda[1], M Fujino[1], K Yamada[2] and N Yamamoto[1]

1 AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan; and 2 Japanese Foundation for AIDS Prevention, Tokyo, Japan

BACKGROUND AND OBJECTIVE: Antiretroviral treatment situation in Japan has been similar to other Western countries, zidovudine became available in 1987 and HAART was introduced in 1997. As has been widely reported, HAART has improved the prognosis of HIV/AIDS significantly. However, treatment failure due to antiretroviral drug resistance is a critical issue that must be overcome. We started HIV drug resistance genotyping in 1996 to support the treatment, and also to understand prevalence and patterns of HIV drug resistance. Here we present a summary of our 7-year surveillance study of HIV drug resistance in Japan.

METHODS: HIV/AIDS patient blood samples sent to our laboratory from November 1996 to December 2003 were analysed. Drug resistance genotyping was performed using *in-house* protocol. Briefly, HIV-1 RNA was extracted from patient serum, and protease and reverse transcriptase fragments were amplified and sequenced. Drug resistance mutations were defined according to IAS-USA drug resistance mutation list.

RESULTS: During the study period, 5561 samples from 1156 HIV/AIDS patients were collected and analysed. The prevalence of NRTI resistance was 45% in the beginning of our study, and remained at 40–45% for throughout the study period. In contrast, the prevalence of PI resistance increased dramatically. There was no resistance case in 1996, but it increased up to 35% in 2000. Subsequently, PI resistance frequency decreased in 2001 to 2002, and reciprocally the prevalence of NNRTI increased from 5% in 2000 to 15% in 2002. The data appears to reflect the availability and trends of antiretroviral treatment in Japan. PI was approved in 1997, prescriptions increased thereafter, and the increase in PI resistance matched this progression. Subsequent changes in the prevalence of PI resistance and NNRTI resistance coincided with the availability of efavirenz, which was approved in 2000 and experienced increased use as a replacement for PIs in HAART.

CONCLUSION: Our data demonstrates significant increase of HIV drug resistance in these 7 years. Further continuation of the surveillance is necessary not only to understand epidemiological status, but also to find effective strategy to overcome the HIV drug resistance issue.

ABSTRACT 2

Antiviral Therapy 2004; 9:S6.

Novel small-molecule compounds which inhibit strand transfer activity of HIV-1 integrase

H Yan[1], T Chiba[1], Y Kitamura[2], M Nishizawa[1], M Fujino[1], N Yamamoto[1] and W Sugiura[1]

1 AIDS Research Center, NIID, Tokyo, Japan; and 2 Division of Infectious Diseases, Institute of Medical Science, University of Tokyo, Japan

OBJECTIVE: Integration of the proviral DNA into the host genome is essential event in the human immunodeficiency virus type 1 (HIV-1) replication life cycle. Therefore, integrase (IN), which plays crucial role in this integration event, has been the attractive target of anti-HIV drugs. Up to now, a number of inhibitory compounds have been reported, yet none has been successful in clinical treatment usage. In this study we attempted to find new IN inhibitory compounds, and screened a small molecule-compound library.

METHODS: In-house strand-transfer assay was constructed to screen IN inhibitory compounds. In brief, biotinylated 31 bp donor DNA was mixed with recombinant IN, followed by incubation with digoxigenin (DIG), labelled 29 bp target DNA and the test compound. After 1 h incubation at 37°C, integrated product was captured by streptavidin-coated 96 well plate, and quantified by alkaline phosphatase-conjugated anti-DIG antibody and CSPD chemiluminescence detection system. Lineweaver-Burk plot analyses and intercalation assays were performed to clarify the mechanism of inhibitions. To evaluate *in vitro* virus replication suppressions, single replication assays using HeLa/CD4/LTR-EGFP cell line were performed.

RESULTS: We tested 12000 small-molecule compounds and discovered one compound, carbazole derivative, with potent strand-transfer inhibitory activity. To analyse structural determinants of the strand transfer inhibitory activity, we chemically synthesized 15 derivatives with different side chains on the carbazole structural backbone. Among these 15 compounds, eight derivatives have shown potent strand-transfer inhibitions. IC_{50}s of these eight compounds ranged from 0.78 to 5.3 μM. The result of Lineweaver-Burk plot analyses indicated the carbazole derivatives as competitive inhibitor of strand transfer. No intercalation activities were observed. In HeLa/CD4/LTR-EGFP cell culture assay, IC_{50}s of the eight compounds ranged from 0.49 to 1.92 μM. However, these eight derivatives demonstrated cytotoxicity (CC_{50}=1.97 to 5.04 μM) in this HeLa cell culture.

CONCLUSION: We have successfully found novel small-molecule IN inhibitory compounds carbazole derivatives. Though their strong cytotoxicity may limit carbazole derivatives to be used in clinical at this moment, it can be the lead compound for developing novel IN inhibitors. In addition, analysing IN inhibitory mechanisms of carbazole may give more detailed information of HIV-1 IN structure and function.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cellular Immunology 229 (2004) 117–129
Cellular Immunology
www.elsevier.com/locate/ycimm

Phenotype and function of GM-CSF independent dendritic cells generated by long-term propagation of rat bone marrow cells☆

Hua Yan[a,b,c], Tohko Miyagi[a], Eigo Satoh[a,d], Wataru Sugiura[b], Naoki Yamamoto[b,c], Hiromitsu Kimura[a,*]

[a] *Department of Research Surgery, National Center for Child Health and Development, Tokyo, Japan*
[b] *Aids Research Center, National Institute of Infectious Disease, Tokyo, Japan*
[c] *Department of Molecular Virology, Graduate School, Tokyo Medical and Dental University, Japan*
[d] *Department of Surgery, Graduate School, Tokyo Medical and Dental University, Japan*

Received 3 February 2004; accepted 24 July 2004

Abstract

GM-CSF is believed to be an essential factor for growth and differentiation of *myeloid* dendritic cells (DC). Employing a low-density fraction of rat bone marrow cells, we attempted to generate DC with human Flt-3/Flk-2 and IL-6. In this culture system, typical DC gradually appeared without exogenous GM-CSF supplement. Phenotypes and functions of the DC were examined. Evidence provided that the most efficient long-term outgrowth of DC progenitors was obtained by *GM-CSF independent* culture systems with the aid of Flt3/Flk-2 and IL-6, not with c-kit ligand and IL-6. Furthermore, CD103 (OX-62), which is widely used for rat DC separation, was found to be insufficient for enriching DC, due to the down-regulation of the marker. However, the most efficient selection of rat DC was made by CD161a (NKR-P1A), a C-type lectin family. The *GM-CSF independent* DC was functionally active in vitro as well as in vivo assays.

Keywords: Rat dendritic cell; GM-CSF; IL-4; TNFα; c-kit ligand; Flt3/Flk2 ligand; IL-6; APC; CD161a

1. Introduction

Klinkert and Bowers [1,2] first described a method to generate specialized antigen presenting cells (APC), dendritic cells (DC) [3], and/or veiled-type cells (VC) [4] from low-density fraction of bone marrow cells under a serum free or conditioned medium. DC are known to bear distinguishing morphology and distinct phenotypes from various types of macrophage (Mϕ) populations judged by their phenotypic characteristics [5–7]. Nevertheless, DC are generally difficult to obtain in a substantially large number for many experimental purposes, due to their paucity in the peripheral lymphoid tissues. In this regard, however, Steinman and his colleagues reportedly generated a substantially large number of DC with an in vitro system employing granulocyte–macrophage colony-stimulating factor (GM-CSF). Hence, GM-CSF was believed to support both growth and differentiation from DC-precursor and/or its progeny in mouse BMC and peripheral blood, respectively [8,9]. Additionally, it has also been reported that co-stimulatory factors such as tumor necrosis factor (TNF-α) [10,11] and/or IL-4 were also effective in enhancing human DC induction from BMC culture. Furthermore, it has been shown that pure human DC

☆ This study was supported by a Research Grant from the Ministry of Health and Welfare of Japan, a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan, and the Human Science Foundation of Japan.
* Corresponding author. Fax: +81 3 3414 8121.
E-mail address: chimera@nch.go.jp (H. Kimura).

0008-8749/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellimm.2004.07.006

colony was generated from CD34+ cells by additional cytokines such as c-kit ligand with GM-CSF and TNF-α for a long-term BMC culture [12].

DC-like or DC-lineage APC, interstitial DC including skin Langerhans cells, found in many non-lymphoid tissues [13], is believed to play a primary role in initiating immune responses accompanying transplanted tissues and organs. However, inasmuch as the most, if not all, DC or DC-related APC are derived from hematogeneous organs such as BMC, it is important to determine the crucial cytokine that induces cell growth, differentiation and migration of DC from its BMC progeny. In particular, the presence of GM-CSF independent DC subset was demonstrated [14]. Heretofore, subsets of the DC, *myeloid*, *lymphoid*, and/or *plasmacytoid* DC, have been proposed by their phenotypes, differential function to produce specific cytokines, and their growth requirements [14,15]. Thus, it is important to identify a specific factor and a general principle to induce growth and differentiation of DC from their progenitor.

DC play an important role in cellular immunity. However, obtaining a large number of DC for experimental as well as clinical settings requires multi-step separations and time-consuming processes. Thus, it is necessary to establish a simple and efficient culture system to generate a large number of well-characterized DC so that wide audiences in cellular immunology as well as clinical medicine are able to use the DC for their applications.

In this study, we examined recombinant cytokines including GM-CSF and IL-4 to induce the growth and/or differentiation of DC precursors in rat BMC. Furthermore, using Flt-3/Flk-2 and c-kit ligand known as hematopoietic cell growth factors (type III membranous tyrosine kinase), with IL-6, we also attempted to determine a minimal requirement to increase the frequency of DC precursor(s) from hematopoietic stem cells to obtain a practical amount of functionally mature DC from rat BMC culture systems.

Our study demonstrates that unlike mouse systems, GM-CSF per se was not able to support a meaningful growth of rat DC progenitor in BMC, regardless of the cytokine sources, murine, or human. However, we did obtain the outgrowth of the precursors that contained DC progenitors by 1000-fold with combined cytokines, either Flt-3/Flk-2ligand with IL-6 or c-kit ligand (stem cell factor: SCF) with IL-6, the former combination produced far better yields than the latter in terms of outgrowing DC-committed progenitor cells. Thus, a relatively large number of rat DC was obtained from the single step long-term (two or more months) BMC culture without the multi-step and time consuming processes of BMC. Furthermore, our study provided evidence that GM-CSF, TNF-α, and IL-4 promote differentiation of DC and hence down-regulate the growth of DC progenitors.

2. Materials and methods

2.1. Animals

Inbred strain of male and female rats that include LEW (MHC: RT1l), DA (MHC: RT1avl), PVG (MHC: RT1c) and its hybrid (DA × LEW) F1, (LEW × PVG) F1 as well as recombinant strain of PVG.1U (MHC: RT1u) where PVG background and its MHC was derived from WF (MHC: RT1u), were originally obtained from Harlan Olac (Blackthorn, Bicester, England). C57BL/6 (MHC: H-2b) mice were obtained from Tokyo Experimental Animal (Tokyo, Japan). Animals were maintained in our animal facility under the specific pathogen free.

2.2. Reagents

Monoclonal antibodies (mAbs) that included mouse anti-rat FITC-CD103 (MRC-OX62), PE-CD103 (MRC-OX62) used for specific rat DC marker [16] were purchased from Serotec Bioproduct, UK (Dainippon Pharmaceutical, Osaka, Japan). Likewise, mouse anti-rat FITC-CD3, FITC-CD4, PE-CD4, FITC-CD8, PE-CD8, FITC-CD80, FITC-CD86, PE-CD161a (NKR-P1A) were purchased from BD Immunocytometry products (BD Pharmingen International, Fujisawa Pharmaceutical, Osaka, Japan), respectively. Mouse anti-rat hybridoma cloned cell lines that included anti-rat class I (MRC-OX18), class II (MRC-OX6, MRC-OX3), ED-1, ED-2, TcR (R73), CD3, W3/25, MRC-OX35, MRC-OX38 (CD4), MRC-OX8 (CD8), MRC-OX39 (CD25), MRC-OX26 (CD71), MRC-OX7 (CD90), MRC-OX43, HIS24 (CD45R, mouse CD45R/B220 equivalent) were obtained from European Collection of Animal Cell Cultures (Salisbury, United Kingdom). Hybridoma cell line IA-29 (mouse anti-rat CD54) was donated from Dr. Masayuki Miyasaka (Osaka University, Osaka, Japan), and 3H5 (mouse ant-rat CD80), 24F (mouse ant-rat CD86) were generously provided by Dr. Hideo Yagita (Juntendo University, Tokyo, Japan), respectively.

Cytokines that included recombinant mouse and human GM-CSF, human IL-6 were generously supplied by Kirin Brewer (Maebashi, Gunma, Japan). Likewise, recombinant rat c-kit ligand (SCF) was generously provided by Amgen (Thousand Oaks, CA). Recombinant rat IL-2 and IL-4 were purchased from R&D Systems, USA (Funakoshi, Tokyo, Japan). Recombinant human Flt3/Flk2 ligand, rat GM-CSF were purchased from PeproTech, USA (IBL, Gunma, Japan).

2.3. Culture medium

Medium for primary cell preparation was performed by Dulbecco's phosphate-buffered saline (D-PBS) (DAB, OXOID, Basingstoke, Hampshire, UK).

For cell cultures, different medium with or without serum (fetal bovine serum: FBS) were employed.

(1) Serum-free RPMI1640 containing 25 mM Hepes (Whittaker, Walkersville, MD) supplemented with 2 mM L-glutamine and antibiotics was used to determine the effect of single cytokine on the growth of BMC.
(2) For screening purpose to analyze the effects of combined cytokines, complete medium (CM) RPMI1640 supplemented with 10% FBS (Hyclone, Logan, UT), 2-mercaptoethanol (5×10^{-5} M), 2 mM L-glutamine and antibiotics was employed.
(3) For generating a large scale of DC from a long-term culture of rat BMC, GIT (NIHON Pharmaceutical, Tokyo, Japan) supplemented with 2 mM L-glutamine, and antibiotics without 2-mercaptoethanol was employed. This medium was already supplemented with well conditioned-serum components.
(4) For mixed lymphocyte culture (MLC), complete medium (CM) RPMI1640 supplemented with 2.5% LEW rat or 10% FBS (Hyclone, Logan, UT), 2-mercaptoethanol (5×10^{-5} M), 2 mM L-glutamine, and antibiotics was employed.

2.4. Cell preparation

Suspensions of spleen, lymph node, thoracic duct lymphocytes (TDL), and BMC were prepared according to standard procedures [17].

2.5. Preparation of lectin-free conditioned medium

Conditioned medium was prepared according to the method by Kilinkert [2], as described before [5], with several modifications. In brief, spleen cells from LEW rat was stimulated by Concanavalin A (Con A) for 2 h, and were further incubated for 120 h after washing out residual Con A.

2.6. Examination of cytokines

Ten to 10,000 units of recombinant murine (mouse, rat) and human GM-CSF or 15% of the conditioned medium was employed in 1.5 ml RPMI-1640 medium supplemented with 10% FCS, 5×10^{-5} M of 2-mercaptoethanol, 2 mM L-glutamine and antibiotics. The initial cell dose was 7.5×10^6 cells/1.5 ml and cultured in 24-well plates (Nunc, Naperville, IL).

To examine the effect of single or combined cytokines on the growth and differentiation of DC from BMC, RPMI-1640 medium supplemented with 10% FBS, 2-mercaptoethanol (5×10^{-5} M), 2 mM L-glutamine and antibiotic was used.

2.7. Cell surface analysis by flow cytometry

An aliquot of cell suspension was stained with monoclonal antibodies and analyzed by flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA).

2.8. Cell separation by autoMACS systems

According to the manufacturer's procedures (autoMACS systems: Miltenyi Biotec, Bergisch Gladbach, Germany), the positive selection of cells was performed. Briefly, cells were first incubated with FcR blocking antibody for 10 min on ice and then stained with PE-conjugated monoclonal antibody for 30 min on ice. After two times washing by D-PBS, the cells were further incubated with anti-PE magnetic microbeads (10^7 cells/20 μl, Miltenyi Biotec, Bergisch Gladbach, Germany), incubated for 30 min at 4 °C temperature. Cells bound by magnetic-beads were likewise washed carefully and re-suspended in 4 ml buffer solution, and followed by magnetic separation by autoMACS systems.

2.9. Cytospin preparation

To assess the morphological characteristics of proliferating DC, cytospin preparations of 1×10^4 cells were made in a cytocentrifuge (Shandon, Pittsburgh, PA, USA) in 800 rpm for 10 min, stained with May–Gruenwald and Giemsa (Merck Japan, Tokyo, Japan), and examined by light microscopy.

2.10. Skin grafting

The procedure of skin grafting was performed according to standard procedures [18]. In brief, full thickness of donor male trunk skin was removed and the skin muscles were trimmed off by a pair of forceps. Size of the donor skin was 2×2.5 cm; approximately 5 cm^2. Female recipient's skin bed was laterally prepared by trimming off the epidermal layer while keeping the skin muscle by fine optical scissors. Graft skin was sutured, gauzed and bandaged by elastic bandage. Nine days after skin grafting, the bandage was removed and inspected by daily bases for first 30 days and at least twice weekly thereafter.

2.11. Graft versus host (GVH) assay

The popliteal lymph node weight assay [19] was employed for screening purposes and single dose assays were performed. In brief parental donor cell suspensions were prepared at 10×10^6/0.1 ml and injected into each foot pad of 4–6-week-old F1 hybrid. Following the foot pad-injection, draining popliteal lymph node was excised at day 7 and weighed.

2.12. Mixed lymphocyte culture

One source of responder LEW T cells was prepared from LEW rat that had been lethally irradiated (10 Gy) and subjected to the thoracic duct drainage. Normal 1.5×10^9 LEW TDL consisting of 70–80% T cells were collected from three to four LEW donors. After intravenous inoculation of the LEW TDL into the lethally irradiated syngeneic LEW recipient, we collected the first 8–30 h thoracic duct lymph from the lethally irradiated LEW rat. This population is known to contain more than 99% T cells. Tentatively, this T cell population was labeled as LEW_{-LEW}. The other source of responder LEW T cells was likewise labeled LEW_{-DA}. Thus, 1.5×10^9 LEW TDL was injected into the lethally (10 Gy) irradiated DA rat that had been subjected to similar thoracic duct drainage. This LEW_{-DA} population is known to be incapable to cause GvH reactivity to DA in vivo as well as MLC reactivity in vitro, respectively. Employing these responder LEW T cells (LEW_{-LEW} or LEW_{-DA}) syngeneic MLC as well as allogeneic MLC was set up as follows: For syngeneic MLC, 2×10^5/0.1 ml responder LEW T cells were stimulated with a graded dosage of syngeneic LEW DC, from 5×10^4/0.1 to 0/0.1 ml cells. Normally, 0.5 μCi methyl-[^{3}H]thymidine (Amersham International, Amersham, UK) was added to 25 μl of cell suspension at day 4 to day 6 in a 6 h pulse. Likewise, for allogeneic MLC, 2×10^5/0.1 ml responder LEW T cells were stimulated with a graded dosage of allogeneic DA DC.

Standard deviations of [^{3}H]thymidine incorporation were determined from a minimum of four replicated micro-cultures.

2.13. Irradiation

To ensure uniformity of tissue distribution of the radiation dose, rats were rotated in a Perspex box at 15 rpm around a vertical axis in the horizontal beam from two-way X-ray irradiation source (MBR-1520A-TWZ; Hitachi Medico, Tokyo, Japan). The beam was filtered with shaped lead disks to a dose uniformity across the beam of >96%. The dose was delivered at approximately 1 Gy/min.

3. Results

3.1. Effect of GM-CSF on rat bone marrow cell cultures

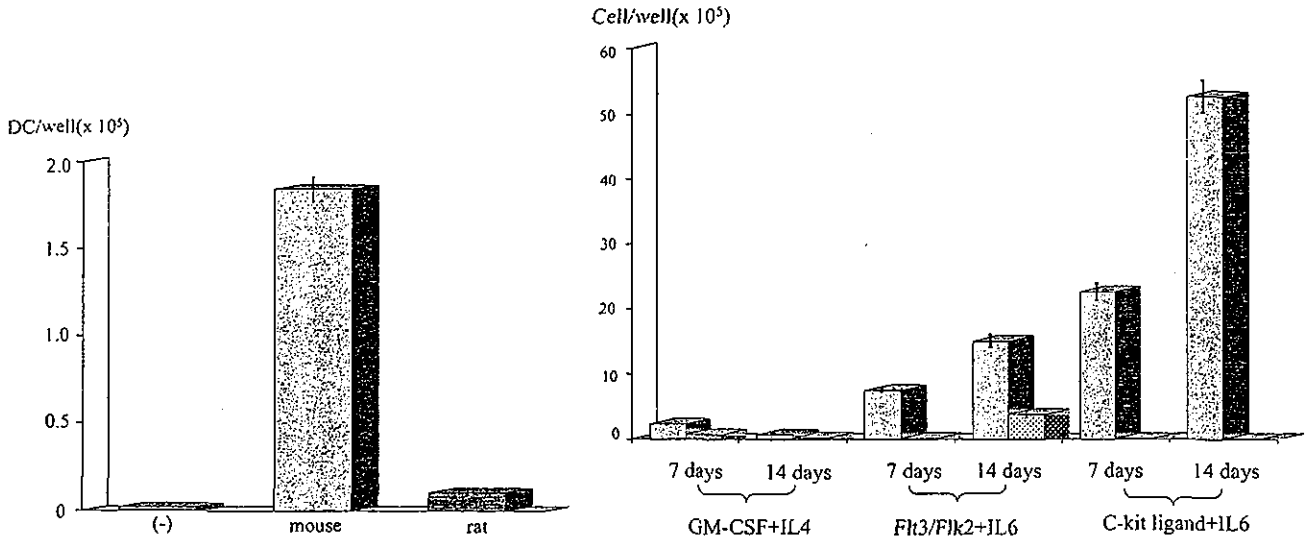
Our attempt to generate rat DC by a serum-free medium supplemented with a conditioned medium [5] based on the early studies [1,2] led to a limited amount of DC recovery, final cell recovery was usually around 0.1% of total BMC input. Hence, attempts were first made to determine whether and to what extent single cytokine GM-CSF is able to increase DC yields in short-term cultures of rat BMC as observed in mouse systems [8]. For this purpose, BMC culture supplemented with single recombinant GM-CSF was compared with those employing serum-free RPMI1640, or those with conditioned medium.

Un-manipulated whole BMC at 7.5×10^6/1.5 ml were cultured for one week. On day 7, free-floating cells were harvested, and counted under phase-contrast microscopy. In this particular experimental setting, unlike mouse systems, even species-matched GM-CSF was unable to generate a significant increase of rat DC recovery, as shown in Fig. 1A. Although we examined a wide range of GM-CSF concentrations from 1 to 1000 ng/ml, the results were not significantly altered, and final DC recovery in mouse systems was found to be, at most, 1.8×10^5 DC/7.5×10^6 BMC/well (final DC recovery was approximately 2.3 ± 0.4% of the initial BMC input compared with that of 0.2 ± 0.03% in rat DC recovery). It should be noted that rat GM-CSF per se has nearly equal activity for granulocyte–macrophage (GM) colony formation under an agar culture system for both mouse and rat BMC, however, the mouse GM-colony was significantly larger than those of rats (data not shown), and most of the BMC appeared to proliferate and adhere to the culture dishes. Despite the growth-promoting activity, final DC recovery by GM-CSF was not as high as was expected.

3.2. Effect of GM-CSF and IL-4 on rat bone marrow cell cultures and comparison of other putative cytokines for DC induction

Based on the studies by others, we examined three sets of culture systems in an effort to seek better culture systems to obtain a large number of DC from rat BMC progenitors. Thus, one culture system adopted an FBS-supplemented RPMI1640 medium containing two cytokine combinations, mouse GM-CSF and rat IL-4 [20]. The other two sets of culture systems were based on the reports that employed type III membranous tyrosine kinase, as a growth factor for hematopoietic stem cell, c-kit ligand or Flt3 ligand. It has been shown that the former type III membranous tyrosine kinase c-kit ligand generated pure human DC colony from CD34+ cells of BMC with GM-CSF and TNFα [12], and the latter Flt3 ligand IL-6 combinations have been shown as growth factors for primitive multipotential hematopoietic progenitor cells [21].

The single step of low-density separation method (*d*; 1.077) was chosen. Inasmuch as our preliminary experiments repeatedly demonstrated that a single step of two-layers separation which employed two layers of Ficoll-based separation medium, i.e., the light density (*d*; 1.077) medium over the high density (*d*; 1.094) medium and each layer was cultured separately, resulted in a



(A) Production of DC with recombinant GM-CSF. (B) Effect of cytokines of BMC cultures and DC induction.

Fig. 1. (A) About 7.5×10^6/1.5 ml rat bone marrow cells (BMC) and mouse BMC was cultured for one week with recombinant rat GM-CSF and mouse GM-CSF under various concentrations of GM-CSF under serum free medium, respectively. At day 7, survived, free-floating cells were harvested and the number of typical veiled shaped DC was counted by phase-contrast microscopy. Each column was expressed by an average of quadruplicated culture wells. Figure showed representative results of BMC culture employing GM-CSF at the dosage of 20 ng/ml. Left column was from serum free RPMI1640 medium supplemented lectin-free condition medium described in Section 2. Central column expressed final DC recovery from mouse BMC culture with mouse GM-CSF. Likewise, right column expressed the number of DC from rat BMC culture supplemented with rat GM-CSF. (B) 7.5×10^6/1.5 ml low-density (*d*; 1.077) rat bone marrow cells were cultured for two weeks with three different conditions under 10% FBS supplemented-RPMI 1640 medium. Namely (1) rat GM-CSF (20 ng/ml) and rat IL-4 (10 ng/ml) (2) human Flt3/Flk2 ligand (100 ng/ml) and human IL-6 (10 ng/ml) (3) rat c-kit ligand (100 ng/ml) and human IL-6 (10 ng/ml). At day 7 and day 14, survived, free-floating cells were harvested and the number of typical veiled shaped DC was counted by phase-contrast microscopy, respectively. Total cells (open column) and content of DC (shaded column) were compared with each experiment. Each column was expressed by average of quadruplicated culture well.

far higher yield of DC recovery in the low-density fraction of BMC (data not shown).

Thus, the low-density (*d*; 1.077) rat bone marrow cells were cultured for two weeks with three different conditions under 10% FBS supplemented RPMI 1640 medium. Namely,

(1) rat GM-CSF and rat IL-4,
(2) human Flt3/Flk2 ligand and human IL-6,
(3) rat c-kit ligand and human IL-6.

At day 7 and day 14, survived, free-floating cells were harvested and the number of typical veiled shaped DC was counted by phase-contrast microscopy, respectively. Total cells and content of DC were compared with each experiment.

As shown in Fig. 1B, the Flt3/Flk2 ligand with IL-6-supplemented culture medium was found to produce the best recovery of veiled-type DC at 14 days, compared with other culture conditions. During the course of BMC culture, the DC recovered by BMC culturing was often dependent upon FBS regardless of the cytokine concentration. Indeed, DC induction was never succeeded by a single cytokine such as Flt3/Flk2 without FBS supplementation (data not shown). To standardize and simplify the culture systems and to avoid the lot difference of FBS, we employed the well-defined culture medium, GIT supplemented with freshly prepared L-glutamine and single antibiotics for further study. Under the conditions, generation of DC from rat BMC appeared to be stable. Thus, we performed phenotypic and functional analyses with GIT-based culture systems instead of the RPMI1640 culture medium.

3.3. Histological examination and FACS analysis of DC

Fig. 2A (fresh BMC) depicts representative flow cytometric analysis of low-density BMC, and Fig. 2B (at eight weeks) depicts that of DC induced by GIT based medium supplemented with human Flt3 ligand and IL-6. When DC were generated by Flt3/Flk2 ligand and IL-6 for eight-week's culturing, the most prominent picture was evident in anti-CD161a (NKR-P1A), a C type lectin family [22], which has been described as a marker for rat NK cells [23]. Additionally, anti-CD70 (thy1.1) with two phases of dull and bright population, CD80 and CD86 of B7 family, and CD54 (ICAM-1) of co-accessory molecules with a single peak, were found to be consistently positive. By contrast, CD11b/c (MRC-OX42) expressed on most of the typical Mϕ, and CD45R/B220 (HIS24) which is considered to be a counter part of mouse B220, a marker for *plasmacytoid* DC, was down-regulated and stained as

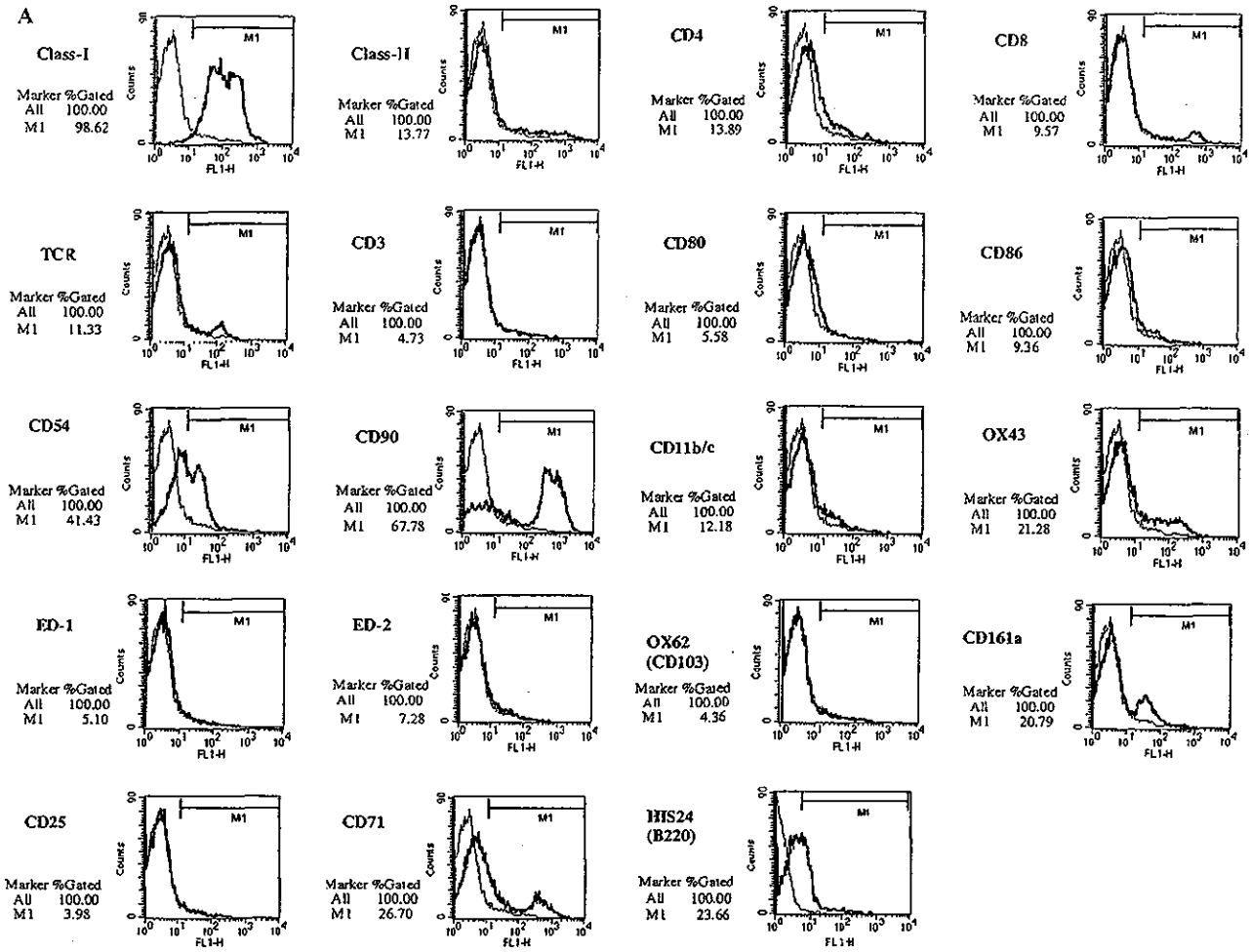


Fig. 2. (A) Cell surface phenotypes of LEW fresh bone marrow cells (BMC) analyzed by various mAbs which are related with antigen presenting cells (APC). These include mAbs specific for class I (MRC-OX18), class II (MRC-OX6) and co-accessory molecules for B7.1 (CD80), B7.2 (CD86) and for functional cell surface molecules of Mϕ for complement $CD11_{b/c}$ and CD161a (NKR-P1A) of the C-type lectin family expressed on NK cells. (B) Cell surface phenotypes of outgrowing DC derived from LEW bone marrow cell cultures for eight weeks supplemented with human Flt3/Flk2 and human IL-6, were analyzed by various mAbs with a unique and restricted region where DC accumulated. Antibodies used are mainly concerning with antigen presenting cells (APC). These include mAbs specific for class I (MRC-OX18), class II (MRC-OX6) and co-accessory molecules for ICAM-1 (CD54), B7.1 (CD80), B7.2 (CD86) and for functional cell surface molecules of Mϕ for complement $CD11_{b/c}$ and one of the C-type lectin family expressed on NK cells, CD161a (NKRP1A). Rat DC specific MRC-OX62 (CD103), rat Mϕ specific CD4, $CD11_{b/c}$, ED1, ED2, OX43 were all negative or extremely dull. Consistent finding was most of DC under the culture conditions expressed CD161a (NKR-P1A) at a relatively high level. (C) Representative flowcytometric analysis of cell surface markers of outgrowing DC derived from LEW BMC; BMC cultures supplemented with human Flt3/Flk2 and human IL-6 at two weeks and three weeks, were positively selected by AutoMACS cell separation systems with PE-CD161a (NKR-P1A) and anti-PE micromagnetic beads described in Section 2. Cells were analyzed by various FITC-mAbs. Antibodies used are mainly concerning with antigen presenting cells (APC). These include mAbs specific for class II (MRC-OX6) and co-accessory molecules for B7.2 (CD86) and for functional cell surface molecules of $CD11_{b/c}$ (MRC-OX42) and rat DC specific MRC-OX62 (CD103), CD4 specific for a subset of rat Mϕ as well as CD8. Consistent finding was that most of CD103+ (MRC-OX62) DC, if not all, was down-regulated or lost the marker during the extended culture and hence became dull or null (see B).

nearly null cells. As for the CD4 as well as CD8 that has been used to define *lymphoid* DC in splenic and thymic *lymphoid* DC in mice, likewise, not expressed. Furthermore, unlike co-accessory molecules that appeared to be up-regulated during the culture, CD103 (OX-62) which has been widely used for one of the rat DC specific marker was found to be down-regulated during the culture (Fig. 2C, at two weeks vs. at three weeks, and B).

Based on these profiles, positive selection of fully mature DC by CD54, CD71, CD80, CD86, and/or CD161a was considered for final purification of DC. Nevertheless, inasmuch as co-accessory molecules such as CD54, CD80 and CD86 play important roles in T cell activation, use of these markers for DC separation may not be suitable for the subsequent examination of purified DC as APC in in vitro as well as in vivo studies. Therefore, a population of $CD161a^+$ DC was enriched in high purity (>98%) by automatic cell separation system as described in Section 2. Their functional aspect as antigen-presenting cells (APC) were analyzed in the