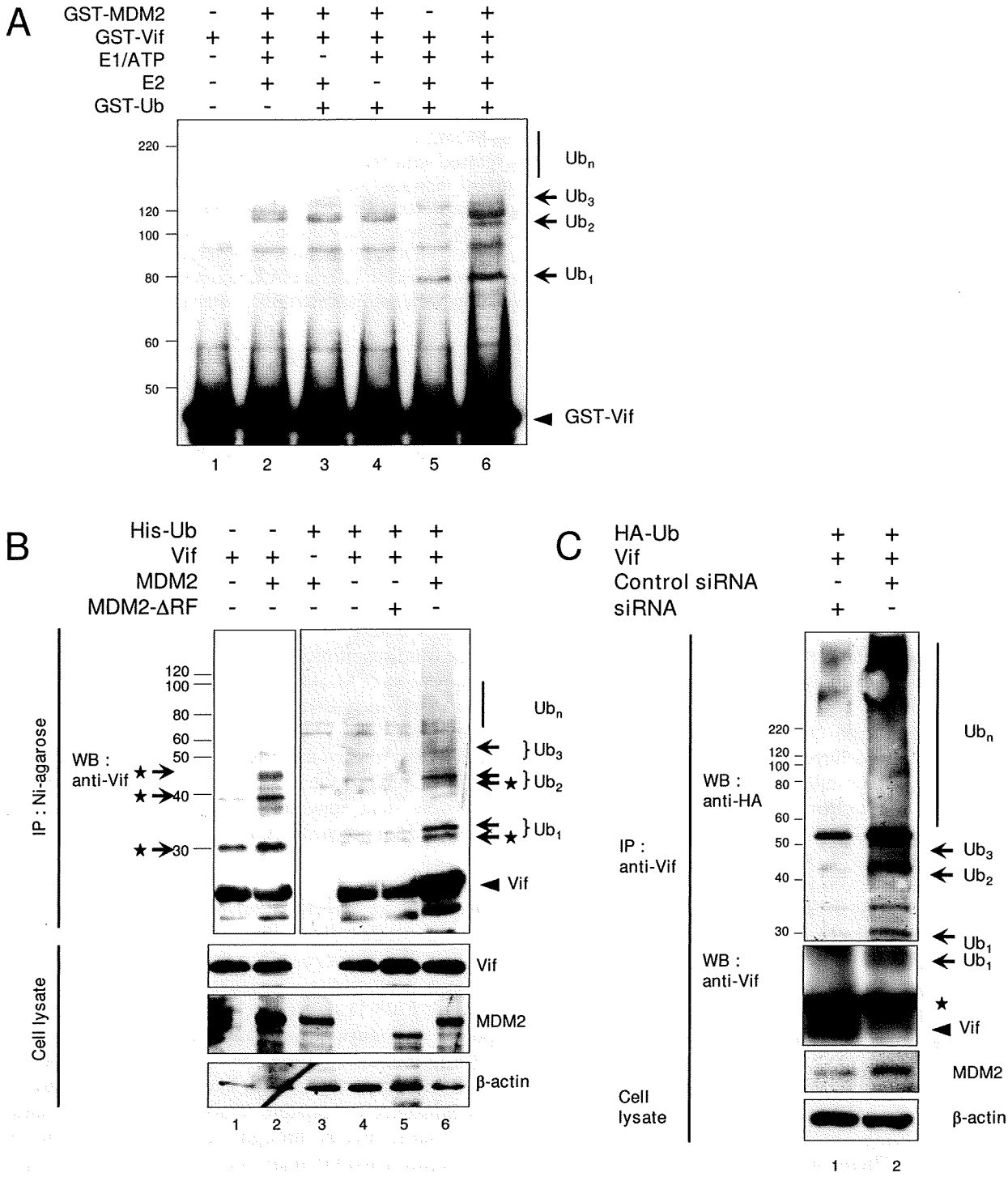

Figure 4 *(see legend on next page)*

Figure 4 *(see previous page)*
MDM2 induced the polyubiquitination of Vif *in vitro* and *in vivo*. (A) GST-MDM2 induced the polyubiquitination of Vif *in vitro*. Bacterially expressed GST-Vif was subjected to *in vitro* ubiquitination assays. The reaction was performed in the presence or absence of E1, E2, GST-MDM2, and GST-Ubiquitin as indicated. Reactions were subjected to immunoblotting with anti-Vif mAb. Arrows indicate GST-ubiquitin-conjugated Vif. (B) Overexpressed MDM2 induced the polyubiquitination of Vif *in vivo*. HEK293T cells were cotransfected with expression vectors for MDM2 Wt and a ΔRF mutant together with expression vectors for Vif and His-Ubiquitin (His-Ub) as indicated. Cells were treated with MG132 for 6 hrs, and cell lysates were precipitated with Ni-NTA agarose beads followed by immunoblotting with the indicated Abs. Since Vif naturally bound to Ni-NTA agarose, we detected a Vif band itself (arrowhead), whereas no signal was detected in cells lacking Vif (lane 3). Arrows indicate His-Ub-conjugated Vif. Arrows with asterisk indicate Vif conjugated with endogenous ubiquitin. (C) Transduction of siRNA reduced cellular levels of endogenous MDM2 and polyubiquitination of Vif. HEK293T cells were cotransfected with expression vectors for MDM2 siRNA and control siRNA together with expression vectors for Vif and HA-Ubiquitin (HA-Ub). Cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblotting with the indicated Abs. Asterisk indicates immunoglobulin light chains from the immunoprecipitation.

sion of exogenous MDM2 efficiently induced polyubiquitination of Vif *in vivo*. Furthermore, the knock-down of endogenous MDM2 expression by introduction of MDM2-specific short interfering RNA (siRNA) resulted in a significant reduction in the amount of polyubiquitinated Vif, commensurate with the extent of reduced MDM2 expression (Fig. 4C). Collectively, these data indicated that MDM2 mediates polyubiquitination of Vif both *in vitro* and *in vivo*.

MDM2 negatively regulates HIV-1 replication in non-permissive cells through ubiqutination and degradation of Vif

Next, we examined the effect of MDM2 on HIV-1 replication. In a single round infection assay (Fig. 5A), in the absence of A3G, viral replication was not affected by expression of MDM2 and/or Vif (lanes 1–6). In contrast, in the presence of A3G in a non-permissive cell setting, without the expression of MDM2, the wild type virus could replicate but the ΔVif virus could not, as previously reported (lanes 7 & 8) [3,8]. Co-expression of MDM2 reduced the cellular level of Vif (Fig. 5B, upper panel, lanes 5 & 11), resulting in the increased virion incorporation of A3G (Fig. 5B, 2nd lower panel, lane 11 as compared with lanes 7) and the greater suppression of viral replication (Fig. 5A, lane 11 as compared with lane 7).

We also tested the effect of MDM2 on HIV-1 replication in the presence of A3F. MDM2 suppressed viral replication in the presence of A3F, similar to results shown for A3G (Additional file 3). These data indicated that the MDM2-mediated Vif downregulation led to upregulated cellular A3G and A3F levels in producer cells, resulting in less infectious HIV-1 virions produced. Since MDM2 was previously reported to upregulate HIV-1 transcription by ubiquitination of Tat, we further examined HIV-1 replication in macrophages knocked down for MDM2 (Fig. 5C). We chose terminally differentiated macrophages as the target, because the knockdown of MDM2 is lethal for proliferating cells. HIV-1 replicated more efficiently in macrophages transfected with MDM2 siRNA than in control siRNA-transfected macrophages. These data indicated that MDM2 negatively regulated HIV-1 replication in non-permissive target cells through the ubiquitination and degradation of Vif.

To obtain further insights into the mechanisms why our MDM2 system did not induce the ubiquitination of A3G which was bound to Vif, we tested the expression levels and the binding affinity of A3G to Vif in transfected cells. Co-expression of MDM2 reduced the cellular levels of Vif and inversely increased the A3G levels in a dose dependent manner (Fig. 5D). Immunoprecipitation assays revealed that the co-expression of MDM2 blocked the binding of A3G to Vif in a dose dependent manner (Fig. 5E). These data suggest that the interaction between MDM2 and Vif precludes A3G from binding to Vif.

Discussion

In this study, we report that MDM2 is a novel E3 ligase for HIV-1 Vif. MDM2 physically interacts with Vif and functions as an E3 ligase for Vif to induce its polyubiquitination and proteasomal degradation. Several E3 ligases including Cul5 [17], Nedd4, and AIP4 [18], have been reported to induce Vif ubiquitination, and the roles of Cul5 for Vif ubiquitination and degradation are especially well documented. Dang et al. have recently reported that Cul5 induces A3G degradation not by direct ubiquination of A3G but indirectly through Vif ubiqutination and that polyubiquitinated Vif might serve as a vehicle to transport A3G into proteasomes for degradation [23]. In this manuscript, we show that MDM2 only targets Vif for degradation but not A3G, although MDM2 and Cul5 both induce Vif ubiquitination (Additional file 2, part A). MDM2 reduced cellular Vif levels and inversely increased A3G levels (Fig. 5B &5D), unlike Cul5. One possible explanation is that the binding of MDM2 to Vif precluded A3G from binding Vif (Fig. 5E), whereas a Cul5-Vif complex

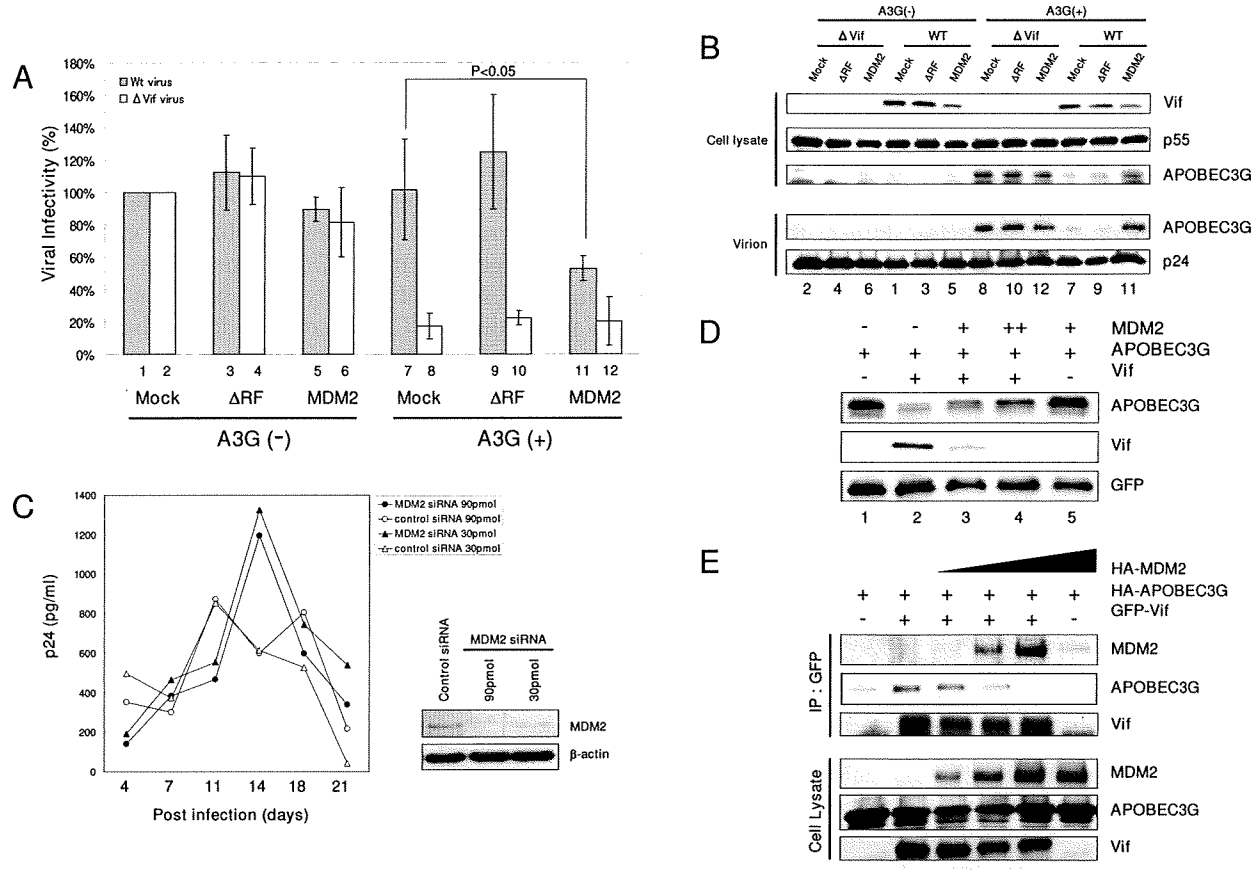

Figure 5

MDM2 negatively regulated HIV-1 replication in non-permissive cells through the degradation of Vif. (A) The overexpression of MDM2 inhibited HIV-1 replication in the presence of A3G. NL-43 Wt and ΔVif viruses were produced from HEK293T cells transfected with expression vectors for MDM2 Wt and a ΔRF mutant in the presence or absence of A3G. The viral infectivity was examined using M8166 cells. Values are presented as averages of more than 3 independent experiments. (B) MDM2 reduced cellular levels of Vif, resulting in more incorporation of A3G into HIV-1 virions. Immunoblotting for cell lysates (upper 3 panels) and precipitated virions (lower 2 panels) was performed with the indicated Abs. Lane numbers correspond to those in Fig. 4A. (C) HIV-1 replication in macrophages transfected with MDM2- and control-siRNA. MDM were transfected with MDM2- and control-siRNA and challenged with R5 HIV-1_{JR-FL} (left panel). Cell lysates were subjected to immunoblotting with the indicated antibodies (right panels). (D) Coexpression of MDM2 reduced cellular levels of Vif and inversely increased A3G levels in a dose dependent manner. HEK293T cells were cotransfected with expression vectors for A3G, Vif, GFP, and MDM2 as indicated. Cell lysates were subjected to immunoblotting with the indicated Abs. (E) Immunoprecipitation assays revealed that the coexpression of MDM2 blocked the binding of A3G to Vif in a dose dependent manner. HEK293T cells were cotransfected with expression vectors for A3G, GFP-Vif, and MDM2 as indicated. Cell lysates were immunoprecipitated with anti-GFP mAb followed by immunoblotting with the indicated Abs.

can bind A3G to form a ternary complex. MDM2 binds the N-terminal region of Vif which does not overlap with, but is close to the A3G/A3F binding domain [25]. This binding might affect the interaction of Vif with A3G and/or A3F. Furthermore, the evidence that an MDM2 ΔRF mutant failed to protect A3G indicated that the ubiquitination and degradation of Vif is necessary to protect A3G and A3F from Vif. These findings suggest that different E3 ligases might play different roles in Vif ubiquitination. Further studies on the different roles of Vif ubiquitination by different E3 ligases and their virological significance should be investigated.

We demonstrate that MDM2 negatively regulated HIV-1 replication through Vif degradation. Through the degradation of target proteins (p53, pRB, etc), MDM2 can exert profound physiological effects on the regulation of cell cycle, cell proliferation, DNA repairs and other processes. To our knowledge, this is the first report to show that MDM2 plays an important role in viral replication

through the degradation of viral proteins. Recently, MDM2 was also reported to ubiquitinate HIV-1 Tat protein and activate its transcriptional activity in a non-proteolytic manner [26]. Our experiment using MDM2 knockdown macrophages showed that HIV-1 replication in these macrophages was more efficient than in control siRNA-transfected macrophages. These data are consistent with MDM2 negatively regulateing HIV-1 replication through Vif ubiquitination (Fig. 5C). However, the replication efficiency of HIV-1 in MDM2 knockdown macrophages was only 2-fold higher and was slower than in control siRNA-transfected macrophages. This suggests the possibilities that the ubiquitination of Tat might work as a positive regulatory factor at an earlier phase of infection and that MDM2 might be involved in both positive and negative regulation of HIV-1 replication at different stages. Further studies on the detailed effect of MDM2 on HIV-1 replication are needed.

We also demonstrated that Vif can bind MDM2 directly. We also mapped the interaction domain of MDM2 with Vif to amino acids 168–320 which is located in its central acidic and Zn finger domains. This central domain is different from the primary p53-binding site of MDM2 which is located in its N-terminal region; however, this central deomain was recently reported as a second p53-binding site and was shown to be important for the regulation of p53 stability [27-30] (Fig. 2B &2C). Interestingly, several proteins including p300, $p14^{ARF}$, and pRB bind to the central domain of MDM2 and regulate the stability and function of p53 via MDM2 [28,31]. Thus, it is possible that Vif might affect the stability and function of p53. Indeed, we confirmed that Vif can stabilize p53 (*Izumi et al., unpublished data*), which could explain why the effect of MDM2 on p53 degradation was weaker than that on Vif as shown in Fig. 1A. A further study is under way to elucidate this new function of Vif (*Izumi et al., HIV-1 Vif induces G2 cell cycle arrest via the p53 pathway, unpublished*).

Finally, expanding evidence suggests that the ubiquitination system plays important roles in many aspects of HIV-1 replication including the degradation of A3G by Vif [9-11], the degradation of CD4 by Vpu [32], HIV-1 viral budding [33], Tat-mediated transactivation [26], and Vpr-induced G2 cell cycle arrest [34,35]. The functional linkage between Vif and MDM2 also suggests that ubiquitin processes such as the A3G/Vif interplay is highly complex. It is obvious that HIV-1 replication in target CD4+ T cells is strongly affected by the interplay of these proteins. From the viral point of view, this interplay might give an advantage to HIV-1 replication. One possibility is that MDM2 regulates cellular Vif levels appropriately, such as not to affect viral replication [36] but just enough to antagonize A3G. Recent studies suggest that the G-to-A mutations induced by A3G may not be the mechanism by which A3G restricts or controls viral replication [37] and that a partially effective Vif inhibitor may actually accelerate the evolution of drug resistance and immune escape [38]. The inhibitory activity of MDM2 toward Vif could be partially effective and therefore could lead to viral evolution of drug resistance and immune escape. More recently, Nathans et al. have reported a small molecule that specifically antagonizes Vif function and inhibits viral replication by targeting the A3G/Vif axis. This compound enhances Vif degradation only in the presence of A3G, but does not induce A3G degradation and rather stabilizes A3G. They suggested the possibility of a new proteolytic enzyme for Vif degradation and that their new compound interferes with Vif interaction with a host protein in a Vif-A3G-host protein complex, thereby making Vif less stable. The precise biological significance of this Vif-A3G-host protein complex requires future elucidation. Nevertheless, modification or intervention of such Vif-A3G-host protein interplay could lead to the development of new therapeutic strategies for HIV-1 infection.

Conclusion

MDM2 is a novel E3 ligase for Vif which induces the polyubiquitination and degradation of Vif to negatively regulate HIV-1 replication.

Methods

Plasmid constructs

Expression vectors for hemagglutinin (HA)- or FLAG-tagged MDM2, pCMV4/HA-MDM2 or pCMV4/FLAG-MDM2, and their mutants were constructed as previously described [19]. An expression vector for HA-tagged human APOBEC3G, pcDNA3/HA-hA3G [39], and HIV-1 reporter plasmids, pNL43/Δenv-Luc (WT) and pNL43/ΔenvΔvif-Luc (ΔVif) [8], were constructed as previously described. Expression vectors for FLAG-tagged Parkin and Cul5 (pcDNA3/FLAG-Parkin and pcDNA3/FLAG-Cul5, respectively) were constructed by the PCR method. Complementary DNA for HIV-1 Vif was also cloned into pDON-AI (TAKARA BIO INC.) and pDON/EGFP for expression of Vif and EGFP-fused Vif (EGFP-Vif). The subgenomic expression vector pNL-A1, which expresses all HIV-1 proteins except for *gag* and *pol* products, and its mutants expressing Vif deletion mutants were kind gifts from Dr. K. Strebel [22].

Co-immunoprecipitation assays

We performed an immunoprecipitation assay for protein-protein interaction *in vivo*, as described previously [8]. HEK293T cells were cotransfected with pCMV4/HA-MDM2 and pNL-A1 by the calcium phosphate method. Two days after transfection, cells were lysed in lysis buffer (25 mM HEPES pH7.4/150 mM NaCl/1 mM $MgCl_2$/0.5% TritonX-100/10% Glycerol) and complexes were immunoprecipitated with anti-MDM2 monoclonal antibody

(mAb) (SMP-14, Santa Cruz Biotechnology, Inc., Santa Cruz, CA and Ab-1, Calbiochem, EMD Biosciences, Inc, Darmstadt, Germany) and Protein A-Sepharose beads (Amersham Biosciences Corp.) at 4°C. The beads were washed with RIPA buffer (50 mM Tris-HCl pH8.0/150 mM NaCl/1% Triton-X 100/0.1% SDS/0.1% DOC) and analyzed by immunoblotting with anti-Vif mAb (#319) (A kind gift from Dr. M. Malim through the AIDS Research and Reference Reagent Program) [40] or anti-HA mAb (12CA5). To map the regions of MDM2 necessary for binding to Vif, HEK293T cells were cotransfected with expression vectors for a series of MDM2 deletion mutants together with pNL-A1. Complexes were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-Vif mAb. To map the regions of Vif necessary for binding to MDM2, HEK293T cells were cotransfected with expression vectors for a series of Vif deletion mutants together with pCMV4/HA-MDM2. Complexes were immunoprecipitated with anti-Vif mAb and analyzed by immunoblotting with anti-MDM2 mAb. In all these experiments, transfected cells were treated with MG132 for 6 hrs prior to harvesting in order to stabilize both Vif and MDM2; otherwise we could not detect the expression of MDM2 because of its rapid degradation, as seen in Fig. 1A.

In vitro and in vivo ubiquitination assays

In vitro ubiquitination assays were carried out in ubiquitin reaction buffer (50 mM Tris-HCl/2 mM ATP/5 mM $MgCl_2$/2 μM DTT) with E1(200 ng), E2(Ubc5c)(150 ng), and GST-tagged ubiquitin (GST-Ub) (10 μg) as described previously [13]. MDM2 and Vif were expressed as GST-fusion proteins in Escherichia coli strain DH5α and BL21, respectively. The reactions were incubated at 30°C for 90 min. The samples were subjected to immunoblotting with anti-Vif mAb to detect GST-ubiquitin conjugated Vif.

For *in vivo* ubiquitination assays, HEK 293T cells were cotransfected with plasmids expressing Vif, FLAG-MDM2 or its mutants, and His-tagged ubiquitin (His-Ub) as indicated. Cells were treated with 10 μM MG132 for 6 hrs prior to harvesting. Forty-eight hours post transfection, cell lysates were affinity-purified with Ni-NTA-agarose beads (Invitrogen corporation, Carlsbad, CA) and analyzed by immunoblotting with anti-Vif mAb.

For production of RNAi within the cells, we used the pSuper vector as described previously [19]. pSuper-MDM2-1 contained the 19 nt derived from the *mdm2* cDNA (nt 404–422) as the target sequence. Double-stranded RNA containing scrambled 19 nt was used as a control. HEK293T cells were transfected with pSuper plasmids together with plasmids expressing Vif and HA-Ub. Cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblottimg with anti-HA mAb.

Single round infection assays with HIV-1 luciferase reporter virus

Luciferase reporter viruses with or without Vif were prepared by cotransfection of pNL43/Δenv-Luc (Wt) or pNL43/ΔenvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or an expression vector for MDM2 or a mutant in the presence or absence of pcDNA3/hA3G by calcium phosphate as previously described [8]. The reporter viruses were adjusted according to p24 values and used to infect M8166 target cells. Productive infection was measured by luciferase activity and values were presented as percent infectivity relative to the value of each virus without the expression of hA3G.

Knockdown of MDM2 in macrophages and replication assays

Monocyte-derived macrophages (MDM) were cultured for 7 days from CD14+ monocytes isolated from the peripheral blood of an HIV-1-negative healthy individual. Electroporation with Stealth Select RNAi for MDM2 or Control (Invitrogen Corporation) was performed using the Nucleofector machine (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's instructions. Twenty four hours after transfection, MDM were challenged with R5 HIV-1_{JR-FL} at multiplicity of infection of 0.1 at 37°C for 3 hrs. The cells were cultured from day 4 to 21 after infection, and the concentration of p24 antigen in the supernatant was measured with an HIV-1 p24 antigen enzyme-linked immunosorbent assay [ELISA] kit (ZeptMetrix, Buffalo, NY).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TI. designed research, performed research, contributed vital new reagents, analyzed data, and wrote the paper. ATK designed research, analyzed data, wrote the paper, and organized the research. KS, KIo, and MM prepared the materials and performed a part of the research. KIwai, HK, TS, MT, SI., and HA contributed vital new reagents. YK contributed vital new reagents, performed a part of the research, and analyzed the data. HH, KItoh, and JF designed the research, contributed vital new reagents, and analyzed the data. TU analyzed the data, drafted the paper, and organized the research.

Additional material

Additional file 1

Supplementary figure 1 – the stability of Vif protein in p53-/- MEF and p53-/-MDM2-/- MEF cells. MEF cells were transfected with pDON/Vif or pcDNA3/HA-A3G. Twenty-two hours after transfection, the cells were treated with cycloheximide (CHX) for the indicated times, and cell lysates were subjected to immunoblotting with the indicated Abs.
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[http://www.biomedcentral.com/content/supplementary/1742-4690-6-1-S1.pdf]

Additional file 2

Supplementary figure 2 – immunopurified MDM2 induced the polyubiquitination of Vif in vitro. *(A) MDM2 as well as Cul5 induced the polyubiquitination of Vif. HEK293T cells were transfected with expression vectors for His-MDM2 and His-Cul5. His-tagged proteins were purified using Ni-NTA agarose and subjected to* in vitro *ubiquitination assays as described in a legend to Fig. 4A. Reactions were subjected to immunoblotting with anti-Vif Ab. Arrows indicate GST-Ub-conjugated Vif. Asterisks indicate non-specific bands associated with GST-Vif protein recognized by anti-Vif Ab, as they are seen in lanes 1 and 3. (B) MDM2 induced the polyubiquitination of Vif Wt but not that of Δ22 that was defective for binding MDM2. Filled asterisks indicate non-specific bands associated with GST-Vif protein, while white asterisks indicate those associated with GST-Vif Δ22.*
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Additional file 3

Supplementary figure 3 – the overexpression of MDM2 inhibited HIV-1 replication in the presence of A3F. Single round infection assays were performed in the presence or absence of A3F as described in a legend to Fig. 5A. Values are presented as averages of more than 3 independent experiments.
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MINI-REVIEW

Non-human primate surrogate model of hepatitis C virus infection

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ABSTRACT

More than 170 million people worldwide are chronically infected by HCV, which is the causative agent of chronic hepatitis C, cirrhosis, and finally liver cancer. Although animal models of viral hepatitis are a prerequisite for the evaluation of antiviral and vaccine efficacy, the restricted host range of HCV has hampered the development of a suitable small animal model of HCV infection. Use of the chimpanzee, the only animal known to be susceptible to HCV infection, is limited by ethical and financial restrictions. In this regard GBV-B, being closely related to HCV, appears to be a promising non-human surrogate model for the study of HCV infection. This review describes the characteristic of GBV-B infection of New World monkeys, and discusses current issues concerning the GBV-B model and its future directions.

Key words GBV-B, HCV, hepatitis C, monkey.

INTRODUCTION

Since HCV was identified as a major causative agent for non-A, non-B hepatitis in 1989 by Choo *et al.* (1), it has become evident that HCV is disseminated worldwide and is carried by an estimated more than 170 million people (2). In most advanced nations, the prevalence of HCV infection is roughly 1–2% and further dissemination is suppressed. By contrast, among developing countries the number of HCV-infected patients is still increasing due to iatrogenic exposure, including blood transfusion from unscreened donors and reuse or inappropriate sterilization of contaminated medical equipment, and injecting drug use (3). After HCV exposure, about 70% of individuals who exhibit acute infection progress to chronic liver disease, and many of these patients develop hepatic cirrhosis and hepatocellular carcinoma (2). Currently, the only treatment available for patients with chronic HCV infections is combination therapy with pegylated interferon and ribavirin. As the standard therapy is effective in only approximately 50% of patients with chronic HCV hepatitis, the other half of affected patients are still threatened by poor prognosis (4). It is therefore urgent to develop more effective therapeutics for HCV infection. At the same time, prophylactic vaccines are indispensable for prevention of further spread of HCV in developing countries, including reduction of the risk to health care workers of occupational transmission.

ANIMAL MODELS OF HCV INFECTION: RODENTS AND CHIMPANZEES

Research in infectious diseases will never progress without animal models. Because conventional small animals are not susceptible to HCV infection due to its limited host range, development of an effective prophylactic vaccine, as well as unveiling of the molecular mechanism of viral pathogenesis, has been hampered. Nonetheless, decades

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List of Abbreviations: ALT, alanine aminotransferase; CTL, cytotoxic T lymphocytes; GBV-B, GB virus-B; GE, genome equivalents; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency viruses; SIVmac, SIV derived from rhesus macaques.

of struggle have resulted in a number of animal models for HCV infection and hepatitis C.

Recently, a number of rodent models have been developed (for a review of this topic see reference 5). Rodent models, which permit HCV replication and involve transplantation of human hepatocytes, include immunotorelated fetal rat (transplantation of human hepatoma cell line Huh7 into newborn rats after prior injection of the same cells into pregnant females) (6) and immunodeficient mice such as trimera mice (7, 8) and uPA mice (9–11). These rodent models are highly useful for evaluating the efficacy of antiviral drugs and neutralizing monoclonal antibodies. In addition, a number of HCV transgenic mice have been developed. These enable direct characterization of the effects of expression of HCV genes on liver injury (5). These small animal models do not require costly facility for primates as mentioned later. While having a number of merits as mentioned above, these rodent models still have some limitations. For example the former models are not suitable for investigation of the pathogenesis of hepatitis C and the development of effective vaccine strategies, while in the latter models the proteins of interest are usually over-expressed as compared with natural HCV infection, and the integration site of the transgene may have an influence on the outcome of the study.

The chimpanzee model is the most straightforward since this animal can be experimentally infected with HCV. One third of HCV-inoculated chimpanzees develop chronic infection, while infection resolves in the remainder after an acute phase lasting 2–3 months, indicating that the chronicity rate in chimpanzees is somewhat lower than in humans (12). The chimpanzee model has been considered the primary choice for studying the relationship between the virus and host anti-viral immune responses, as well as for evaluating immunopathogenesis and the efficacy of prophylactic vaccination. However, irrespective of its benefits, many obstacles need to be overcome in order to use this model. For example in many countries it is illegal to employ the chimpanzee as an experimental animal, primarily due to ethical, (and secondly to financial), reasons. In fact, in 2004 the Dutch government decided to stop all research with chimpanzees at the biomedical primate research center in Rijswijk, Netherlands. As this was the only primate center in Europe where chimpanzees were used for biomedical research, this decision made chimpanzees unavailable as experimental animals in Europe. It is still possible to employ chimpanzees for biomedical research in some other countries, including the USA. However, the National Center for Research Resources of the National Institute of Health in the USA has recently decided not to continue to breed chimpanzees for research (13). It is estimated that the existing chimpanzees in the National Center for Research Resources will die within 30 years.

AIDS-related research has been one of the major purposes for using chimpanzees. However, due to the reasons mentioned above, as well as the endangered status of chimpanzees, nowadays AIDS scientists mainly make use of macaque monkeys infected with SIV as a non-human primate surrogate AIDS model (14, 15). The macaque/SIV model is useful since SIV is highly related to HIV-1, and induces AIDS-like diseases that are comparable to those of humans infected with HIV-1. Taking this into consideration, an alternative surrogate model which employs New World monkeys infected with GBV-B may be promising for future HCV/hepatitis C research.

GBV-B AS A NON-HUMAN PRIMATE SURROGATE MODEL OF HCV INFECTION

Among viruses so far known, GBV-B is the most closely related to HCV. However, due to a lack of epidemiological information as discussed below, GBV-B has been tentatively classified in the Hepacivirus genus of the Flavivirus family. Originally, Deinhardt *et al.* (16) found that some tamarins (genus *Saguinus*) developed hepatitis after inoculation with an inoculum obtained from a surgeon with the initials GB who had contracted hepatitis. After 11 passages in tamarins they obtained serum including GB agent(s), and were then able to achieve molecular cloning of GBV-A and GBV-B as flavivirus-like genomes (17) and to demonstrate GBV-B as an agent which could cause hepatitis in tamarins (18). Although it was unclear whether GBV-B originated from the GB inoculum or the tamarins themselves, later animal studies demonstrated that GBV-B is infectious for tamarins but not chimpanzees (19) and reciprocally that HCV is infectious for chimpanzees but not tamarins (20). These findings led to the retrospective conclusion that at least one of the tamarins employed for the *in vivo* passage study was persistently infected with GBV-B, and therefore GBV-B is probably a virus that originated in tamarins (20). However, GBV-B has not so far been isolated from additional tamarins, probably due to limited epidemiological analyses. Thus the natural host(s) and prevalence of GBV-B are yet to be determined.

CHARACTERISTICS OF GBV-B INFECTION OF NEW WORLD MONKEYS

Previous data have shown that a number of New World monkeys (parvorder *Platyrrhini*) including tamarins, the common marmoset (*Callithrix jacchus*) and the owl monkey (*Aotus trivirgatus*) are susceptible to GBV-B infection, as summarized in Table 1, although in tamarins peak concentrations of viruses in plasma are higher (10^7–10^{10} GE/ml) than in other monkeys (10^5–10^8 GE/ml) (21–29). In general, in any monkey species viremia

Table 1 Summary of characteristics of acute GBV-B infection in monkeys

Monkeys permissive of experimental infection	Tamarins (Genus *Saguinus*) Common marmoset (*Callithrix jacchus*) Owl monkey (*Aotus trivirgatus*)
Appearance of viremia	1–2 weeks post infection
Peak levels of viremia	Tamarins; 10^7–10^{10} GE/ml Marmoset and owl monkey; 10^5–10^8 GE/ml
Peak ALT levels	Approximately 200–500 IU/ml
Duration of viremia	2–3 months
Timing of seroconversion	A couple of weeks before clearance of viremia

persists for 2–3 months and is followed by clearance. GBV-B-infected monkeys with viremia usually develop self-resolving subacute hepatitis, as indicated by increases in the concentrations of serum enzymes such as ALT, gamma-glutamyltranspeptidase, and isocitrate dehydrogenase. Pathologically, degeneration and apoptosis of hepatocytes, as well as disruption and dilation of sinusoids, have been observed in the livers of GBV-B-infected tamarins with higher viremia and ALT activity (29). It is possible that GBV-B-specific CTL may cause the liver damage. However, a recent study reported that CTL are induced at a late stage of subacute GBV-B infection, and are inversely correlated with reduction in viremia (30). Since liver damage is usually found very early (1–2 weeks) after infection, when specific CTL are not observed, it is likely that viral replication in the hepatocytes leads directly to the early onset of cytopathic effects, while lower numbers of CTL may also contribute to cytotoxicity.

The clearance of viremia in the acute phase of GBV-B infection should require an effective antiviral immune response. In particular, in both GBV-B and HCV intrahepatic CTL appear to play a major role in viral clearance (30, 31). In addition, secondary GBV-B infection after clearance of the primary viremia induces a strong T cell response, leading to virtual absence of viremia, indicating that efficient memory is a key to protection from chronic viral infection (30, 32). In pre-immune chimpanzees antibody-mediated depletion of either CD4 or CD8 T lymphocytes affects their ability to control viral replication, resulting in prolonged viremia, demonstrating essential roles for both CD4 and CD8 memory in protection from viral persistence (33, 34).

On the other hand, the significance of humoral immunity in controlling GBV-B replication is still unclear. It is reasonable to assume that neutralizing antibodies also play important roles in the clearance of subacute viremia and protection from viral persistence. In the case of HCV, in one well characterized single-source outbreak of hepatitis C, viral clearance was associated with rapid induction of neutralizing antibodies in the early phase of infection, while chronic HCV infection was characterized by absent or low-titer neutralizing antibodies in this phase. Patients with resolution of infection were shown to exhibit broader cross-neutralizing activity of antibodies in the early phase of infection (35). In one chronic HCV patient who was followed up for 30 years, it has also been shown that HCV continuously escaped the host's immune system by repeated mutational changes, resulting in loss of recognition of the HCV envelope glycoproteins by antibodies (36). The fact that the sequences of envelope glycoprotein and specificity of neutralizing antibody change over time suggests that neutralizing antibodies exert selective pressure on HCV evolution. Thus, although neutralizing antibodies (and/or CTL) are not necessarily capable of controlling chronic viral infection, frequent escape from the antibodies needs so called fitness cost, resulting in the partial suppression of viral loads. Indeed, HCV-infected patients with primary antibody deficiencies have accelerated rates of disease progression (37).

Although features of the subacute phase of GBV-B infection are similar to that of HCV, a major defect of GBV-B infection as a surrogate model for HCV is that it is difficult to chronically infect monkeys. While as many as 70% of humans with HCV infection become chronically infected, only approximately a third of chimpanzees do so (2, 12). By contrast, only a few cases regarding chronic GBV-B infection have been reported so far. The best example was a case of a tamarin persistently infected with GBV-B (24); the monkey exhibited acute mild hepatitis with viremia (peak level; $\sim 10^9$ GE/ml), which reduced to a set point level (less than 10^4 GE/ml) at 16 weeks post infection, followed by a gradual increase in viremia which reached $>10^7$ GE/ml at 112 weeks post infection, along with a significant ALT increase. However, the viremia suddenly declined thereafter and became undetectable, in association with a reduction in antibody titer, and subsequent *in vivo* passage of virus obtained from the tamarin failed to reproduce persistent infection in other tamarins (24). In addition, immunosuppression of a GBV-B-infected tamarin by FK506 treatment, or infection of GBV-B with deletion of poly(U) tract in the 3′ UTR, reportedly resulted in relatively long-term persistent infection of GBV-B for up to 46 and 90 weeks, respectively (23, 27). These results indicate that GBV-B may have the potential for establishing chronic infection.

Furthermore, our recent study has demonstrated that among four common marmosets infected with GBV-B derived from a molecular clone pGBB (21), two developed long-term chronic infection for up to three years, with recurrent viremia in which plasma viral RNA levels fluctuated between undetectable and 10^5 GE/ml, which is equivalent to the case of chimpanzees chronically infected with

HCV (Iwasaki *et al.*, manuscript in preparation). Notably, the induction of antiviral antibody response as measured by anti-Core and -NS3 antibodies was delayed in both cases, followed by a gradual increase, and then sustained high antibody titers. This was in contrast with an abrupt and transient increase at the end of periods of subacute viremia in marmosets and tamarins with viral clearance. Whether a delayed antibody response is associated with persistent GBV-B infection remains to be determined.

Taken together, these findings indicate the similarity between HCV and GBV-B in regard to their ability to induce chronic infection, and also shed light on the further potential of GBV-B as a surrogate model for HCV.

FUTURE PROSPECT OF GBV-B SURROGATE MODEL

Although many questions are still to be addressed, accumulating evidence from extensive studies to date has greatly advanced the usefulness of the GBV-B as a surrogate model for HCV. The GBV-B model may be applicable for evaluating the feasibility and safety of anti-HCV vaccines employing novel viral vectors and gene therapy which creates RNA interference. For example, in a recent pilot study we showed that systemic administration of cationic liposome-encapsulated small interfering RNA to marmosets resulted in efficient regulation of GBV-B replication, indicating the usefulness of the surrogate model for proving the feasibility of RNA interference technology for future clinical application (38). This GBV-B model will also be helpful in identifying the virological and immunological factors which determine whether the outcome is acute resolving or chronic infection. While the GBV-B model appears to be valuable, development of an HCV/GBV-B chimeric virus would greatly expand the utility of the surrogate model, since it would enable us to directly evaluate antiviral vaccines and chemicals for HCV as a preclinical study. Rijnbrand *et al.* have reported that a chimeric GBV-B with 5′ untranslated region from HCV is infectious and causes hepatitis in tamarins (39). As recently demonstrated by Chevalier *et al.* (40), this will be a good model for evaluating the potential of small interfering RNA specific to HCV genome for future clinical application.

In regard to this, we may refer to an elegant precedent in the case of the macaques AIDS model. SIVmac is well known to efficiently infect, and result in the development of AIDS in macaques. Furthermore HIV-1, of which only 7% of the entire genome is derived from SIVmac, has been demonstrated to overcome the host range of authentic HIV-1, and to acquire the ability to productively infect macaque cells (41, 42). Instead of endangered chimpanzees, tamarins/marmosets which can be chronically infected with an HCV/GBV-B chimera (hopefully capable of inducing chronic hepatitis) should be the next generation of a promising non-human primate surrogate model for HCV infection, one which is similar to the macaques AIDS model. Whatever animals are used for pre-clinical study, it is important to keep in mind that results obtained from monkey models using either GBV-B or HCV/GBV-B chimera (as well as SIV or HIV/SIV chimera) may not necessarily be applicable to humans, because of potential differences in the molecular structure and/or mechanism by which antivirals and/or viral and host proteins function. Further characterization and understanding of the molecular biology and immunology of virus-host interactions will help in developing novel antiviral strategies.

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Efficient inhibition of SDF-1α-mediated chemotaxis and HIV-1 infection by novel CXCR4 antagonists

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CXC chemokine receptor-4, the receptor for stromal cell-derived factor-1α as well as human immunodeficiency virus type 1, belongs to the chemokine receptor family and has been shown to play a critical role in directing the migration of cancer cells to sites of metastasis as well as human immunodeficiency virus type 1 infection. We had previously reported that a duodenally absorbable CXC chemokine receptor-4 antagonist, KRH-1636, showed a potent anti-human immunodeficiency virus type 1 activity both *in vivo* and *in vitro*. In this study, we initially examined the effect of the compound and its derivatives on stromal cell-derived factor-1α-mediated chemotaxis of cancer cells in order to evaluate if they could be applicable as a novel inhibitor of cancer metastasis. We found that both KRH-2731 and KRH-3955 were highly potent antagonists of stromal cell-derived factor-1α-mediated chemotaxis, i.e. the derivatives exhibited 50% effective concentrations of less than 10 nM, for more than 1000-fold efficacy improvement over the prototype KRH-1636. We further demonstrated the greater anti-human immunodeficiency virus type 1 efficacy of the derivatives compared with the original KRH-1636. Taken together, the KRH-1636 derivatives KRH-2731 and KRH-3955 may be promising as a novel inhibitory drug for cancer metastasis as well as for human immunodeficiency virus type 1 infection. (*Cancer Sci* 2009; 100: 778–781)

Chemokines are secretory proteins with a molecular weight of about 8–14 kDa, and are generally alkaline and heparin-bound. The small chemokine proteins are classified into four highly conserved groups, i.e. CXC, CC, C, and CX3C (X indicates the number of amino acids between the cysteine residues) on the basis of the position of the first two cysteines that are adjacent to the amino terminus.[1] An established role for several members of the CXC and CC chemokine families is to provide directional cues for the movement of leukocytes in development, homeostasis, and inflammation.[2] At the time of the movement of leukocytes, chemokine concentration gradually increases at the inflammatory site because the chemoattractants released from the luminal surface of the endothelium, the inflammatory site of the lymphocyte, are rapidly diluted and swept downstream by blood flow. Leukocytes in the mainstream of blood flow may make contact with the endothelium via a group of molecules called selectins,[3] and may then roll along the endothelial surface.

The cell surface molecule CXC chemokine receptor-4 (CXCR4) is a 7-transmembrane-spanning, G-protein-coupled receptor for the CXC chemokine stromal cell-derived factor-1α (SDF-1α)/pre-B-cell growth stimulating factor (PBSF)/CXCL12.[2] The open reading frame of the *CXCR4* gene encodes a peptide of 352 amino acids and is interrupted by one intron in the region encoding the N-terminal segment.[4]

CXCR4 is a receptor for the SDF-1α. SDF-1α interacts with CXCR4 to play a variety of physiological roles: B-cell formation in liver and bone marrow at the fetal stage, homing of bone marrow cells in the developmental process, formation of the interventricular septum, regulation of movement of the cerebellum granule cell in neurogenesis, and large vasculogenesis that nourishes the gastrointestinal tract.[2] Since both CXCR4 and SDF-1α knockout mice do not survive, the interaction between these molecules is essential in the developmental process.[5–7] It has been reported recently that CXCR7 binds with high affinity to SDF-1α and to interferon-inducible T-cell α-chemoattractant (I-TAC, also known as CXCL11).[8] However, unlike other chemokine receptors, ligand activation of CXCR7 induces neither Ca^{2+} mobilization nor cell migration.[8]

CXCR4 is also shown to be one of the coreceptors for human immunodeficiency virus type 1 (HIV-1).[9] Entry of HIV-1 into target cells involves interactions of the viral envelope protein (Env) with CD4 and a coreceptor, mainly either CXCR4 for T-cell–tropic HIV-1,[10,11] or CCR5 for macrophage–tropic HIV-1.[12,13] In acute HIV-1 infection, primarily macrophage-tropic strains are involved in transmission of the virus, whereas T-cell–tropic strains emerge later and are associated with the rapid progression to AIDS.[9]

Importantly, cancer cells originating from the pancreas, brain, breast, prostate, kidney, ovaries, thyroid, and malignant melanoma express CXCR4; however, normal tissues scarcely express CXCR4. Increasing CXCR4 promotes metastasis of these tumor cells toward SDF-1α-expressing organs including the lungs, liver, lymph nodes, bone marrow, and adrenal glands.[14–17] Further, interaction between CXCR4 and SDF-1α promotes progression of chronic and acute lymphocytic leukemia,[3] and exacerbation of chronic rheumatoid arthritis.[18]

We previously reported that a duodenally absorbable CXCR4 antagonist, KRH-1636, competitively blocked the association of the Env protein of HIV-1 with CXCR4 both *in vivo* and *in vitro* as well as the interaction of SDF-1α with CXCR4.[19] We therefore hypothesized that KRH-1636 could be a promising chemical for offering protection from both cancer metastases induced by SDF-1α and from CXCR4-tropic HIV-1 infection. In order to assess this possibility, we sought to evaluate whether the CXCR4 antagonist KRH-1636 and its derivatives could potently inhibit SDF-1α-mediated chemotaxis of cancer cells as well as HIV-1 infection.

Materials and Methods

Reagents. SDF-1α (R&D systems, Minneapolis, MN, USA) was dissolved in phosphate-buffered saline (PBS) at 1 μM. KRH-1636,[19] and its derivatives KRH-2731, -3148, and -3955 were synthesized at Kureha Chemical Industry (Tokyo, Japan). These

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Abbreviations: CXCR4, CXC chemokine receptor-4; DMSO, dimetyl sulfoxide; EC_{50}, 50% effective concentration; Env, envelope protein; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HIV-1, human immunodeficiency virus type 1; mAb, monoclonal antibody; OD, optical density; PBS, phosphate-buffered saline; PBSF, pre-B-cell growth stimulating factor; PE, phycoerythrin; SDF-1α, stromal cell derived factor-1α.

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compounds were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1%.

Cell culture. Jurkat and its subline Jurkat E6-1 were used in this study. The cells were cultured in a complete medium (CM) composed of RPMI-1640 (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1% 2-Mercaptoethanol at 37°C in a humidified environment with a 5% CO_2 atmosphere.

Fluorescence-activated cell sorter (FACS) analysis. Expression of CXCR4 and CD4 on Jurkat cells was measured by flow cytometry. The cells were suspended at 1×10^5 cells/mL in PBS containing 1% FCS. The cells were reacted with phycoerythrin (PE)-labeled mouse monoclonal antibodies (mAbs) to human CXCR4 (12G5; eBioscience, San Diego, USA) and CD4 (Leu3a; Becton Dickinson, Tokyo, Japan) as a positive control at 4°C for 1 h. The treated cells were washed and fixed with 1% formalin in PBS. Fluorescence of the stained cells was detected by a FACSCalibur (Becton Dickinson), followed by the analysis of fluorescence intensity by CellQuest software (Becton Dickinson).

Cytotoxic assay. Jurkat cells were treated with CXCR4 antagonists at 37°C for 1 h. The cells were harvested and resuspended in a 96-well plate. The viability of the treated cells was measured using a Cell Counting Kit-8 (Dojindo, Tokyo, Japan).

Chemotaxis assay. Cellular chemotaxis was investigated using a 24-well culture plate with 8-μm-pore filters (Transwell; Corning, Tokyo, Japan). Jurkat cells were washed three times in a FCS-free medium and suspended at 3×10^6 cells/mL in RPMI-1640 containing 0.1% bovine serum albumin (control medium). The control medium (0.2 mL) containing 3×10^5 cells was added to the upper well; the control medium (0.6 mL) with or without SDF-1α (100 ng/mL) or CXCR4 antagonists (10 μM) was added to the lower well. The culture plate was incubated for 3 h at 37°C; thereafter, the cells in the upper or lower well were then harvested and resuspended in a 96-well plate. The number of cells in each well was measured using a Cell Counting Kit-8. Optical density (OD) (455 nm/650 nm) values were measured on a microplate reader. The chemotaxis index was calculated as follows: [(OD of treated cells in the lower well – OD of control medium in the lower well)/(OD in sum of the lower and upper wells – OD of control medium in the lower well)] × 100.

For evaluating the inhibitory effect of the CXCR4 antagonists on chemotaxis, cells were pretreated with CXCR4 antagonists at 37°C for 1 h, followed by the chemotaxis assay as stated above.

Anti-HIV-1 assay. Human peripheral blood mononuclear cells, which were activated with immobilized anti-CD3 mouse mAb in RPMI-1640 medium supplemented with 10% FCS for 3 days, were infected with NL4-3 at a multiplicity of infection of 0.001. After 3 h of adsorption, the cells were washed, and cultured in CM supplemented with recombinant human interleukin-2 (50 U/mL), in the presence or absence of the test compounds. Amounts of HIV-1 capsid (p24) antigen produced in the culture supernatants were measured by an enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., Buffalo, NY, USA) 7–10 days after infection.

Results

The initial purpose of this study was to evaluate whether a series of CXCR4 antagonists could inhibit cancer metastasis, which is promoted by the interaction between SDF-1α and CXCR4. In order to evaluate the antagonistic effect of the compounds, we sought to develop an assay system for quantitatively detecting SDF-1α-mediated chemotaxis induced by the interaction. In this experiment, we employed $CD4^+$ leukemic cell line Jurkat as a $CXCR4^+$ indicator.[20] Since Jurkat sublines have different characteristics, we compared CXCR4 expression in the original Jurkat cells and its subline E6-1 by using flow cytometry. As expected, CXCR4 expression was comparable in both cell lines, while CD4 expression was greater in the Jurkat cells (Fig. 1a).

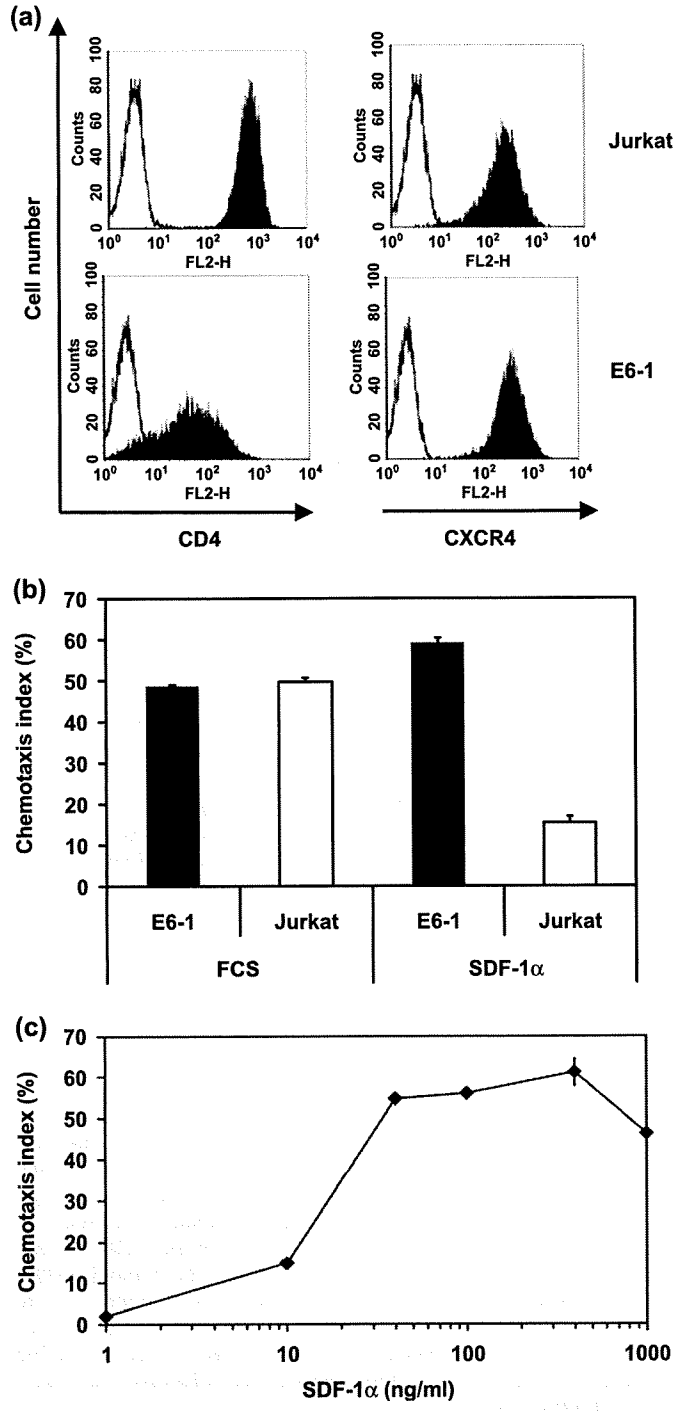

Fig. 1. A quantitative assay system for stromal cell-derived factor-1α (SDF-1α)-mediated chemotaxis. (a) Evaluation of CD4 and CXC chemokine receptor-4 (CXCR4) expression on Jurkat and its subline E6-1. The cells were stained with phycoerythrin-labeled anti-CXCR4 or anti-CD4 mouse monoclonal antibodies. Open and closed lines indicate fluorescence of the control and stained cells, respectively. (b) Effect of SDF-1α on chemotaxis of Jurkat and its subline E6-1. The cell lines were incubated with the control medium including 400 ng/mL of SDF-1α or 10% fetal calf serum (FCS) for 24 h at 37°C. The results are shown as a chemotaxis index and standard deviation. The calculation of the chemotaxis index is described in 'Materials and Methods'. (c) Dose-dependent effect of SDF-1α on the chemotaxis of E6-1 cells. Increasing amounts of SDF-1α were treated with E6-1 cells for 3 h and the levels of migration to the lower well are indicated as a chemotaxis index.

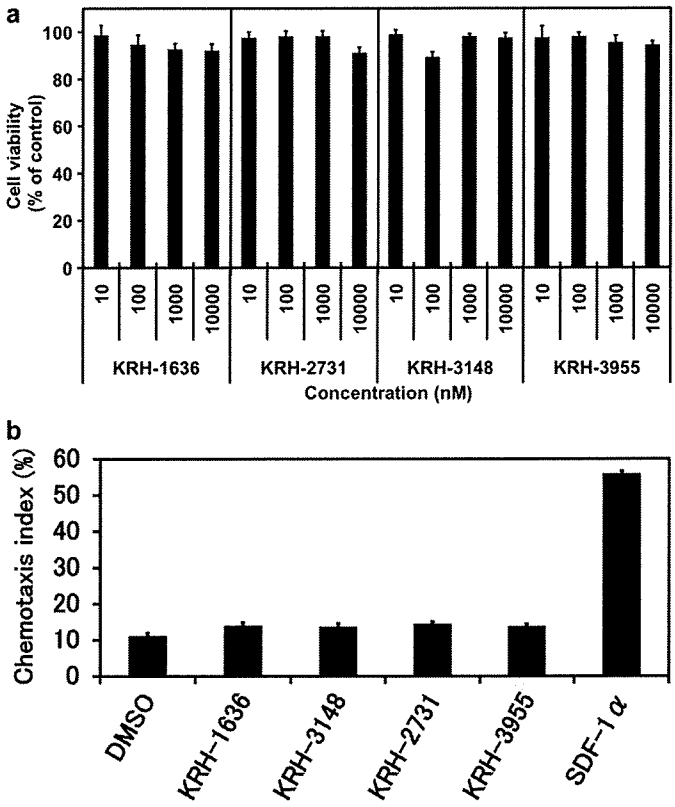

Fig. 2. CXC chemokine receptor-4 (CXCR4) antagonists exhibited neither cytotoxic nor agonistic effects. (a) Increasing amounts of CXCR4 antagonists were examined for their cytotoxic effect on E6-1 cells. (b) CXCR4 antagonists (10 μM) or stromal cell-derived factor-1α (SDF-1α) (100 ng/mL) were added to the lower wells in a chemotaxis assay and were incubated at 37°C for 3 h. The treated E6-1 cells were evaluated for the chemotaxis index. DMSO, dimethyl sulfoxide.

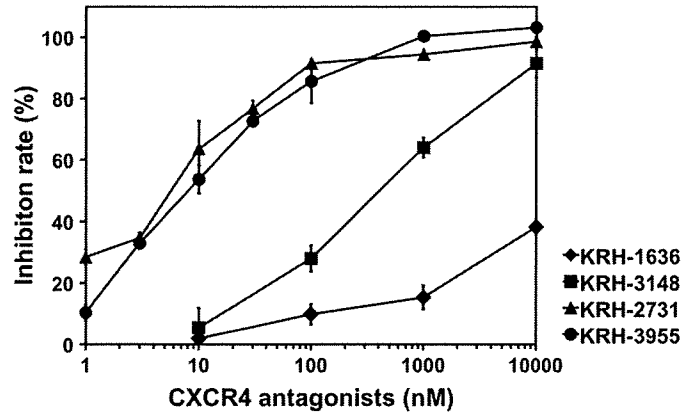

Fig. 3. Dose-dependent effect of CXC chemokine receptor-4 (CXCR4) antagonists on inhibition of stromal cell-derived factor-1α (SDF-1α)-mediated chemotaxis. E6-1 cells were pretreated with each concentration of CXCR4 antagonists at 37°C for 1 h, followed by incubation with 100 ng/mL of SDF-1α for 3 h. The cells were evaluated for the chemotaxis index. The inhibition rate was calculated as the percentage inhibition of chemotaxis by the antagonists.

Table 1. Inhibitory effects of CXCR4 antagonists on SDF-1α-mediated chemotaxis and HIV-1 infection

CXCR4 antagonists	CXCR4 (EC_{50}, nM)	
	Chemotaxis	HIV-1
KRH-1636	>10 000	42
KRH-3148	396.7	4
KRH-2731	9.2	0.9
KRH-3955	5.3	1

Next, the two cell lines were analyzed for SDF-1α-mediated chemotaxis activity; after 24 h of incubation, about 30% of both Jurkat and E6-1 migrated to the lower wells in the presence of the control medium. Since the value was the background for this chemotaxis assay, we subtracted this value from the subsequent experiments. We decided to use 400 ng/mL of SDF-1α for the chemotaxis assay as previously described by Liang *et al.*[21] It was found that SDF-1α induced a four-fold increase in the migration efficiency of E6-1 cells compared to the original Jurkat cells (Fig. 1b). Therefore, we decided to use E6-1 cells for the subsequent experiments.

Next, we attempted to optimize the experimental conditions for the SDF-1α-mediated chemotaxis assay. The chemotaxis index plateaued at approximately 60% after 3 h incubation of E6-1 cells with 400 ng/mL of SDF-1α (data not shown). We then examined the effect of increasing concentration of SDF-1α on the chemotaxis index and found that the level of chemotaxis was augmented in a dose-dependent manner and plateaued when more than 40 ng/mL of SDF-1α was used (Fig. 1c). Accordingly, the optimal condition for the chemotaxis assay in subsequent experiments was 100 ng/mL of SDF-1α for a 3h incubation period.

Next, we analyzed the cytotoxicity of CXCR4 antagonists to E6-1 cells. As indicated in Figure 2(a), the CXCR4 antagonists were not cytotoxic for E6-1 cells at a 10 μM concentration. To ascertain the possibility of these antagonists also exhibiting agonistic activities, we examined the chemotaxis activity of the antagonists. We observed that 100 ng/mL SDF-1α efficiently induced migration of E6-1; however, none of antagonists induced migration even at 10 μM (Fig. 2b). This indicated that the CXCR4 antagonists did not possess agonistic properties.

The effect of CXCR4 antagonists on the chemotaxis was investigated under the same conditions as described above. The prototype antagonist KRH-1636 inhibited the SDF-1α-mediated chemotaxis up to approximately 40% at a maximal concentration (10 μM). By contrast, KRH-3148 almost completely inhibited the chemotaxis at the maximal concentration; moreover, KRH-2731 and KRH-3955 showed the maximum inhibition rate even at 1 μM (Fig. 3). In order to quantitatively compare these efficacies, 50% effective concentration (EC_{50}) was calculated (Table 1). The results from this study clearly showed that KRH-2731 and KRH-3955 were effective at >1000-fold as compared with KRH-1636.

We further evaluated the effect of the compounds on HIV-1 infection. Anti-HIV-1 activities in nM of KRH-1636, KRH-3148, KRH-2731, and KRH-3955, which were shown as EC_{50}, were 42, 4, 0.9, and 1, respectively (Table 1). The efficacy of the antagonists was highly correlated with their inhibitory effects on HIV-1 infection by interrupting the association of the Env with CXCR4. Interestingly, inhibition of chemotaxis by KRH-1636 and KRH-3148 was relatively lower than that of HIV-1 infection compared with KRH-2731 and KRH-3955. The difference may be because action sites of KRH-2731 or KRH-3955 against CXCR4 are somewhat different from those of KRH-3148 (Sei Kumakura, unpublished data). In summary, these results demonstrate that both KRH-2731 and KRH-3955 are capable of efficiently inhibiting SDF-1α-mediated chemotaxis as well as infection of T cell–tropic HIV-1.

Discussion

The present study demonstrated that the novel CXCR4 antagonists efficiently inhibited SDF-1α-mediated chemotaxis as well as

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infection of T cell-tropic HIV-1. Two compounds KRH-2731 and KRH-3955 were found to be highly potent inhibitors for both efficacies without any cytotoxicity or agonistic activity, indicating that they may be promising as anti-cancer metastasis and anti-HIV-1 drugs. In particular, both KRH-2731 and KRH-3955 efficiently inhibited calcium signaling induced by SDF-1α at a concentration of 10 nM, while KRH-3148 and KRH-1636 inhibited at 100 nM and at greater than 10 μM, respectively (Sei Kumakura *et al.*, unpublished results). This indicated that their antagonistic effects were highly correlated with their abilities to inhibit chemotaxis and HIV-1 infection.

While the Jurkat cell line expressed a smaller but almost comparable level of CXCR4 compared with E6-1 cells (Fig. 1a), their migration levels in the presence of SDF-1α were quite different (Fig. 1b). It is possible that the original Jurkat cells express non-functional CXCR4 with regard to signal transduction that is required for chemotaxis.

Tumor cells from various types of human cancers of epithelial, mesenchymal, and hematopoietic origins express high levels of CXCR4.[14,16] The interaction of SDF-1α with its receptor CXCR4 contributes to metastasis of breast cancer as well as a number of other malignancies in the lung, brain, and prostate. Furthermore, patients with cancers expressing high levels of CXCR4 have more extensive metastasis at lymph nodes compared with low CXCR4-expressing ones.[22] On this basis, the efficient CXCR4 antagonists demonstrated in this study may be highly valuable for the regulation of cancer metastasis. In fact, a synthetic peptide against CXCR4 efficiently inhibited metastasis of breast cancer in a mouse model,[21] thus providing support to our notion. However, a hurdle remains for the delivery of the peptide inihibitor to the primary focus of cancer in patients, thus impeding the clinical application of the inhibitor. In this regard, our low molecular weight CXCR4 antagonists are promising because they are non-cytotoxic and can be administered orally. In fact, KRH-3955 showed oral bioavailability of 25.6% in rats and its oral administration blocked X4 HIV-1 replication in the human peripheral blood lymphocytes and in severe combined immunodeficiency mouse system (Tsutomu Murakami *et al.*, manuscript in preparation). It is notable that AMD3100, another small non-peptide CXCR4 antagonist, has been shown to inhibit metastasis of cancer cells *in vitro* and *in vivo*.[23,24] Moreover, our preliminary data suggested that injection of the breast cancer cell line MDA-231 produced a huge tumor at the inoculated site as well as aggressive metastasis in the lungs of mice, and that our compounds partially inhibited both the primary tumor growth and the metastasis (data not shown).

In conclusion, CXCR4 antagonists, which can be orally administered, are promising agents for SDF-1α-mediated metastasis of cancer cells and also for the treatment and prophylaxis of a number of diseases related to the interaction between CXCR4 and SDF-1α, the best example of which would be an anti-HIV-1 drug.

Acknowledgments

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インタヴュー：霊長類飼育実験施設におけるバイオセーフティ

アンケート(10～11ページ)の結果から、多くのユーザーが供給される個体の健康状態に深い関心を寄せ、ニホンザルを飼育する上で注意すべき病原体および獣医学的管理に関する情報提供を望んでいることが明らかになりました。推進室もこれまで以上に供給個体の質の向上および最新情報の収集に力を注いでいきたいと考えています。また、供給先が拡大するにつれて判ってきたことですが、それぞれの受け入れ機関が対応を想定している病原体のリスク評価には相当のばらつきがあります。現在移送前に実施している検疫にも、基本項目(結核、赤痢、サルモネラ、Bウイルス)に加えて、多様な検査の追加を希望されるユーザーが少なくありません。今後、供給検討先が脳神経生理学研究から周辺の先端医科学研究へとさらに拡大していくことも予想され、それに伴って、より多様な感染症リスクへの対応を求められる可能性があります。さらに、今春から世間を騒がし、現在も猛威を振るっている新型インフルエンザのような新興感染症対策も課題の一つになってくることでしょう。

今号は「バイオセーフティ特集」として、まず今春京都大学霊長類研究所人類進化モデル研究センターに新設された比較免疫微生物領域の明里宏文(あかりひろふみ)教授に、感染症およびバイオセーフティ管理の専門家としてお話を伺いました。　　(編集部)

1. バイオセーフティをめぐるコンプライアンス

ユーザーの立場として、健康で安全な個体が安定供給されることを期待するのは当然のことでしょう。しかし、研究責任者としてバイオセーフティ管理を考える場合には、それ以上に広い視野で捉えることが求められると思います。霊長類を飼育する機関が守らなくてはならない、感染症予防に関わる規制を定めているのは感染症法ですが、この法律の主旨は、ヒトにとって危険な感染症の発生にどう対処するか、またはバイオテロに利用される危険性がある病原体の管理(流出防止、輸送時の安全対策)をどうするか、といった、かなり限定されたリスクしか想定されていない、最低限の規制でしかありません。その結果、狭義のコンプライアンス(＝法令遵守)ができているからといって、研究・飼育に携わるスタッフ、サル、さらに周辺住民にとって、十分な安全性を確保できている保証にはならない可能性があります。法規制対象外の病原体に関する安全性については、各機関が自発的に、機関内規程やマニュアルなどの形で、安全な取り扱いルールを整備し、周知徹底する努力を怠らないことで守られているのです。

ここで重要になってくるのは、管理運営の責任者、研究者、技術者、飼育スタッフなど、立場や背景の異なる人々の間の意思疎通ですが、これがとても難しいのです。例えば、これまで高リスクとは認識されず、しかも幸運なことにその機関では深刻なアウトブレークが起こっていない病原体について、リスク評価を改め、より厳しい安全性対策を講じる必要があるかもしれない、という事態になることがあります。このとき、自分たちの機関ではどう対処していけば良いか、それぞれの立場から細部まで議論を尽くすことが必要になるのですが、日本ではついそれを先送りしてしまう例が少なくないのではないかと危惧しています。たしかに、より厳しい安全性管理を実施することになれば、設備の拡張や維持にかかるコスト、訓練や作業にかかる手間が増大し、利便性も低下します。差し迫った問題が起こっているわけでもない状態では、うまく機能しているルールをあえて厳しくしようと提案することで、「いらぬ波風を立てる」ことになっては、と議論を抑制する心理が働きがちです。米国などでバイオセーフティ管理が推進されてきた背景の一つとして、組織管理者には現場の安全性を確保するために必要なコストを負担する義務があり、その保証がなければ、研究者も技術者も働くことを拒否できる、それが当然の権利として広く認められているという事情があります。一方、日本では、互いの立場を慮って、対立を避けてしまう傾向が強いと感じています。その結果、最終的な判断は管理責任者の判断・良識にゆだねられることになりますが、ぜひより広い視野でコスト感覚を働かせ、必要な対策がとられているかを検討して頂きたいと思います。昨今は、リスクに気づきながら安全対策を講じていなかった、広義のコンプライアンス(＝社会的責任)を果たすことを怠っていたと糾弾され、訴訟などの形で責任を追及された場合に、組織が被る社会的なダメージの大きさが無視できなくなっています。バイオハザードにつながりかねない事故(サルの逸走、スタッフの病原体暴露など)を起こさないための日常の安全確保体制、万一事故が起こった場合も被害を最小に食い止めるための危機管理体制、両方を整備しておくことが社会からも求められているのです。

今春まで私がバイオセーフティ管理に取り組んできたつくばの(独)医薬基盤研究所・霊長類医科学研究センターでは、厚生労働省の管轄であるという背景と、過去に経験したSVV(サル水痘様ヘルペスウイルス)感染症アウトブレークのような事態を二度と起こしてはならないという共通認識の下、バイオセーフティレベル3(BSL-3)の病原体まで取り扱いできる体制を築きあげてきたという経緯があります。是非参考にして頂きたいと思います。

2. 霊長類を用いる研究における二つのバイオハザード

一つは現場スタッフや周辺住民、つまりヒト側の安全性という観点から、より世間の注目を集めやすいのですが、サルが持っている(かもしれない)人獣共通感染症(Zoonosis)、または研究目的で使用、保管している病原体がヒトに伝播してしまうというタイプのバイオハザードです。もう一つは、サルまたはヒトが持っている(かもしれない)病原体が、サルに伝播してしまうタイプです。こちらはヒトに直接危険が及ぶとは限らないため、一般の方には問題視されにくいのですが、病原体の侵入を許してしまうと、隔離や治療が難しい場合も多く、やっかいな事態になりかねません。前述のSVVはこちらのタイプ(サルからサルへの伝播)でした。発症個体が増えていく中、原因究明・対処に時間がかかってしまい、その間現場スタッフ自身も安全を確信できない、不安な状況で作業を続けることになりました。ヒトからサルへ伝播する可能性がある病原体も、結核、麻疹、風疹、インフルエンザなど、数多く候補が存在します。サルと接触する可能性のあるスタッフの健康チェック、感染症予防教育が重要です(＊1)。とくに今年は新型インフルエンザ・パンデミックという新しいリスクが生じています。8月にNatureに掲載された報告(＊2)によれば、カニクイザルにも新型ウイルスへの感受性があり、従来の季節性インフルエンザ以上に、肺で増殖したウイルスによる肺炎を引き起こす可能性が高いという結果が出ています。霊長類を飼育している施設では、ヒトからサルへの伝播をどう防ぐか、万一伝播した場合、感染個体の隔離・治療の準備はできているか、治療に当たるスタッフの安全確保はどうするか、などを事前に検討しておく必要があります。体制・設備や飼育規模の異なる複数の機関で足並みをそろえることは難しいかもしれませんが、施設間で最新情報を共有し、スタッフに感染者が出た場合→感染が疑わしいサルが見つかった場合→サル間で感染拡大した場合と複数のフェーズに対応した危機対応マニュアルを準備しておきたいものです。

3. バイオセーフティを確保するハードウェアとソフトウェア

すでに実験動物施設の多くはその方向へ進んでいると期待していますが、霊長類を飼育する実験施設、検疫施設ではABSL-2(P2A)レベルの設備を維持管理できることが望ましいと考えています。理想を言えば、病原体を封じ込めるハードウェアとして、二重扉、HEPAフィルタを設置した空調施設(少なくとも排気)、排水を消毒するシステム、廃棄物を滅菌するオートクレーブ等の設備を備えておきたいものです(詳しくは＊3)。さらに、オートクレーブ、安全キャビネットなどのバイオセーフティ機器、空調施設には、日常・定期点検と計画的な設備更新が欠かせませんし、災害時も自家発電などで必要な機能が維持できるようにしておく必要がある

写真1:バイオセーフティ対応型解剖台

でしょう。写真1はバイオセーフティ対応型解剖台です。ABSL-3レベルの設備ですが、霊長類医科学研究センターでは、通常飼育エリアに感染症が疑わしい個体が見つかった場合に、その検査、治療、剖検などの処置にも使用できるよう、ABSL-2エリア内にも設置され、処置が終わるたびに台上や周辺を完全消毒するようになっています。

ハードウエア整備だけでなく、それを使いこなすためのソフトウェアの充実も重要です。機器の正しい使用方法、病原体やサルの取り扱いルール、検疫規程、日常の作業マニュアル、病原体ごとの暴露事故対応マニュアル、事故報告書のひな形などを整備しておくこと、最新情報を取り入れ定期的にアップデートすること、更新部分については、なぜ変更が必要になったかの説明も含め

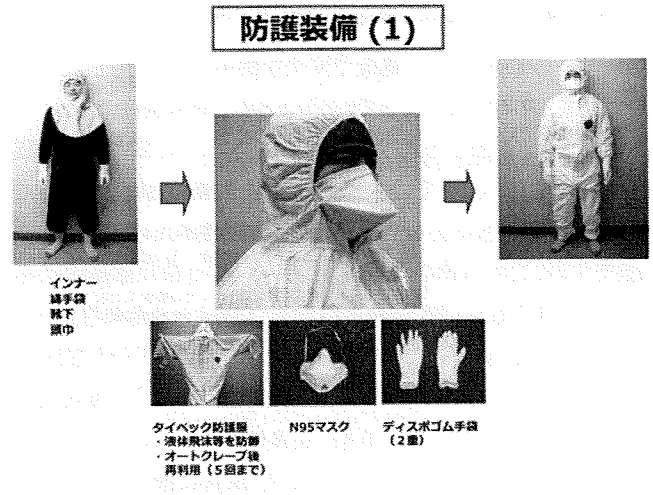

写真2:霊長類医科学研究センター(ABSL-2以上)で使用されている防護装備の着装手順(1)

(4 ページに続く)

(3 ページから続く)

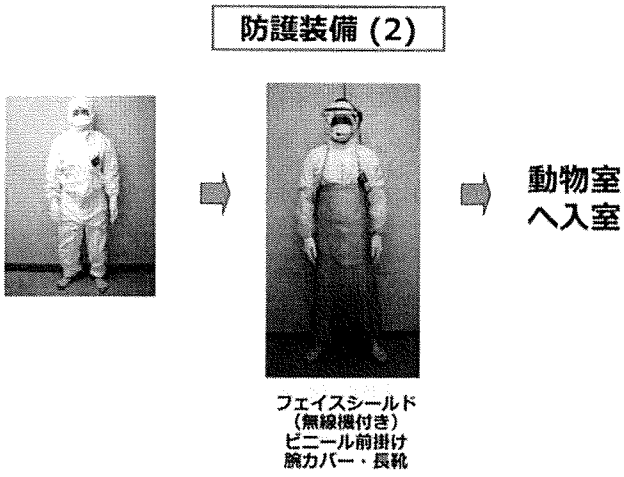

写真3:霊長類医科学研究センター(ABSL-2以上)で
使用されている防護装備の着装手順(2)

て周知徹底を怠らないことが重要です。説明が腑に落ちるまでは、とくに経験の長いスタッフから「これまでの方法ではなぜ不十分なのか、今以上のコストや手間をかける必要があるのか」という反発が生じることは避けられないかもしれません。新しい措置がスタッフ自身だけでなく、サル、機関全体の安全をも守るためのものであることを根気よく説得し続ける努力が必要となるでしょう。また、サルに接するスタッフを対象に、抗体価測定検査を含む定期的な健康診断、血清採取を実施し、検診記録と血清を保存しておくこと、必要十分かつ操作性の高い防護装備(写真2, 3及び ＊4参照)を準備し、正しい着脱手順を徹底させることなども重要です。日常の作業だけでなく、災害、停電などの非常事態に備えた模擬訓練も定期的に実施し、危機対応マニュアルの手順が安全性を確保する上で有効かつ現実的か、事前に検討しておきたいものです。多くの機関ではバイオセーフティ講習、訓練を受けた者に証明書を発行し、一定期間(3年など)以内に更新を受けた者でなければ動物の取り扱いができないシステムになっていると思います。今後、霊長類を受け入れた経験のない機関からNBR事業に供給が申請されるケースが増えてくることも予想されます。その場合にはニホンザルでとくにリスクの高い病原体について、暴露程度による感染・発症の危険性についてのデータや具合的な対処法など、安全な飼育管理システム作りに役立つ資料を提供し、各機関のバイオセーフティ管理体制との連携を図っていくことが必要になると思います。

4. それでも起こるかもしれない事故から身を守るために

安全対策を徹底したとしても、暴露事故が起こってしまう可能性はゼロにはなりません。そのときには未知の病原体への感染も想定して指定医療機関を受診し、事故直後とその一ヶ月後の血清を保管するよう、事前に担当医療機関、医療従事者に説明しておく必要があります。事故の報告はより高い安全性を確保するために必要な措置であること、受診結果と血清サンプルは、労災申請をするとき、事故との因果関係を立証するための重要な記録にもなることを当事者によく説明し、個人情報の管理規程も明確にしておきます。例えば霊長類医科学研究センターでは、血清サンプルは番号のみで保管、対応する事故の記録、検査結果、個人情報については別の部署で厳重に管理し、事故を報告した者がそのために噂を広められたり、否定的な評価を受けたりして不利益を被る恐れがないよう、十分注意して取り扱います。事故の記録が規程通り安全に管理されるという保証がなくては、軽微な事故や「ヒヤリハット」事例がきちんと報告されないまま放置され、重大なリスクの発見・対処が遅れ、被害をさらに大きくしてしまう危険があるからです。さらに、感染の恐れがある病原体については、ワクチン接種や抗血清、治療薬の投与など、必要な処置を一刻も早く受けられるよう、医療機関の協力を取り付け、投与が早いほど効果が高い薬は、処方箋の問題もクリアして現場に常備して置く必要があるでしょう。治療の参考として、事故に関わったサルの過去の検査記録なども速やかに参照できることが望ましいです。

5. マカク類コロニーのSPF化とは

マカク類の繁殖コロニー管理で「SPF化」という言葉は、結核菌を始めとするリスクの高い特定の病原体について、感染個体の排除と定期検査を徹底させ、感染リスクを低く保つよう管理が行き届いているという意味で使われています。つまり病原体が100%存在しないという保証にはなりません。細菌や寄生虫などは比較的排除しやすいですが、難しいのはウイルスです。NIHでは、マカクを使うHIV-1/AIDS関連研究上の必要性を重視して、サル免疫不全ウイルス(SIV)に加え、サルレトロウイルス/タイプD(SRV:SPFレベル1)、サル白血病ウイルス(STLV:SPFレベル2)、Bウイルス(CHV-1:SPFレベル3)、サルフォーミーウイルス(SFV)及びその他のヘルペスウイルス(SPFレベル4)という順で、排除できたコロニーを格付けしています(＊5)。高レベルのSPFコロニーをいったんは確立できた機関でも、ウイルスの侵入や潜在を検出し、防ぎきることができずに、格下げせざるをえなくなった例も少なくありません。とくにSRVで知られているケースですが、抗体検査値が上昇しないまま、ウイルスを排出し続けていた個体(母子感染の結果、免疫寛容になってしまったコザル)の症例もあります。複数の病原体で、ELISA法などの一般的な抗体検査だけでなく、病原体の遺伝子を検出できるPCR法など、複数の検査法を併用して検出率を高めることが推奨されています。

今後NBR事業が目標の一つとしているニホンザルのSPF化を実現していくためには、ニホンザルでとくにリスクの高い病原体を優先的に考えた独自のガイドラインを定めること、それぞれの病原体の性質を熟知し、未知の病原体の発生にも対処できる専門家による検査、治療体制を整備していくことが重要でしょう。これから事業が発展し、供給する分野が広がることも考慮して、より広い分野のニーズに適う、安全な個体を安定供給していけるように、「量」以上に「質」を重視した計画的なコスト配分を考えていく必要があると思います。

6. 霊長類モデル研究が期待されている感染症

霊長類を研究モデルとして使うには、今回お話ししたバイオセーフティ管理以外にも、動物福祉(動物愛護管理法)や生物多様性保全(ワシントン条約、外来生物法、カルタヘナ法)など、多様なコンプライアンスに気を配り、大きなコストを払っていかなくてはなりません。それでも、そのコストを上回る大きなベネフィットのある研究、つまりマウスなど霊長類以外の実験動物モデル、培養細胞などの代替法を用いる研究ではヒトの病態を突き止め、新たな治療・予防法、ワクチンなどの安全性・有効性を確認することが難しく、しかも世界的規模で非常に多数の罹患者が成果を待ち望んでいる、そのような疾患研究は脳神経科学分野だけでなく、感染症にもたくさんあります(＊6)。私が取り組んできたものは、HIV-1/AIDS、C型肝炎ウイルス(HCV)、デング熱ウイルス、マラリア原虫の4つですが、どれも全世界で数千万人から数億人の罹患者が存在し、毎年数万人、数十万人という方が亡くなっているにもかかわらず、感染から発症、重症化にいたる機序が十分に解明されていないために、有効な予防ワクチン・治療薬の開発が進んでいない感染症です。デング熱やマラリアなどは遠い熱帯の病気と思われるかもしれませんが、地球温暖化の影響で、病原体を媒介する蚊の分布域が拡大してきており、日本でものんびり構えてはいられない状況になりつつあります。さいわい優れた霊長類モデルの開発が進み、治療法やワクチンを開発している研究グループとの連携が可能な段階に入ろうとしています。優れた研究成果が社会に還元されていけば、大きなコストを払って霊長類を医科学研究に使用する意義を広く認めて頂けるでしょう。

(2009年9月2日　京都大学霊長類研究所にて)

補足:

＊1:最近では類人猿の野外観察やエコツアーでも、ヒトからの感染症伝播が疑われる事例が発生しているため、観察に訪れる研究者、観光客、ガイドなどのスタッフとサルたちとの接触を制限するルール作りが進められています(編集部注)。

＊2:Itoh Y et al.(2009) In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature 460/20 Aug 2009:1021-1025

＊3:明里宏文(2007) 医学実験用霊長類を用いた病原体感染実験施設の管理運営におけるコンプライアンスとバイオセーフティ JVM(獣医畜産新報) 60(8):641-645

＊4:Roberts JA et al.(2008) Nonhuman Primate Quarantine: Its Evolution and Practice. ILAR Journal 49(2):145-156

＊5:Morton WR et al.(2008) Specific Pathogen-Free Macaques: Definition, History, and Current Production. ILAR Journal 49 (2):137-144

＊6:Gardner MB et al.(2008) Macaque Models of Human Infectious Disease. ILAR Journal 49(2): 220-255

(ILAR Journal Vol.49 No.2には, Microbial Quality Control for Nonhuman Primates特集として、他にも多くの最新情報が掲載されています。http://dels.nas.edu/ilar_n/ilarjournal/49_2/html/)

霊長類への遺伝子導入技術

バイオセーフティに関係する国内法の一つとして、近年重要性を増しているものに、2004年から施行された「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律(カルタヘナ法　＊1)」があります。さまざまな分野で応用が広がっている遺伝子組換え技術によって加工された核酸を導入された生物(細胞やウイルス)が生態系に影響を及ぼす可能性を想定し、その防止措置を定めたものです。神経生理学研究分野でも、光によって活性化するタンパク質の遺伝子を神経細胞に導入することで、今まで以上に精緻な神経ネットワークの解析が可能になる手法など、遺伝子導入技術による新たな進展が大いに期待されていますが、実験室の立ち上げや管理には、文部科学省・環境省の「研究開発等に係る遺伝子組換え生物等の第二種使用等に当たって執るべき拡散防止措置等を定める省令(研究開発二種省令　＊2)」によって定められた拡散防止措置をとることが求められます。

特集後半は、自然科学研究機構岡崎3機関に新たな共同研究施設として立ち上げられた霊長類遺伝子導入実験施設におけるバイオセーフティ対策を生理学研究所・生体システム部門の南部篤教授にご紹介頂きました。　(編集部)

(6 ページに続く)