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Original article

Intranasal immunization with H5N1 vaccine plus Poly I:Poly $C_{12}U$, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge

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Abstract

The avian H5N1 influenza virus has the potential to cause a new pandemic. Since it is difficult to predict which strain of influenza will cause a pandemic, it is advantageous to produce vaccines that confer cross-protective immunity. Mucosal vaccine administration was reported to induce cross-protective immunity by inducing secretion of IgA at the mucosal surface. Adjuvants can also enhance the development of fully protective mucosal immunity. Here we show that a new mucosal adjuvant, polyI:polyC$_{12}$U (Ampligen®), a Toll-like receptor 3 agonist proven to be safe in a Phase III human trial, is an effective adjuvant for H5N1 influenza vaccination. Intranasal administration of a candidate influenza vaccine with Ampligen resulted in secretion of IgA, and protected mice that were subsequently challenged with homologous A/Vietnam/1194/2004 and heterologous A/HK/483/97 and A/Indonesia/6/2005 virus.

Keywords: Influenza virus; H5N1 subtype; Ampligen; Adjuvant; Immunoglobulin A

1. Introduction

The first outbreak of the highly pathogenic avian influenza virus H5N1 was reported in humans and birds in Hong Kong in 1997, during which 6 out of 18 infected people died [1]. Subsequently, re-emergence of H5N1 highly pathogenic influenza viruses associated with a high fatality rate (greater than 60%) has been reported in Southern China, Vietnam, Thailand, Cambodia, Indonesia, Turkey, and Iraq. From January 2003 to June 2007, 310 laboratory-confirmed H5N1-infected human cases were reported to the World Health Organization. Although most human H5N1 infections have been caused by the direct transmission of virus from infected poultry, there is fear that a pandemic could result if subsequent transmission of H5N1 virus occurred between infected humans [2]. Therefore, there is an urgent and important public health need to develop effective vaccines against this highly pathogenic strain of avian influenza virus.

H5 Vaccine candidates must be continually updated to match the antigenicity of circulating viruses because of the difference in HA antigenicity among 1997, 2003, and 2004 H5 viruses [3]. In addition, it is difficult to predict which strain of virus (H5 or other avian-associated hemagglutinin) will be responsible for a pandemic. In such circumstances, the ideal

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approach is to prepare a vaccine that confers strong cross-protective immunity against variants of a particular virus strain. Mucosal immunity induced through natural infection by influenza virus has potent cross-protective activity, compared to subcutaneous vaccination-induced systemic immunity. Cross-protective activity is correlated with mucosal secretory IgA, which is not induced following subcutaneous vaccination [4]. In order to induce cross-protective mucosal immunity through influenza vaccination, we have examined the effect of intranasal administration of an inactivated viral vaccine with various adjuvants, and found that mucosal IgA plays an important role in cross-protection against variant influenza A and B virus infection [5–8]. Nicholson et al. reported that the H5N1 vaccine is poorly antigenic in humans, and requires adjuvant to elicit a detectable antibody response [9]. Several groups looking at avian influenza H5N1 vaccines have reported that intranasal administration of a formalin-inactivated whole virus vaccine with or without mutant *Escherichia coli* heat-labile toxin (LT) adjuvant (R192G), or an adenoviral vector-based influenza vaccine, protected mice from lethal challenge by a heterologous H5N1 virus [10–12]. Although LT is an effective adjuvant for the production of mucosal IgA, it has adverse clinical side effects, such as the facial paralysis of Bell's palsy [13]. New, clinically safe and effective adjuvants are necessary for the administration of intranasal influenza vaccines to humans.

We previously demonstrated that the synthetic double-stranded RNA (dsRNA) poly(I:C), a Toll-like receptor 3 (TLR-3) agonist, has mucosal adjuvant activity when co-administered intranasally with an influenza hemagglutinin (HA) vaccine, and increases both the mucosal and systemic humoral response, resulting in complete protection against challenge by homologous avirulent (H1N1) and highly pathogenic (H5N1) influenza viruses in mice [7,14]. Sloat et al. [15] also reported that mice immunized intranasally with recombinant anthrax protective antigen adjuvanted with poly(I:C) developed strong systemic and mucosal anti-anthrax antigen responses with lethal toxin neutralization activity. However, while TLR agonists, including poly(I:C), are potent mucosal adjuvants that induce type I interferons (IFNs), and have the potential to bridge the gap between innate and adaptive immunity [16], they have been associated with serious adverse events during clinical trials. Poly(I:C) induced a number of side effects in human, including renal failure and hypersensitivity reactions in some patients in a previous clinical trial using as high as 75 mg of poly(I:C)/m^2 on day 0 and then daily from day 7 to a maximum of 35 days [17].

PolyI:PolyC_{12}U (Ampligen®) is similar to dsRNA and has a good safety profile based on clinical trials, including a recently conducted double-blind placebo-controlled Phase III clinical trial [18]. To date, >75,000 doses of Ampligen have been administered to humans, at an average dose of 400 mg, and it has been generally well tolerated. Recently, it was shown that PolyI:PolyC_{12}U was as effective as poly(I:C) in inducing the maturation of human monocyte derived dendritic cells in vitro [19].

In this study, we examined the protective effect of intranasal administration in mice of an H5N1 vaccine together with synthetic dsRNAs (PolyI:C and Ampligen) as powerful TLR-3 agonists. The H5N1 vaccine used was NIBRG14, which was derived from a highly pathogenic influenza virus (A/Vietnam/1194/04) isolated from a patient with H5N1 influenza, and was prepared by the UK National Institute for Biological Standards and Control (NIBSC) [20]. We demonstrate that co-administration of the vaccine with either poly(I:C) or Ampligen as mucosal adjuvants elicited protective immunity not only against homologous virus (A/Vietnam/1194/04), but also heterologous viruses, including past (A/HK/483/97) and recent (A/Indonesia/6/05) clinical highly pathogenic H5N1 influenza virus isolates.

2. Materials and methods

2.1. Mice

Six- to eight-week-old female BALB/c mice were purchased from Japan SLC. Mice were kept under specific-pathogen-free conditions approved by the Institution Animal Care and Use Committee of National Institute of Infectious Diseases.

2.2. Viruses

The strains of H5N1 viruses used in this study were A/Hong Kong/483/97 (A/HK/483/97), A/Vietnam/1194/2004 (A/Vietnam/1194/04), A/Indonesia/6/2005 (A/Indonesia/6/05) [21]. The HK/483/97 virus, isolated from patient with fatal influenza A H5N1 disease in the Government Virus Unit, the Queen Mary Hospital, Hong Kong, was prepared in Mardin–Darby canine kidney (MDCK) cells without any special step for mouse adaptation. The A/Vietnam/1194/04 virus and A/Indonesia/6/05 from patients with H5N1 disease were propagated in 10-day-old embryonated chicken eggs for 2 days at 37 °C. These viruses were stored at −80 °C and viral titers were quantified by plaque assay using MDCK cells.

2.3. Preparation of vaccine and adjuvants

The vaccine used in these studies was a formalin-inactivated whole virus vaccine, NIBRG14, derived from a recombinant avirulent avian virus containing modified HA and neuraminidase (NA) from the highly pathogenic avian influenza strain A/Vietnam/1194/2004 virus, and other viral proteins from influenza strain A/PR/8/34 (H1N1) [20]. Modified HA lacks the multibasic amino acids at the cleavage site. The HA vaccine (split-product virus vaccine) from A/PR/8/34 (A/PR8; H1N1) was used in the examination of RNA expression experiments. Synthetic poly(I:C) dsRNA was kindly provided by Toray Industries Inc. (Kamakura, Kanagawa, Japan). Poly I:Poly C_{12}U (Ampligen®) was kindly provided by Hemispherx Biopharma (Philadelphia, PA).

2.4. Immunization and virus challenge

Five mice for each experimental group were anaesthetized with diethyl ether and immunized intranasally with 1 μg of total protein amount of vaccine, with or without adjuvant. Four weeks later, they were re-immunized in the same manner. According to a modification of the procedure of Yetter and coworkers [22], each mouse was anesthetized and infected by intranasal administration of 4 μl of PBS containing virus suspension with 1000 PFU of H5N1 virus into each nostril (2 μl/nostril). As 2 μl of the virus suspension remained in the local nasal area and could not enter the lung tissue, the initial viral infection was limited to the nasal area. H5N1 virus infection experiments were carried out in Biosafety Level 3 containment facilities, approved by the Guides for Animal Experiments Performed at National Institute of Infectious Diseases.

2.5. Measurement of the virus titer and antibody titers

Serum and nasal wash fluid were collected for measurement of the virus titer and antibodies against vaccine from mice that were sacrificed under anesthesia with chloroform. The levels of IgA and IgG Abs against vaccine determined by enzyme-linked immunosorbent assay (ELISA) as described previously [5]. Standards for vaccine-reactive IgA and IgG antibody titration were prepared from the nasal wash or serum of survived mice after H5N1 virus challenge, and expressed as the same arbitrary units (160-unit). The antibody titers of unknown specimens were determined from the standard regression curve constructed by twofold serial dilution of the 160-unit standard for each assay.

Virus neutralization tests by antisera were performed as described previously [23].

The virus titer was measured as follows. One milliliter of nasal wash was harvested from each mice, and aliquots of 200 μl of serial 10-fold dilutions of the nasal wash fluid were inoculated into MDCK cells in six-well plates. After 1 h incubation, each well was overlaid with 2 ml of agar medium according to the method of Tobita and coworkers [24]. The number of plaques in each well was counted at 2 days after inoculation. All of the experiments were repeated independently at least three times. The data are presented as means ± standard error (S.E.).

2.6. RNA isolation, cDNA synthesis, and real-time PCR

The mRNA levels of IRF-3, IRF-7, RIG-I, MDA5, Fas, and TRAIL in nasal-associated lymphoid tissues (NALTs) in vaccinated or influenza virus-infected mice was measured by real-time quantitative PCR after reverse transcription. Mice were inoculated with influenza virus or intranasally administered influenza HA vaccine (A/PR8) with or without poly (I:C). The NALTs were collected sequentially up to 72 h after administration. The NALTs from three mice in each group was pooled and total RNA was extracted using an SV-Total RNA Isolation kit (Promega, Madison, WI) and cDNA were synthesized using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Real-time quantitative PCR was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) with a QuantiTect Probe PCR kit (Qiagen), TaqMan probes (Applied Biosystems), and primers (Sigma Genosys, Ishikari, Japan) (Table 1) designed with Primer Express (Applied Biosystems). The system uses two dye layers to detect the presence of target and control sequences. The FAM dye layer yields results for quantification of the target mRNA. The detection of RIG-I and MDA5 mRNAs was performed with TaqMan Gene Expression Assays (Applied Biosystems). PCR was carried out in a volume of 20 μl; initial denaturation at 50 °C for 2 min and 95 °C for 15 min, was followed by 45 cycles of 94 °C for 15 s and 60 °C for 1 min. Relative mRNA abundance was calculated using the comparative delta-Ct method [25].

2.7. Statistics

Comparisons between experimental groups were made with the *t*-test for paired observations, and $P < 0.05$ was considered significant.

3. Results

3.1. Intranasal immunization of H5N1 vaccine with poly(I:C) protects against highly pathogenic avian influenza virus infection

To determine the efficacy of poly(I:C) as a mucosal adjuvant for H5N1 vaccines, the antibody response to NIBRG14, a whole virus vaccine derived from virus strain A/Vietnam/1194/04, was examined in mice immunized intranasally with

Table 1
Primers for quantitative PCR (probes labeled 5′ FAM, 3′ TAMRA)

Target	Sequences	
IRF3	Forward:	TGA CAC CAA TGG CAA AAG CA
	Reverse:	CCC AAG ATC AGG CCA TCA A
	Probe:	CCT CAC TCC CAG GAA AAC CTA CCG AAG TTA
IRF7	Forward:	CAG CCT TGG GTT CCT GGA T
	Reverse:	CCC ACC ACT GCC TGT AGC A
	Probe:	CCA TCA TGT ACA AGG GCC GCA C
Fas	Forward:	GCG ATT CTC CTG GCT GTG A
	Reverse:	GGC TCA AGG GTT CCA TGT TC
	Probe:	CAC TGT GTT CGC TGC GCC T
TRAIL	Forward:	CCT TAG GCC AGA AGA TTG AAT CC
	Reverse:	CCT AAA GAG CAC GTG GTT GAG A
	Probe:	TCC TCT CGG AAA GGG CAT TCA T
β-actin	Forward:	CAC CGA TCC ACA CAG AGT ACT TG
	Reverse:	CAG TGC TGT CTG GTG GTA CCA
	Probe:	CAG TAA TCT CCT TCT GCA TCC TGT CAG CAA

mRNA expression levels of RIG-I and MDA5 were measured using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA).

0.1–1 μg of NIBRG14 and various amounts (0 to 10 μg) of poly(I:C). Mice were immunized with vaccine and poly(I:C) twice, 6 and 2 weeks before infection. Mice immunized with vaccine alone (no adjuvant), or adjuvant alone had a small or an undetectable Ab response (Fig. 1). Animals immunized with 1 μg of vaccine and 10 μg of poly(I:C) had the highest concentration of anti-NIBRG14-specific IgA and IgG Abs in their nasal wash and serum, respectively. Next, we examined the protective effect of intranasal administration of NIBRG14 and poly(I:C) against homologous (A/Vietnam/1194/04) H5N1 influenza virus infection. The mean virus titer in the nasal washes of control mice was $10^{2.3}$ PFU/ml 3 days post inoculation (d.p.i.) with 1000 PFU, and none of the control mice survived more than 14 d.p.i. Intranasal immunization with vaccine alone had a partial protective effect, when comparing nasal wash virus titers to that of controls, and 80% of the mice in this group survived. However, intranasal immunization with H5N1 vaccine and poly(I:C) adjuvant resulted in a marked protective effect against viral infection (Fig. 1). Thus, intranasal administration of H5N1 vaccine with poly(I:C) adjuvant protected mice against homologous H5N1 influenza virus infection.

3.2. Antibody responses against H5N1 influenza virus in BALB/c mice immunized intranasally or subcutaneously with NIBRG14 vaccine and Ampligen as an adjuvant

We next examined the efficacy of Ampligen (PolyI:Poly$C_{12}U$) as a mucosal adjuvant for H5N1 vaccines. Mice were immunized twice by intranasal or subcutaneous administration of A/Vietnam/1194/04 whole virus vaccine (NIBRG14) with or without Ampligen, and their antibody response was examined by ELISA. In nasal washes, the highest concentration of anti-NIBRG14 IgA Ab was observed in animals immunized intranasally with 1 μg of NIBRG14 and 10 μg of Ampligen (Fig. 2). A small IgA response was elicited by intranasal administration of NIBRG14 without adjuvant, and no IgA response was evident in mice that received intranasal administration of Ampligen alone (control group), or in any of the mice who received a subcutaneous vaccination (Fig. 2). The neutralization antibody titer to A/Vietnam/1194, A/HK/483/97, and A/Indonesia/6/05 viruses was examined *in vitro* using sera from the same group of mice. The neutralizing activity to homologous virus (A/Vietnam/1194/04) was detected in the sera from immunized mice intranasally or subcutaneously with or without adjuvant. However, no neutralizing activity to heterologous viruses (A/HK/483/97, A/Indonesia/6/05) was detected in the sera from all immunized mice group.

3.3. Intranasal administration with H5N1 vaccine with Ampligen protected mice from variant H5N1 influenza virus challenge

We next examined the protective effect of intranasal administration of vaccine and Ampligen adjuvant against homologous (A/Vietnam/1194/04) and heterologous (A/HK/483/97 and A/Indonesia/6/05) H5N1 influenza virus challenge (Fig. 3). Two groups of mice were immunized either intranasally or subcutaneously with 1 μg of NIBRG14 and 10 μg of Ampligen, then challenged by intranasal administration of 1000 PFU of H5N1 influenza virus at 2 weeks after the final immunization. A third group of control mice was immunized intranasally with 10 μg of Ampligen alone. In response to homologous viral challenge, the mean nasal wash virus titer of control mice was $10^{3.04}$ PFU/ml 3 d.p.i., and none of the mice survived more than 12 d.p.i. (Fig. 3A). In mice immunized subcutaneously with vaccine

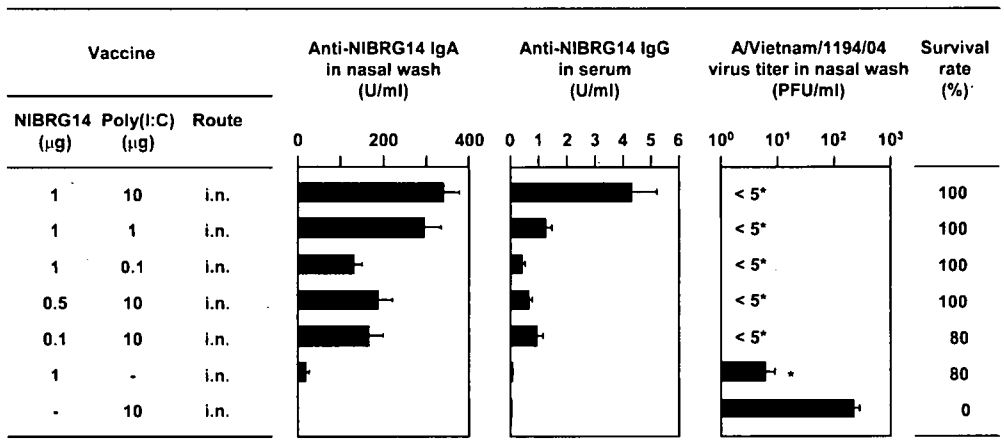

Fig. 1. Anti-NIBRG14-specific IgA and IgG antibody titers, A/Vietnam/1194/04 virus titers, and survival rate of mice after lethal challenge with A/Vietnam/1194/04 virus. Anti-NIBRG14-specific IgA and IgG responses in BALB/c mice immunized twice intranasally with the indicated doses of vaccine (NIBRG14) and/or poly(I:C) adjuvant. Nasal washes and serum samples were collected 14 days after the second immunization. Antibody titers of five mice from in each group were measured by ELISA. The same groups of mice were then infected intranasally with 1000 PFU of A/Vietnam/1194/04 virus 14 days after the second immunization. Nasal washes were collected 3 d.p.i., and virus titers were measured by plaque assay. Each column represents the mean ± standard error (S.E.) of five animals. For statistical analysis, virus titers were compared to control mice that received intranasal administration of 10 μg of poly(I:C) without vaccine. Survival rates were monitored for 16 d.p.i. $*P < 0.05$.

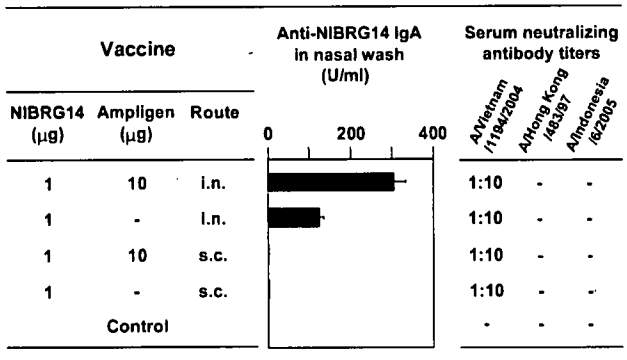

Fig. 2. Anti-NIBRG14-specific IgA and IgG responses in BALB/c mice immunized twice intranasally or subcutaneously with vaccine alone, or in combination with Ampligen®. Nasal washes and serum samples were collected 14 days after the final immunization. Antibody titers were measured by ELISA; data represent the means ± S.E. of five mice. The serum collected at 2 weeks after the booster was analyzed for the presence of neutralizing antibodies against homologous or heterologous influenza virus. Inhibition of the virus was assessed by the additional reduction in infectivity beyond the background of naïve mice. Sample were run in duplicate, and data are presented per group, where the ability to inhibit 100% of infection at the indicated dilution is shown. Dashes (−) indicate a lack of reduction of infectivity.

and Ampligen, there was a 1 log reduction in nasal wash viral titer compared to control mice, and all mice survived up to 18 d.p.i. (Fig. 3A). In contrast, none of the mice immunized intranasally with vaccine and Ampligen had virus titers in their nasal washes, and 100% of the mice survived up to18 d.p.i. (Fig. 3A). In the heterologous, A/HK/483/97 virus challenge group, the mean nasal wash virus titer of control mice was $10^{3.44}$ PFU/ml 3 d.p.i., and none of the mice survived more than 10 d.p.i. (Fig. 3B). In mice immunized subcutaneously with vaccine and Ampligen, there was no evidence of a protective effect based on nasal viral titers, and none of the mice survived more than 10 d.p.i. (Fig. 3B). However, in mice immunized intranasally with vaccine and Ampligen, there was a significant reduction in virus titer in the nasal washes compared to the subcutaneous vaccination group, and 80% of mice survived up to 18 days following virus challenge (Fig. 3B). In the group challenged with heterologous A/Indonesia/6/05 virus, the mean nasal wash virus titer of the control group was $10^{4.44}$ PFU/ml 3 d.p.i., and 20% of the mice survived up to 18 d.p.i. (Fig. 3C) In addition, 1000 PFU of A/Indonesia/6/05 was not 100% lethal. In mice immunized subcutaneously with vaccine and Ampligen, there was a significant reduction in nasal wash virus titer compared to control mice, and 40% of the mice survived up to 18 d.p.i. (Fig. 3C). In mice immunized intranasally with vaccine and Ampligen, there was a significant reduction in virus titers compared to the subcutaneous vaccination and control groups, and 100% of the mice survived up to 18 d.p.i. (Fig. 3C). These results clearly indicated that intranasal administration of H5N1 vaccine and Ampligen adjuvant is more effective than subcutaneous vaccination against homologous and heterologous H5N1 influenza virus challenge.

3.4. Expression levels of IRF-3, IRF-7, RIG-I, MDA5, Fas, and TRAIL mRNA in the NALT after intranasal administration of vaccine with dsRNA

Lastly, we examined mRNA expression levels of IRF-3, IRF-7, RIG-I, MDA5, Fas, and TRAIL to define the mechanism of adjuvant activity when dsRNA was administered intranasally with influenza vaccine (Table 1). The expression of IRF-7 in the NALT was up-regulated in mice administered vaccine and poly(I:C) without infection, or mice challenged with A/PR8 influenza virus, while that of IRF-3 was unchanged under either condition (Fig. 4A and B). The mRNA level of RIG-1, a cytoplasmic dsRNA receptor, but not MDA5 was rapidly induced in mice administered vaccine and poly(I:C) without infection, or mice challenged with A/PR8 influenza virus, compared to the group receiving vaccine alone (Fig. 4C). The mRNA levels of Fas and TRAIL were unchanged among the three groups (Fig. 4E and F). These results suggested that up-regulation of IRF-7 and RIG-I, which are key molecules in the recognition of viral dsRNA and the induction of type-I IFNs in the early stages of viral replication, mediate the adjuvant effect of dsRNA, and enhance the immune response in the nasal mucosa.

4. Discussion

In the current study, we evaluated the immunogenicity and cross-protective effect of the NIBRG14 H5N1 influenza vaccine co-administered intranasally with poly(I:C) or Ampligen adjuvant. It has been suggested that the most effective immunization strategies for protection against influenza virus infection should involve the induction of a mucosal immune response at the nasal mucosal epithelium, which is the initial site of virus infection [5,26]. Single subcutaneous immunization with an inactivated H5 influenza vaccine with incomplete Freund's adjuvant or aluminum hydroxide adjuvant conferred protection against heterologous H5N1 influenza virus challenge in a lethal mouse model [27]. In the current study, we demonstrated that intranasal immunization with adjuvant-combined vaccine, but not subcutaneous vaccination, induced an A/Vietnam/1194/04-specific IgA response in the nasal mucosa (Figs. 1 and 2), and conferred a broad spectrum of cross-protective immunity against heterologous A/HK/483/97(H5N1) and A/Indonesia/6/05(H5N1) viruses (Fig. 3). In intranasal vaccination group, A/Vietnam/1194/04 HA-reactive IgA and IgG were detected in the nasal wash and serum, respectively. However, the neutralizing activity of the serum against homologous A/Vietnam/1194/04 virus but not heterologous A/Hong Kong/483/97 and A/Indonesia/6/05 was observed exclusively (Fig. 2). No neutralizing activity was detected in the nasal wash in any group in the present study against both homologous and heterologous viruses. The concentration of anti-A/Vietnam/1194/04 HA IgA in the nasal wash was much lower than that of anti-A/Vietnam/1194/04 HA IgG in the serum, because the nasal wash was diluted with PBS for collection from the nasal mucosa. It seems that the concentration of anti-A/Vietnam/1194/04 HA IgA is

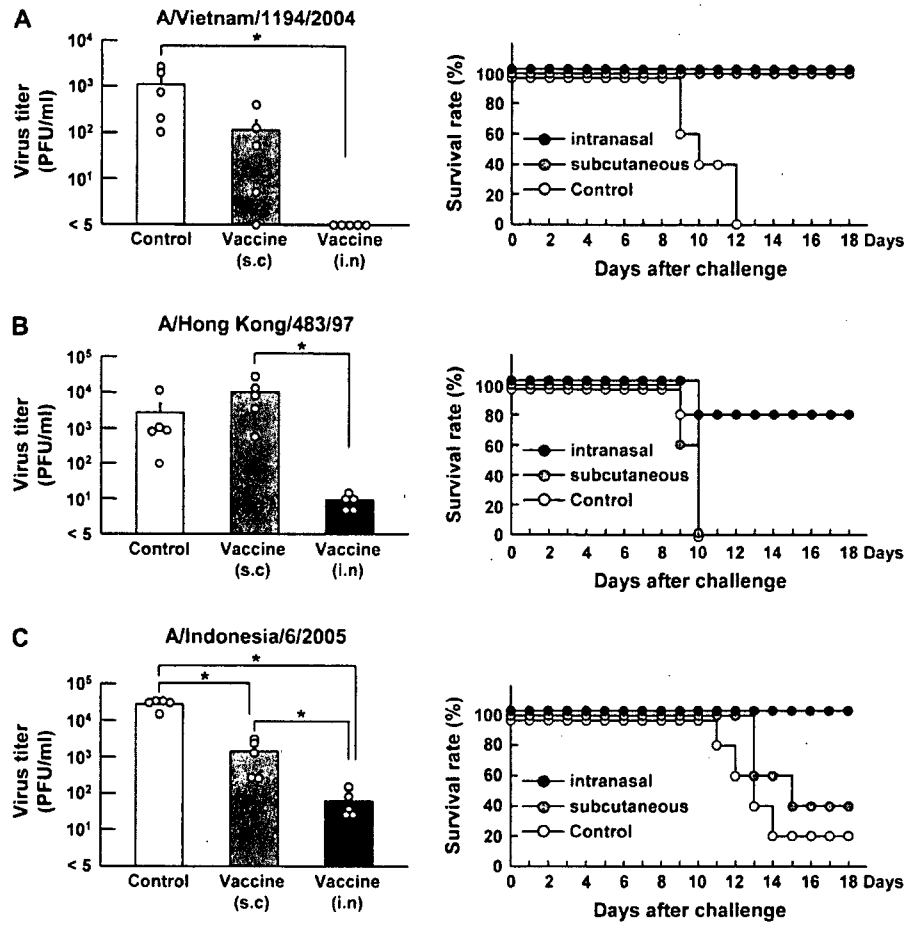

Fig. 3. H5N1 virus titers in nasal washes and survival rates after lethal challenge with homologous A/Vietnam/1194/04, heterologous A/HK/483/97, or heterologous A/Indonesia/6/05 viruses. Mice were immunized intranasally (black column) or subcutaneously (gray column) with vaccine and Ampligen, then challenged by intranasal administration of 1000 PFU of A/Vietnam/1194/04 (A), A/HK/483/97 (B), or A/Indonesia/6/05 (C) virus 14 days after the final immunization. Nasal washes were collected 3 d.p.i., and virus titers were measured by plaque assay. Each column represents the mean ± S.E. of five mice and open circles indicate individual animals. For statistical analysis, virus titers were compared to those from control mice (open column) that received intranasal administration of 10 μg of Ampligen alone. Survival rates were monitored for 18 days. $^*P < 0.05$.

much lower than the physiological concentration in the nasal mucosa, and therefore neutralizing activity in the nasal wash may not be detected. In this experiment, we used the same PFU value of each strain of H5N1 viruses to compare the virus titer in the nasal wash and survival rate after the challenge between subcutaneously vaccinated and intranasally vaccinated group within the same challenging strain. Although A/Indonesia/6/05 was not 100% lethal at this challenging dose, intranasal administration of the vaccine improved the survival rate from 20% to 100%, while subcutaneous vaccination modified the survival rate from 20% to 40%. These results indicate that intranasal vaccination is more effective than subcutaneous vaccination in protecting against heterologous H5N1 influenza virus infection.

Cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) have been used as adjuvants to enhance mucosal immune responses [28]. Although CT and LT are effective adjuvants, they have several undesirable side-effects in humans, including dysfunction of the VIIth cranial nerve [13]. Therefore, the development of other, safer adjuvants is required. Previously, we demonstrated that poly(I:C) has mucosal adjuvant activity in combination with a split-product influenza vaccine (A/PR8, H1N1), and does not have any adverse effects on the central nervous system [7]. Although poly(I:C) is a potent mucosal adjuvant, that induces type I IFNs and could potentially bridge the gap between innate and adaptive immunity [16], its poor safety record in clinical trials imposes a significant regulatory barrier [17].

In this study, we also explored the potential mechanism underlying the adjuvant effect of dsRNA. Intranasal administration of poly(I:C) and the split-product vaccine, or A/PR8 virus, but not vaccine alone (no adjuvant), induced mRNA expression of IRF-7 and RIG-I in NALT. Previously, we showed that TLR-3, which recognizes dsRNA, was upregulated, and IFN-α/β was induced, as were Th1- and Th2-related cytokines, after intranasal administration of poly(I:C) and the split-product HA vaccine [7]. dsRNA is recognized not only by TLR-3 and RNA helicase, RIG-I and MDA5, and induces activation of NF-κB and production of type-I IFNs [29,30]. Thus, it appears that the mucosal adjuvant effect of dsRNA results in

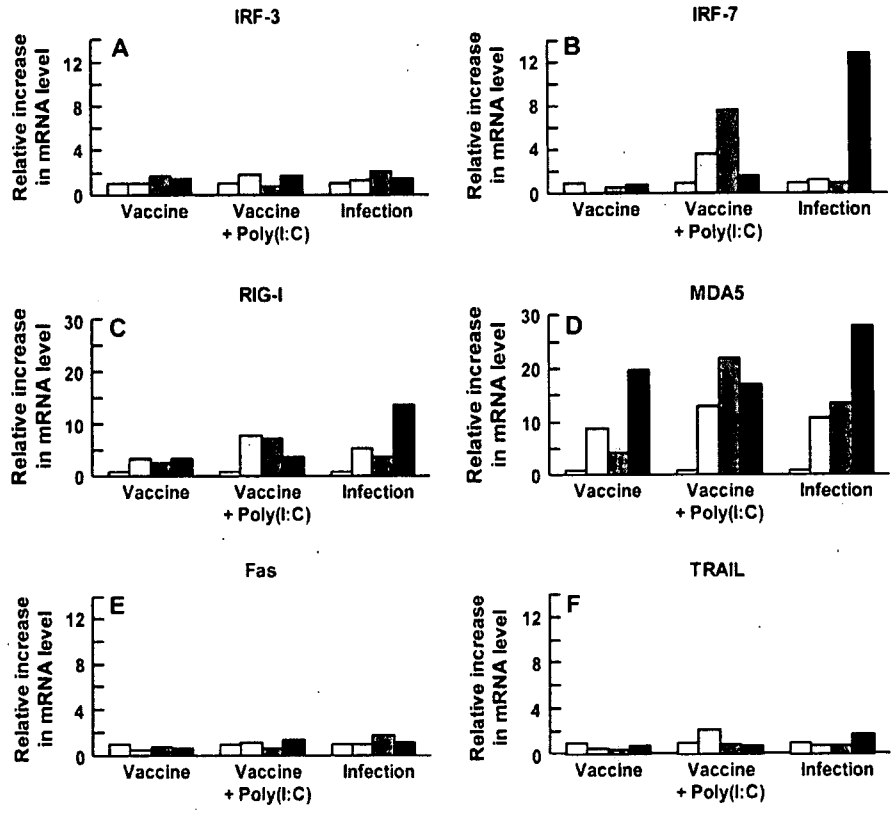

Fig. 4. Expression of mRNAs of IRF-3 (A), IRF-7,(B), RIG-I (C), MDA5 (D), Fas (E), and TRAIL (F) in NALT. Total RNA was extracted from NALT at 0 h (open column), 6 h (light gray column), 24 h (dark gray column), and 72 h (closed column) after intranasal administration of HA vaccine (A/PR8) alone, HA vaccine plus Poly (I:C), or infected with 1000 PFU of A/PR8 viruses. Real-time quantitative RT-PCR was used to determine mRNA expression levels of pooled total RNAs extracted from NALT of three mice.

marked antibody responses, together with enhanced expression of TLR-3, RIG-I, IRF-7, type-I IFNs, and Th1- and Th2-related cytokines. It also confers a cross-protective effect against lethal challenge with heterologous H5N1 influenza viruses. The expression of Fas and TRAIL did not change upon virus infection or intranasal administration of vaccine with dsRNA adjuvants.

In summary, intranasal administration of Ampligen combined with H5N1 vaccine derived from a highly pathogenic influenza virus clinical isolate induced cross-protective mucosal immunity against heterologous H5N1 influenza virus infection. Intranasal vaccination co-administered with the TLR-3 agonist, Ampligen, appears to offer an effective strategy against an influenza pandemic, regardless of the strain of H5N1. The potential shortage of vaccine during a pandemic might also be addressed by preparing a stock of this vaccine in advance using the United States Food and Drug Administration "animal rule" as a global model for regulatory approval by various governmental regulatory agencies.

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Original article

Integration of HIV-1 caused STAT3-associated B cell lymphoma in an AIDS patient

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Abstract

Signal transducer and activator of transcription 3 (STAT3) is a DNA-binding transcription factor activated by multiple cytokines and interferons. High expression of STAT3 has also been implicated in cancer and lymphoma. Here, we show a case of B cell lymphoma in which a defective human immunodeficiency virus 1 (HIV-1) integrated upstream of the first STAT3 coding exon. The lymphoma cells with anaplastic large cell morphology formed multiple nodular lesions in the lung of an acquired immunodeficiency syndrome (AIDS) patient with Kaposi's sarcoma. The provirus had a 5′ long terminal repeat (LTR) deletion, but the 3′ LTR had stronger promoter activity than the STAT3 promoter in reporter assays. Immunohistochemistry showed increased expression of STAT3 in the nuclei of lymphoma cells. Transfection of STAT3 resulted in transient cell proliferation in primary B cells in vitro. Although this is a very rare case of HIV-1-integrated lymphoma, these data suggest that up-regulation of STAT3 caused by HIV-1 integration resulted in the development of B cell lymphoma in this special case.

Keywords: HIV-1; Integration; AIDS-related lymphoma; STAT3

1. Introduction

Malignant lymphoma is an important complication of patients with acquired immunodeficiency syndrome (AIDS). A large part of AIDS-related lymphomas are of B cell linage, and positive for Epstein–Barr virus (EBV) or Kaposi's sarcoma-associated herpesvirus (KSHV) [1–4]. Since human immunodeficiency virus 1 (HIV-1) is not usually detected in AIDS-related lymphoma cells, HIV-1 infection plays an indirect role in lymphomagenesis by impairing host immune surveillance. However, proviral DNA can either disrupt expression of tumor suppressor genes or enhance expression of cellular oncogenes. Alternatively, retroviral promoters can integrate into the host genome in such a manner that expression of a nearby oncogene is enhanced by a strong promoter within the proviral 3′-long terminal repeat (3′LTR). In humans, abnormal T cell proliferation following gene therapy for severe combined immunodeficiency resulted from retroviral integration into the intron of the *LMO2* proto-oncogene [5].

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In AIDS patients, some cases of lymphomas had HIV-1 integration within the *fur* gene, just upstream from the *c-fes/fps* proto-oncogene [6]. That report, however, did not investigate the functional effect of this integration event. These observations suggest that HIV-1 may contribute directly to lymphomagenesis by inserting an active promoter into a cellular oncogene [6]. In the present study, we report a case of AIDS-related lymphoma in which HIV-1 integrated upstream of the STAT3 gene. The association of HIV-integration and lymphomagenesis was investigated.

2. Materials and methods

2.1. Samples

Lymphoma tissues in the lung of a patient with HIV-1 infection were obtained at autopsy. Formalin-fixed pathological samples of lymphoma, including nine unrelated cases of AIDS-related lymphoma and 15 cases of non-Hodgkin lymphoma in HIV-1-uninfected individuals, were studied. All samples were obtained with informed consent according to the Declaration of Helsinki. The study protocol was approved by the institutional review board of National Institute of Infectious Diseases (Approval No. 93).

2.2. Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as described before [7,8]. Primary antibodies were: anti-CD3 (Dako, Copenhagen, Denmark), CD20 (Dako), CD30 (Dako), CD45 (Dako), CD45RO (Dako), CD79a (Dako), CD138 (Serotec, Oxford, UK), and $p80^{NPM/ALK}$ (Nichirei, Tokyo, Japan), STAT3 (sc8019, Santa Cruz Biotechnology, Santa Cruz, CA), pSTAT3 (sc8059, Santa Cruz), KSHV-encoded LANA [8], and vIL-6 [7] antibodies. In situ hybridization for EBERs was performed as described before [9].

2.3. PCR and DNA sequences

PCR detection for KSHV-encoded open reading frame (ORF) 26, EBV W region, HIV-1 V3, and β-globin gene was performed as described previously [9,10]. For PCR amplification of HIV-1 3′LTR and STAT3 junction, HIV3LTR-F (5′-TCTGAGCCTGGGAGCTCTCT-3′, 9561–9580 in GenBank K03455) and Stat3intron-R (5′-AGTGCATGGCACATAACAGA-3′, 41131–41150 in GenBank AY572796) were used. For amplification of HIV-1 5′LTR and STAT3 junction, 6 reverse primers of 5′LTR (55R 5′-TCAGGGAAGTAGCCTTGTGTGTGGT-3′, 78R 5′-GCCCTGGTGTGTAGTTCTGTCAATC-3′, 348R 5′-GAAAGTCCCCAGTGGAAAGTCCCTT-3′, 495R 5′-GCAGTGGGTTCCCTAGTTAGCC-3′, 563R 5′-TTACCAGAGTCACACAACAGACGGG-3′, and 612R 5′-CACTGCTAGAGATTTTCCACACTGAC-3′), and a reverse primer positioning between 5′LTR and gag (676R 5′-CGAGTCCTGCGTCGAGAGATCTCCT-3′) were used with a forward primer of Stat3-intronF2 (5′-CATTTTTCTTTCCTTCTCTGTTGTC-3′, 40881–40905 in GenBank AY572796). These primers for HIV-1 were designed based on the sequence of HIV-1 IIIB (GenBank K03455).

2.4. Cloning of HIV-1 integration sites

The methods used were essentially as described for the Gene Walker Kit (BD Clontech, Palo Alto, CA). Lung tumor DNA was cleaved with four different blunt cutting enzymes (*Dra*I, *Eco*RV, *Pvu*II and *Ssp*I). Gene specific primers for HIV-1 LTR were 5′-ACCACACACAAGGCTACTTCCCTGA-3′ (GSP-1) and 5′-AAGGGACTTTCCACTGGGGACTTTC-3′ (GSP-2).

2.5. Real-time PCR

Copy numbers of HIV-1 integration site and STAT3 gene were measured with real time PCR as described previously [11]. Two probe and primer sets were used (Set 1: forward primer: 5′-CTAGAGATCCCTCAGACCATTTTAGTC-3′, reverse: 5′-AAAAGTATAAATGAGGATCCAGGAAGAT-3′, probe: 5′-6FAM-TGTGGAAAATCTCTAGCAGAATCTCAGG-TAMRA-3′; Set 2: forward primer: 5′-GCAGCTTGACACACGGTACCT-3′, reverse: 5′-AAACTGCCGCAGCTCCATT-3′, probe: 5′-6FAM-AGCAGCTCCATCAGCTCTACAGTGACAGC-TAMRA-3′).

2.6. Plasmids

For the promoter assay, genes of the HIV-1 3′LTR, STAT3-intron (40951–41959 of GenBank AY572796), and STAT3-promoter (1–1998 of GenBank AY572796) were amplified from DNA of the HIV-1-integrated lymphoma using the LTR-*Mlu*I-F, 5′-GAG*ACGCGT*TGGAAGGGCTAATTCACTCCC-3′ and LTR-*Xho*I-R, 5′-GTG*CTCGAG*TGCTAGAGATTTTCCACACT-3′, the Intron-*Mlu*I-F, 5′-GAG*ACGCGT*GAATCTCAGGCAGATCTTCC-3′ and Intron-*Xho*I-R, 5′-CAC*CTCGAG*CCTGCTAAAATCAGGGGTCCC-3′, and, the Stat3prom-*Mlu*F1, 5′-GAG*ACGCGT*ACCCATAGTCGCAGAGGTAGA-3′ and Stat3prom-*Xho*R1, 5′-GAG*CTCGAGCGCTGAATTACAGCCCCTTCA-3′, respectively. Enzyme sites are indicated in italics. A fragment of the HIV-1 3′LTR was amplified also from HIV-1 pNL4-3 (GenBank AF324493). The PCR product was subcloned into *Mlu*I-*Xho*I site of pGL3-basic vector (Promega, Madison, WI). For the STAT3-expression plasmid, STAT3 cDNA was amplified from the mammalian gene collection-human (MGC-1607, American type culture collection, Manassas, VA) using forward primer (STAT3-HpaI-F10 5′-CACCGTTAACGGATCCTGGACAGGCACCC-3′) and reverse primer (STAT3-R24 5′-CATGTCAAAGGTGAGGGACTCAAA-3′). The PCR product was TA cloned using pcDNA 3.1 Directional TOPO Expression kit (Invitrogen, Carlsbad, CA). For cell proliferation experiment, the STAT3 expression vector was digested with *Hin*dIII and *Eco*RV and ligated into *Bsm*BI and *Eco*RV sites of pMACS 4-IRES.II vector, which is a bicistronic expression vector containing multiple cloning site followed by an internal ribosome entry site (IRES) element

from encephalomyocarditis virus and the truncated (non-functional) CD4 cDNA (Miltenyl Biotec, Auburn CA).

2.7. Promoter assay

Plasmids were transiently transfected into HeLa cells with a renilla reporter gene construct using Lipofectamine Plus (Invitrogen). Luciferase activity was measured with a dual luciferase assay system (Promega). In the HIV-1-Tat (+) group, an HIV-1-Tat expression vector, kindly provided by Dr. Kenzo Tokunaga, National Institute of Infectious Diseases, Tokyo, Japan, was cotransfected.

2.8. DNA methylation analysis

Methylation of the cytosine residue of the CpG site was analyzed by the bisulfite genomic sequencing method, as described previously [12]. The primer pair for selective analysis was as follows: sense primer, 5′-TATAAACCAGCATGG GATGGATGA-3′; antisense primer, 5′-CCCAGGCTCGGA TCTGGTCTAACC-3′.

2.9. Cell proliferation assay for primary lymphocytes

Primary B cells were negatively selected from whole blood of healthy volunteers using RosetteSep B cell enrichment (StemCell Technology, Vancouver, BC, Canada) [13]. Cell proliferation assay was performed using BrdU Cell proliferation ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN).

3. Results

3.1. HIV-1 was concentrated in lymphoma cells in a case of AIDS-related lymphoma

A 59-year-old, homosexual, HIV-1-positive male with a CD4 cell count of 6/mm^3 showed high fever and multiple KS skin lesions. Computed tomography scanning revealed multiple nodules in the lung (Fig. 1A). Despite treatments with antibiotics and combined chemotherapy, with intensive care, he died 30 days after admission. The clinical course of the patient was also reported previously [14]. At autopsy, multiple nodules were present in the lung (Fig. 1B). Histologically, these nodules were composed of large atypical cells with anaplastic large cell morphology infiltrating into interstitial and alveolar areas in the lung tissue (Fig. 1C). Immunohistochemistry demonstrated that the tumor cells were CD3$^-$, CD20$^-$, CD30$^+$, CD45$^+$, CD45RO$^+$, CD79a$^-$, CD138$^-$, and p80$^{NPM/ALK-}$, suggesting that the lung tumor was composed of lymphoma cells (Fig. 1D and data not shown) [14]. Southern blot hybridization of DNA extracted from the lung tumor with an immunoglobulin junction hinge (JH) probe demonstrated immunoglobulin gene rearrangement, confirming a B

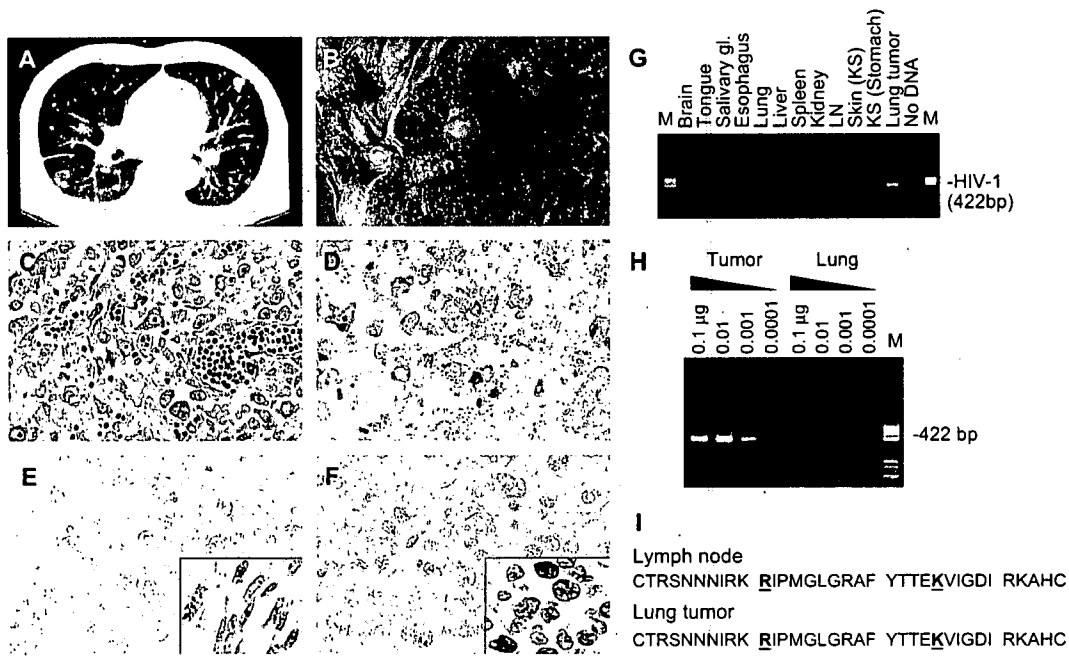

Fig. 1. Pathological findings of tumors in the lung of a patient with AIDS. CT scan (A), macroscopic view (B) and Hematoxylin and eosin staining (C) of the lung tumor. (D) Immunohistochemistry of CD45RO. (E) Immunohistochemistry for KSHV-LANA in the lung tumor cells. Inset shows gastric KS cells from the patient. (F) In situ hybridization for EBV-EBER in the lung tumor cells. Inset shows a positive control of EBV-positive lymphoma from an unrelated patient. (G) PCR detection for HIV-1 V3 region in various organs of the patient. LN, lymph node; M, DNA molecular weight marker (pBR322/*Hae*III). (H) Semi-quantitative PCR for HIV-1. DNA quantities are indicated at the top of the panel. DNA extracted from the lung tumor and surrounding lung tissues was tested. (I) Predicted amino acid sequence of HIV-1 gp120 V3 loop of HIV-1 amplified from the lymph node and lung tumor by PCR. Positions 11 and 25 are indicated by bold letters with underlines. DNA sequences are deposited in GenBank under accession numbers DQ116951 to DQ116954 (HIV-1 envelope from LN and lung tumor).

cell linage (data not shown). Since KS lesions were found in the oral cavity, stomach, sole and some lymph nodes at autopsy, we examined KSHV positivity in the lymphoma (lung tumor). KSHV-encoded ORF26 was amplified in both gastric KS lesions and lung tumor by PCR (data not shown). However, immunohistochemistry demonstrated that expression of KSHV LANA was very weak or absent in the lymphoma cells, whereas KS cells in the stomach strongly expressed LANA (Fig. 1E). Immunohistochemistry also demonstrated that the lung tumor cells were negative for KSHV-encoded vIL-6 (data not shown). The lymphoma cells were positive for EBV by PCR (data not shown), but in situ hybridization failed to detect EBERs (Fig. 1F). Thus, these data suggest that KSHV and EBV were present in the lymphoma at low copy numbers. Surprisingly, HIV-1 DNA was detected in the lymphoma cells by PCR, but not in other organs besides the lymph nodes (Fig. 1G). Semi-quantitative PCR revealed that there was a 100-fold higher copy number of HIV-1 DNA from the lymphoma than from surrounding lung tissue (Fig. 1H). PCR products of HIV-1 V3 region were TA-cloned and each 10 clones were sequenced. Although two (clones L2 and T3) and three (clones T1, T3, and T6) kinds of sequences were obtained from the lymph nodes and lymphoma, respectively, all sequences coded the same amino acid sequence in the V3 loop (net charge = + 7). Basic amino acids at positions 11 and 25 of the gp120 V3 loop and a high positive net charge strongly suggest that fusogenic X4 viruses were detected in the lymphoma cells and lymph nodes (Fig. 1I) [15].

3.2. HIV-1 integration in the STAT3 gene

A high copy number of HIV-1 in the lymphoma suggested integration of HIV-1 into the genome of lymphoma cells. Genome walking PCR produced a 400 bp fragment which contained a 300 bp fragment with >99% identity with the HIV-1 IIIB 3′LTR sequence (GenBank K03455) and A 40 bp genomic segment just before the first coding exon of STAT3 (Fig. 2A). PCR using primers in HIV-1 3′LTR and STAT3-intron yielded an independent amplicon with HIV-1 3′LTR and the predicted STAT3 genomic sequences from DNA of the lymphoma cells (Fig. 2B). These data confirmed that HIV-1 had integrated into the intervening sequence just before the first coding exon of STAT3. PCR using a primer pair binding to the STAT3 intron and upstream of HIV-1 gag demonstrates that the 5′LTR of the integrated HIV-1 was truncated (Fig. 2C). The sequence analysis revealed that the integrated HIV-1 lacked a fragment at the position of 1–587 in the 5′LTR (Fig. 2A,D, GenBank AF538307). Compared with the sequence of the 3′ integration site, HIV-1 integration resulted in duplication of the cellular 5 bp (GAATC) and addition of a dinucleotide at the integration site by HIV-1 integrase, which is commonly seen among retrovirus integrases [16,17]. Consequently, the integration event was produced by a defective virus (Fig. 2A,D). The absence of p24-staining of the tumor is consistent with this conclusion (data not shown).

3.3. Copy number of the integrated HIV-1 in the lymphoma tissue

Generally, pathological tissues obtained from lymphoma lesions contain not only lymphoma cells, but also surrounding CD4-positive T cells or alveolar macrophages. Although immunohistochemistry demonstrated no or rare CD4-positive cells in the lymphoma tissue, we tried to determine a copy number of the integrated HIV-1 in the lymphoma tissue by a real time PCR targeting genes near the integration site to deny the possibility that HIV-1 integration was originated in the contaminated CD4-positive cells (Fig. 3). A fragment of HIV-1-integration site was amplified at 12,570 copies/100 ng of DNA by the real time PCR, whereas exon 1 of STAT3 gene was amplified at 121,597 copies/100 ng. Since each cell has two copies of STAT3 gene on two alleles, these data suggest that HIV-1 integration occurred about 20% of the population that the DNA was extracted from. As shown in Fig. 1C, the lymphoma tissue contained many cells other than lymphoma cells, such as alveolar epithelial cells, macrophages, and endothelial cells. However, CD4-positive T cells were rare in the tissue, and the HIV-1 was X4 virus. Therefore, these data suggest that the HIV-1 might be detected from lymphoma cells, not from contaminated T cells or macrophages, and integrate into more than 20% of the lymphoma cells.

3.4. Promoter activity and methylation of HIV-1 3′LTR

LTRs of HIV-1 usually have a promoter activity in HIV-1-infected T cells and macrophages [18]. To investigate if the HIV-1 3′LTR contained a functional promoter, we constructed a plasmid containing the patient's HIV-1 3′LTR or upstream intron sequence of STAT3 before a luciferase reporter gene. Transfection of the plasmid to HeLa cells revealed that the sequence of 3′LTR derived from the patient had significant promoter activity at a similar level to that of 3′LTR in HIV-1 NL4-3, but the upstream intron sequence of STAT3 did not (Fig. 4A). 3′LTR was a stronger promoter than the STAT3 promoter derived from the patient. Cotransfection with a plasmid expressing HIV-1-Tat enhanced the activity of the patient's 3′LTR 31-fold, whereas the activity of the STAT3 promoter was not enhanced. These data suggest that the HIV-1 3′LTR contains promoter activity. It is known that DNA CpG methylation inactivates retroviral promoter including HIV-1 LTR [12,19]. However, a bisulfite genomic sequence revealed that the fragment of HIV-1 3′LTR did not have any CpG or non-CpG methylation in the DNA extracted from the lymphoma (Fig. 4B,C). These data suggest that methylation might not reduce or inhibit the transcriptional activity of HIV-1 3′LTR in the HIV-1-integrated lymphoma cells.

3.5. Expression of STAT3 in the HIV-1-integrated lymphoma

We investigated expression of STAT3 in the case of HIV-1-integrated lymphoma. Immunohistochemistry demonstrated a high level of STAT3 expression predominantly in the nuclei

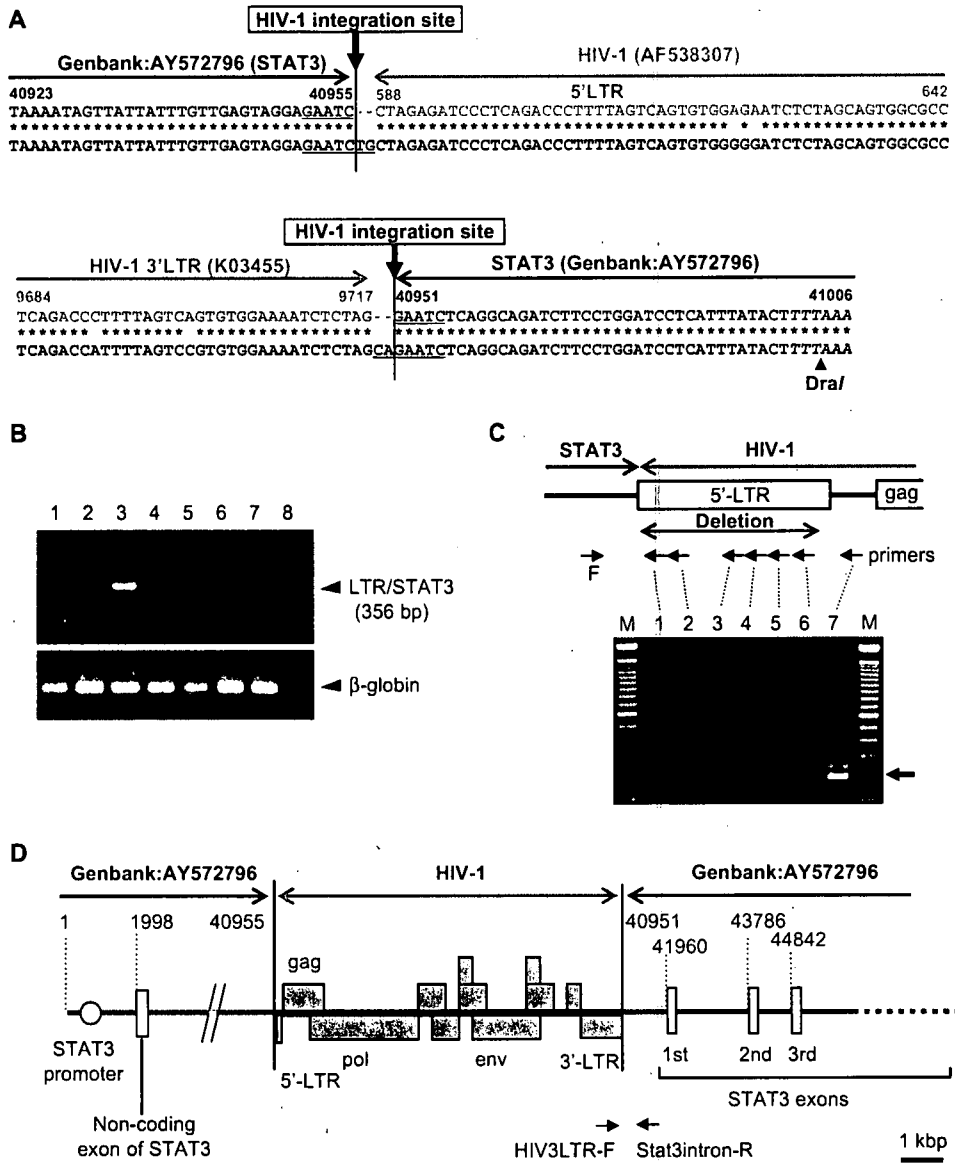

Fig. 2. Identification of HIV-1 integration site in the lymphoma cells with genome walking. (A) Sequence of the HIV-1 5′-LTR (upper panel) and 3′-LTR (lower panel) insertion site in the lymphoma genome. Whole sequences of PCR products are registered as GenBank DQ355432 (5′-LTR, 190 bp) and DQ117603 (3′-LTR, 1.5 kbp), respectively. The sequence of the lymphoma genome is shown in the lower line in black letters. The upper colored line indicates the HIV-1 LTR sequence (blue, GenBank K03455 or AF538307) and STAT3 genomic sequence (violet, GenBank AY572796). HIV-1 intervening sequence between 5′LTR and gag is indicated by green. Duplication of the cellular 5 bp (GAATC) and additional dinucleotides (TG in 5′-LTR and CA in 3′-LTR) by HIV-1 integrase are underlined. *DraI* site is indicated by italics. (B) PCR for the junction region of 3′LTR and STAT3 gene using HIV3LTR-F and Stat3intron-R primers (see Fig. 3D). 1, PBMCs from a healthy donor; 2, HIV-1-positive Molt4 cell line; 3, lymphoma cells with HIV-1integration; 4, KS lesion from the patient; 5, AIDS-related lymphoma from an unrelated patient; 6, lymphoma from a non-HIV-1infected patient; 7, BCBL-1 (KSHV-positive B cell line); 8, No DNA. The lower panel shows the results of an internal control (β-globin gene). (C) PCR of genomic DNA with a STAT3-intron forward primer (F in this figure, Stat3-intronF2) in combination with 5′ LTR reverse primers (lanes 1–6, 55R, 78R, 348R, 495R, 563R and 612R), and a reverse primer positioning between 5′LTR and gag (lane 7, 676R). The upper panel shows the positions of these primers. A 188 bp product was identified when the 676R primer was used with the STAT3 intron primer (lane 7). If the 5′LTR was intact, the predicted size of this amplicon would have been 777 bp. (D) Map of the defective HIV-1 insertion site in the STAT3 gene. Violet numbers indicate the number in GenBank AY572796 (STAT3). Blue boxes are HIV-1 genomes.

of the HIV-1-integrated lymphoma cells (Fig. 4D). To know the phosphorylation status of STAT3, we immunostained the slide using an anti-pSTAT3 (Tyr-705) antibody as a primary antibody. However, any signal was not found in the lymphoma cells (data not shown). We also examined STAT3 expression in 24 cases of lymphoma, including nine cases of AIDS-related lymphoma and 15 of non-AIDS-related lymphoma, normal tonsilar tissues and lymph nodes derived from unrelated patients. The nine cases of AIDS-related lymphoma contained seven of EBV-positive diffuse large B cell lymphoma

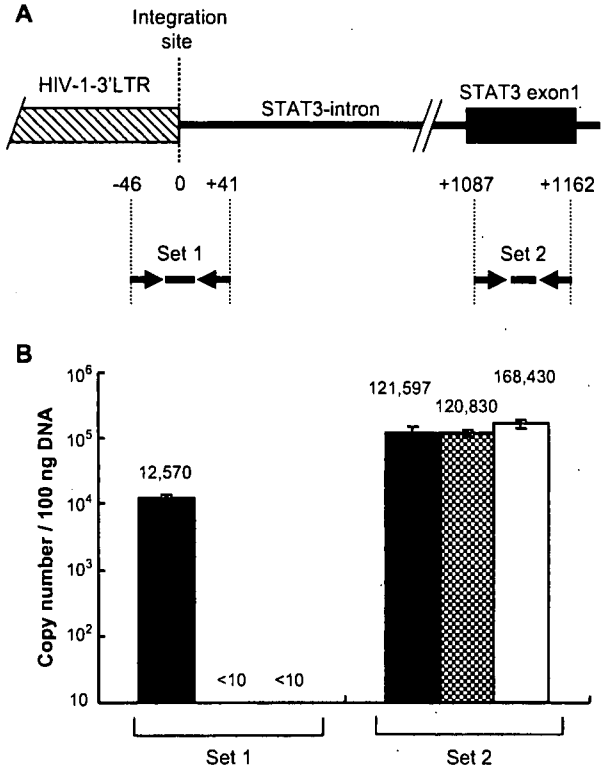

Fig. 3. Quantitative analysis of genes for HIV-1 integration site. (A) Probe-primer sets for real time PCR. The top line with boxes is a genome map around HIV-1 integration site of HIV-1 3'LTR. Numbers with plus and minus under the genome map indicate distances (bp) from the integration site. Arrows and heavy lines are probe-primer sets of real time PCR. (B) Copy numbers of HIV-1 integration site and STAT3 gene. Black, gray and white bars indicate mean copy number per 100 ng DNA of this case, HIV-1-positive Molt4 cell line, and TY-1 (HIV-1-negative, KSHV-positive B cell line), respectively. Copy numbers per 100 ng DNA are indicated on the top of each bar. Error bars indicate standard errors of triplicate samples.

(DLBCL), and two cases of Hodgkin's disease. The 15 cases of non-AIDS-related lymphoma contained 12 EBV-positive or EBV-negative DLBCL and three cases of Hodgkin's disease. Immunohistochemistry revealed that several cases of AIDS-related lymphoma and one of HIV-unrelated lymphoma expressed STAT3 predominantly in the cytoplasm (Fig. 4E); however, no case expressed STAT3 predominantly in the nucleus (Table 1). STAT3 expression was not found, or was weak, in other cases examined (Fig. 4F). These data suggest that the integration of HIV-1 induced high expression of STAT3 in the lymphoma cells of the patient.

3.6. Transfection of STAT3 expression plasmid to primary B cells in vitro

To investigate if expression of STAT3 induces cell growth, we constructed an expression plasmid for STAT3 and transfected the plasmid to B cells. At first, to confirm expression of STAT3 by Nucleofector transfection, His-tagged STAT3 was expressed in TY-1, a KSHV-positive B cell line. Immunofluorescence assay using anti-STAT3 and anti-6x His antibodies revealed that transfection efficiency to lymphocytes was 30–40% in this experiment (Fig. 5A). Addition of IL-6 to culture medium of transfected TY-1 altered the localization of STAT3 from the cytoplasm to the nucleus, suggesting that the transfected STAT3 reacted with IL-6 stimulation (Fig. 5B). Then, we investigated the proliferation of STAT3-transfected primary B cells. Cell proliferation assay after 48 h transfection showed that the proliferation of STAT3-transfected primary B cells were slightly higher than that of vector-transfected primary B cells (Fig. 5C, Mann–Whitney test, $p < 0.01$). However, 4 days after transfection, the difference was not statistically significant (data not shown). The transfection of STAT3 to B cells was repeated 4 times with similar results. These data suggested that transfection of STAT3 might induce a transient proliferation in the primary B cells in vitro.

4. Discussion

In the present study, we present a case of AIDS-related B cell lymphoma with HIV-1 integration. HIV-1 with defective 5'LTR integrated into the upstream region of the first STAT3 coding exon. The 3' LTR had strong promoter activity, resulting in increased expression of STAT3 in the nuclei of lymphoma cells. This is the first case report describing dysregulation of STAT3 by HIV-1 integration, resulting in B cell lymphoma development.

STAT3 is an important molecule for IL-6-type cytokines that signal and stimulate proliferation and terminal differentiation of B cells [20]. STAT3 also plays some oncogenic roles. Activated and phosphorylated STAT3 has been observed in a variety of experimental and numerous human malignancies [21–23]. A recent study reveals that high expression of unphosphorylated STAT3 results in up-regulation of oncogenes, suggesting that overexpression of either form of STAT3, phosphorylated and unphosphorylated, might induce cancer [24]. Although we failed to detect phosphorylated STAT3, high expression of STAT3 in the nucleus implies that activated STAT3 may bind to DNA and activate some genes constitutively. Alternatively, it implies that overexpression of unphosphorylated STAT3 in the nucleus might induce various oncogenes such as cdc2, cyclin B1 and mras [24]. However, our transfection study of STAT3 resulted in transient cell proliferation in the primary B cells (Fig. 5), suggesting that additional factors other than STAT3 expression might be required for complete transformation of primary B cells. HIV-1 integrated into *c-fes/fps* in other reported cases of AIDS-related lymphoma [6], and it has been demonstrated that *c-fes* activates STAT3 [25]. Thus, STAT3 may play some roles in the lymphomagenesis in the cases of HIV-1-integrated lymphoma.

This case was B cell lymphoma. HIV-1 usually infects and integrates into T cells or macrophages, and it is uncommon for HIV-1 to infect B cells. In the report by other group, HIV-1 provirus was frequently detected in macrophages infiltrating lymphomas, not in lymphoma cells [6]. However, in our case, we concluded that the HIV-1 integration occurred in the lymphoma cells, not in T cells or macrophages infiltrating

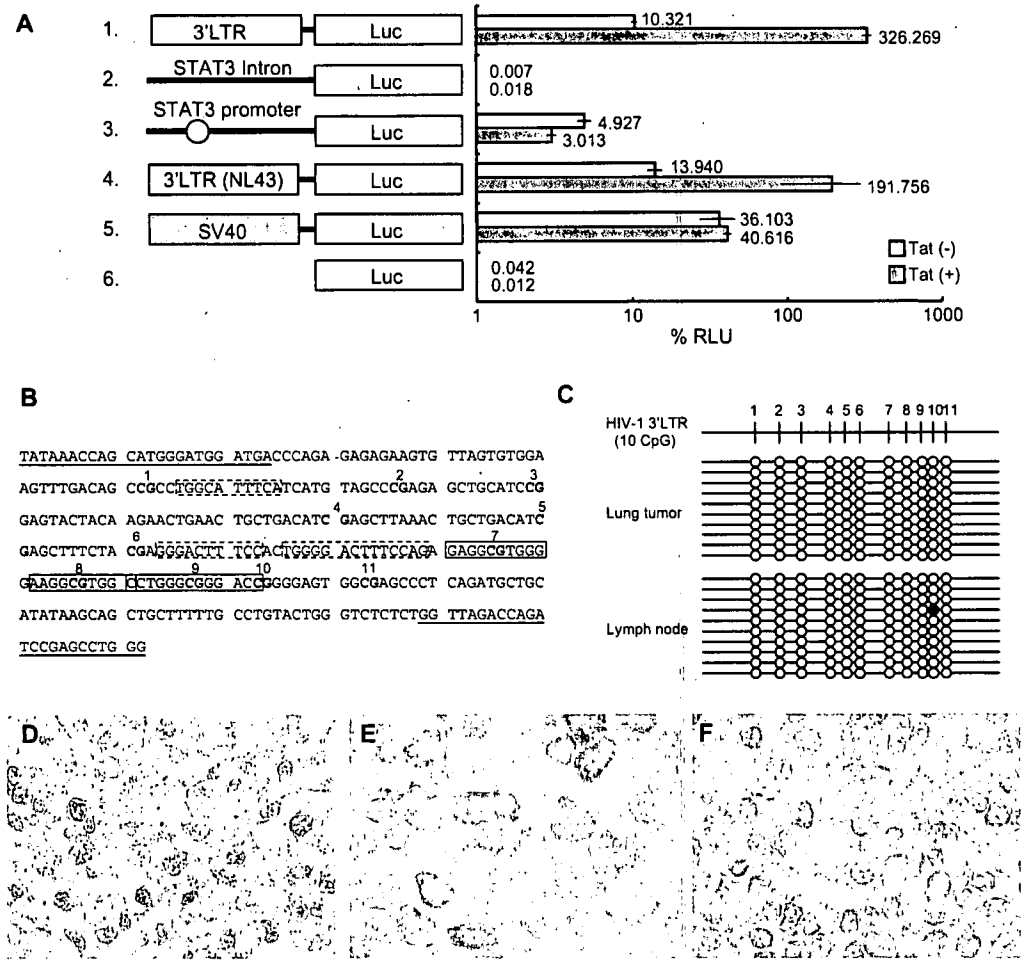

Fig. 4. Promoter activity of HIV-1 3′LTR and STAT3 expression in the lymphoma. (A) Promoter activity of HIV-1 3′LTR by reporter assay. Schematic representation of promoter constructs used in transient transfection assays is shown on the left. Forty-eight hours after transfection, cells were collected and the luciferase activity was measured. The percentage relative luminescence units (RLU) were calculated by dividing firefly activity by renilla activity. Horizontal bars indicate standard deviations of three independent experiments. (B and C) No methylation in a promoter enhancer region of HIV-1 3′LTR in the HIV-1-integrated lymphoma. (B) CpG sites in the promoter enhancer region of 3′LTR of the HIV-1 provirus in the patient with HIV-1-integrated lymphoma (218–529 in GenBank DQ117603). CpG sites are in boldface and numbered from the 5′ end of the LTR (1–11). Nuclear factor-κB and Sp1 sites identified with Motif Search (Kyoto University Bioinfomatics center, Kyoto, Japan, http://motif.genome.jp/) at a 75% cut-off value are indicated by boxes with broken and solid lines, respectively. Sequences used for primers are indicated by underlining. (C) Levels of CpG methylation of the promoter enhancer region of HIV-1 3′LTR in the HIV-1-integrated lymphoma and lymph nodes in the patient. Results of bisulfite genomic sequencing coupled with TA cloning are shown. The methylation status of 10 clones for each sample is presented; methylation of each CpG site is expressed as a filled circle, and unmethylated sites are shown as open circles. Top, schematic description of CpG sites in the 3′LTR of (B). (D–F) Immunohistochemistry of STAT3. The HIV-1-integrated lymphoma cells expressed STAT3 predominantly in the nucleus (D), however, signals of STAT3 were weak and localized in the cytoplasm in the other case of KSHV-positive, AIDS-related lymphoma (E), and were very weak in a case of EBV-positive, AIDS-related lymphoma (F). Original magnification is ×400.

in the lymphoma, because of following reasons: (1) there were few T cells in the lymphoma tissue by immunohistochemistry for CD3 (data not shown); (2) HIV-1 DNA was detected in the lymphoma at a high copy number, that is very rare or none in AIDS-related lymphoma [26]; (3) HIV-1 sequences suggested the possibility of X4 viruses, which leads the integrated HIV-1 sequences are usually not found in the macrophages; (4) some different HIV-1 V3 sequences were identified between the lymphoma and lymph node; and (5) the titer of HIV-1 DNA in the lymphoma were higher than that in the lymph node (Fig. 1G). Then, how did HIV-1 infect B cells in the patient? Although detail mechanism of HIV-1 infection to B cells in this case was still unknown, we presume that KSHV played an important role in HIV-1 infection to B cells. This case of lymphoma was positive for KSHV and EBV by PCR, however, KSHV and EBV did not play a direct role in the oncogenesis of the lymphoma because of the low or absent expression of

Table 1
STAT3 expression in AIDS-related and unrelated lymphoma

STAT3 expression	Nucleus	Cytoplasm	No expression	Total
AIDS-related lymphoma	1*	7	2	10
Non-AIDS-related lymphoma	0	1	14	15

*HIV-integrated lymphoma reported in the present study.

LANA and EBERs. It is possible that KSHV infection might increase susceptibility of B cells expressing CD4 and CXCR4 to infection with the X4 genotype of the HIV-1 [27]. Moreover, it is demonstrated that KSHV-encoded ORF50 protein increases susceptibility of B cells to infection with HIV-1 [28]. Although ORF50, CD4 and CXCR4 were not detected in the lymphoma cells by immunohistochemistry (data not shown), it is possible that KSHV-infected B cells might be infected and integrated by HIV-1 in the early stage of lymphoma development.

Although an intensive study revealed that there were many hot spots of HIV-1 integration [29], the STAT3 gene was not included in the list of hot spots. Thus, the STAT3 gene is a novel target of HIV-1 integration. Since HIV-1 DNA has not been detectable in DNAs extracted from AIDS-related lymphoma cases by Southern blot hybridization, so far [26], HIV-1 integration should be rare in AIDS-related lymphoma. A recent study demonstrates a decrease in EBV-positive lymphoma among patients with AIDS because of introduction of highly active antiretroviral therapy (HAART) [30]. Therefore, novel mechanisms other than oncogenesis by EBV or KSHV may have been involved in the lymphomagenesis of AIDS-related lymphoma recently. There is no report describing a frequency of HIV-1 integration among AIDS related lymphoma. HIV-1 usually infects T cells or macrophages in AIDS patients, however, T cell lymphoma is still rare among AIDS-related lymphoma in the HAART era [30]. In addition, HIV-1 infection to B cells would occur in a very special condition, such as under KSHV infection. Taken together, although the case we described in the present study contained an important scientific phenomenon on STAT3, HIV-1-integrated lymphoma should be very rare among AIDS-related lymphoma.

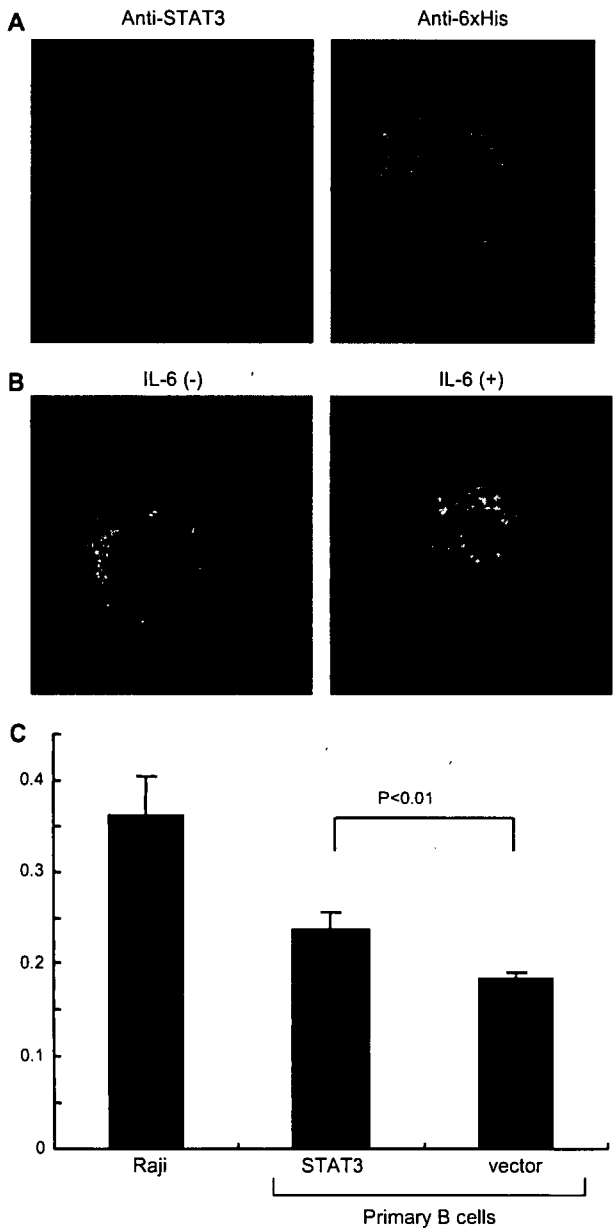

Fig. 5. Transfection of STAT3 into B cells in vitro. (A) STAT3 expression in the STAT3-transfected TY-1, a KSHV-positive B cell line. The cells were transfected with STAT3 expression vector by Nucleofector (Amaxa, Cologne, Germany) using O-06 program. STAT3 expression was detected by anti-STAT3 mouse monoclonal antibody (green in left panel) and anti-6xHis antibody, followed by Alexa 488-conjugated anti-mouse IgG antibody (molecular probe, green in right panel). Red color indicates nuclear counterstaining of propidium iodide. (B) Localization of transfected STAT3 in TY-1. His-tagged STAT3 was detected by anti-6x His antibody in the cytoplasm of B cells (left panel). In the presence of IL-6 (Peprotech, Rocky Hill, NJ, 0.1 ng/ml), transfected STAT3 localizes in the nucleus predominantly (right panel). (C) Cell proliferation assay for STAT3-transfected primary B lymphocytes. Primary B cells were isolated from PBMC. The purity of B cell (CD19+) was >95%. The cells were transfected with STAT3 expression vector expressing STAT3 and CD4 by Nucleofector using U-15 program. Transfection efficiency to primary B cells was around 20%. To increase the proportion of transfected cells, the transfected B cells were separated with CD4 microbeads after 16 h of the transfection (Miltenyl Biotec, Auburn, CA). 48 h after transfection of STAT3 or vector to primary B cells, the proliferation rate was measured with BrdU ELISA (Roche). Raji is an EBV-positive Burkitt lymphoma cell line (untransfected). Numbers in Y-axis indicates absorbance in ELISA. Error bars indicate standard errors of 8 independent experiments.

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Original article

TRAF activation of C/EBPβ (NF-IL6) via p38 MAPK induces HIV-1 gene expression in monocytes/macrophages☆

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Abstract

C/EBPβ plays a pivotal role in activation of human immunodeficiency virus type 1 (HIV-1) in monocytes/macrophages. However, mechanisms for functional regulation of C/EBPβ remain uncharacterized. Previous studies indicated that NF-κB activation by tumor necrosis factor (TNF) receptor family, which activates TNF receptor associated factor (TRAF), induces HIV-1 expression. We found that TRAF signals activate HIV-1 LTR with mutations of NF-κB sites in promonocytic cell line U937, suggesting existence of an alternative HIV-1 activating pathway. In this study, we have characterized the signal transduction pathway of TRAF other than that leading to NF-κB, using U937 cell line, and its subline, U1, which is chronically infected by HIV-1. We show that signals downstream of TRAF2 and TRAF5 activate p38 MAPK, which directly phosphorylates C/EBPβ, and that activation of p38 MAPK potently activates C/EBPβ-mediated induction of HIV-1 gene expression. We also show TRAF2 and TRAF5 are expressed in monocytes/macrophages of spleen samples from HIV-1 infected patients. Identification of TRAF-p38 MAPK-CEBPβ pathway provides a new target for controlling reactivation of latent HIV-1 in monocytes/macrophages.

Keywords: TRAF; p38 MAPK; C/EBPβ (NF-IL6); HIV-1

1. Introduction

Nowadays, highly active antiretroviral therapy (HAART) can successfully decrease and control human immunodeficiency virus type 1 (HIV-1) replication, however, complete eradication of the virus is impossible [1]. Infection by HIV-1 often results in a period of viral latency after the virus integrates into the host cell chromosome that is characterized by low levels of virus production [2]. These latently infected cells are a permanent source for virus reactivation and lead to the rebound of the viral load after interruption of HAART. Therefore, controlling virus reactivation from reservoirs after interruption of HAART is an urgent problem to be addressed.

Activation of viral gene expression can occur in response to a variety of stimuli including mitogens, cytokines and environmental stresses such as UV light, heat shock, and oxygen radicals [3]. The exact mechanism by which these stimuli activate gene expression is not completely understood. Cytokines and

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mitogens such as phorbor ester activate HIV-1 gene expression in part through the core enhancer present in the HIV-1 long terminal repeat (LTR), which contains two consensus NF-κB binding sites. Deletions or mutations in the NF-κB enhancers abolish transactivation by these stimuli [4,5]. Previous studies revealed the important roles of tumor necrosis factor (TNF) receptor family leading to activation of NF-κB in the reactivation of HIV-1 [6–8]. NF-κB activation by TNF receptor family is mediated via tumor necrosis factor receptor associated factor (TRAF) proteins [9]. Therefore, above reports underscore the importance of TRAF signals in the reactivation of HIV-1.

Monocytes/macrophages serve as a major reservoir for HIV-1 even after cytopathic treatment. Unlike T cells, monocytes/macrophages are non-lytically infected. These cells serve as viral reservoirs and vectors for virus transmissions to target cells. Therefore, the control of the reactivation from these reservoirs is an important issue to be addressed for the treatment of HIV-1 infection [10,11]. However, mechanisms of HIV-1 reactivation in monocytes/macrophage are not fully elucidated. Cytokines and cellular stress also activate p38 mitogen-activated protein kinase (p38 MAPK) leading to activation of the HIV-LTR, although the target molecule(s) of this kinase in the activation of HIV-1 has not been characterized [12]. Replication of HIV-1 in macrophages/monocytes but not in T cells requires CAAT enhancer binding proteins (C/EBP) including C/EBPβ (NF-IL6) and their binding sites present in the HIV-LTR [13,14] .

We found that TRAF signals activate HIV-1 LTR with mutations of NF-κB sites in promonocytic cell line U937, suggesting existence of an alternative HIV-1 activating pathway. In this study, we have characterized the signal transduction pathway of TRAF proteins other than that leading to NF-κB, using a promonocytic cell line, U937 and its subline, U1, which is chronically infected by HIV-1. We examined the involvement of p38 MAPK and C/EBPβ in this pathway. We also examined the expression of TRAF proteins in monocytes/macrophages of spleen samples from HIV-1 infected patients.

2. Materials and methods

2.1. Plasmids and chemicals

An expression vector for mouse C/EBPβ, pMTV-C/EBPβ, was kindly provided by Dr. S.L. McKnight (Tularik Inc. South San Francisco, CA). The luciferase reporter genes were constructed using pGL3 vector (Promega, Madison, WI). HIV-1 LTR sequence was amplified by PCR using genomic DNA of ACH-2 cell line. HIV-1 LTR sequence (8897–9621) was introduced into pGL3 vector (Promega) and resultant plasmid was named pHIV LTR-Luc. The pHIV LTR mκB-Luc construct having mutations in both NF-κB sites (GGGACTTTCC to TCTACTTTCC) was prepared with Gene Editor in vitro mutagenesis system (Promega). The pHIV LTR mC/EBPβ-Luc construct having mutations in both C/EBPβ sites (ATTT CATC in 281–288 to TGCAGGGG and GCTTGC in 339–344 to CAGCTG) was prepared according to the previous paper [15] based on the method by Kunkel et al. [16]. MAPK kinase 6 (MKK6) expression vector was constructed using pME18S vector and cDNA amplified by PCR from a control peripheral blood mononuclear cell (PBMC) cDNA. The expression vector for human CD30 and its deletion mutant, pCR-CD30 Δ, which lacks C-terminal TRAF domains consisting of 57 amino acids, was described elsewhere [17]. The p38 MAPK inhibitor SB203580 was a generous gift of Dr. J.C. Lee, King of Prussia, PA. Anisomycin was purchased from Sigma (St. Louis, MO).

2.2. Cell culture

U937 is a human promonocytic cell line, and HEK 293 cells are derived from the human embryonic kidney. Both of them were obtained through the Japanese Cancer Research Resources Bank (Tokyo, Japan). ACH-2 is a human T cell line chronically infected with HIV. U1 is a derivative of U937 line carrying latently infected HIV-1. HEK 293 cells were cultured in DMEM supplemented with 10% FCS and kanamycin. Other cell lines were cultured in RPMI 1640 supplemented with 10% FCS and kanamycin.

2.3. Transfections and luciferase assays

Activation of HIV-1 promoter was measured by reporter gene assay in U937 cells using various HIV-LTR driven Luc reporter plasmid. Renilla luciferase expression vector driven by the herpes simplex virus thymidine kinase promoter (pRL-TK) was co-transfected to standardize each experiment. Briefly, 1×10^5 cells were transfected with 25 ng of the reporter plasmids, 100 ng of pRL-TK and indicated amounts of various effector plasmids, using DMRIE-C reagent (Invitrogen, Carlsbad, CA) or Lipofectamine reagent (Invitrogen) according to the procedures provided by the manufacturer. The total amount of DNA transfected was always adjusted with an empty expression vector. About 30 h after transfection, cells were harvested and lysed, then, the activity was measured using dual luciferase assay system (Promega) according to the procedures provided by the manufacturer. Levels of activation are expressed as fold activation compared with the basal luciferase activity of the reporter constructs. Transfection was always done in triplicate and performed more than three times. The representative data were presented with the mean and standard deviation (s.d.).

2.4. Analysis of kinase activity

For in vitro kinase assays using C/EBPβ as substrate, we prepared a GST fusion protein. A fragment of C/EBPβ cDNA corresponding to nucleotide position from 575 to 887 was amplified by PCR and subcloned into pGEX5X-2 (GE Healthcare UK Ltd. Buckinghamshire, UK). The fusion protein named GST-C/EBPβ was expressed and purified by a standard procedure [18]. Using U937 cells, activation of p38 MAPK by TRAF2, TRAF5, CD30 and its deletion mutant (CD30Δ), was studied by in vitro kinase assay. Transfection

was done by DMRIE-C (Invitrogen) using 5 μg of the each expression plasmid or a control plasmid. Forty-eight hours after transfection, cells were harvested. As controls, U937 cells treated with or without anisomycin (10 μg/ml) for 20 min were included for the assay. The cell lysates were immunoprecipitated by anti-p38 MAPK antibody (New England Biolabs, Inc. Ipswich, MA). The Immunecomplex was incubated with 2.5 μg of GST-C/EBPβ in the presence of γ-^{32}P-ATP at 37°C for 60 min and analyzed by 8% SDS-PAGE.

2.5. Measurement of HIV p24 production in U1 cells

Anti-CD30 monoclonal antibody, Ber-H2, a control monoclonal antibody against glucose oxidase (*Aspergillus niger*) (mouse-IgG1 negative control), and affinity purified anti-mouse immunoglobulin goat antibody were all purchased from DAKO (Kyoto, Japan). Cross-linking of cell surface CD30 was performed as follows: harvested U1 cells were washed in RPMI 1640 medium without fetal calf serum for 2 h, then 1 × 10^6 of U1 cells were incubated for 20 min at room temperature with Ber-H2 or the mouse-IgG1 negative control at the concentration of 10 μg/ml, and were washed with PBS before addition of goat anti-mouse immunoglobulin at a final concentration of 10 μg/ml. After washing with PBS and adding the cross-linking antibody, cells were cultured in RPMI 1640 medium without fetal calf serum. 1x 10^6 of U1 cells were treated with 10 ng/ml of TNFα. Samples were incubated for 48 h and harvested. ELISA assays for HIV p24 expression in U1 cells were performed using RETRO-TEK HIV-1 p24 antigen ELISA kit (Cellular Products Inc. Buffalo, NY) according to the manufacturer's instruction.

2.6. Immunohistochemistry

Sections of formalin fixed and paraffin-embedded samples were deparaffinized and treated with microwave in 10 mM sodium citrate buffer (pH7.0). Sections were treated with 5% skimmed milk in TBS and then incubated with anti-CD68 mouse antibody (Dako Cytomation, Glostrup, Denmark) at 37°C for 40 min. After wash with TBS, the sections were incubated with anti-mouse-IgG Texas Red antibody (GE Healthcare UK, Ltd.) at 37°C 40 min. For double staining with anti-TRAF2 (C-20) rabbit monoclonal antibody or anti-TRAF5 (C-19) goat polyclonal antibody (both from Santa Cruz), a modification of the tyramide signal amplification (TSA) system (NEN Life Science) was used in order to facilitate use of streptavidine–FITC instead of peroxidase-conjugated streptavidine. Signals were detected, using confocal microscopy.

Gene Bank Accession Number.

The national Cancer for Biotechnology Information human genome sequence for HIV-1 (IIIB) is K03455.2.

2.7. Statistical analysis

Differences between mean values were assessed by two-tailed *t*-test. A *P*-value < 0.05 was considered to be statistically significant.

3. Results

3.1. TRAF signals activate HIV-LTR with mutations of NF-κB sites

Signaling from TNF receptor family leads to the induction of HIV gene expression by activating NF-κB in T cells and monocytes/macrophages [6–8]. We tried to examine whether an alternative pathway is involved in activation of HIV-LTR. For this purpose, we did reporter gene assays using a luciferase reporter construct with mutations in both NF-κB sites in HIV-LTR (pHIV LTR mκB-Luc) and a human promonocytic cell line, U937. Transient overexpression of TRAF2 or TRAF5 did activate pHIV LTR mκB-Luc, although the magnitudes were smaller than when pHIV LTR-Luc was used as the reporter (Fig. 1). The results suggested a possibility that signals emanated from TRAF proteins other than those activating NF-κB might mediate activation of HIV-LTR in monocytes/macrophages.

3.2. TRAF signals potentiate the C/EBPβ-mediated activation of HIV-LTR

We next studied whether TRAF signals can regulate C/EBPβ-mediated transactivation of HIV-1 LTR in U937 cells. In the reporter gene assays, overexpression of TRAF2 or TRAF5 along with C/EBPβ showed marked synergistic effects on HIV-LTR activation (Fig. 2a). Overexpression of TRAF2 with C/EBPβ potentiated C/EBPβ activation of HIV-LTR more than two-fold compared with C/EBPβ alone. Similarly, TRAF5 signals enhanced HIV-LTR activation by C/EBPβ up to about 50-fold compared to the basal LTR activity, which is significantly more than additive effects of C/EBPβ and TRAF5. The same effects were seen when two NF-κB sites in the construct (HIV-LTR-Luc) were mutated (HIV-LTR

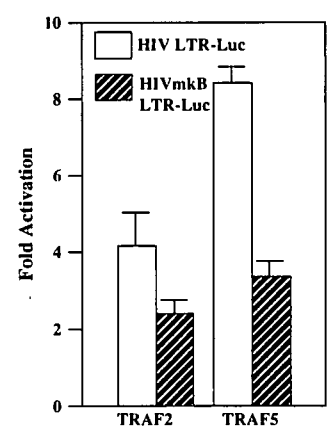

Fig. 1. Activation of HIV-LTR with mutations of NF-κB sites by TRAF signals. Transient co-transfection reporter gene assay was done in U937 cells using indicated luciferase constructs and expression plasmids of TRAF2 and TRAF5. Levels of activation are expressed as fold activation compared with the basal luciferase activity of the reporter constructs. Bars indicate standard deviation. *$P < 0.05$.