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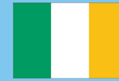
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World Journal of Virology (*World J Virol* , *WJV*, online ISSN 2220-3249, DOI: 10.5501) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJV covers topics concerning arboviral infections, bronchiolitis, central nervous system viral diseases, DNA virus infections, encephalitis, eye infections, fatigue syndrome, hepatitis, meningitis, opportunistic infections, pneumonia, RNA virus infections, sexually transmitted diseases, skin diseases, slow virus diseases, tumor virus infections, viremia, zoonoses, and virology-related traditional medicine, and integrated Chinese and Western medicine. Priority publication will be given to articles concerning diagnosis and treatment of viral diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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ORIGINAL ARTICLE

Nuclear factor κB represses the expression of latent membrane protein 1 in Epstein-Barr virus transformed cells

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Abstract

AIM: To investigate the role of nuclear factor κB (NF-κB) in the regulation of Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) in EBV transformed cells.

METHODS: LMP1 expression was examined in EBV transformed human B lymphocytes with modulation of NF-κB activity.

RESULTS: EBV infection is associated with several human cancers. EBV LMP1 is required for efficient transformation of adult primary B cells *in vitro*, and is expressed in several pathogenic stages of EBV-associated cancers. Regulation of EBV LMP1 involves both viral and cellular factors. LMP1 activates NF-κB signaling pathway that is a part of the EBV transformation program. However, the relation between NF-κB and LMP1 expression is not well established yet. In this report, we found that blocking the NF-κB activity by Inhibitor of κB stimulated LMP1 expression, while the overexpression of NF-κB repressed LMP1 expression in EBV-transformed IB4 cells. In addition, LMP1 repressed its own promoter activities in reporter assays, and the repression was associated with the activation of NF-κB. Moreover, NF-κB alone is sufficient to repress LMP1 promoter activities.

CONCLUSION: Our data suggest LMP1 may repress its own expression through NF-κB in EBV transformed cells and shed a light on LMP1 regulation during EBV transformation.

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Key words: Nuclear factor κB; Epstein-Barr virus; Latent membrane protein 1; Latency; Transformation

Core tip: We find a classical feedback inhibition of Epstein Barr virus (EBV) Latent membrane protein 1 (LMP1) and nuclear factor κB (NF-κB): LMP1 activates NF-κB, and NF-κB inhibits LMP1 expression. The regulatory loop may benefit EBV transformation processes.

Cao M, Wang Q, Lingel A, Zhang L. Nuclear factor κB represses the expression of latent membrane protein 1 in Epstein-Barr virus transformed cells. *World J Virol* 2014; 3(4): 22-29 Available from: URL: http://www.wjgnet.com/2220-3249/full/v3/i4/22.htm DOI: http://dx.doi.org/10.5501/wjv.v3.i4.22

INTRODUCTION

Epstein-Barr virus (EBV) is a human-herpesvirus that infects most humans without causing an obvious disease. However, EBV is associated with nasopharyngeal carcinoma, Hodgkin's lymphoma, Burkitt's lymphoma, post-transplantation lymphoproliferative diseases, and central nervous system lymphoma in certain healthy and

immune-compromised hosts[1-3].

EBV transforms adult primary B cells into continually growing lymphoblastoid cell lines (LCLs) and concomitantly establishes type Ⅲ latency *in vitro*[1]. Nine viral proteins are expressed, including six nuclear proteins (EBNA1, -2, -3A, -3B, -3C, and -LP) and three integral latent membrane proteins (LMP1, -2A, and -2B) plus EBV-encoded RNAs[3,4].

EBV LMP1 is an integral membrane protein and acts as a constitutively active, receptor-like molecule[5]. LMP1 is required for the efficient transformation of primary B cells *in vitro*[6-8]. Also, LMP1 is able to transform rodent fibroblasts[9-11]. In addition, LMP1 seems to be a central effector of altered cell growth, survival, adhesive, invasive, and antiviral potential in EBV transformed cells[12-18].

The nuclear factor κB (NF-κB) molecule plays a pivotal role in regulating a variety of biological processes, such as immunity, cell survival, and proliferation[19,20]. The NF-κB pathway can be regulated by many stimuli, and its activity is tightly controlled to ensure a transient response to infection or other stimuli. NF-κB transcription factors are homodimers or heterodimers of REL homology domain proteins p50, p52, RelA, RelB, or cREL[19,20]. LMP1 activates both canonical and noncanonical NF-κB pathways by the use of cellular signaling proteins, such as tumor necrosis factor-receptor-associated factors[21]. The functional importance of NF-κB is exemplified by the fact that the blockade of NF-κB triggers apoptosis of EBV-transformed lymphoblastoid cells[22].

Previously, there are several conflicting reports about the relation between NF-κB and LMP1[23-25]. During our study on NF-κB and LMP1, we found that NF-κB is likely to be the negative regulator for the LMP1 expression in EBV-transformed cells. LMP1 may negatively regulate its own expression through NF-κB, which is a classical feedback loop. Our results may provide an insight in NF-κB's role in viral transformation and add the complexity of viral gene regulation and their relation to transformation.

MATERIALS AND METHODS

Plasmids, antibodies, and reporter assays

Expression plasmids of LMP1 and its signaling defective mutant, LMP-DM, were described previously[26]. The NF-κB expression plasmids (p65 and p50), and NF-κB reporter construct with two consensus recognition sites were gifts from Albert Baldwin. pCMV-beta-gal and renilla luciferase expression plasmids were purchased from Clontech. CD4 expression plasmid was provided by Dr. Jenney Ting. LMP1 promoter reporter construct, LMP-ISRE-luc and LMP-GAS-luc were described previously[27]. LMP1 (CS1-4) antibody was purchased from Dako. Glyceraldehyde-3-phosphate dehydrogenase (0411) and inhibitor of κB (IκB) (SC-371) antibodies were purchased from Santa Cruz Biotechnology. Tubulin antibody was purchased from Sigma (T6557). The luciferase and beta-galactosidase assays were performed by standard methods[27,28].

Cell culture, transfection, and inducible expression of IκBα

The IB4 cell line was an EBV-transformed B cell line with type Ⅲ latency and were maintained in RPMI 1640 plus 10% fetal bovine serum (FBS). 293T cells were human fibroblast cell line. The cells were maintained in Dulbecco's Modified Eagle's Medium plus 10% FBS. The inducible IκB expression IB4 line was the gift from Cahir-McFarland *et al*[22] and were maintained in RPMI160 + 10% FBS plus 1 μg/mL tetracycline. For the induction of IκBα, cells were washed three times with RPMI without tetracycline and suspended in the media with or without tetracycline at a concentration of 10^5 cells per milliliter as described[22]. Cells were analyzed within 24 h after the initial inductions. The Electroporation (320 V; 925 μF) was used for the transfection of IB4 cells as described previously[11,29-31]. For transfection of 293T cells, the attractene transfection reagent (Qiagen) was used following manufacturers recommendations.

Isolation of CD4 positive cells

Enrichment for CD-4-positive cells was performed with the use of anti-CD-4-antibody conjugated to magnetic beads according to the manufacturer's recommendation (Dynal, Inc.). IB4 cells were transfected with CD-4 expression and other plasmids. One day after the transfection, the cells were used for isolation of CD-4-positive cells with the use of Dynabeads CD4 (Dynal, Inc.) The transfected cells were incubated with Dynabeads CD4 at 72 μL of beads/10^7 cells for 15-30 min at room temperature with gentle rotation. CD4-positive cells were isolated by placing the test tube in a magnetic separation device (Dynal magnet). The supernatant were discarded while the CD4-positive cells were attached to the beads. The CD4-cells-attached beads were washed 3 times in phosphate-buffered saline plus 2% FBS. The isolated cells were used to prepare cell lysates immediately. Total time for isolation was approximately 30-40 min. No tetracycline was used in the process.

Western blot analysis with enhanced chemiluminescence

Separation of proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out following standard protocol. After the proteins were transferred to a nitrocellulose or Immobilon membrane, the membrane was blocked with 5% nonfat dry milk in TBST (50 mmol/L Tris-HCl pH 7.5, 200 mmol/L NaCl, 0.05% Tween-20) at room temperature for 10 min. It was then washed briefly with TBST, and incubated with the primary antibody in 5% milk in TBST for 1 h at room temperature, or overnight at 4 ℃. After washing with TBST three times (10 min each), the membrane was incubated with the secondary antibody at room temperature for 1 h. It was then washed three times with TBST, treated with

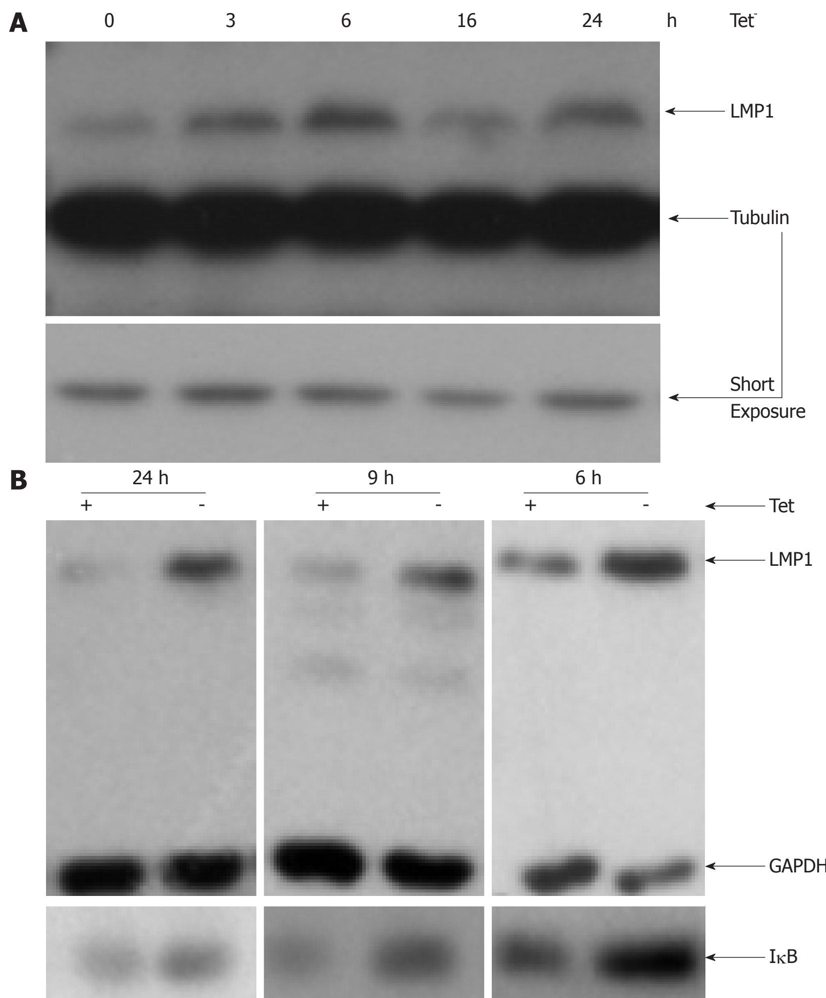

Figure 1 Blockage of nuclear factor-κB increases the expression of latent membrane protein 1 in Epstein-Barr virus-transformed cells. Inducible IκB-expression IB4 line were washed three times with fresh RPMI1640 medium, and re-suspended in tetracycline (Tet) plus or minus medium. Cells were isolated at indicated time, and used for Western blot analysis with indicated antibodies. The expression of latent membrane protein 1 (LMP1) was shown in (A) and the expression of IκB was shown in (B). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

enhanced chemiluminescence detection reagents, and exposed to Kodak XAR-5 film.

Statistical analysis

Student *t* tests between groups of data were carried out with the use of Excell 2013. The statistically significant differences between the indicated samples were assessed by *P* values.

RESULTS

Inhibition of NF-κB increased the expression of LMP1

To determine the role of NF-κB in LMP1 expression in EBV-transformed cells, we used an EBV-transformed LCL (IB4 cell line) in which expression of a degradation-resistant mutant IκBa that was regulated by tetracycline (Tet)[22]. We found that LMP1 was induced upon Tet removal (Figure 1). Moreover, the potential correlation between the induction of IκB and the expression of LMP1 was examined. Within six hours after culture in media lacking tetracycline (Tet-media), IκBa was induced to stable levels (Figure 1; Panel B), however at three hours post removal of Tet, the IκBa induction was not consistently detected (data not shown). Increase in IκBa expression was also detected during 6-24 h after induction. The endogenous LMP1 was associated with Tet-removal and IκBα inductions (Figure 1). Therefore, LMP1 is increased upon IκBa induction in IB4 cells.

Overexpression of NF-κB decreased the expression of LMP1

Next, we tested whether the activation of NF-κB itself would affect the expression of endogenous LMP1 in EBV transformed cells. NF-κB can be activated by many stimuli, however, the specificity of the treatment might vary significantly. Therefore, we chose to use the ectopic expression of NF-κB, or p65 and p50 simultaneous, in IB4 cells. The reason to choose IB4 as it is a parental line for the inducible IκB line. IB4 cells were transfected with the expression plasmids p65 and p50 at 1:1 ratio, and the transfected cells were enriched by CD4 selection (see “Materials and Methods” for detail). No tetracycline was

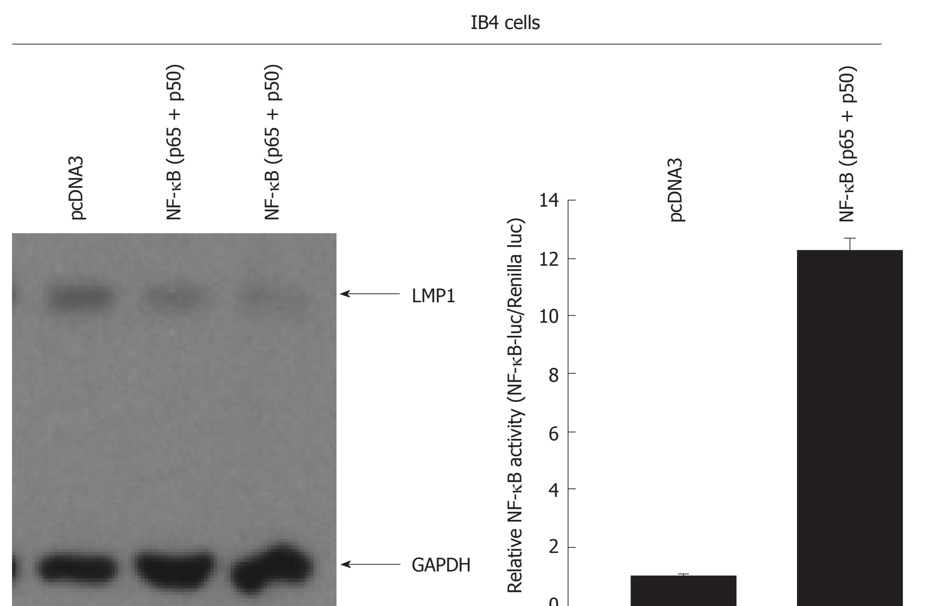

Figure 2 Overexpression of nuclear factor-κB decreases the expression of latent membrane protein 1 in Epstein-Barr virus-transformed cells. IB4 cells were transfected with CD4 expression plasmid along with pcDNA3 (vector) or nuclear factor κB (NF-κB) expression plasmids (p50 and p65 expression plasmids at 1:1 ratio). One day after the transfection, the transfected cells were enriched using the CD4 magnetic beads, and the cell lysates were used for Western blot analysis. The identity of the proteins is as shown. The right panel: IB4 cells were transfected with the indicated plasmid as shown at the top along with NF-κB specific reporter construct and Renilla luciferase reporter plasmid. One day later, luciferase and Renilla luciferase activities were measured. Relative promoter reporter activities are shown. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; LMP1: Latent membrane protein-1.

involved as plain IB4 cells are used. Western blot analyses were used for detection of the expression of LMP1 in enriched transfected cells. The NF-κB activity was increased and LMP1 was reduced in the NF-κB-transfected IB4 cells (Figure 2). Therefore, the endogenous LMP1 is reduced upon NF-κB activation in EBV-transformed cells.

LMP1 represses its own promoter activity

It is well established that there is one functional NF-κB recognition site in LMP1 promoter and NF-κB binds to the site in the LMP1 promoter[23-25]. In addition, LMP1 activates NF-κB pathway through at least two independent domains[32]. We examined if LMP1 could repress the LMP1 promoter reporter constructs (Figure 3A). LMP-DM is an expression plasmid that has mutations in two functional domains of LMP1 for NF-κB activation[26]. The promoter reporter constructs and LMP1 or LMP-DM expression plasmid were co-transfected into 293T cells and the reporter activities were measured. As shown in Figure 3B, LMP1 was able to repress the LMP1 promoter reporter constructs. However, LMP-DM failed to repress the same reporter constructs, which correlated with that data that LMP-DM failed to activate NF-κB pathway (Figure 3C). Therefore, LMP1 represses its own promoter reporter constructs.

NF-κB represses LMP1 promoter reporter construct

Next, we tested whether the activation of NF-κB alone would affect activities of the LMP1 promoter reporters. The promoter reporter construct and NF-κB (p65 + p50) expression plasmids were co-transfected into 293T cells and the reporter activities were measured. As shown in Figure 4, NF-κB activation alone was able to repress the LMP1 promoter reporter construct. The NF-κB-specific reporter construct was activated by the co-transfection of p65 and p50 expression plasmids, suggesting the NF-κB was functional (Figure 4B).

DISCUSSION

Both viral, such as EBNA2 and EBNA-LP, and cellular factors, such as IRF7, RBP-jκ, PU.1, and STAT are involved in the regulation of LMP1 in various EBV latencies and transformation processes[33-36]. LMP1 needs to be tightly regulated during viral transformation processes because LMP1 itself is a perplex protein with multiple functions, such as proliferative and anti-proliferative activities. The higher amounts of LMP1 may convert itself from a proliferative function to an anti-proliferative one[37].

Activation of NF-κB and the consequence of the action during viral transformation have been established clearly. NF-κB is required for the maintaining of the growth phenotypes of the transformed cells, and NF-κB seems to be responsible for most of the cellular changes during the transformation[38]. However, the relation between the NF-κB and LMP1 expression is somewhat unclear. While it is clear that there is a NF-κB recognition site in LMP1 promoter and NF-κB is able to physically bind to the site, the exact function of NF-κB on LMP1 expression is in debate[23-25]. During our research on LMP1 and other cellular factor interactions, we find that: (1) inhibition of NF-κB enhances the endogenous LMP1

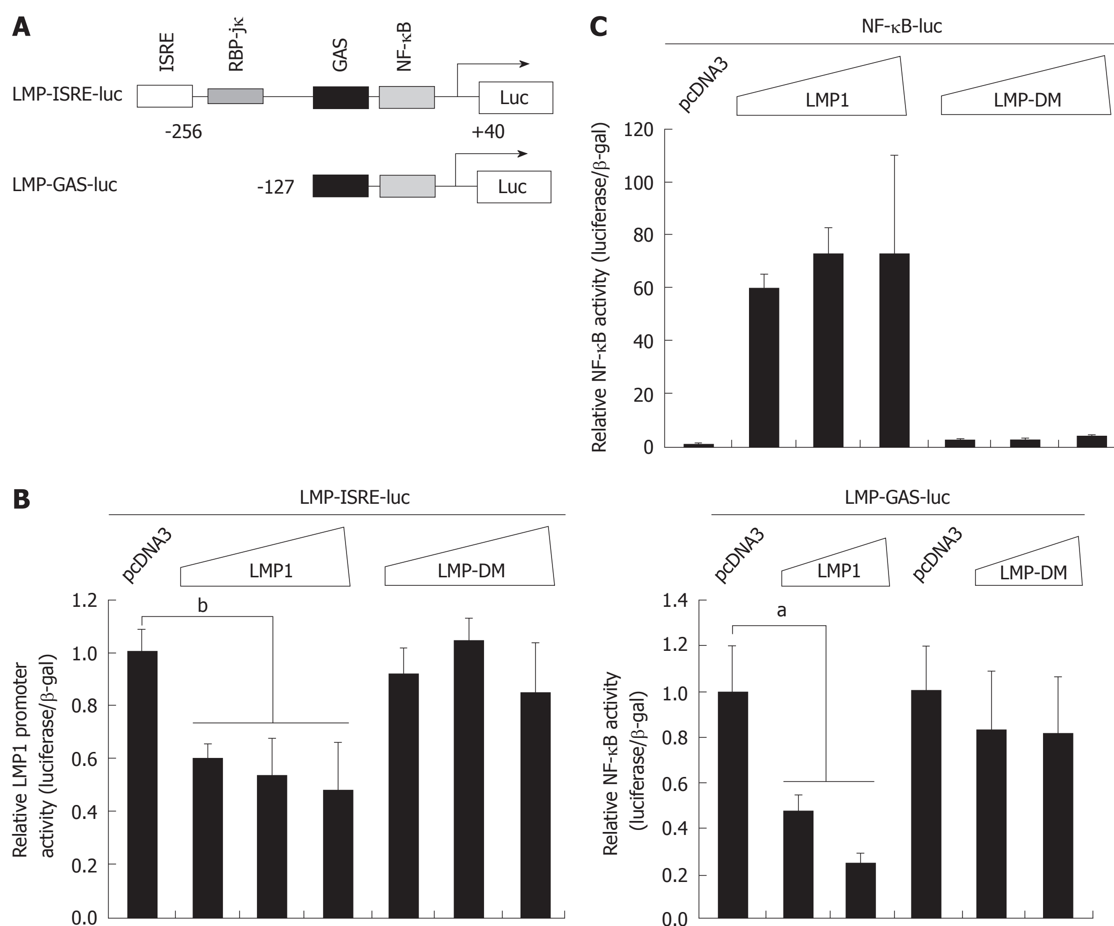

Figure 3 Latent membrane protein 1 negatively regulates its own promoter activity. A: Schematic diagram of Epstein-Barr virus (EBV) latent membrane protein-1 (LMP1) promoter reporter constructs. RNA start site is shown. The drawing is not to scale; B: 293T cells were transfected with LMP1 promoter reporter construct and expression plasmid (0.01, 0.05, and 0.1 μg) as shown at the top. The LMP-DM has mutations in the critical domains in LMP1 for signaling. Cell lysates were used for the luciferase and β-galactosidase assays. Relative promoter reporter activities (luciferase/β-galactosidase) are shown. The results represented an average of triplicate transfections; Standard error bars are shown. Results from one representative experiment are of shown. The statistically significant difference between the indicated samples is denoted as $^{a}P < 0.05$; $^{b}P < 0.01$; C: 293T cells were transfected with the indicated plasmid as shown at the top. Nuclear factor κB (NF-κB) specific reporter construct was used. One day later, luciferase and β-galactosidase activities were measured. Relative promoter reporter activities are shown.

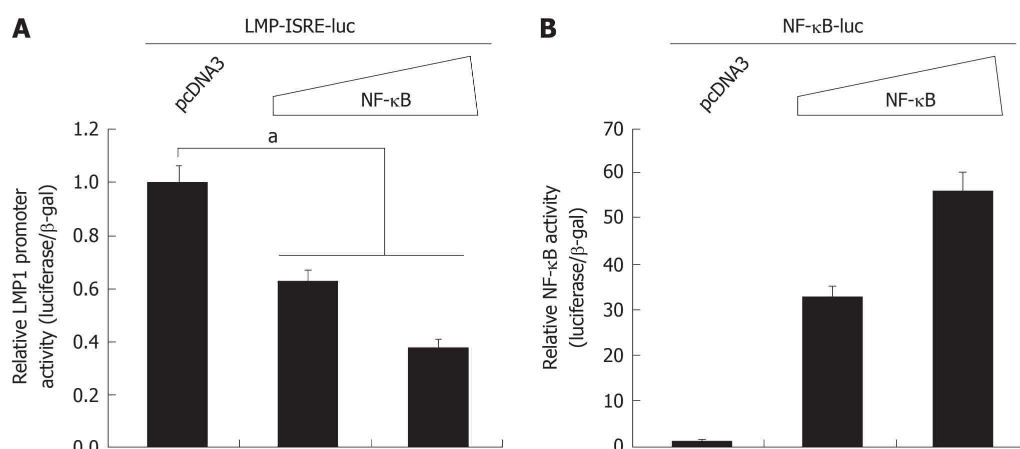

Figure 4 Nuclear factor κB represses latent membrane protein 1 promoter activity. A: 293T cells were transfected with latent membrane protein-1 (LMP1) promoter reporter construct and nuclear factor κB (NF-κB) expression plasmid (0.05 and 0.1 μg; p65 and p50 at 1:1 ratio) as shown at the top. Cell lysates were used for the luciferase and β-galactosidase assays one day later. Relative promoter reporter activities (luciferase/β-galactosidase) are shown. Standard error bars are shown. The statistically significant difference between the indicated samples is denoted as $^{a}P < 0.05$; B: 293T cells were transfected with the indicated plasmid as shown at the top. NF-κB specific reporter construct was used. One day later, luciferase and β-galactosidase activities were measured. Relative promoter reporter activities are shown.

expression, and activation of NF-κB leads to reduced expression of endogenous LMP1 in EBV transformed cells (Figures 1 and 2); (2) LMP1 repressed the LMP1-promoter reporter construct activities, and the repression was correlated with NF-κB activities (Figure 3); and finally (3) NF-κB itself repressed the LMP1-promoter activities (Figure 4). From all the results, it seems that NF-κB is a negative regulator of LMP1 in type Ⅲ latency or EBV transformed cells. Interestingly, when we used NF-κB inhibiting drug (BAY11-7082) for the LCL and found that the effects on LMP1 expression were not obvious (unpublished observations). We reasoned the side effects of chemical NF-κB inhibitor might influence the end results. Because our experimental approaches were based "solely" on NF-κB activation/inactivation, the results about NF-κB activation and LMP1 expression might be more reliable than a chemical activator and inhibitor of the NF-κB. Moreover, IB4 is a cell line transformed by EBV *in vitro* and has been used extensively in EBV research[22,39-43]. The results based on the IB4 cells might be more comparable to others researches. Of note that induction of IκB in terms of time course and LMP1 expression seems to be slightly different from the previous report[22]. We think the discrepancies might be due to the growth condition for the cells in various laboratories and passages numbers of the cells might be slightly different. Interestingly, we have found that the detection of the IκB induction by IκB antibody was more sensitive than the FLAG antibody (data not shown).

As we mentioned above, there are several conflicting reports about LMP1 and NF-κB[23-25]. It is hard to reconcile with all the conflicting reports. We think the endogenous LMP1 levels, genetic differences in cell line used, the presence of other viral factors, type of assays, and promoter construct differences may all collectively caused the two quite different conclusions. We have used lines with high endogenous or ectopic LMP1 expression, therefore, the results may be most suitably extrapolated to type Ⅲ latency or EBV transformed cells *in vitro*. Because high LMP1 is detrimental for growth, the observed negative effects might make sense in the LMP1-high situations. Furthermore, NF-κB might have dual roles in various backgrounds. Our results are in line with one previous report[24].

Because the response activated by NF-κB is so potent, tight regulation of the NF-κB activity is needed. There are many mechanisms for NF-κB signaling to be terminated to prevent potential tissue pathology due to prolonged expression of inflammatory mediators[44]. Fortunately, many of the NF-κB target genes encode inhibitors of the signaling pathways, which allow the inflamed tissues to reset to normal function once the danger has passed[44]. LMP1-mediated NF-κB activation seems to be the major mediator affecting cell expression programs in EBV-infected cells. The negative regulation of LMP1 expression by NF-κB would offer EBV a feedback inhibition to fine adjust NF-κB activity.

As NF-κB, LMP1 molecule *per se* is also need to be tightly regulated as it has both pro- and anti-proliferative effects[37]. The negative roles of NF-κB in regulation of LMP1 may offer EBV a feedback inhibition for its own LMP1 expression. The feedback loop between NF-κB and LMP1 might be important for the control of NF-κB as well as LMP1 activities, and eventually EBV transformation as a whole.

ACKNOWLEDGMENTS

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COMMENTS

Background

Epstein-Barr virus (EBV) is a human herpesvirus with increasing medical significances. EBV is associated with many cancers and able to transform adult primary B cells into continually growing lymphoblastoid cell lines. EBV Latent membrane protein-1 (LMP1) is an integral membrane protein and acts as a constitutively active, receptor-like molecule. LMP1 is required for the efficient transformation of human primary B cells *in vitro* and possibly *in vivo*. In addition, LMP1 seems to be a central effector of altered cell growth, survival, adhesive, invasive, and antiviral potential in EBV transformed cells. The nuclear factor κB (NF-κB) molecule plays a pivotal role in regulating a variety of biological processes, such as immunity, cell survival, and proliferation. The NF-κB pathway can be regulated by many stimuli, and its activity is tightly controlled to ensure a transient response to infection or other stimuli. The functional importance of NF-κB is exemplified by the fact that the blockade of NF-κB triggers apoptosis of EBV-transformed cells.

Research frontiers

The relation between EBV and NF-κB, especially LMP1 and NF-κB, has been extensively studied. Other than the involvement in the EBV transformation, it is apparent that NF-κB regulates LMP1 expression *via* a well conserved binding site in LMP1 promoter region, however how the end results of the regulation is confusing. There are several conflicting reports about the relation between NF-κB and LMP1.

Innovations and breakthroughs

During the authors study on NF-κB and LMP1, The authors found that NF-κB is likely to be the negative regulator for the LMP1 expression at least in EBV-transformed cells. LMP1 may negatively regulate its own expression through NF-κB, which is a classical feedback loop.

Applications

The authors results provide an insight in NF-κB's role in viral transformation and add the complexity of viral gene regulation and their relation to transformation. This will be useful to determine the therapeutic potential and benefit of drugs that targeting NF-κB and/or LMP1 in EBV-associated cancers.

Terminology

Both EBV LMP1 and NF-κB are proteins involved in the process called viral transformation in which normal cells are converted into cancerous ones by a virus. Such a mechanism is crucial in viral pathogenesis. Non-surprisingly, EBV LMP1 and NF-κB has a classical regulatory loop.

Peer review

The study design and paper writing are OK. The methods provided in this study are correct.

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ORIGINAL ARTICLE

Substrate specificity of avian influenza H5N1 neuraminidase

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Abstract

AIM: To characterise neuraminidase (NA) substrate specificity of avian influenza H5N1 strains from humans and birds comparing to seasonal influenza virus.

METHODS: Avian influenza H5N1 strains from humans and birds were recruited for characterising their NA substrate specificity by using a modified commercial fluorescence Amplex Red assay. This method can identify the preference of α2,6-linked sialic acid or α2,3-linked sialic acid. Moreover, to avoid the bias of input virus, reverse genetic virus using NA gene from human isolated H5N1 were generated and used to compare with the seasonal influenza virus. Lastly, the substrate specificity profile was further confirmed by high-performance liquid chromatography (HPLC) analysis of the enzymatic product.

RESULTS: The H5N1 NA showed higher activity on α2,3-linked sialic acid than α2,6-linked (P < 0.0001). To compare the NA activity between the H5N1 and seasonal influenza viruses, reverse genetic viruses carrying the NA of H5N1 viruses and NA from a seasonal H3N2 virus was generated. In these reverse genetic viruses, the NA activity of the H5N1 showed markedly higher activity against α2,3-linked sialic acid than that of the H3N2 virus, whereas the activities on α2,6-linkage were comparable. Interestingly, NA from an H5N1 human isolate that was previously shown to have heamagglutinin (HA) with dual specificity showed reduced activity on α2,3-linkage. To confirm the substrate specificity profile, HPLC analytic of enzymatic product was performed. Similar to Amplex red assay, H5N1 virus showed abundant preference on α2,3-linked sialic acid.

CONCLUSION: H5N1 virus maintains the avian specific NA and NA changes may be needed to accompany changes in HA receptor preference for the viral adaptation to humans.

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Key words: H5N1 avian influenza virus; Neuraminidase; Sialic acid; Adaptation; Substrate preference

Core tip: We analyzed neuraminidase (NA) substrate specificity of avian influenza H5N1 strains from humans and birds using a modified fluorescence assay, and the substrate specificity profile was further confirmed by

high-performance liquid chromatography analysis of the enzymatic product. The H5N1 NA showed higher activity on α2,3-linkage. Interestingly, NA from an H5N1 human isolate that was previously shown to have heamagglutinin (HA) with dual specificity showed reduced activity on α2,3-linkage. These suggest that the H5N1 virus maintains the avian specific NA activity and that changes in the NA may be needed to compensate for changes in the HA specificity for the viral adaptation to human hosts.

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INTRODUCTION

Neuraminidase (NA) is a tetrameric type Ⅱ transmembrane glycoprotein on the envelope of influenza virus. NA molecule consists of three domains: globular head, stalk and transmembrane domains[1-3]. The function of NA is to cleave terminally bound sialic acid on carbohydrate chains of glycans on cell surface and viral envelope in order to release newly budded virions from host cells[2]. If the function of NA is impaired, sialyl residues on the surface of virus particles and infected cells will be bound by heamagglutinin (HA), which leads to virus aggregation at the cell surface preventing the dissemination of infection[4,5].

HA of avian and human influenza viruses bind preferentially to α2,3- and α2,6-linked sialic acid, respectively. This difference is believed to play an important role in the interspecies barrier of influenza transmission between avian species and human. A change in the receptor preference is required for emergence of a new pandemic strain from avian influenza viruses[6]. HA and NA counteract each other, and their activities need to be balanced for the efficient viral replication and respiratory- droplet transmission[4,7]. NA activities on α2,3- and α2,6-linked sialic acid have been previously characterized for some avian and seasonal influenza viruses[8-10]. NA of N2 subtype from human and avian influenza viruses had been studied for substrate specificity[8,9]. Avian and early human isolated N2 showed much more activity on α2,3-linked sialic acid than α2,6-linked. However, late human N2 isolation trended toward increase substrate specificity for α2,6-linked while maintaining the α2,3-linked activity. The N1 substrate specificity had also been studied[10]. Similar to N2 activity, N1 isolated from avian hosts showed much higher activity on α2,3- than α2,6-linked substrate, while human viruses showed reduced activity to α2,3- and increased activity to α2,6-linked sialic acid. From these finding we can conclude that human isolated NA shows the increased substrate specificity on α2,6-linked,which is found in human respiratory tract, while maintaining specificity on α2,3-linked sialic acid[8,9]. Because α2,3-linked sialic acid is expressed on the intestines of aquatic birds which is believed to be the primordial reservoir for all subtype of influenza A virus[11]. Occasionally viruses are transmitted to other host species and introduce avian viral gene to non-avian hosts like human. This situation can lead to severe outbreaks or pandemics[11]. Moreover, a recent study showed that replacing *NA* gene of North American triple reassortant swine influenza virus with that of 2009 pandemic H1N1 virus altered the enzymatic activity and led to an enhanced efficiency of respiratory-droplet transmission in ferrets[7]. Therefore, the monitoring of NA activity on substrate specificity is needed.

Highly pathogenic H5N1 avian influenza virus is causing a wide-spread epidemic in poultry with occasional transmission to humans and poses a serious pandemic threat. While receptor preference of H5N1 HA has been extensively studied[12-14], data on their NA substrate specificity are scarce. We therefore characterised NA activity of H5N1 viruses in comparison to NA of a seasonal influenza virus.

MATERIALS AND METHODS

Cell and virus culture

Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) with 10% fetal bovine serum (FBS) in the present of Gentamicin, Penicillin G and Fungizone. 293T cell were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS, antibiotics and antifungal. Viruses used in this study are shown in Table 1. Viruses were cultured in MDCK cells in MEM without phenol red to avoid the interference with the fluorescent assay[15,16].

Generation of reverse genetic virus

Reverse genetic viruses were generated by DNA transfection as described by Hoffmann *et al*[17]. The *NA* genes were extracted from A/Thailand/KAN-1A/2004, A/Thailand/676/2005, A/Thailand/3(SP-83)/2004 and seasonal influenza virus, A/Thailand/AW10/2010 (H3N2), respectively and cloned into pHW2000. Then, 1 μg of pHW2000 expressing NA-DNA was transfected into the co-cultured of MDCK and 293 T cell in Opti-MEM (Gibco, United States) with the other seven genomic segments of A/Puerto Rico/8/34(H1N1) in the presence of TransLT according to the manufacturer's instructions. Thirty hours post transfection, fresh Opti-MEM containing TPCK-trypsin was added to the cells at the final concentration 0.5 μg/mL in the cell suspension. The HA titer of the NA reverse genetic virus was determined by Hemagglutination test.

NA Amplex Red® assay

NA activity was assayed using Amplex Red® assay following the instruction provided by the manufacturer (Molecular Probe, Inc.). This assay utilizes Amplex Red to detect H_2O_2 generated by oxidation of desialylated galactose which is the end product of neuraminidase action. In the presence of horseradish peroxidase, H_2O_2 reacts with 1:1

Table 1 Virus strains and sources

Virus	Subtype	Passage	Source
A/Thailand/KAN-1A/2004	H5N1	MDCK8	Human
A/Thailand/676/2005	H5N1	MDCK8	Human
A/Thailand/3(SP-83)/2004	H5N1	MDCK8	Human
A/Openbill stork/Thailand/VSMU-4-NSA/2004	H5N1	MDCK4	Avian
A/Openbill stork/Thailand/VSMU-5-NSA/2004	H5N1	MDCK4	Avian
A/Chicken/Bangkok/VS-MU-1/2006	H5N1	MDCK4	Avian
A/Chicken/Thailand/BF2037/2007	H5N1	MDCK4	Avian
A/Openbill stork/Thailand (Nakhonsawon)/VSMU-32/2005	H5N1	MDCK4	Avian

MDCK: Madin-Darby canine kidney.

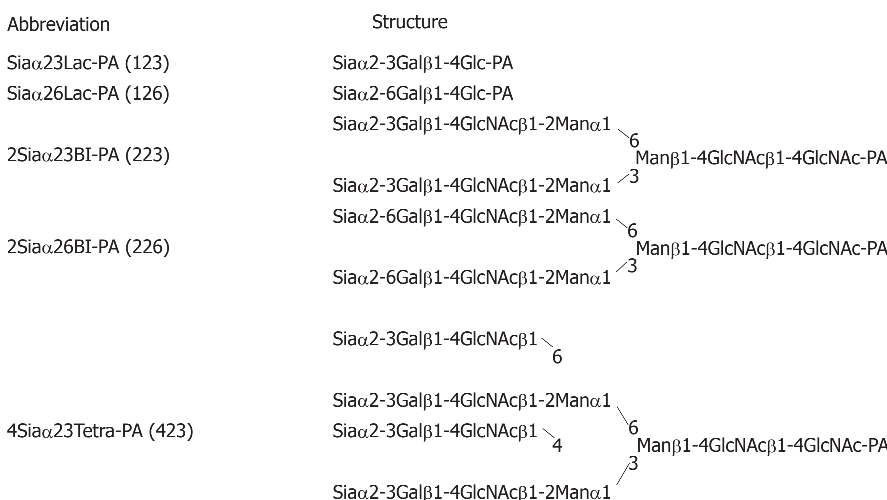

Figure 1 Pyridylamino oligosaccharide that used for high-performance liquid chromatography neuraminidase assay.

stoichimetry with Amplex Red reagent, then, generates Resorufin, the red-fluorescent oxidation product, which is detected at 640 nm. The method had been modified in order to study the substrate specificity by using 2 types of glycopolymer instead of fetuin. The substrates which were applied for this assay was Neu5Acα2,3LacNAcb-p-Aminophenyl (pAP) and Neu5Acα2,6LacNAcb-pAP which contained α2,3-linked sialic acid and α2,6-linked sialic acid, respectively[12,18]. Briefly, 10 μL of 64 HA unit of virus was mix with 10 μL of Amplex red reaction mixture in the present of 0.5 μg of either Neu5Acα2,3LacNAcb-pAP or Neu5Acα2,6LacNAcb-pAP for virus and 2 μg of each for reverse genetic virus. The NA activity on each substrate was detected at 640 nm after incubation at 37 ℃ for 110 min. Percentage of fluorescence correlated to NA activity of each virus was subtract with mock and plotted and analysed by using GraphPad Prism version 4.0 for windows (GraphPad software, San Diego, California; http://www.graphpad.com). Mean ± SEM from triplicate experiments were calculated for NA activity. One-way Anova were used to determine *P*-value for the significant difference between viruses. A *P*-value of $\leqslant$ 0.05 was considered significant.

NA assay by high-performance liquid chromatography

To confirm the NA activity by Amplex Red® assay, NA was determined the activity by using high-performance liquid chromatography (HPLC) as previously described[19]. Viruses was incubated at 37 ℃ for 2 h with buffer and 10 pmole of each Neu5Acα2,3- or Neu5Acα2,6-pyridylamino (PA)-glycopolymer shown in Figure 1. Twenty-five microlitres of saturated $NaHCO_3$ were added, then heated at 100 ℃ for 10 min to inactivate virus. The reaction then concentrated by using CentriVap (Labconco, United States) prior analysis with HPLC which was performed on a Shodex NH2P-50 4E column (4.6 mm × 250 mm) at a flow rate of 0.6 mL/min. PA-glycopolymers were detected by fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm). Two eluents were used, A and B. Eluent A was acetonitorile:water:acetic acid (930:70:3, v/v/v) adjusted to pH 7.0 with aqueous ammonia; Eluent B was acetonitorile:water:acetic acid (200:800:3, v/v/v) adjusted to pH 7.0 with aqueous ammonia. The column was equilibrated with Eluent A:Eluent B (95:5). After injecting Eluent A:Eluent B (86:14) in 3 min, to Eluent A:Eluent B (73:27) in 17 min, to Eluent A:Eluent B (52:48) in 59 min, and then to Eluent A:Eluent B (25:75) in 6 min.

RESULTS

NA substrate specificity of H5N1 viruses from humans and animals

The substrate specificity NA from H5N1 using two syn-

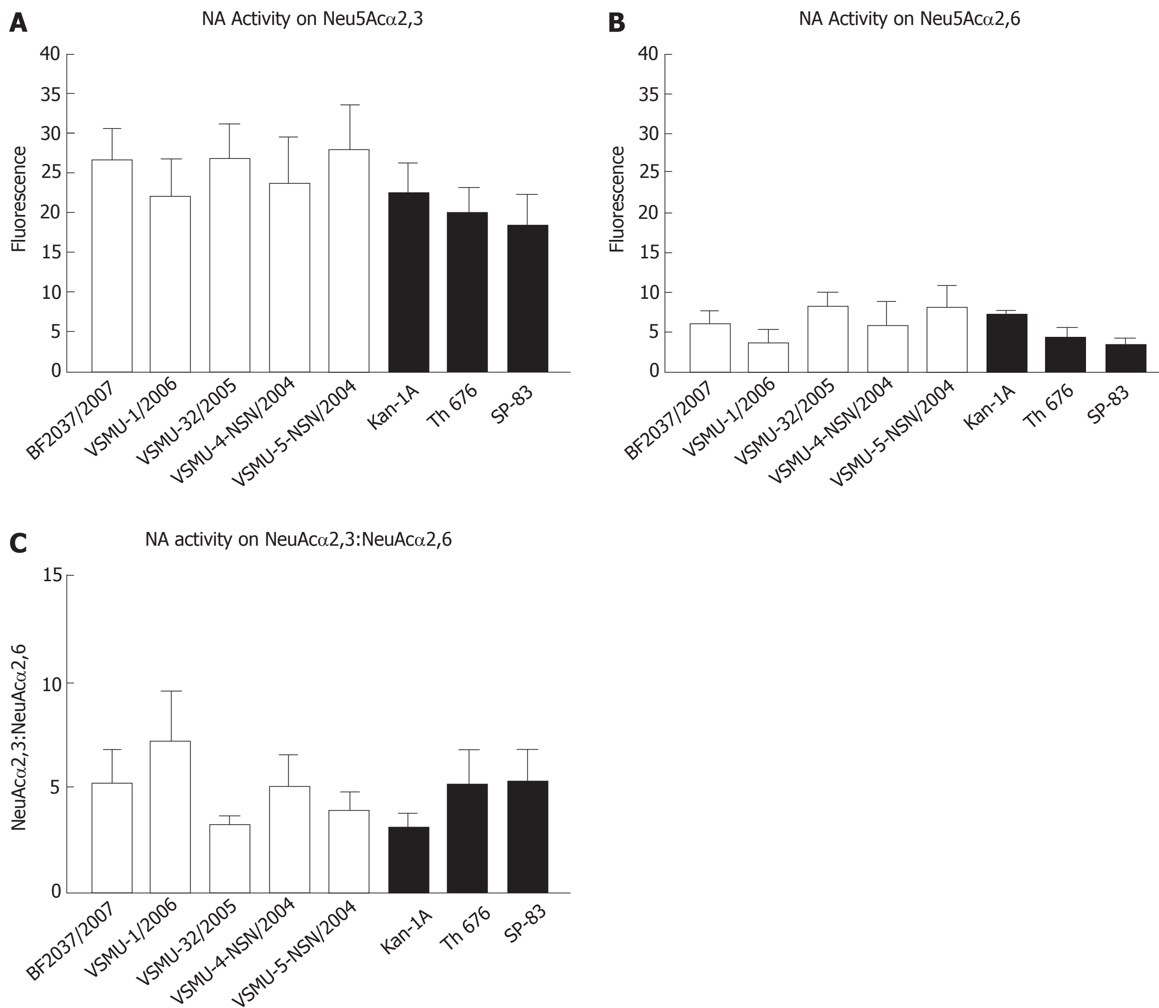

Figure 2 The N1 activity on substrate specificity by Amplex Red® assay. Substrate-specific neuraminidase activity of H5N1 avian influenza viruses isolated from animal (white bars) and human (black bars) was measured by a modified Amplex Red® assay. The α2,3-linked sialoside specific activity was measured using Neu5Acα2,3LacNAcb-pAP as substrate (A), whereas the α2,6-linked sialoside specific activity was measured using Neu5Acα2,6LacNAcb-pAP (B). The fluorescence related to neuraminidase activity from triplicate experiments had been shown as mean ± SEM. In order to show substrate preference, ratios between the α2,3- and α2,6-specific activity are shown (C).

thetic glycopolymers as substrate in Amplex Red® fluorescence assay was shown in Figure 2. The NA activity was 10-30 fluorescence unit, average 23.55 ± 1.489 (mean ± SEM), on α2,3-linked sialosides (Figure 2A) and 5-10 fluorescence unit, average 6.133 ± 0.667 (mean ± SEM) on α2,6-linked sialosides (Figure 2B). Comparing between the two substrates, H5N1 isolates from humans and animals showed higher activity on α2,3-linked sialic acid than α2,6-linked ($P < 0.0001$) with the ratio of activity on α2,3-linked sialosides to the activity on α2,6-linked sialosides of 4.685 ± 0.2092 (mean ± SEM) (Figure 2C).

Moreover, the NA activity of the reverse genetic viruses on 2,3- and 2,6-linked sialosides were shown in Figure 3A and 3B, respectively. While the NA from the H3N2 virus showed low activity on both α2,3- and α2,6-linkage, the reverse genetic viruses with the NA from the H5N1 viruses showed markedly higher activity on α2,3-linkage than on α2,6-linkage giving a high α2,3- to α2,6-ratio with P-value = 0.0249 (Figure 3C).

NA substrate specificity by HPLC analysis

To confirm the NA specificity profile, another assay using PA-glycopolymers and HPLC analysis of the enzymatic products was performed on a human isolate of the H5N1 virus (KAN-1) (Table 2). In concordance with the Amplex Red® assay, the H5N1 NA showed robust activity on α2,3-linked glycopolymers and undetectable activity on α2,6-linked glycopolymers. The two assays thus together conclusively showed that NA activity of the H5N1 virus had a α2,3-linkage preference.

DISCUSSION

Although, thiobarbituric acid method is the gold standard to detect NA activity, it is time-consuming and sensitive to interference by complex culture media[20]. Moreover, these methods use NANA, 4-methylumbellifery or fetuin as the substrate, which could not distinguish the substrate specificity because fetuin contained both α2,3-linked

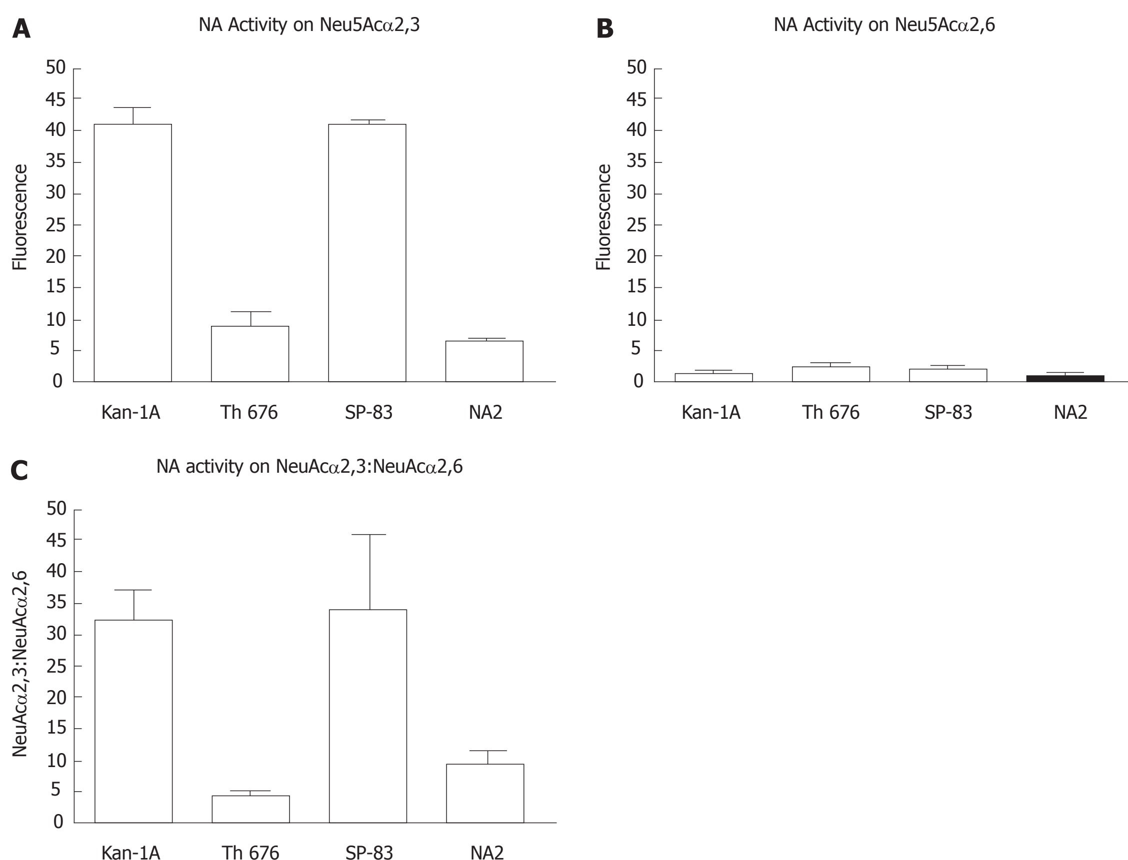

Figure 3 The neuraminidase activity on substrate specificity of reverse genetic virus by using Amplex Red® assay. The neuraminidase (NA) activity on α2,3- and α2,6-sialosides was shown in term of mean ± SEM from individual triplicate experiments on (A) and (B), respectively. The ratios of α2,3- and α2,6-substrate specific NA activity are shown on (C).

Table 2 Neuraminidase activity measured by high-performance liquid chromatography analysis

Linkage type	Siaαx Lac-PA	DiSiaαx BI-PA		4Siaαx Tetra-PA
	Digested	One sialic digested	Two sialic digested	Completely digested
α2,3	100	3.5%	96.5%	100%
α2,6	0	0	0	ND

ND: Not done.

and α2,6-linked sialosides. In order to detect the NA substrate specificity, there are several proposing methods to differentiate the substrate specificity, *i.e.,* BODIPY-labeled substrate, glycan array and library screening format[10,21-23]. These methods required modification and purification on neuraminidase which is not the original forms of neuraminidase from influenza virus[21,22]. To avoid the modification on influenza neuraminidase, the commercial Amplex Red® assay was modified by changing the substrate. In this assay all viruses had to be cultured in phenol red free medium to avoid the interference of fluorescent assay as previously described[15]. Similar to Amplex Red® assay, NA activity by HPLC also use the fluorescent labeled substrates and can detect the NA activity from the virus directly. Moreover, HPLC can also separate the size of digested substrates[19] which reflect to NA activity whether it completely or partially digest substrates with more than one sialic acid molecules.

Not only alpha-linkage that affect the substrate specificity, but the sialygalactoside; the basic form of sialic acid also effected the substrate specificity because their variations between species[24]. There are 3 forms of sialic acid, N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) and deamineuraminic acid[21,24,25]. Several studies showed that most of human viruses prefer to cleave Neu5Ac, which is predominantly expressed on human upper respiratory tract and is the most abundant sialic form in nature[21,24-26]. Therefore, this study used the Neu5Acα2,3- and Neu5Acα2,6-linkage which are the majority form of sialic acid in human on for substrate specificity.

The NA substrate specificities of this study is in agreement with previously published data showing predominant NA activity on α2,3-linked sialosides in avian viruses[8,21,22]. However, the input virus in the NA assay of this study

was normalized by their HA unit, a difference in HA activity may bias the amount of the input virus. To avoid this problem, reverse genetic viruses carrying NA from either human isolated H5N1 or a seasonal influenza virus (H3N2) with the rest of the genome including HA from PR8 strain was generated. Therefore, the viral input can be reliably normalized and the NA activity between the H5N1 NA and the H3N2 NA could be reliably compared.

The general patterns of NA substrate preference of avian and seasonal influenza viruses are in agreement with previously published data[10,27-29]. The similar sialic acid preference between HA and NA of the same group of viruses suggests that compatibility between HA and NA is important for optimal viral infection. On the other hand, NA function is not only to release progeny virions from producer cells but also to help virions penetrate mucus layer of respiratory mucosa[24,30]. This may explain why seasonal influenza viruses maintain α2,3-linkage specific NA activity despite their HA specificity to only α2,6-linked sialic acid. Human mucin is rich in α2,3-linked sialic acid, and NA activity against this type of sialic acid may be required for virions to reach target cells underneath the mucus layer[31]. The high α2,3-linkage specific NA activity of H5N1 avian influenza virus may help the virus penetrate the mucus layer and enhance the viral infection in humans.

Although the change in NA substrate preference does not seem to be a prerequisite for emergence of a pandemic virus, the NA substrate preference of H3N2 seasonal influenza virus and the H5N1 isolate with dual-specific HA (A/Thailand/Th676/2005, which was previously shown to have a dual specific HA conferred by two mutations at position 129 and 134[12]) suggested that the adaptation by decreasing α2,3-specific activity may help balance the HA adaptation toward human receptor specificity. The balance between HA and NA play a crucial role in the viral fitness and the emergence of pandemic virus[23,32]. The NA mutations, A138S, E259D, N325T and A343T, were observed. These mutations were located near either framework or active site of the NA[9,33-35]. Therefore, the adaptation of NA function may be resulting from either each or combination of these mutations.

COMMENTS

Background

Neuraminidase (NA) is a glycoprotein on the envelope of influenza virus. NA cleaves viral receptor on the cell surface in order to release virions from host cells. If the NA function is impaired, virions will aggregate on the cell surface hindering the dissemination of infection. NA activities on bird-type and human-type receptor substrate have been previously characterised for some avian and seasonal influenza viruses. It can be concluded that NA from human viruses shows the increased substrate specificity on human-type sialic acid, which is found in human respiratory tract, while maintaining specificity on bird-type sialic acid. Occasionally viruses are transmitted to other host species and introduce avian viral gene to non-avian hosts like human and this can lead to severe outbreaks or pandemics. Therefore, the monitoring of NA activity on substrate specificity is required.

Research frontiers

Highly pathogenic H5N1 avian influenza virus is causing a wide-spread epidemic in poultry with occasional transmission to humans and poses a serious pandemic threat. The authors therefore characterised NA activity of H5N1 viruses in comparison to NA of a seasonal influenza virus.

Innovations and breakthroughs

In order to study the NA substrate specificity, there are several proposing methods to differentiate the substrate specificity which required modification and purification on neuraminidase which is not the original forms of neuraminidase from influenza virus. To avoid the modification on influenza neuraminidase, the commercial Amplex Red® assay was modified by changing the substrate, instead. Similarly, HPLC used fluorescent labelled substrates and can detect the NA activity from the virus directly. The authors' data showed that H5N1 avian influenza isolates from both humans and birds maintained the NA activity profile with preference for bird-type receptor, except for a human isolates that was previously shown to have HA with dual specificity. This H5N1 virus showed reduced activity on bird-type substrate suggesting a requirement for compatibility with its HA that gained binding to human-type receptor. A138S, E259D, N325T and A343T mutations were found in the NA of this virus.

Applications

This study suggests that NA substrate specificity must be monitored for assessing the risk of cross-species transmission.

Terminology

α2,3-linked sialic acid or bird type substrate is the sialic acid that is mostly found in avian gastrointestinal and respiratory tract while α2,6-linked sialic acid is abundant in human respiratory tract.

Peer review

The authors have performed a good study, the manuscript is interesting.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/2220-3249/g_info_20100725073806.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.

Biology: *H. pylori*, *E coli*, *etc*.

Examples for paper writing

All types of articles' writing style and requirement will be found in the link: http://www.wjgnet.com/esps/NavigationInfo.aspx?id=15

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