Short Communication

Insertion of a multibasic cleavage site in the haemagglutinin of human influenza H3N2 virus does not increase pathogenicity in ferrets

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A multibasic cleavage site (MBCS) in the haemagglutinin (HA) protein of influenza A virus is a key determinant of pathogenicity in chickens, and distinguishes highly pathogenic avian influenza (HPAI) viruses from low pathogenic avian influenza viruses (LPAI). An MBCS has only been detected in viruses of the H5 and H7 subtypes. Here we investigated the phenotype of a human H3N2 virus with an MBCS in HA. Insertion of an MBCS in the H3N2 virus resulted in cleavage of HA and efficient replication in Madin–Darby canine kidney cells in the absence of exogenous trypsin in vitro, similar to HPAI H5N1 virus. However, studies in ferrets demonstrated that insertion of the MBCS into HA did not result in increased virus shedding, cellular host range, systemic replication or pathogenicity, as compared with wild-type virus. This study indicates that acquisition of an MBCS alone is insufficient to increase pathogenicity of a prototypical seasonal human H3N2 virus.

In wild birds and poultry throughout the world, avian influenza viruses expressing 16 antigenically distinct subtypes of haemagglutinin (HA) and nine subtypes of neuraminidase have been described (Fouchier et al., 2005; Roehm et al., 1996). Only subtypes H1, H2 and H3 have circulated extensively in humans (Nicholson et al., 2003). Avian influenza viruses can be classified, based on pathogenicity in chickens, into highly pathogenic avian influenza (HPAI) and low-pathogenic avian influenza (LPAI) viruses. Virtually all known HPAI viruses are of the H5 or H7 subtypes. Direct transmission of HPAI H5N1 viruses to humans was first detected in 1997 (de Jong et al., 1997) and has continued to be reported since (WHO, 2010). In addition, various human cases of HPAI H7 virus infection have been reported (Fouchier et al., 2004; Tweed et al., 2004; Webster et al., 1981).

Pathogenicity of avian influenza viruses in chickens is directly associated with the cleavability of the HA glycoprotein (Chen et al., 1998; Horimoto & Kawaoka, 1994; Klenk & Garten, 1994; Webster & Rott, 1987). HA is initially synthesized as an HA0 precursor protein that is subsequently cleaved into the two functional subunits HA1 and HA2. This cleavage step is essential for virus infectivity since uncleaved HA is able to mediate virus attachment but is unable to perform the fusion step necessary for the initiation of infection (Steinhauer, 1999). HA proteins of LPAI viruses have a single arginine at the cleavage site that is recognized by trypsin–like proteases expressed predominantly in the respiratory and intestinal tract. HAs of HPAI viruses contain a multibasic cleavage site (MBCS) that can be cleaved by ubiquitously expressed furin and related furin-like proprotein convertases (PC5/6) (Bertram et al., 2010; Stieneke-Gröber et al., 1992). The cleavage properties of HA and the expression patterns of proteases in the host are considered to be the major determinants of systemic influenza virus replication and pathogenesis in chickens.

The association between HA cleavability and replication outside the respiratory tract is less straightforward for influenza viruses in mammals. Human influenza viruses of subtypes H1, H2 and H3 do not have an MBCS in their HA and virus replication is generally restricted to the respiratory tract. For humans infected with HPAI H5 and H7 viruses, virus detection outside the respiratory tract has been reported, but it is not a general phenomenon (Korteweg & Gu, 2008). In Cynomolgus macaques infected with HPAI H5N1 virus, detection of virus antigen was limited to the respiratory tract (Rimmelzwaan et al., 2001). Systemic virus replication and associated pathogenesis has been reported for several HPAI H5 and H7 viruses in mice, ferrets and cats, but certainly not for all viruses tested in these mammalian model systems (Belser et al., 2007;
Govorkova et al., 2005; Kuiken et al., 2010; Maines et al., 2005; van Riel et al., 2010). In mice, it has been shown that removal of the MBCS from some HPAI H5 and lab-adapted H7 virus HAs resulted in reduced virulence (Gabriel et al., 2005; Hatta et al., 2001). Here, we followed an opposite -- gain of function -- approach, by inserting an MBCS into a human H3N2 virus and testing virus replication and pathogenesis in the ferret model. The goal of this work was to increase our basic understanding of the role of the MBCS and trypsin/furin-dependent cleavage of HA in influenza virus pathogenesis in mammals.

Influenza virus A/Netherlands/16190/68 (H3N2) was isolated from an individual in the Netherlands during the 1968 pandemic. Influenza virus A/Hong Kong/156/97 (H5N1) was isolated from the human index case during the 1997 H5N1 outbreak in Hong Kong (de Jong et al., 1997). The eight gene segments of these two viruses were amplified by RT-PCR and cloned in the BsmBI site of a modified version of plasmid pHW2000 (de Wit et al., 2004; Hoffmann et al., 2000). For the generation of H5N1 virus without an MBCS (H5N1AMBCS), the cleavage site PQIETR↓G in the H5 HA plasmid was changed to PQIETR↓G by RT-PCR with specific primers as described previously by Webby et al. (2004). For the generation of H3N2 virus with an MBCS (H3N2MBCS), the cleavage site PEKQTR↓G in the H3 HA plasmid was changed to PEKQRRKKR↓G, which is identical to that of the MBCS of the control virus A/Hong Kong/156/97 H5N1. Viruses H3N2wt, H5N1wt, H3N2MBCS and H5N1AMBCS were generated by reverse genetics as described previously (Munster et al., 2010). The genotypes of all plasmids and viruses were confirmed by sequencing. The risk potential of the H3N2MBCS virus was assessed prior to the start of the experiments and it was determined that the anticipated risk of generating this virus would be equivalent to that of a mammal-transmissible HPAI virus. Therefore, all in vivo and in vitro experiments were performed under ABSL3+ containment conditions. Animal studies were approved by an independent animal ethics committee (Stichting Dieren Experimenten Commissie consult).

First, the requirement of trypsin for the cleavability of the H3 HA proteins was tested. To this end, 293T cells were harvested 48 h after transfection with plasmids expressing HA of H3N2wt and H3N2MBCS, using HA of H5N1wt and H5N1AMBCS as controls. Cells were treated with either PBS (pH 7.4) or 2.5 μg trypsin ml−1 (Lonza) for 1 h at 37 °C. Cell lysates were subjected to electrophoresis in SDS-polyacrylamide gels (10% acrylamide) and immunoblotting. Western blots were incubated with rabbit serum reactive against A/Hong Kong/1/68 (H3) or A/Hong Kong/156/97 (H5) and a peroxidase-labelled swine anti-rabbit antibody as previously described (Munster et al., 2010). In the presence of trypsin, the HA of H3N2wt and H3N2MBCS were cleaved to completion (Fig. 1a). In contrast, in the absence of trypsin, the HA of H3N2wt remained uncleaved while a large fraction of the HA of H3N2MBCS was cleaved. Thus, upon insertion of an MBCS into H3 HA the cleavage of HA was no longer dependent on exogenous trypsin. H3 HA with and without an MBCS thus behaved essentially the same as H5 HA with and without an MBCS (Fig. 1a, b).

Next, virus replication of reverse genetics-derived H3N2wt and H3N2MBCS viruses in the presence and absence of 1 μg trypsin ml−1 was tested in Madin–Darby canine kidney (MDCK) cells, again using H5N1wt and H5N1AMBCS as controls. Cells were inoculated in duplo at an m.o.i. of 0.01 TCID50 per cell and supernatant samples were harvested 6, 12, 24 and 48 h later. Supernatant samples were titrated in MDCK cells. Geometric mean titres and so were calculated from two independent experiments. Data for Fig. 1(b and d) were taken with permission from Munster et al. (2010) (Copyright, American Society for Microbiology).

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Next, we investigated whether the trypsin-independent cleavage of H3N2MBCS resulted in enhanced pathogenicity in ferrets. The ferret model is generally thought to be a good animal model for influenza in humans, because ferrets are naturally susceptible to infection and develop respiratory disease and lung pathology similarly to humans when infected with seasonal, avian or pandemic influenza viruses (Bodewes et al., 2010; Maher & DeStefano, 2004; van den Brand et al., 2010). Groups of six influenza-seronegative female ferrets (Mustella putorius furo) were inoculated intranasally with $10^6$ TCID$_{50}$ of H3N2wt or H3N2MBCS virus, divided over both nostrils ($2 \times 250 \mu l$). Animals were observed for the development of clinical symptoms and weighed daily. The percentage mean maximum weight loss was $8.8 \pm 1.4\%$ (SD) for animals inoculated with H3N2wt virus. Animals inoculated with H3N2MBCS had a maximum weight loss of $7.0 \pm 1.4\%$ (SD) (Fig. 2a). No differences were observed for clinical parameters, such as lethargy, sneezing, ruffled fur, interest in food and runny nose, between the two groups of ferrets. Thus, H3N2MBCS does not appear to cause more severe disease as compared with H3N2wt.

Throat and nasal swabs were collected daily to determine virus excretion from the upper respiratory tract. Infectious

![Fig. 2. Weight loss and virus replication in ferrets inoculated with H3N2 wt and H3N2 MBCS virus. (a) Weight loss of ferrets inoculated with H3N2 wt (○) and H3N2 MBCS (■) virus. Mean body weight and SD were calculated as percentages of body weight at time of inoculation for each group. After day 3, only three animals remained in each group. (b and c) Virus shedding from the nose (b) and throat (c) of ferrets inoculated with H3N2 wt (black bars) and H3N2 MBCS (grey bars). (d) Virus detection in tissues of ferrets inoculated with H3N2 wt (black bars) and H3N2 MBCS (grey bars) virus at day 3. Geometric mean titres are shown, with error bars indicating the SD. The lower limit of detection is indicated by the dotted line. The number of positive tissues from the ferrets are shown.](image-url)
virus shedding from the nose peaked at day 2 for both viruses and continued until days 7 and 6 for H3N2wt and H3N2MBCS, respectively (Fig. 2b). Shedding from the throat peaked at day 1 and continued until day 6 for both viruses (Fig. 2c). We observed a consistent decline in viral shedding from the throat, compared with the nose. Nucleotide sequencing was performed to confirm that the HA cleavage site remained unchanged during the experiment.

Three animals from each group were euthanized at 3 and 7 days post-inoculation (p.i.), and the nasal turbinates, trachea, lungs, liver, spleen, kidney, colon, and brain were collected to study virus distribution. Necropsies and tissue sampling were performed according to a standard protocol and infectious-virus titres were determined by end point titration in MDCK cells (Munster et al., 2010). At 7 days p.i., virus was undetectable in samples from the organs of all groups of ferrets. At 3 days p.i., virus was not detected in lungs, spleen, liver, kidney and intestine of ferrets inoculated with H3N2wt or H3N2MBCS. Both groups of inoculated ferrets revealed low virus titres in the trachea. Virus titres in the nasal turbinates were high and similar for both groups of ferrets. Virus was detected in the brain of 1 of 3 animals inoculated with H3N2wt and 3 of 3 animals inoculated with H3N2MBCS, but only at low titres (Fig. 2d). Immunohistochemistry was performed to confirm virus replication in nasal turbinate, trachea, and brain at 3 days p.i. After fixation in 10 % neutral-buffered formalin and embedding in paraffin, tissue sections were stained using a mAb against the nucleoprotein of influenza A virus (Munster et al., 2010). Influenza virus antigen expression was not detected by immunohistochemistry in the brain and trachea of either group of ferrets. In contrast, virus antigen was readily detected in the nasal turbinates of all three ferrets in both groups at 3 days p.i. There was no evidence for different cellular tropisms between H3N2MBCS and H3N2wt virus in the nasal turbinates of infected ferrets (Fig. 3). These results indicated that replication of H3N2MBCS, like replication of H3N2wt, is mostly restricted to the upper respiratory tract of ferrets.

The current study demonstrates that, with the insertion of an MBCS identical to that of currently circulating HPAI H5 viruses, a human H3N2 virus becomes trypsin-independent in vitro. The introduction of an MBCS into other human and LPAI HA subtypes (H3 and H6) also resulted in trypsin-independent cleavage of HA0 in vitro (Kawaoka, 1991; Munster et al., 2010; Ohuchi et al., 1991; Stech et al., 2009). Introduction of an MBCS into an LPAI H6N1 virus resulted in an increased intravenous pathogenicity index in chickens (Munster et al., 2010). However, the consequences of the introduction of the MBCS for human H3N2 virus replication and pathogenesis in animal models have not been investigated previously. Surprisingly, the trypsin independence of a human H3N2 virus did not result in enhanced pathogenicity, tissue tropism or cellular tropism in the ferret model, as compared with wild-type H3N2 virus. If anything, the H3N2MBCS virus was slightly attenuated compared with H3N2wt virus, based on observations of weight loss and virus shedding from the upper respiratory tract.

Both groups of inoculated ferrets revealed no virus replication in the lower respiratory tract. This is not unusual, as strain-to-strain variability among human H3N2 viruses with respect to infection of the lower respiratory tract of ferrets has been demonstrated (Jackson et al., 2009; Parks et al., 2007; Svitech et al., 2008; Zitzow et al., 2002). Although the H3N2MBCS virus was detected in the brain of ferrets, virus titres were very low and virus-infected cells were not detected by immunohistochemistry. Given that low virus titres were also detected for a ferret inoculated with H3N2wt virus, and that neither of the groups of infected ferrets developed neurological symptoms, we conclude that the MBCS in the context of H3 HA did not result in systemic virus replication and enhanced disease. In ferrets infected with more recent wild-type human H3N2 viruses, low virus titres were also detected in the brains (Zitzow et al., 2002).

The goal of the present work was to increase the basic understanding of the role of the MBCS and trypsin- or furin-dependent cleavage of HA in influenza virus
pathogenesis in mammals. Our results indicate that an MBCS is not sufficient to increase the virulence of a human H3N2 virus. For HPAI H5N1 viruses, additional virulence determinants in HA and other viral proteins have been shown to affect virulence independently of the MBCS in ferrets and chickens (Bogs et al., 2010; Gohrbandt et al., 2011; Govorkova et al., 2005; Imai et al., 2010). It is possible that an MBCS in H3 HA in combination with other genetic changes, such as changes in the receptor binding site, could result in increased pathogenicity of human H3N2 virus as well, by facilitating replication in extra-respiratory tissues.

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