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The anti-papillomavirus activity of human and bovine lactoferricin

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Abstract

Human papillomavirus (HPV) cause common warts, laryngeal papilloma and genital condylomata and is necessary for the development of cervical cancer. We have previously found that lactoferrin has antiviral activity against HPV-16 and others have demonstrated that lactoferricin, an N-terminal fragment of lactoferrin, has inhibitory activities against several viruses. Two cell lines and two virus types, HPV-5 and HPV-16, were used to study if lactoferrin and lactoferricin could inhibit HPV pseudovirus (PsV) infection. We demonstrated that bovine lactoferrin (bLf) and human lactoferrin (hLf) were both potent inhibitors of HPV-5 and -16 PsV infections. Among the four lactoferricin derivatives we analyzed, a 15 amino acid peptide from bovine lactoferricin (bLfcin) 17–31 was the most potent inhibitor of both HPV-5 and HPV-16 PsV infection. Among the other derivatives, the human lactoferricin (hLfcin) 1–49 showed some antiviral activity against HPV PsV infection while bLfcin 17–42 inhibited only HPV-5 PsV infection in one of the cell lines. When we studied initial attachment of HPV-16, only bLfcin 17–42 and hLfcin 1–49 had an antiviral effect. This is the first time that lactoferricin was demonstrated to have an inhibitory effect on HPV infection and the antiviral activity differed depending on size, charge and structures of the lactoferricin.

Keywords: Lactoferrin; Lactoferricin; Human papillomavirus; Antiviral activity

1. Introduction

Human papillomavirus (HPV) is an 8 kb naked DNA virus belonging to the family of *Papillomaviridae*. HPV infect basal cells in mucosa or skin, possibly through micro lesions, and is strongly dependent on the differential status of the epithelium for its viral life cycle and is necessary for the development of cervical cancer. HPV-16 belongs to the α-papillomavirus genus, species 9 (de Villiers et al., 2004) and is the most common type found in cervical cancer but does also cause condyloma and other infections of the genital and respiratory tracts (Stubenrauch and Laimins, 1999). HPV-5 is the most common type found on normal skin all around the world (Antonsson et al., 2003). It belongs to species 1 of the β-papillomavirus genus (de Villiers et al., 2004) and is associated with skin cancers in patients diagnosed with e*pidermodysplasia verruciformis* (Berkhout et al., 1995). Propagating HPV has been very difficult but the development of virus like particles (VLP) produced in recombinant expression systems (Zhou et al., 1992) and pseudovirus (PsV) produced inside cells (Buck et al., 2004; Roden et al., 1996; Unckell et al., 1997; Zhao et al., 1998) have simplified the studies of the papillomavirus life cycle. VLP and PsV have been used in several studies of HPV binding and entry (Day et al., 2004; Drobni et al., 2003; Evander et al., 1997; Giroglou et al., 2001; Joyce et al., 1999) and PsV have also been used for high-throughput screening of compounds with the potential to block papillomavirus infectivity *in vitro* (Buck et al., 2006b). The first step of HPV infection is believed to be binding of the major capsid protein L1 to the cell surface. The cell surface glycosaminoglycan (GAG) heparan sulfate is important for the initial attachment to the cell surface of certain α-papillomaviruses found more frequently in mucosal lesions (Combita et al., 2001; Drobni et al., 2003; Giroglou et al., 2001; Joyce et al., 1999; Shafti-Keramat et al., 2003). Heparan sulfate is also a receptor for several other viruses such as herpesvirus, norovirus, hepatitis C virus and foot-mouth disease virus (Spillmann, 2001).

Lactoferrin is an 80 kDa monomeric glycoprotein present in secretions such as breast milk, saliva, semen and tears. The highest concentration of lactoferrin is found in colostrums (Cohen et al., 1987). Lactoferrin plays important and multifunctional

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roles in host defence and some of these functions are to inhibit infection of bacteria, fungi and viruses (Bellamy et al., 1993). Lactoferrin acts as an antiviral protein against herpes simplex virus (HSV), human cytomegalovirus (HCMV), hepatitis C virus (HCV), poliovirus, enterovirus 71 (EV71), BK polyomavirus, HIV and HPV (Andersen et al., 2003; Andersen et al., 2004; Andersen et al., 2001; Drobni et al., 2004; Harmsen et al., 1995; Ikeda et al., 1998; Jenssen, 2005; Lin et al., 2002; Longhi et al., 2006; Marchetti et al., 1996; Marchetti et al., 1999). Lactoferrin exhibits its antiviral activity early in the infection cycle and for HSV (Andersen et al., 2004; Hasegawa et al., 1994; Marchetti et al., 2004), HPV-16 (Drobni et al., 2004) and hepatitis B virus (Hara et al., 2002) the lactoferrin interaction with heparan sulfate on the cell surface seem to block the attachment of the virus. The antiviral activity could also be mediated by a direct interaction between the virus and lactoferrin as seen for poliovirus (Marchetti et al., 1999), rotavirus (Superti et al., 1997), HIV (Swart et al., 1996), HCV (Yi et al., 1997), EV71 (Ammendolia et al., 2007; Weng et al., 2005), and BK polyomavirus (Longhi et al., 2006).

Lactoferricin is generated by proteolytic cleavage of the N-terminal part of lactoferrin by pepsin. Bovine lactoferricin (bLfcin) fragments have been described to be composed of either amino acid 17-41 (Bellamy et al., 1992a) or 17–42 (Dionysius and Milne, 1997). One cysteine–cysteine disulfide bond between residue 19 and 36, creates a loop structure (Hwang et al., 1998), though this loop structure is not essential for antibacterial activity (Bellamy et al., 1992a). In the homologus human lactoferricin (hLfcin), composed of amino acid 1–49, a loop is created with two disulfide bridges between residue 20–37, and 10–46 respectively (Hunter et al., 2005). Lactoferricin has broad host defense properties against bacteria, fungi, parasites and viruses (Gifford et al., 2005) and some of the antimicrobial properties of lactoferricin can be explained by its ability to form amphipathic structures with clear hydrophobic and positively charged faces. Other peptides with antimicrobial activity also display these characteristics (Epand and Vogel, 1999). Different lactoferricin types have antiviral effects against viruses such as HSV-1 and -2 (Andersen et al., 2003), HCMV (Andersen et al., 2001), HIV (Berkhout et al., 2002), HCV (Ikeda et al., 2000) and echovirus 6 (Pietrantoni et al., 2006), but have also been shown to have tumor inhibitory effects (Eliassen et al., 2002; Yoo et al., 1997). We have previously demonstrated the antiviral effect of lactoferrin on HPV internalization and binding to HaCaT cells in vitro using HPV VLPs (Drobni et al., 2004). In this study we wanted to better understand the inhibitory effect of human and bovine lactoferrin and four derived peptides on HPV-5 and HPV-16 PsV infection.

2. Materials and methods

2.1. Virus-like particle production

The HPV VLPs were produced using a baculovirus expression system. Sf-21 insect cells were infected with recombinant baculovirus expressing the HPV-16 L1 capsid protein under the control of the polyhedrin promoter. The recombinant baculovirus was a kind gift from Ian Frazer, CICR, Brisbane, Australia. The VLPs were produced as previously described (Drobni et al., 2003). Briefly, the infected cells were grown until 80% cell death, and nuclei were prepared using different steps of mechanical disruption, sonication and centrifugations. The VLPs were extracted by sucrose gradients and CsCl gradients before dialysis against extensive amounts of PBS. The integrity of the particles was assessed using electron microscopy.

2.2. Pseudovirus production

Plasmids and 293TT cells (Buck et al., 2005) used for pseudovirus production were a kind gift from Chris Buck (National Cancer Institute, Bethesda, Maryland, USA) and Martin Müller (German Cancer Research Center, Heidelberg, Germany). Detailed protocols are available at the website (http://home.ccr.cancer.gov/lco/default.asp). HPV-5 and HPV-16 GFP-expressing pseudoviruses were produced according to previously described methods (Buck et al., 2005). Briefly, 293TT cells engineered to express high levels of SV40 large T antigen were transfected with plasmids expressing the papillomavirus major and minor capsid proteins, L1 and L2, together with a green fluorescent protein (GFP)-expressing reporter plasmid, pfwB. All PsV were produced using codon-modified L1 and L2 genes. HPV16 PsV was produced using a bicistronic L1/L2 expression plasmid, p16sheLL and HPV-5 PsV was produced using the p5L1w and p5L2w plasmids. Capsids were allowed to mature overnight in cell lysate and were then purified using OptiPrep® gradients (Axis-Shield).

2.3. Proteins and peptides

Lactoferrin of bovine origin was purchased from Sigma Chemical Co. (St. Louis, MO). BLfcin and hLfcin was purchased from Centre for Food Technology (Brisbane, Australia), except for hLfcin 18–42, which was provided by MedProbe (California, USA). Four different lactoferricin peptides were used to investigate the possible differences between human and bovine peptides. BLfcin 17–41 (FKC_1RRWQWRMKKLGA-$PSITC_1$VRRAF). BLfcinB 17–42 (FKC_1RRWQWRMKKL-GAPSITC_1VRRAFA). BLfcin 17–31 (FKCRRWQWRMKK-LGA) with acetamidomethyl thiol protecting group on the cysteine. HLFcin 1–49 (GRRRRSVQWC_1AVSQPEATKC_2-FQWQRNMRKVRGPPVSC_2IKRDSPIQC_1IQA). HLfcin 18–42 (TKC_1FQWQRNMRKVRGPPVSC_1IKRDS). Cysteines forming disulfide bonds are numbered with subscript numbers to indicate their pairing.

2.4. Internalization assay

Lactoferrin and lactoferricin were tested using a previously described PsV-based papillomavirus inhibition assay (Buck et al., 2006b). Briefly, the human epithelial cell line HaCaT, from adult trunk skin (Boukamp et al., 1988) and the human cervical cell line C33A (Crook et al., 1991) were plated at 5×10^4 cells/well in 400 μl of DMEM supplemented with 10% FCS (Life technologies, Inc., Gaitersburg, MD) in 24-well