Chemically modified bovine beta-lactoglobulin inhibits human papillomavirus infection

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Abstract

Previous studies have shown that 3-hydroxyphthalic anhydride-modified bovine beta-lactoglobulin is a promising anti-HIV microbicide candidate. Here we found that this chemically modified protein, designated JB01, exhibited highly potent antiviral activity against infection by human papillomaviruses (HPV), including HPV6, HPV16 and HPV18. Its anti-HPV activity was correlated with the percentage of modified lysine and arginine residues in JB01. This modified milk protein had no cytotoxicity at the concentration of 1 mg/ml, and it is highly stable at room temperature and 37 °C for at least 12 weeks. These results suggest that JB01 has good potential to be developed as an effective, safe and inexpensive antiviral agent for treatment and prevention of HPV infection.

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1. Introduction

Cervical cancer is the third most common cancer for women worldwide, and an estimated 274,000 women die from it each year [1]. This is more serious in the developing countries where cervical cancer is the second leading cause of cancer mortality in women 15–44 years old [2]. In China, about 135,000 women develop cervical cancer, and 50,000 die from this disease each year, or about 18% of total worldwide mortality [3]. Especially in this decade, the incidence of cervical cancer has rapidly increased, making this disease a great threat to Chinese women’s health. Therefore, development of an effective and safe biologic to prevent cervical cancer is urgently needed.

Human papillomavirus (HPV) is now well recognized as the causal agent of cervical cancer [4]. HPV is a small non-enveloped virus, about 55 nm in size, containing double-stranded DNA. The whole genome consists of three regions. E and L regions control viral replication at the early and late stages, respectively, while the LCR region regulates gene functions [5]. So far, over one hundred different subtypes of HPV have been isolated, which could be further divided into high-risk and low-risk groups [6]. Since HPV types 6 and 11 mainly cause benign genital warts and mild cervical epithelial necrosis, they are considered as low-risk types. On the other hand, HPV16 and HPV18 are high-risk types since they are responsible for more than 70% of malignant tumors [7,8].

HPV usually infects the basal cells of stratified epithelium on human skin and mucosal tissues, particularly around the mouth, hand, feet and genitals. Direct contact between virus and human cells is a necessary requirement for viral invasion. When the skin or mucosa is damaged, free mature HPV particles have the opportunity to penetrate the microtrauma site and interact with tissue cells. Generally speaking, this is the beginning of the HPV lifecycle, which is also the best stage for prevention of the infection. Vaccines against HPV have now been licensed in over 100 countries [9]. However, their
application in low-income, developing countries is limited by high cost and the lack of effectiveness against all HPV types. Furthermore, no effective antiviral agent is available for inhibiting HPV infection topically, necessitating the development of an anti-HPV agent for topical application.

Our previous studies have shown that 3-hydroxyphthalic anhydride-modified bovine beta-lactoglobulin, herein designated JB01, showed potent inhibition activity against HIV, HSV-1, HSV-2, as well as some Chlamydia [10—13]. In this study, we tested the potential inhibitory activity of this chemically modified milk protein against HPV infection. We found that JB01 exhibited potent antiviral activity against infection by HPV6, HPV16 and HPV18. Our previous studies have shown that this chemically modified bovine protein, which is very inexpensive, is highly stable in aqueous solution and can be easily formulated into a topical gel [10,14]. Therefore, we believe that JB01 has good potential to be further developed as an effective, safe and affordable topical biologic for prevention and treatment of HPV infection.

2. Materials and methods

2.1. Reagents

3-Hydroxyphthalic anhydride, bovine beta-lactoglobulin, trypsin–agarose beads, XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino) carbonyl-2H-tetrazolium hydrazide], and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma (St. Louis, MO), and pHyphenylglyoxal (p-HPG) was purchased from Fisher Scientific (Valley Park, VA). TZM-bl cells, which are HeLa-cell derivatives expressing high levels of CD4 and both co-receptors CXC4 and CCR5, and are stably transduced carrying a LTR-driven firefly luciferase, and 293FT cells, a fast-growing variant of 293T cell line that contains the SV40 large T antigen, were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. VK2/E6E7 cells (an immortalized vaginal epithelial cell line) were purchased from American Type Culture Collection (ATCC) (Manassas, VA).

2.2. Preparation and identification of JB01

The JB01 protein was prepared based on a previously described method [10]. Briefly, bovine beta-lactoglobulin was dissolved in 0.1 M phosphate buffer (pH 8.5) to final concentration of 20 mg/ml. Then 3-hydroxyphthalic anhydride saturated in dimethylformamide was added slowly to this solution with gentle shaking. Five aliquots with the final concentrations of 3-hydroxyphthalic anhydride at 0, 10, 20, 40 and 60 mM, respectively (as indicated in Table 1), were prepared. The mixtures were kept for another 1 h at room temperature, extensively dialyzed against phosphate buffer saline (PBS), and filtered through 0.45 μm syringe filters (Acrodisc; Gelman Sciences, Ann Arbor, MI). Protein concentrations were determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

<table>
<thead>
<tr>
<th>Concentration of HP (mM)</th>
<th>% of modified residues</th>
<th>IC50 (μM) for inhibiting infection bya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lysine</td>
<td>Arginine</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>35.2</td>
<td>47.3</td>
</tr>
<tr>
<td>20</td>
<td>51.3</td>
<td>66.2</td>
</tr>
<tr>
<td>40</td>
<td>79.6</td>
<td>85.9</td>
</tr>
<tr>
<td>60</td>
<td>90.1</td>
<td>95.2</td>
</tr>
</tbody>
</table>

a Each sample was tested in triplicate, and the experiment was repeated twice. The data are presented in means ± SD of triplicate determinations from a representative experiment.

To quantify lysine residues in modified or unmodified proteins, a TNBS assay was used as previously described [15]. Briefly, 25 μl of anhydride-modified or -unmodified proteins (90 μM) were treated with 25 μl Na2B4O7 (0.1 M) for 5 min at room temperature (RT). Then 10 μl TNBS were added to the mixture. After another 5 min, 100 μl stop solution (0.1 M NaH2PO4 and 1.5 mM Na2SO3) was added to terminate the reaction. The absorbance at 420 nm (A420) was measured using a microplate reader (Ultra 384; Tecan, Research Triangle Park, NC). The percentage of arginine residues modification was also detected using a previously described method [16]. In brief, 90 μl of anhydride-modified or -unmodified proteins (90 μM) in 0.1 M sodium phosphate (pH 9.0) were treated with 10 μl of 50 mM p-HPG for 90 min at RT in the dark. The absorbance at 340 nm (A340) was measured.

2.3. Detection of inhibitory activity of JB01 on HPV infection

The HPV pseudoviruses were generated by co-transfection of plasmid containing codon-modified HPV L1 and L2 genes into 293FT cells as described previously [17]. The pseudoviruses of HPV6, HPV16 and HPV18 at 100 TCID50 (50% tissue culture infective doses) were incubated with JB01 or control proteins for 30 min at 37 °C. Then the mixture was added into 1 × 105/ml 293FT cells in DMEM medium containing 10% FBS overnight. The culture supernatants were removed, and fresh media were added the next day. The cells were harvested and lysed on the third day postinfection with 50 μl of lysing reagent. The luciferase activity was analyzed using a luciferase kit (Promega, Madison, WI) and a luminometer (Ultra 386; Tecan, Durham, NC) according to the manufacturer’s instructions. The IC50s of JB01 were calculated using the CalcuSyn software [18].

2.4. Analysis of cytotoxicity

The potential cytotoxicity of JB01 on TZM-b1 and VK2/E6E7 cells was measured by using the colorimetric XTT assay as previously described [19,20]. Briefly, 100 μl of a compound at graded concentrations were added to equal...
volumes of cells (10^5 cells/ml) in wells of 96-well plates. After incubation at 37 °C for 4 days, 50 µl of XTT solution (1 mg/ml) containing 0.02 µM phenazinemethosulfate was added. After 4 h, the absorbance at 450 nm was measured with an ELISA reader, and the percentage of cytotoxicity was calculated.

3. Results

3.1. The percentage of positively charged lysine and arginine residues modified by 3-hydroxyphthalic anhydride is correlated with JB01’s anti-HPV activity

Beta-lactoglobulin consists of 162 residues, with a molecular weight of 18.4 kDa and contains 18 positively charged residues, including 15 lysine residues and 3 arginine residues. We have previously demonstrated that the number of chemically modified lysine residues in JB01 is an important determinant of its anti-HIV-1 activity [21]. Here, we found that the number of both lysine and arginine residues is important for JB01’s anti-HPV activity. As shown in Table 1, with increased concentration of 3-hydroxyphthalic anhydride, more lysine and arginine residues were modified, resulting in an increased anti-HPV activity. When 60 mM of 3-hydroxyphthalic anhydride was used, about 90% and 95% of the lysine and arginine residues were modified, respectively, leading to the highest inhibition of HPV16 infection (IC_{50} = 0.027 µM). Therefore, we used this concentration of 3-hydroxyphthalic anhydride to modify beta-lactoglobulin in subsequent studies.

3.2. JB01 was effective in inhibiting infection by HPV6, HPV16 and HPV18

Subsequently, we tested the inhibitory activity of JB01 against infection by HPV6 that can cause benign genital warts, and HPV16 and HPV18, which are recognized as high-risk types causing cervical cancer [7,8]. As shown in Fig. 1, JB01 exhibited highly potent antiviral activity against all three HPV subtypes, HPV6, HPV16 and HPV18, with IC_{50} values of 0.33, 0.04, 0.065 µM, respectively. These results suggest that JB01 is effective against the major HPV subtypes that cause diseases in humans.

3.3. JB01 was stable and had low cytotoxicity

The stability and safety of an anti-HPV agent are important issues for its development as a clinically usable product. To study JB01’s stability, we kept JB01 protein at room temperature (24 °C) and human body temperature (37 °C) for 12 weeks, followed by testing its anti-HPV activities at the week 1, 2, 3, 4, 6, and 12, respectively. The results showed that JB01 was highly stable since its anti-HPV activity showed no significant changes during the 12-week storage period (Fig. 2A). Then, we further tested the cytotoxicity of JB01 to TZM-b1 cells, which were derived from a human cervical cell line (HeLa cells), and VK2/E6E7 cells (vaginal epithelial cells), using unmodified beta-lactoglobulin as a control. As shown in Fig. 2B, JB01 exhibited no significant cytotoxicity on these cells.

Fig. 1. The inhibitory activity of JB01 against infection by HPV, including viral HPV6 (A), HPV16 (B) and HPV18 (C). The measurements were performed in triplicate, and the experiment was repeated at least twice. The data are presented as the means ± SD of triplicate determinations from a representative experiment.
human cervical cells and vaginal epithelial cells, suggesting that it is as safe as the unmodified beta-lactoglobulin.

4. Discussion

HPV does not infect intact, but rather damaged epithelium of mucosa. Using its capsid proteins L1 and L2, it infects the basal cells of stratified epithelium via a unique mechanism. The L1 protein first binds to heparan sulfate proteoglycan (HSPG) on the basement membrane (BM), which is exposed after epithelial trauma, resulting in furin cleavage of the L2 protein and binding of L1 to an as yet undetermined receptor on surface of keratinocytes to cause a new replication cycle [22]. Therefore, a topical formulation containing anti-HPV agent that covers the wounds in genital mucosa is expected to stop HPV new infection, thus being useful for preventing and treating HPV infection.

Since the expression of L1 and L2 proteins requires cellular differentiation in the upper layers of the stratified squamous epithelial tissues [22], HPV cannot replicate in conventional monolayer cell cultures. However, the HPV-based pseudoviruses can be used for investigating the early phase of the HPV lifecycle [17]. Here we used a pseudotyped HPV for testing the inhibitory activity of JB01 on HPV infection in 293FT cells. We found that JB01 is highly effective in inhibiting infection by several types of HPV, including HPV6, HPV16 and HPV18. The percentage of modified lysine and arginine residues in JB01 was correlated with its anti-HPV activity, indicating that the net negative charges in JB01 play an important role in JB01-mediated inhibition of HPV infection. These results suggest that JB01 may block HPV entry into the target cell through the interactions between the negatively charged residues on beta-lactoglobulin and the positively charged residues on the L1 and/or L2 proteins. We speculate that the C-terminal region of L1 protein and the N-terminal region of L2 protein may be the target sites for JB01 since both regions contain the positively charged residues that are exposed on the surface of viral capsid [23,24]. Indeed, we found that JB01 could strongly bind to the positively charged peptides derived from the L1 and L2 proteins of HPV, while the unmodified beta-lactoglobulin exhibited no significant binding to these peptides (data not shown).

In conclusion, our study has demonstrated that JB01, a chemically modified beta-lactoglobulin with relatively increased surface negative charges (due to the chemical modification of the positively charged residues), inhibits HPV infection, possibly by targeting the early stage of viral replication, particularly the viral entry process. As an inexpensive, safe and stable anti-HPV agent, JB01 can be formulated in a topical gel formulation for prevention and treatment of HPV infection in genital mucosa, and consequently reduce the occurrence of cervical cancer.
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References


