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The antiviral activity of the milk protein lactoferrin against the human immunodeficiency virus type 1

Ben Berkhout[1,*], René Floris[2], Isidra Recio[3] & Servaas Visser[2]
[1]*Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands*
[2]*Department of Product Technology, NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands*
[3]*Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain*
[*]*Corresponding author (Tel.: +31-20-566-4822; Fax: +31-20-691-6531; E-mail: b.berkhout@amc.uva.nl; http://www.berkhoutlab.com)*

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Abstract

Milk forms a rich source of biologically interesting components and the protein fraction is known to facilitate many different biological functions. In this manuscript, we review the antiviral properties of the milk protein lactoferrin (LF). In particular, we will describe its antiviral activity against the human immunodeficiency virus type 1 (HIV-1).

Introduction

Milk provides a rich source of valuable proteins, minerals and vitamins. The nutritional significance of the protein fraction has macronutritient and physiological aspects (Hambraeus 1992), which are important for their use in dietary and pharmaceutical applications. Besides bioactive proteins, milk also provides bioactive peptides. These are essentially formed by enzymatic hydrolysis of intact proteins, which themselves are not necessarily bioactive. Such bioactive fragments within the amino acid sequence of milk proteins have been studied extensively (for reviews, see Meisel 1997, Dziuba *et al.* 1999). A large range of bioactivities has been reported for milk protein components, some of these components showing more than one kind of biological activity. In this paper, we concentrate on the antiviral properties of LF against HIV-1.

HIV-1 biology

Most drugs that are currently used in the treatment of HIV infections belong to one of the three following classes: nucleoside/nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and protease inhibitors (Volberding 1999, Moyle 2001, Squires 2001, De Clerq 2002, Van Heeswijk *et al.* 2002). These compounds inhibit crucial processes: reverse transcription of the viral RNA genome, and processing of the viral proteins (Gag and Gag-Pol polyprotein precursors) that are needed for viral assembly. There are other important events in the replicative cycle of HIV that form potential targets for therapeutic intervention: viral adsorption to the cell (blocking the viral envelope proteins gp120 and gp41); viral entry (blocking cell membrane receptor CD4 or chemokine co-receptors CXCR4 and CCR5); virus-cell fusion (blocking viral envelope protein gp41); viral assembly and disassembly (targeting NCp7 zinc finger); proviral DNA integration (inhibiting integrase); viral mRNA transcription (inhibiting the transcription/ transactivation process).

Of these possibilities, interference with the infection process by binding to either the virus envelope proteins (gp120 and gp41) or the cell membrane (co)-receptors (CD4, CXCR4, and CCR5) seems a relevant mechanism for the design of anti-HIV drugs, in part because the drug does not have to penetrate into the cells for inhibition to occur. For this reason, the entry process is also a possible target for therapeutic milk

proteins. These entry blockers could be combined in therapies with drugs that inhibit processes in later stages of infection. An advantage of such drug combinations could be the reduced possibility of resistance development, and the drugs could also potentially exhibit synergistic effects (D'Souza *et al.* 2000, Blair *et al.* 2000).

It is thought that electrostatic interactions play an important role in the interaction between the HIV-1 virion and the host cell. There are solid indications that a positively charged domain on gp120, which faces away from the virus, forms the actual co-receptor binding site (Rizzuto *et al.* 1988, Kwong *et al.* 2000). This domain includes the so-called V3 loop of gp120, which has been shown to determine co-receptor usage. A strongly basic V3 loop is frequently correlated with usage of CXCR4 (De Jong *et al.* 1992, Fouchier 1992). It is of interest that the electrostatic potential of extracellular domains of CXCR4 is much more pronounced than that of the CCR5 co-receptor (Jiang 1997, Berkhout *et al.* 1998). The importance of several negatively charged residues in CXCR4 for HIV-1 entry was confirmed (Kajumo *et al.* 2000). In addition, the conserved part of gp120, which is also very basic (Moulard *et al.* 2000), is important in the interactions with co-receptors, and negatively charged residues are critical for functioning of the CCR5 co-receptor (Kajumo *et al.* 2000, Cormier *et al.* 2000). Sulfonation of CCR5, which increases the overall negative charge, is important for HIV-1 entry (Farzan *et al.* 1999, Cormier *et al.* 2000). Electrostatic interactions have also been shown to be of importance in contacts of HIV-1 with accessory cell-surface molecules, such as in the interaction between the V3 loop and heparan sulfate (Rodriquez *et al.* 1995).

Naturally occurring negatively charged polymers such as heparin and dextran sulfate have been shown to display antiviral effects (Ueno *et al.* 1987, Baba *et al.* 1988, Lederman *et al.* 1989). Consistent with the idea of blocking virus-cell interaction via (non-specific) charge-charge interactions, HIV-1 replication is also blocked by positively charged molecules, including the highly cationic ALX40-4C (nonapeptide) and AMD3100 (Schols *et al.* 1997, Doranz *et al.* 1997). Milk was also shown to be a source of highly positively charged macromolecules that could inhibit the binding of HIV-1 to the CD4 receptor (Newburg *et al.* 1992). This finding demonstrates that milk and plasma proteins, as well as modified forms thereof such as some charge-modified proteins and protein fragments, may be used as antiviral compounds.

LF-mediated HIV-1 inhibition

During microbial infections and autoimmune diseases, the level of plasma LF increases from 4 to up to 200 μg/mL (Lash *et al.* 1983, Nuijens *et al.* 1992). Based on this physiological fluctuation of the protein concentration, it was hypothesized that LF may display antiviral activities. The first report of antiviral activity of LF against HIV-1 was published in 1995 (Harmsen *et al.* 1995). The same group reported that LF does not inhibit HIV-2 replication. Native bovine LF and human LF inhibit HIV-1 infection of MT-4 cells. Bovine LF ($IC_{50} = 40$ μg/mL) was a more potent inhibitor than human LF ($IC_{50} = 75$ μg/mL). Most studies have been performed with the bovine protein, which we will refer to as LF. LF isolated from milk, colostrum or serum did not show large differences in inhibitory activity. Charge-modified LFs were also prepared: succinylated (Suc)-LF, sulfitolized-LF, aconitilated (Aco)-LF and asialo-LF (removal of sialic acid residues) (Harmsen *et al.* 1995, Swart *et al.* 1999). Of these modified proteins Suc-LF and Aco-LF showed an increase in anti-HIV-1 activity (2–4 times more active). The anti-HIV-1 activities of the other molecules were decreased and sulfitolized LF was more toxic to MT-4 cells. The amino acid sequences of bovine and human LF are 70% identical, and this may explain the differences in anti-HIV-1 activity. It has been reported that LF exerts its effect in the early phase of infection, at the level of virus adsorption and penetration. Since the protein can exist in an iron-saturated and apo form, the effect of metal ions bound to bovine LF on HIV-1 infection was studied (Puddu *et al.* 1998). Apo-LF, Fe^{3+}-LF, Mn^{2+}-LF and Zn^{2+}-LF were all shown to be potent and selective inhibitors when evaluated in the C8166 T-cell line. Iron-saturated LF appeared to be the most potent inhibitor and apo-LF the least potent.

Since surface charges were found to be important for the inhibitory effect of LF, this protein was also cationized using ethylene diamine (Swart *et al.* 1999). The cationized LF derivatives did not show any significant anti-HIV-1 effect, in contrast to some acylated anionized forms of LF. This result shows that an increased positive charge of LF leads to a destruction of its antiviral activity. Bovine LFcin is a highly cationic peptide domain on the surface of the intact LF molecule, and it shows antiviral activity at 100 μM (Berkhout *et al.* 1997). However, this peptide shows little anti-HIV activity when compared to intact

LF, indicating that other parts of the LF molecule are important.

To get more insight into the mechanism of inhibition, the binding of LF and Suc-LF to gp120-derived peptides was studied (Swart *et al.* 1996, Swart *et al.* 1998). Synthetic peptides from the V2 and V3 domains of gp120 from a T cell-tropic and a monocytotropic HIV-1 isolate were used to study the binding of LF to gp120 in peptide scanning experiments. Native LF interacts weakly with both V3 loops, which differ in amino acid sequence and in total net charge (+0.63 and +2.38 at pH 8.0, respectively). A binding constant of 5.2 μM could be determined for this interaction. However, cleavage of the GPGRAF domain in the V3 loop by thrombin resulted in a complete loss of the LF-peptide interaction, indicating that LF has a strong affinity for the intact V3 domain. Binding of the positively charged LF to these peptide domains was rather unexpected, since these peptides are positively charged. Binding of LF to the V3 domain peptides could be blocked by other charged molecules, indicating that charge interactions may be involved. Both human and bovine LF contain a cluster of negative charges between residues 210–240. This peptide sequence contributes to two β-sheets, with a net negatively charged loop (residues 231–245). This loop is relatively easily accessible and may interact with the positively charged domains in the V3 loop. Differences between human and bovine LF in net charge densities of this loop may explain the differences in the anti-HIV-1 effects that were observed. These findings also confirm that LF inhibits viral replication at an early stage (virus-cell fusion and/or binding) by binding to the gp120 molecule, thus inhibiting subsequent interaction with the CD4 receptor and possibly with the CXCR4 and CCR5 co-receptors.

A recent study (Berkhout *et al.* 2002) analyzed the antiviral activity of LF in a spreading virus infection assay (primary HIV-1 LAI isolate and the SupT1 T cell line), by which the accumulation of virus particles is monitored after multiple replication rounds. Bovine LF was able to completely block the spreading infection at a concentration of 10 μM ($IC_{50} = 0.4$ μM) and significant inhibition of virus replication was already observed at 0.1 μM. Since it was suggested that LF, apart from its interaction with the V3 loop, could possibly inhibit virus-cell interaction through (competitive) binding to the CXCR4 and CCR5 co-receptors, the antiviral activity of LF against HIV-1 variants with different V3 domains and co-receptor usage was tested. Bovine LF was capable of inhibiting different HIV-1 strains that use the CXCR4 and CCR5 co-receptor, confirming the broad activity spectrum of LF (Moriuchi & Moriuchi 2001). This result suggests that it is possible that LF binds to both the V3 loop of gp120 and the co-receptor CXCR-4 or CCR5. To obtain further insight into the mechanism of inhibition, an LF-resistant virus was selected in prolonged infection experiments in the presence of 10 μM LF (Berkhout *et al.* 2002). The further analysis of the LF-resistant HIV-1 variant indicated that LF blocks the process of virus entry. There are also indications that LF is able to inhibit viral processes within the host cell. LF was shown to inhibit the reverse transcriptase, protease and integrase enzymes (Ng *et al.* 2001, Wang *et al.* 2000). Interestingly, it was also found that LF resistance coincided with a loss of viral replication capacity or fitness (Berkhout *et al.* 2002).

Conclusions

In recent years there has been an increasing industrial interest in the application of functional food proteins and peptides because of their potential as health-promoting food additives or as therapeutically interesting proteins of biological origin. For instance, antibacterial proteins may be useful in food preservation, and a well-known example is LF. Besides antimicrobial activity, other biological effects have been reported for LF, which includes regulation of the immune response, cellular functions and antiviral activity. Milk and plasma proteins have been investigated in particular with respect to their antiviral activity against HIV-1 and human cytomegalovirus (HCMV). Although LF shows antiviral activity in its native form, this activity may be strongly enhanced by chemical modification. The successful application of these bioactive proteins requires the eventual demonstration of *in vivo* beneficial effects in animal models and in humans. Some clinical trials have already been conducted to evaluate the antibacterial effect of orally administered LF in humans. The work on antiviral effects of food proteins is at the stage of intensive laboratory research and has already yielded promising results by providing a better insight into the mechanistic aspects of viral inhibition. This research may eventually lead to the development of useful antivirals for therapeutic treatment or prevention of viral transmission, which would be of particular importance for developing countries.

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