AN OVERVIEW ON THE ANTIVIRAL ACTIVITY OF LACTOFERRIN

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Lactoferrin (Lf) is a glycoprotein present in most biological fluids which exerts a plethora of biological properties, including anti-inflammatory, anti-tumoral and antiviral activities. The antiviral effect of Lf has been demonstrated against a broad range of RNA and DNA viruses that infect humans and animals. The mechanism underlying this activity is complex, multifaceted, and not yet fully characterized. To date, it is generally accepted that Lf prevents entry of virus in the host cells either by blocking cellular receptors or by direct binding to the viral particles. This paper summarizes the current knowledge about the antiviral mode of action of Lf.

Key words: lactoferrin, antiviral, lactoferricin.

INTRODUCTION

Lactoferrin (Lf) is an iron-binding glycoprotein of the transferrin family, present in almost all mammalian secretions and in neutrophils, which plays an important role as a modulator component of the immune system (1–3). It is a single polypeptide chain (~ 692 amino acids) folded into two globular lobes, N and C, linked by a short α-helix (4). All Lfs characterized so far are glycosylated, but the number, location, and sites actually occupied depend on the origin of protein. Although the glycan structures vary, most of them are of the N-acetyl lactosamine type. Besides conferring an increased resistance to proteolysis, the role of the glycans in the biological functions of Lf is not well defined.

Although human Lf is ~60% identical to human transferrin, the two proteins differ in their physicochemical and biological properties. Thus, in contrast to transferrin, an acidic molecule (pI ~5.4), Lf has a strongly cationic nature (pI ~9) and it binds Fe^{3+} with higher affinity than transferrin (5).

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A variety of biological properties have been ascribed for Lf, including anti-inflammatory, antitumoral and antiviral effects (6, 7). Some of these depend on the iron-chelating capacity of Lf, others are related to its ability to interact with molecular and cellular components of both host and pathogens.

Lactoferricin (Lfcin) is a peptide derived from the N-terminus region of Lf after pepsin digestion (residues 1–47 of human and 17-41 of bovine Lf) (Fig. 1) (8, 9). Lfcin has been shown to preserve many functions of Lf, in some cases being even more potent than the parental molecule (4).

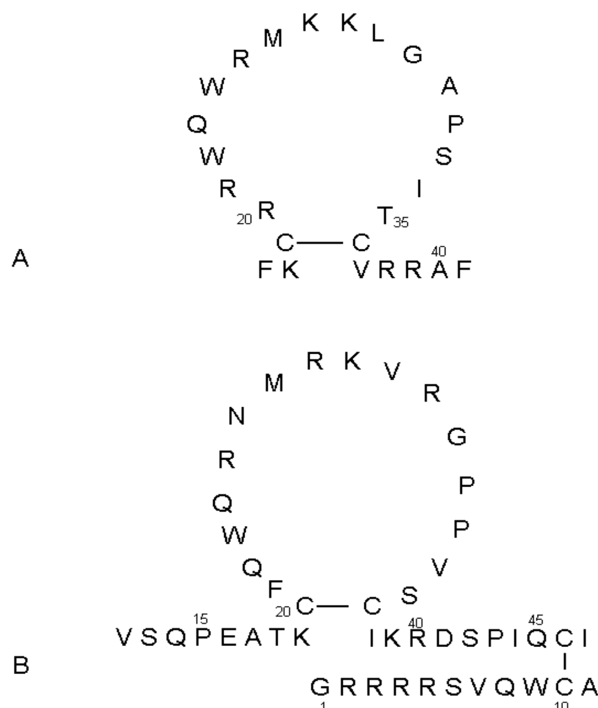

Fig. 1. – The primary structures of bovine (A) and human (B) Lfcin. Single letter codes are used to represent the amino acid sequence of each peptide.

The antiviral activity of Lf has been extensively studied and it was reported against both naked and enveloped human and animal viruses (10–12). As for other functions, the antiviral activity of Lf is a multifaceted property. The antiviral effect occurs in early phases of viral infection, mainly by hindering virus adsorption and internalization into cells through specific binding to cell receptors and/or viral particles. The present paper is an overview on the current knowledge of the mechanisms proposed to explain the antiviral activity of Lf.

BINDING TO HOST CELLS

The property of Lf to bind to most cells confers protection against infection with some viruses such as herpes simplex-1 (HSV-1), herpes simplex -2 (HSV-2)

and human hepatitis B (HBV). Some of receptors for Lf, such as heparan sulphate (HS) glycosaminoglycans (GAGs), the low-density lipoprotein receptor (LDLR), nucleolin and the asialoglycoprotein receptor (13–15), are used by viruses as receptors/co-receptors at the plasma membrane of the host cells during the infection process. The competition between Lf and virus for common binding sites prevents the internalization into cells and accounts for the decreased infectivity.

Viral entry of HSV-1 is effectively blocked by Lf (16), most likely as a result of the interaction of Lf with cell surface GAGs (17). Anti-HSV activity has been studied on many cell lines, both lacking and expressing different GAGs at the cell surface. The results have shown that the presence of HS at the cell surface is important for Lf to exert its antiviral activity (18). As Lf can block viral entry when added to the cells prior to infection, this suggests that cells are able to adapt a type of long-lasting antiviral immunity after exposure to Lf.

In addition to interfering with viral entry, it has been recently shown that Lf /Lfcin delay the HSV-1 traffic towards the nucleus along the microtubules, thus affecting viral replication in Vero cells (19). Since intact microtubules are important for successful viral replication these observations may help to explain the mode of action of Lf.

Both bovine and human Lf effectively prevented HBV infection in a susceptible human hepatocyte cell line, PH5CH8 (19). Pre-incubation of the cells with Lf is required for Lf to exert its antiviral effect. Lf binds to the asialoglycoprotein receptor in rat liver (13) and this molecule has also been proposed to act as a possible HBV receptor (21).

The antiviral effect of Lf against cytomegalovirus (CMV), originally demonstrated in 1994 (22) and later confirmed by a number of other groups (23–26), is due probably to the interference with the entry step of the viral infection (27), since pre-incubation of Lf with cells is crucial for the antiviral activity of the protein. The docking of the virus to the target cell is prevented by the low affinity binding of Lf to HS (28, 29). The N-terminal region of Lf has been proved to be essential for its antiviral activity. Thus, sequential deletion of the sequence of arginine residues, the region responsible for the HS binding, gradually diminishes the antiviral activity of Lf (25, 26). The potency of Lf was increased when the positive charge of the protein was increased by amination, whereas addition of negative charges abolished the antiviral effect of Lf.

Lf binds to some of the co-receptors of human immunodeficiency virus (HIV), such as surface nucleolin, and the dendritic cell-specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) receptor. The interaction of Lf with surface nucleolin was shown to block the initial attachment and entry of HIV particle into HeLa P4 cells (15). The DC-SIGN receptor is one of the best studied C-type lectin receptors on the surface of the dendritic cells that mediate

HIV internalization. Pre-incubation with bovine Lf could inhibit HIV-1 capture by blocking the adhesion of soluble DC-SIGN to gp120 and subsequent transmission of the virus (30). Interestingly, bovine Lf is a much more efficient inhibitor of transmission than human Lf. Both bovine Lf and the C-lobe fragment bind equally well to DC-SIGN, but the C-lobe fragment is less efficient in blocking transmission of HIV-1. The full length bovine Lf may be more efficient than C-lobe in shielding DC-SIGN due to steric hindrance.

GAGs mediate the attachment of both bovine Lf and adenovirus to target cells, and the competition between protein and virus for the common receptor resulted in the inhibition of the viral infection (31). The cationic N-terminus of Lf, reported to be its major GAG binding region, is also important for the anti-adenovirus activity. The C-lobe lacked any inhibitory effect. Human Lf and bovine Lfcin showed comparable dose-dependent inhibition of adenovirus infection, but in both cases less than the bovine protein. Three hypotheses could be proposed for the higher activity of bovine Lf as compared to LFcin peptide: (i) a minor steric hindrance exerted by the polypeptide in the competition with viral particles for binding to GAGs; (ii) the involvement of sequential interactions in adenovirus infection between various cellular and viral components, so that the inhibition of this event could have several targets; (iii) the involvement of other domains, in addition to those involved in GAG binding, which could be important for the antiviral activity of bovine Lf (32).

Binding of Lf to cell surface-expressed heparan sulfate, one of possible receptors for the Japanese encephalitis virus (JEV), has been postulated to be the possible mechanism of anti-JEV activity (33). The LDLR, involved in the entry of several RNA viruses, also binds to bovine Lf. Both rLDLR and anti-LDLR antibodies reduced the effectiveness of bovine Lf inhibition of JEV infection. This observation provided evidence to suggest that cell surface-expressed LDLR may play a role in JEV infection, especially for non HS-adapted strains.

DIRECT INTERACTION WITH VIRAL PARTICLES

The direct interaction of Lf with viral particles was proposed to explain the antiviral effect against HSV, HCV, HIV, rotaviruses and adenoviruses and would take place in an early phase of infection.

Bovine Lf was reported to target the entry process of HSV-1 by interacting with the structural viral proteins ICP-5 (major capsid protein) and VP-16 (viral tegument protein) (34). Studies performed on two human-derived cell lines-PH5CH8 and MT-2C-revealed an anti-HCV effect of Lf by its binding to the envelope proteins E1 and E2 (35). Hepatitis G virus infection in PH5CH8 was also inhibited by Lf *via* the same mechanism as HCV infection (36). The viral envelope

protein E2 was reported to be important for the entry step, being involved in the interaction with the host cell-surface receptors such as CD81, SR-B1, LDL, DC-SIGN, L-SIGN (37). Unlike Lf, Lfcin proved to be ineffective against HCV. Recently, a 33-residue peptide known as the Nozaki peptide, derived from C-terminal region of Lf, was found to specifically prevent HCV infection in human hepatocytes by binding to the E2 protein. However, it possesses weaker E2-binding capacity and anti-HCV activity than those of the parent protein. Using different synthetic helical peptides derived from the Nozaki fragment Beleide *et al*. (37) demonstrated the importance of the helical secondary structure for the E2-peptide interaction, the affinity of binding increasing with the helicity.

Both human and bovine Lf are also potent inhibitors of HIV-infection *in vitro* (38, 39). The antiviral action of Lf against HIV takes place in an early phase of infection, probably during adsorption of the virus to target cells (23, 39). The antiviral effect decreases when Lf is added at increasing time-points after HIV infection. It was shown that Lf is able to bind to the GPGRAF domain in the V3 loop of the gp120 glycoprotein (38) and since gp120 is very important in the adsorption and entry of HIV into target cells, it was suggested that the binding of Lf to gp120 is responsible for the antiviral effect of protein (40–42). Only very low inhibition of HIV infection was observed with LFcin, indicating that other domains within the native Lf protein may be required for the inhibition of the entry step (43).

The anti-rotavirus effect of Lf was tested in cultured human intestinal cells (HT-29 cells) infected with the SA-11 rotavirus (44). Experiments carried out by adding the protein to the cells before, during or after the viral attachment step, suggested that Lf possesses a dual role, both preventing virus attachment to intestinal cells and inhibiting an unknown post-adsorption step. The antiviral activity, linked to the N-lobe, takes place at the viral attachment step, and not as a result of competition with the virus for common binding sites on HT-29 cells, since SA-11 rotavirus binds to glycidic residues different from GAGs. The Lf inhibition in the post-adsorption step could be attributed to the withholding of calcium, which is important for the assembly of new viral particles. Tryptic fragments of Lf identified as a large fragment (residues 86-258) and a small peptide (residues 324-329: YLTTLK) could inhibit rotavirus, although to a lower extent than full-length Lf (45).

A strong interaction of Lf with two structural proteins of molecular mass 86 and 66 kDa, corresponding to the viral polypeptides IIII and IIIa responsible for the virus attachment to integrin cell receptor and for internalization, was demonstrated in the case of adenovirus-2 (46).

Lf is thought to prevent the early steps of BK polyomavirus infection in Vero cells (47) at the level of the adsorption phase, probably through the interaction with capsidic structures, although a competition between Lf and BK virus for cell-surface receptors cannot be ruled out. However, immunoelectron microscopy clearly demonstrated that Lf is capable of binding specifically to BKV particles (48).

UP-REGULATION OF THE IMMUNE SYSTEM RESPONSE

Binding of Lf to the cells might involve an active process such as activation of the immune system response.Virus-induced inflammatory responses can be modulated by Lf by what is thought to be a complex mechanism.

Infectivity of Friend virus complex (FVC) is associated with the DNA-synthesis phase of the cycle of the target cell (49). Since Lf had no direct effect against FVC infection *in vitro*, it was hypothesized that the antiviral mechanism probably resides in the regulation of the immune system, especially in myelopoiesis (50).

In a mouse model for CMV infection, the protective effect of Lf was due to an up-regulation of NK cells, leading to the elimination of the infection. The stimulation of not only NK cells but also monocytes and granulocytes by Lf both *in vivo* and *in vitro* has already been reported (51, 52).

In vivo studies have demonstrated an increase in serum levels of IL-18 and splenocyte production of INF-gamma and IL-12 upon orally administration of Lf (53). These ILs have the ability to protect the host from infections caused by HSV (54). The infectivity of respiratory syncytial virus (RSV) and the RSV-induced IL-8 secretion by Hep2 cells were decreased by Lf through its direct interaction with the F surface viral protein (55). In mouse peritoneal macrophages infected with vesicular stomatitis virus (VSV), the antiviral activity of Lf is related to its capacity to induce IFN–α/β expression which in turn inhibits VSV replication (56).

METAL AND ION BINDING

The iron-binding capability of Lf is not thought to be involved in its antiviral action. However with some viruses, such as rotaviruses**,** iron-free Lf (apo-Lf) was more efficient as an inhibitor of virus replication than iron-saturated Lf (44). In this case, Lf probably modulates the hemagglutination of the virus and its binding to the host cells. As early as in 1976 it was demonstrated that the increased availability of Zn^{2+} ions is likely a cause of impaired poliovirus replication (57). Marchetti *et al.* (58) showed that the addition of Zn-loaded Lf after the viral adsorption phase resulted in the inhibition of viral replication. The authors proposed that, due to the binding of Zn-loaded Lf to the target cell, Zn^{2+} ions were more efficiently delivered to the cell. Some studies have demonstrated the potent antiviral capacity of Zn- and Mn-loaded bovine Lf against HSV-1 and HSV-2 through their binding to Vero cells or virus particles (59). Both HIV-1 replication and syncytium formation were efficiently inhibited in a dose-dependent manner, by Fe-, Mn- or Zn-saturated bovine Lf when added to the C8166 T-cell line, prior to HIV infection or during the viral adsorption step (39). Saturation of Lf with iron, zinc or manganese was also found to strongly inhibit poliovirus cell attachment (58).

CONCLUSIONS

It is difficult to get a clear picture of Lf antiviral activity since the mechanism of action often differ from one cell line/organism to another. However, almost all studies have indicated that Lf prevents viral infection mainly through its binding to host cells and/or viral particles (Fig. 2) (60). The iron-binding property of Lf is apparently of no importance for its antiviral activity. However, for some viruses such as rotaviruses, apo-Lf was more potent in inhibiting viral infection than the iron-saturated protein. Zn-and Mn-loaded Lf also demonstrated potent antiviral capacity against HIV, HSV and poliovirus infection. Finally, Lf is thought to exert an indirect antiviral activity through the up-regulation of the immune system.

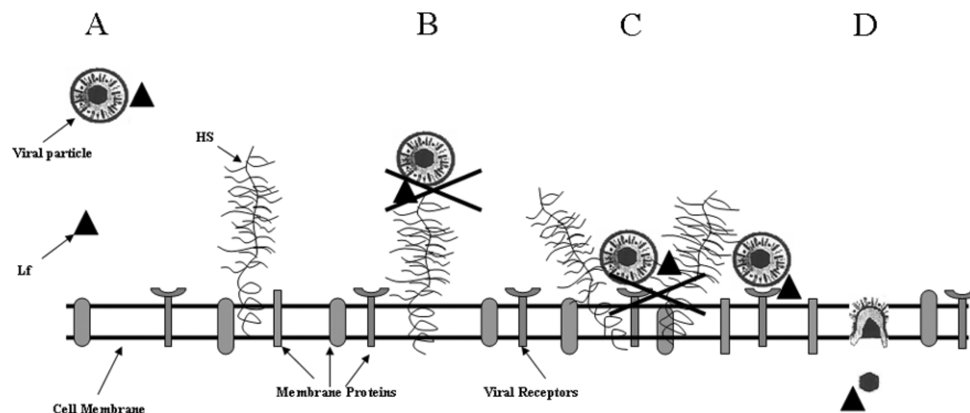

Fig. 2. – Representation of different antiviral modes of action of Lf. Lf could prevent viral infection of the host cells either by the direct binding to virus particles (A), or by competing with virus for common receptors/co-receptors (B, C) at the surface of the target cells. Finally, an intracellular activity of Lf has been postulated (D).

Compared to Lf, LFcin moderately inhibits *in vitro* multiplication of a number of viruses. The peptide might directly inactivate the virus particles, or it is possible to exert its effect inside the cell. As Lfcin contains a DNA-binding region, it could up-regulate the host cell defense response to viral attack.

It has to be mentioned that many clinical studies indicate a synergistic effect of common antiviral drugs with Lf/Lfcin. For example, the combination of Lf with cidofovir resulted in the enhanced inhibition of CMV, and that one with azidovudine proved to be more potent in HIV infections. Lfcin helps to increase the anti-HSV activity of acyclovir (ACV), a nucleoside analogue used for inhibition of viral replication. Clinical studies have also demonstrated the beneficial effect of combined triple therapy of Lf, interferon and ribavirin in patients with chronic hepatitis C.

Further work on *in vitro* mechanisms and *in vivo* effects will be required to define the role of Lf in the pathogenesis of viral infections.

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