Studies On Antimicrobial Activity Of Lactoferrin Having Potential For Hepatitis C Virus Inhibition

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Abstract: Hepatitis c virus is associated with the development of cirrhosis and hepatocellular carcinoma. Bovine lactoferrin (bLF), a milk glycoprotein belonging to the iron transporter family, was found to prevent hepatitis C virus (HCV) infection in human hepatocyte PH5CH8 cells that are susceptible to HCV infection, which demonstrated that the anti-HCV activity of bLF was due to the interaction of bLF and HCV. In this study we further characterized the anti-HCV activity of bLF and the mechanism by which bLF prevents HCV infection. We found that bLF inhibited viral entry to the cells by interacting directly with HCV immediately after mixing of bLF and HCV inoculum. The anti-HCV activity of bLF was lost by heating at 65°C, and other milk proteins (mucin, β-lactoglobulin and casein) did not prevent HCV infection, indicating that bLF prevented HCV infection in a rather specific manner. Furthermore, we found that bovine lactoferricin, a basic N-terminal loop of bLF that is an important region for antimicrobial activity, did not exhibit any anti-HCV activity, suggesting that some other region is involved in anti-HCV activity. In conclusion, lactoferrin is a natural glycoprotein which effectively protects against HCV infection in hepatocytes and lymphocytes by neutralizing the virus.

Keywords: Bovine lactoferrin, Glycoprotein, Hepatitis C virus, Hepatocytes.

I. INTRODUCTION

The term 'viral hepatitis' refers to a primary infection of liver by any one of a heterogeneous group of 'hepatitis viruses', which currently consists of types A, B, C, D, E, G. (The designation 'type F' had been proposed for a putative virus be lived to cause transfusion-associated hepatitis).

Chronic infection with HCV can lead to cirrhosis liver failure or hepatocellular carcinoma over a period of10 to 20 yrs. The numbers of chronically infected Americans are approx.4.5million & 200million from all over the world. HCV infection is over 4 times as prevent as HIV infection. Thus chronic HCV infection represents important public health problems through the world. Treatment of chronic HCV infection with IFα leads to sustain viral clearance in approximately 12% of patients. New therapeutic regimes, such as combination of IFα and ribavirin can lead to 38% to 43% of patients having sustained virological response. Treatment with IFα, ribivirin leads to significant toxicities. Therefore there still remains a great need for improved therapeutic models.

II. HEPATITIS C VIRUS

HCV is an enveloped positive single standard RNA (9.6kb) virus belonging to flaviviridae. Hepatitis C is a flavivirus (of which yellow fever is the prototype) that causes non-A, non-b hepatitis. Flavivirus are icosahedral in shape and gain an envelope from their host cell. The virus particle is about 30 to 60nm across. In many ways flavivirus are similar to picorna virus with prominent exception that they are enveloped. The viral RNA has a 5'cap but no 3'polyA tract. The incubation period is long 15-160 days, with a mean of 50 days. The HCV genome encodes a large polyprotein precursor of about 3000 amino acids residues which is cleaved by the host viral proteases to generate at least 10 proteins: The core, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

HCV infection is seen only in humans. Infection mainly by blood transfusion and other modes of contact with infected blood or blood products. Injectable drug abusers, transplant recipients and immunocompromised persons are at high risk. Several retransmissions is probably less important. Vertical transmission from mother to baby may take place. The most characteristic feature of HVC genome is its remarkable sequence heterogeneities and variations to date at least 6 major HCV genotypes, which have being further, grouped into more than 50subtypes have being identified. The genetic complexity of HCV is thus a major hindrance to the development of vaccines.

III. LACTOFERRIN

Lactoferrin, a milk glycoprotein belongs to the iron transporter family. Lactoferrin has molecular mass of 80 kilo Daltons and consist of two homologous globular lobes (N lobe and C lobe) each with a single iron (Fe^{+3}) biding site. There is a notable degree of internal homology between the lobes that is 35%identical amino acid residues have being identified in the corresponding portions. The three dimensional structures of human and bovine LF have been clarified by crystallographic studies. Although the overall structure of LF is similar to that of transferrin (~60% amino acid sequence homology to LF). LF has 2 distinct features that may be functionally important. First the association constant of LF for iron is 300 times that of transferrin (TF) second

lactoferrin possesses strong inhibitory activity against bacterial growth. The ant microbial activity of LF has been ascribed to the basic N terminal region (Lactoferrin). Lactoferrin (20amino acids residues) shows activity against a wide range of microorganisms. LF present in the milk of most mammals and the concentration of LF in mature milk are 0.1 to 0.4mg/ml in bovines and 1 to 3 mg/ml in humans and LF is especially enriched in colostrums (0.8mg/ml in bovines and 10 mg/ml in humans).It is well established that LF plays an important role in the new born as primary non-specific defense against pathogenic micro-organisms. Also, it has been reported that rats fed a 2%bovine LF diet displayed no significant side effects. Hence clinical pilot studies have being performed recently. The results have shown that BLF was effective in some patients with chronic hepatitis C. This low risk of severe side effects presents a major clinical advantage of BLF.

IV. ANTIMICROBIAL ACTIVITY OF LACTOFERRIN

Lactoferrin has been reported to affect growth and developed of a wide range of infectious agents, ranging from viruses to protozoa, by a number of different mechanisms.

A. Antibacterial activity

The ability of lactoferrin to inhibit bacterial growth in vitro was one of the earliest functions described for the protein and was shown to be due to sequestration of the iron in the medium required for microbial metabolism. Subsequent research has shown that iron sequestration by lactoferrin can inhibit growth of bacteria in vitro, although some can counteract the inhibitory effect through synthesis of low molecular weight high affinity chelators (siderophores) or by production of specific lactoferrin receptors that can facilitate iron removal from the protein.

More recently, a second antibacterial mechanism has been described, which is independent of iron-binding and involves the basic N terminal region of lactoferrin. Originally described in 1977 as a bacterial activity against *Streptococcus mutants* and *Vibrio chorlerae* the mechanism was clarified by studies showing that lactoferrin can disrupt or possibly even penetrate bacterial cell membrane and that the isolated N terminal basic peptides, named lactoferricins (Belllamy *et al*, 1992), were more potent than the intact protein. The lactoferrin derived from bovine lactoferrin was, if anything more potent than human lactoferrin, while mouse lactoferrin contains additional acidic residues in its N terminus and cannot give rise to an active basic lactoferricin.

Despite the large amount of research on the mechanisms of these antibacterial effects in vitro, their role in vivo remains controversial. In vitro experiments showing inhibition of growth through iron sequestration cannot mimic the complex interactions occurring during infections in vivo, when iron is available from a much wider range of sources, including hemoglobin, to mimic the gut flora of breast-fed infants by feeding lactoferrin supplemented cow's milk formulas have had little success (Roberts *et al*.1992) and bacterial infection is not a major cause of mortality in patients with primary haemochromatosis (Powel *et al*.2001). However, a recent in vivo study on mice reported a protective effect against Helicobacter infection, though this is probably not dependent upon iron sequestration. A protective effect against Helicobacter has important implications for the development of stomach cancer, which is associated with this organism. The bactericidal mechanism mediated by the basic N terminal region of lactoferrin is sensitive to ionic strength and pH and may not operate well, if at all, under physiological conditions (Bortner *et al*.1989).It is also uncertain whether physiologically relevant concentrations of active lactoferrin peptides can be generated in vivo. However, there remains a potential pharmacological interest in the therapeutic use of lactoferricins produced on an industrial scale.

B. Antiviral activity

Lactoferrin can reduce infectivity of a number of different viruses, predominantly in *in vitro* systems. Again, mechanisms are uncertain, but probably involve blocking of cell virus interactions as a result of lactoferrin's propensity to bind to acidic molecules, rather than iron mediated effects on host cells. For example, both lactoferrin and lactoferricin block entry of cytomegalovirus into fibroblasts (Andersen *et al*.2001), whereas only the intact protein inhibits hepatitis C, this being achieved by virus neutralization (Ikeda *et al*.2000), Anti-herpes virus activity is mediated mainly by the N-lobe, and even other members of the transferring family also demonstrate some activity, suggesting that antiviral activity may be a property that developed early in the evolution of the transferring family of proteins. Further work on mechanisms and in vivo effects will be required to determine whether lactoferrin genuinely has a role in the pathogenesis of viral infections.

C. Anti-parasitic activity

The role of lactoferrin in parasitic diseases is not well defined and may involve multiple mechanisms. Pre incubation of *Toxoplasma gondii* and *Eimeria stiedal* sporozoites with bovine lactoferrin peptides reduce their ineffectively in animal models (Omata et al.2001), suggesting an effect of basic peptides on parasite membrane integrity and/or interaction with host tissues. Likewise, incubation of fibroblasts with lactoferrin reduces the ability of *Plasmodium berghen* to bind to surface acidic molecules. Other reported ant parasitic activities appear to involve interference with parasite iron acquisition, e.g., by *Pneumocyitis carinii*, while for other parasites such as *Tritrichomonas foetus*, lactoferrin appears to act as a specific iron donor and could thus be expected to enhance infection. Given that many parasitic infections involve mucosal tissues where lactoferrin is likely to be present, further studies of the role of lactoferrin in parasitic infections would be worthwhile.

V. EXPERIMENTAL STUDIES ON ANTIVIRAL ACTIVITY OF LACTOFERRIN

The non-neoplastic human hepatocyte derived PH5CH8 cells supported HCV replication although, HCV proliferation

was at fairly low level. By using human hepatocyte PH5CH8 cells culture system it became to know that bovine and human LF especially prevented HCV infection in these cells was because of interactions of LF with HCV rather than with cells. LF inhibits the viral entry into cells by interacting directly with HCV. Yi *et al*, demonstrated that the HCV envelop proteins E1 and E2 could bind to human and bovine LF.

HCV has 2 envelope proteins E1 &E2 (31 and 70kDa respectively) are expensively post translation ally modified by Asn-linked glycosylation both proteins contain hydrophobic domains in their C-terminal portions these domains presumably act as membrane anchors E1 and E2 form non-covalent heteromers. Heterodimer formation is facilitated by the ER chaperon calnexin. The E1 and E2 heterodimers is believed to expression of the HCV polyprotein in hepatoma cell lines or insect cells in trans-cellular virus-like particles have been detected by electron microscopy. Human as well as BLF a multifunctional immunomodulator binds two HCV envelop proteins N terminal loop of LF. The region important for antibacterial activity has a little role in the binding ability to HCV to E2 but effected the secretion or stability of LF as determined. By the Far-western blotting the bacterially expressed E1&E2 could bind to LF in human milk directly separated or immuno purified and separated by SDS PAGE. The bindings of LF and HCV envelop proteins in vitro were confirmed by another method, the pull down assay with immuno precipitated LF bound protein A resin. By the same assay, mammal expressed recombinant E1 and E2 were also demonstrated to bind human LF was proved in vivo. Since anti human LF antibody efficiently co immuno precipitated with secreted intracellular forms of E2 protein.

33 human LF-derived amino acids were possessing binding activity to the E2 protein of HCV, which leads to inhibition of HCV infection in target cells. By the de-glycosylation of humans and bovine LFs enhanced E2 protein binding activity. This observation suggests that a certain N-linked oligosaccharide chain interferes with interactions between LF and E2 protein .Two regions of humans LF (N – lobe andC-s3) shows ~35%homology in the aa sequence (aa 256 to 287 of N-s3 and aa 600 to 632 of C-S3) and 12aa are shared in common between NS3 and CS3 identify as the critical domine for binding to E2 protein. However, a cys residue at 628 appeared to be essential for binding to the E2 protein and it is not present in NS3 region.

The anti HCV activity of BLF was lost by heating at 65 degree and other milk proteins (mucin, β lactoglobulin and casein) did not prevent HCV infection indicating BLF prevented HCV infection in the rather specific manner. Prevention of HCV infection BLF was a general phenomenon, because BLF inhibited HV infection in all inoculums examine by Ikeda et al by BLF inhibited HCV infection in human MJ-2C T cells that were susceptible to HCV infection. In addition with HGV, which distantly related to HCV was prevented by BLF.

VI. RESULTS

Mammal expressed recombinant E1 and E2were successfully demonstrated to bind human LF efficiently in vivo. For western blot using LF fragments and the E2 protein, expressed in Chinese hamster ovary cells, revealed 93 carboxyl amino acids of LF specifically bound to the E2 protein in addition the site directed mutagenesis to the ala residue in both terminals residues of the 33 amino acids revealed that cys-amino acid at 628 was determined to be critical to the binding to the E2 protein. The studies by the Tanaka A. *et al*, on patients with chronic hepatitis C LF inhibits HCV viremia in 64 patients with chronic hepatitis C received an 8 week course of BLF (1.8-3.68/day) at the end of LF treatment HCV RNA concentration was apparent in 51 of 64 patients (80%)with low pretreatment serum concentrations of HCV RNA.

VII. CONCLUSION

Lactoferrin is a natural glycoprotein, which effectively protects against HCV infection in hepatocytes and lymphocytes in neutralizing the virus. The binding specificity between LF and E2 protein was identified 33 amino acid residue human LF that are primarily responsible for E2 protein binding activity and inhibiting HCV infection of target cells. Pilot studies suggest that LF is one potential candidate as the anti HCV reagent that may be effective for treatment of patients with chronic hepatitis.

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