Detection of Lactoferrin in the Neutrophils and Plasma of the Patients Suffering from Hepatitis C

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ABSTRACT

Lactoferrin (LF) has antimicrobial properties against bacteria, fungi and several viruses including herpes virus, HIV and hepatitis C virus.

The aim of this study was to detect LF in PMNs and plasma of the patients suffering from hepatitis C and the healthy persons.

The sonicated solutions of PMNs of two groups were evaluated by SDS-PAGE (10%), isoelectric focusing (30%) and dot blotting. The level of LF in plasma was measured by ELISA.

The results confirmed the presence of LF in PMNs of the two groups. ELISA showed that the level of LF in plasma of patients was higher than normal persons.

Based on these findings we conclude that not only the production of LF was not reduced in the patients but also its level was significantly increased compared with the normal persons (P<0.0001).

Keywords: Electrophoresis; Enzyme-Linked Immunosorbent Assay; Hepatitis C; Isoelectric focusing; Lactoferrin; Neutrophil; Plasma

INTRODUCTION

Hepatitis C virus is the main cause of post–transfusion non–A, non-B hepatitis.1 There are two drugs for the treatment of HCV: Alpha-interferon and Ribavirin. Alpha-interferon has been used as an effective anti-HCV since 1989, but its effectiveness is limited to only 30% of the patients. Ribavirin is more effective in combination with interferon.2 Recent studies indicated that HCV cellular infection is controlled by bovine lactoferrin (bLF) effectively. LF is a glycoprotein (70-80 kD) which is one of the molecules of the iron transporter family. Each molecule of LF is able to bind two iron atoms.3 It plays many functions including iron absorption, the immune system modulatory, inhibitory action against viruses, fungi, protozoa and bacteria and is also used as an antioxidant and anticancer.

Human LF (hLF) has the same functions.4 LF exists in mucosal secretions such as tears, milk, saliva, seminal and vaginal fluids, as well as secondary granules of the neutrophils.5 Its level in plasma is approximately about 0.2 ug/ml and is thought to be released from the granulocytes. In vitro antiviral activities of LF have been studied against the viruses such as hepatitis B, C, HIV, polio, Rota and Cytomegaloviruses.6 These reports revealed two distinct mechanisms of antiviral activity of bLF. The first mechanism observed in human herpes simplex virus-1, human cytomegalovirus and human immuno-deficiency virus-1 infections was due to the direct interaction of bLF with the cells.7 However, a second mechanism was involved for the interaction of bLF with the virus in HCV and rotavirus infection.8

Studies showed that bLF can bind to the two envelope proteins E1 and E2 of HCV preventing binding the virus to hepatic cells.9 It is thought that the levels of LF in the plasma of patients are less than its levels in plasma of normal persons.2,10,11

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In this research, the production of LF in the neutrophils and its level in plasma of the patients suffering from hepatitis C were studied and compared with the healthy persons.

MATERIALS AND METHODS

Samples of Patients and Normal Subjects

The blood samples of the normal subjects were provided from the blood donor volunteers of the Tehran Blood Transfusion Organization and the patients samples (PCR+) from hepatitis consultation center (Tehran). The patients were pre-stage cirrhotic regarding laboratory markers (e.g. levels of PT, SGOT, SGPT and platelet count). 30 cases from each group were selected and 14 ml of blood was collected in the tubes containing heparin. The samples in each of the two groups were negative from the view point of HIV and hepatitis B by Biotest kit (Germany) and Diasorin kit (Italy), respectively. Hepatitis C in the normal group was evaluated with Avicenna kit (Russia, Cat No.02EM93-2) which was negative, and in patients with RT-PCR which was revealed to be positive.

RT-PCR

It was performed in three stages:
1) RT stage: at 40 °C for 1 hour (1 cycle)
2) Intermediate stage: at 95 °C for 10 minutes (1 cycle)
3) Amplification stage: it was carried out by MMV and tag polymerase enzymes within 40 cycles at five substages: 5 minutes at 95 °c, 50 minutes at 95 °c, 50 minutes at 60 °c, 50 minutes at 70 °c, 5 minutes at 74 °c.

Neutrophils Solution

Neutrophils were isolated with 6% Dextran and Ficoll (1.077) and were then counted and divided in two parts. One part activated in phorbol myristate acetate (PMA) solution and another part was sonicated under the condition of 80 W and 20 KHz for three times. Antiprotease phenyl methyl sulfonyl fluoride (PMSF) was added before and after sonication. Sonicated cells were observed with an optical microscope and then ultracentrifuged at 27000g. Finally protein concentration was determined by Bradford method.

The solution of neutrophils was evaluated by the following methods:

a) Electrophoresis (SDS-page):
In this method, the separating gel 10%, stacking gel 4% and protein marker 20-93kD (Pharmacia) were used.

b) Dot Blotting:
Positive control consisted of Standard lactoferrin (sigma). bovine serum albumin was used as negative control.
Antilactoferrin antibody (1:100) (Sigma) and rabbit antilactoferrin antibody conjugate (1:1000) were also used in this technique.

c) Isoelectric Focusing (IEF):
Thirty percent Acrylamid gel, anodic buffer (20 mM phosphoric Acid), cathodic buffer (25 mM sodium hydroxide), lactoferrin standard, protein marker(5.20-8.65 pl), ampholite (3.5-9.5 pl) and pharmacia standard (5-9 pl) were used. The gels were stained with coomassie brilliant blue R-250 and followed by silver stain.

Measurement of Plasma Lactoferrin Level

An enzyme-linked immunosorbent assay (ELISA) was developed for quantification of LF In plasma (sensitivity of 1 ng/ml). Samples of each of the two groups were tested in duplicate. 24 and 16 subjects of the 30 patients and normal subjects were studied, respectively.

Statistics

Data were statistically analyzed using student's t test. Results are presented as means±SD. The P-values less than 0.05 were considered significant.

RESULTS

a) Electrophoresis (SDS-Page)

The neutrophils solutions in two groups were electrophoresed, separately.

The different bands were obtained in gel (Figure 1) in which, M column was indicative of the markers (20, 43, 63, 93 kD). Comparison of the neutrophil bands in the column M showed the 70-80 kD band. It can be inferred that LF was present in the neutrophil solutions.

b) Dot Blotting

Standard lactoferrin and bovine serum albumin were used as the positive and negative controls,
Figure 1. SDS–PAGE with 10% acrylamide of the neutrophils sonicated solution obtained from the patients.

respectively. The results obtained from dot blotting and their comparison with the above mentioned two controls indicated that the solutions contained LF which reacted with the special anti LF antibody, produced a color similar to standard (Figure 2).

c) -Isoelectric Focusing (IEF)
The solution of neutrophils in each of two the groups were studied separately by IEF. There were specific bands compared with LF standard and the special marker bands of IEF in the gel. Lactoferrin appeared clearly in the range of PI 8.5 (Figure 3).

d) ELISA
The levels of LF in plasmas of the two groups were determined using ELISA (Cal Biochem-Merk-Germany). The obtained data showed a significant difference between the two groups using the statistical independent t-test (P<0.0001) . Level of LF in the plasma of patients was 1534.79 ± 217 ng/ml compared to its level in the normal subjects which was 187.8±22 ng/ml (Table 1).
Lactoferrin in Hepatitis C

Table 1. Mean±SD of lactoferrin in plasmas of normal subjects and patients suffering from hepatitis C

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>Mean±SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>24</td>
<td>1534.76+217</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Normal</td>
<td>16</td>
<td>187.8+22</td>
<td>&lt;0.00001</td>
</tr>
</tbody>
</table>

DISCUSSION

Studies by a number of researchers have shown that LF prevents attachment of viruses such as HIV,15 hepatitis B16 and C,10 Rota17 and Cytomegalovirus21 onto the target cells. It binds HCV envelope proteins E1, E2 and prevents virus binding to the liver cells.18 These findings inspired us to compare the levels of LF in the patients suffering from Hepatitis C and the normal subjects.

We assumed that LF levels in patients are low or there is a disorder in its production. Therefore, the virus may bind to the surface of the liver cells through two proteins E1, E2 and may cause chronic hepatitis. Our study has been qualitative but for obtaining complete results, through the quantitative studies the levels of plasma LF in both groups were also assayed. In the qualitative study, SDS-PAGE electrophoresis, dot blotting and isoelectric focusing were used. Although in the SDS-PAGE electrophoresis, the method was not quantitative, but we could show the presence of LF (MW.70-80kD) according to bands from the protein and lactoferrin standards. The results showed that LF is found in the neutrophils of both patients and normal subjects.

Isoelectric focusing (IEF) showed a band 8.5 PI for LF. In this technique, the migration of molecules was studied according to PI. Presence of this protein in the solutions of sonicated neutrophils of each of the two groups in comparison with PI of special standard was clear (Figure 3). Dot blotting provided further evidence for the presence of LF in the two groups.

The levels of plasma LF of each group were determined using ELISA, results showed a significant difference between the two groups. The average level of LF in patients was more than its average in the normal subject (P<0.0001).

Tohru et al (1999), showed that in hepatitis C patients lacrymal glands the level of LF is lower. They concluded that there was a disorder in the production of tears.11 However our study provided evidence for its presence in the neutrophils as the most important source of LF production in plasma, using SDS-PAGE electrophoresis, Isoelectric focusing and dot blotting. A study by Tegtmeyer FK et al (1991) showed that the level of LF in the patients suffering from viral infections, systemic and local bacterial infections was increased.19

Since LF in combination with the two enveloped proteins E1 and E2 of hepatitis C prevent the virus (HCV) to bind to the liver cells.20 It seems that this molecule plays a defending role in patients and LF is a molecule of innate immunity and its production is increased in the body soon after an infection such as a viral invasion. The results obtained in this research project showed that LF was produced in the patients just before the cirrhosis stage, which seems to be a defensive reaction against HCV.

Binding of LF to the molecules of E1 and E2 of the hepatitis C virus, prevents the binding of this virus to the liver cells. It is considered as an important immune mechanism for a patient and probably most of the infected individuals do not reach the cirrhosis stage and therefore are relieved from the disease and return to a healthy life.

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REFERENCES