Relation of insulin resistance (IR) with viral etiology and blood level of cytokines in patients with liver diseases

M. Irshad*, Asif Iqbal, M.A. Ansari and Raghavendra L.

Clinical Biochemistry Division, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi-110029, INDIA.

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Patients with liver diseases usually show a rise in insulin resistance (IR) leading to occurrence of DM2. Although, IR level depends on etiology, however, there is very little information available on IR in liver diseases caused by hepatitis viral infections. Present study was designed to investigate the occurrence of level of IR in patients with different liver disease and relate its level to viral etiology and cytokines level to explain the underlying mechanism. IR level was calculated using HOMA-IR formula. Blood analysis for insulin, C-peptide and cytokine level and hepatitis serology were done using EIA procedure. Our observations demonstrated raised IR level in all disease groups in comparison to healthy population. However, we could not find a definite relation of IR with viral etiology, C-peptide and cytokines level in these patients. The random changes in some parameters are not adequate for any explanation. IR is raised in liver diseases, however, appears to be related more to disease condition than etiology or cytokines level.

Keywords: Hepatitis, HBV, HCV, Cytokines, Insulin Resistance, HOMA-IR

INTRODUCTION

Insulin resistance (IR) is defined where a normal or elevated insulin level produces an impaired biological response i.e. less sensitivity to insulin mediated glucose disposal (Biddinger and Kahn, 2006). IR is presented as ratio of fasting plasma insulin to glucose. HOMA IR index (Homoeostasis model assessment of insulin resistance) is calculated using fasting insulin level (U/ml) x fasting glucose (mM/L) divided by 22.5 (Matthews et al., 1985). It is < 3 in normal persons but usually increased in patients' populations of various categories. There are several factors including infections and metabolic changes leading to increase in IR. Role of hepatitis C virus (HCV) infection causing IR has been studied quite extensively (Negro and Alaei, 2009). HCV disturbs many metabolic pathways leading to insulin resistance, steatosis, fibrosis, inflammation and apoptosis (Fartoux et al., 2005; Pekow et al., 2007). Oxidative stress, IR and steatosis caused by HCV are assumed to be interconnected and play an important role in HCV-pathogenesis. Structural and non-structural proteins of HCV are reported to have a major role in causation of all these metabolic changes in liver cells during HCV infection (Abdalla et al., 2005; Dionisio et al., 2009). Like HCV, other etiological agents causing chronic liver diseases such as hepatitis B virus (HBV), alcohol etc. also have their role in inducing insulin resistance in patients (Custro et al., 2001; Mohammad et al., 2006; Kim and Kim, 2012). In all these situations, prior occurrence of steatosis has been described to be responsible for IR inducement (Fartoux et al., 2005; Wang et al., 2008). However, the effects of various infections or factors on IR still need extensive investigations.

The occurrence of chronic liver diseases caused by both hepatitis virus(es) and alcohol are quite common in
India (Irshad and Acharya, 1994; Ray et al., 2000). However, there is very little information available on the status of insulin resistance / Type 2 diabetes prevalent in these patients and the relation of insulin resistance with etiology of diseases. Also the possible relations between IR and various cytokines / adipokines in liver disease patients, has not been well studied. DM-2 is a serious public health problem of India and so, it appears essential to understand the underlying mechanism of IR in different categories of patients. Since IR is an added problem to chronic liver diseases, its in-depth understanding becomes a tool to frame suitable strategy in treatment plan of chronic liver diseases in different stages of diseases.

Present study was planned to investigate the occurrence of IR in patients with chronic liver diseases caused by blood transmitted hepatitis viruses, i.e. hepatitis virus B and C and assess the frequency of IR in these patients’ populations. The level of IR was also related to various insulin antagonists including blood level of cytokines in these cases. Finally, an attempt was made to explain the value of IR in relation to viral etiology and blood level of cytokines in these patients’ population.

MATERIALS AND METHODS

Patients and blood samples

A total number of 79 patients including 35 patients (age range 23-49 years) with chronic viral hepatitis (CVH), 33 patients (age range 34-58 years) with cirrhosis (CIR) and 11 patients (age range 43-54) with hepatocellular carcinoma (HCC) of both gender and in adult age group were included in this study plan. These patients either attended outpatient department or were admitted to the Gastroenterology Unit / Haemodialysis Unit of All India Institute of Medical Sciences, New Delhi for treatment. The patients with CVH and liver cirrhosis were diagnosed by histopathological criteria. CVH patients had persistent elevation of transaminases level for more than six months and histologic evidence of chronic hepatitis on liver biopsy at the beginning of follow-up and exclusion of other possible causes of chronic liver diseases. Cirrhotic patients had no previous history of alcohol intake. Patients with HCC were diagnosed on the basis of liver histology. After clinical evaluation, their blood samples were tested for hepatitis viral markers. Twenty, age and both gender matched healthy subjects were included as controls in this study. The Ethics Committee of All India Institute of Medical Sciences (AIIMS), New Delhi, India, approved this study.

The venous blood (6-10 mL) was collected after an overnight fast and transferred in tubes with and without anticoagulants depending on nature of various investigations. Serum was separated after centrifugation of blood and then stored at -80°C. These sera samples were used to analyze liver function tests (LFT), hepatitis markers, fasting insulin level and cytokine estimation. The plasma was used for measuring fasting glucose level and whole blood, for hemogram. Plasma glucose concentration was measured using the Hitachi chemistry analyser obtained from M/S Roche using hexokinase method.

Detection of hepatitis viral markers

Sera were investigated for hepatitis B surface antigen (HBs-Ag) and total antibodies against hepatitis C virus (anti-HCV). The serological analysis was done using enzyme immunoassay kits of high sensitivity and specificity obtained from internationally known firms. Kit for HBs-Ag was purchased from Abbot Laboratories, USA. Anti-HCV was tested using highly sensitive third generation ELISA kit from Ortho Clinical Diagnostics, UK. This anti-HCV kit used peptides versus CORE, NS3, NS4 and NS5 regions of HCV genome, as antigen to coat the ELISA plate.

RNA extraction and cDNA synthesis

Total nucleic acid was extracted from 200 μL serum sample using High Pure Viral Nucleic Acid Kit (Roche Applied Science, Germany) following manufacturer’s instructions. The RNA was eluted into 50 μL Elution buffer, aliquoted and stored at -70°C till further used. For cDNA synthesis, 4 μL of extracted RNA in 20 μL reaction volume was denatured at 65°C for 10 min before the reverse transcription step. cDNA was synthesized using high fidelity reverse transcriptase enzyme (20 Units) (Roche Applied Science, Germany) at 50°C for 60 minutes with 60 μM of random hexamer primer.

HCV- RNA detection on real time PCR

Patient sera were screened for HCV-RNA on real time PCR (LightCycler 2.0; Roche, Germany) using primers and probe for a highly conserved region of HCV genome. HCV RNA was detected using 5’ NCR region specific primers (5’-CGG GTG TAC TCA CGG GTG CCG-3’ and 5’-AGC GTC TAG CCA TGG CGT-3’) and fluorescent labeled probe (MCY CCC CCT YCC GGG AGA GCAT_ _DB). All positive and negative controls were tested in parallel with test samples throughout the entire procedures, starting with RNA extraction. Sera samples and controls were run in duplicate.

Serum insulin assay

Fasting serum insulin was determined using a
commercially available enzyme immunoassay kit (DRG Insulin ELISA, Germany) based on the sandwich principle. The wells of micro ELISA plate were coated with monoclonal antibodies specific to insulin molecule. Patient’s specimen containing endogenous insulin was added in the coated wells and incubated with secondary antibody labeled with biotin. The unbound antibody was washed out according to manufacturer’s instructions. Now peroxidase enzyme having streptavidin was added to the well. Steptavidin binds to biotin-anti-insulin antibody. Colour was developed by adding substrate solution and the intensity of colour measured at 450 A standard curve was generated using varying concentrations of insulin. All the standards were run in duplicate. The intra-assay and inter-assay percentage coefficient variables were found to be 2.6 and 2.9 percent, respectively.

HOMA-IR

Insulin resistance (IR) was measured by two surrogate measures: fasting insulin and homeostasis model assessment (HOMA). The value of HOMA was calculated by the following equation: [fasting insulin (µIU/mL) X fasting glucose (mmol/L)] / 22.5 and depicted as HOMA-IR value.

Estimation of cytokines

The estimation of proinflammatory cytokines including a interleukin-2 (IL-2), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) in serum was done using Enzyme-linked immunosorbent assay (ELISA). The kits for IL-2, IL-6 and TNF-α were obtained from Gen-Probe Diaclone SAS, (France). The antibodies specific to cytokines coated in the microtiter plates were allowed to bind the cytokines present in serum during incubation. Subsequently, biotinylated secondary antibodies bind to specific cytokines. Finally, HRP streptavidin conjugate was added that produced blue coloured complex with chromogen substrate. The intensity of produced coloured complex is directly proportional to the concentration of cytokine present in the samples and standards. The absorbance (OD) of the coloured complex was measured at 450 nm wavelength and a standard curve was generated. All the standards were run in duplicate.

Routine laboratory investigations

All routine investigations including serum chemistry, hemogram and other necessary tests were performed on routine basis in the laboratories of Laboratory Medicine department.

Statistical analysis

Data were presented as either mean ± and frequency percentage as appropriate. In case of categorical variables Chi-square/Fisher exact test were used to compare the proportions among the group(s). The averages in continous variables were compared between the groups by Kruskal wallis test and internal comparison was done by Wilcoxon Ranksum with Bon Ferroni correction. P value <0.05 is taken as statistically significant.

RESULTS

Present investigation describes the presence of insulin resistance (IR) in patients with liver diseases caused by hepatitis virus(es) HBV and HCV and the relation of IR with causative viral agents. It also mentions IR in relation to blood level of cytokines in these patients groups. The study was initiated with enrolling of patients in out-patient department with their evaluation for complete clinical assessment simultaneous with their blood investigations for routine base-line parameters. At the same time, these patients were also investigated for their blood level of insulin, C-peptide and cytokines. The sera were also tested for different viral markers including hepatitis B virus surface antigen (HBsAg), anti-HCV and HCV-RNA as described earlier. The IR level in each case was computed using HOMA-IR formula and the level of IR was related to various parameters. A total number of 79 patients were included in this study plan. Based on clinical evaluation and routine diagnostic criteria, these patients were diagnosed and categorised in the group of chronic viral hepatitis (CVH), cirrhosis of liver (CIR) and hepatocellular carcinoma (HCC). Each group was further sub-categorised on the basis of causative hepatitis B or C virus infection to relate IR with presence of viral markers.

Analysis of data of this study demonstrated that majority of patients had abnormal test-profile depending on their disease conditions. In order to investigate a relation of IR with viral etiology, C-peptide and cytokine level, their blood samples were tested for all these parameters and analysed for the relationship. Analysis of data indicated that IR is significantly raised in all disease groups as compared to control group (p<0.05) (Figure 1). However we could not find a significant difference in IR level on mutual comparision of any two disease groups (Figure 1). This implies that difference in IR values obtained for CVH vs Cirrhosis, CVH vs HCC and Cirrhosis vs HCC was non-significant. The values of IR was also analysed in different disease groups comparing simultaneously in relation to HBV and HCV infection. We observed that as such, there was not a definite trend of IR level obtained in relation to disease or causative virus. Of course, majority of HCC pateints (72.7%) had IR level...
Figure 1. Level of high Homeostasis Model Assessment of Insulin resistance (HOMA-IR) among different diseases groups. Data evaluated by Kruskal–Wallis followed by Bon-Ferioni post test. Data expressed as mean ± SD. IR relationship for CVH vs CIR, CVH vs HCC and CIR vs HCC are non-significant.

CVH : Chronic Viral Hepatitis
CIR : Cirrhosis of liver
HCC : Hepatocellular carcinoma.

Table 1. Comparative level of HOMA-IR in various liver diseases caused by HBV and HCV infections:

<table>
<thead>
<tr>
<th>Disease groups</th>
<th>HOMA-IR &lt; 3</th>
<th>HOMA-IR &gt; 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hepatitis B</td>
<td>Hepatitis C</td>
</tr>
<tr>
<td>CVH</td>
<td>12/35</td>
<td>3/35</td>
</tr>
<tr>
<td></td>
<td>(34.285%)</td>
<td>(8.571%)</td>
</tr>
<tr>
<td>CIR</td>
<td>9/33</td>
<td>7/33</td>
</tr>
<tr>
<td></td>
<td>(27.272%)</td>
<td>(21.212%)</td>
</tr>
<tr>
<td></td>
<td>(18.181%)</td>
<td>(9.090%)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Percent values given in parenthesis indicates the figure obtained by computing it for total no. of cases tested.

CVH : Chronic Viral Hepatitis
CIR : Cirrhosis of liver
HCC : Hepatocellular carcinoma.

of more than 3, though it was not significantly different in HBV and HCV induced HCC (Table 1). When IR was assessed in relation to HBV and HCV infection in other groups also, there was no significant change in IR level with respect to HBV and HCV infection (Table 1).

The serum level of C-peptide and cytokines including IL-2, IL-6 and TNF-α was estimated by ELISA and analysed in relation to IR in different disease groups. C-peptide level was found to be relatively raised in all disease groups in comparison to control. However, it shows a significant rise in patients with only cirrhosis (p<0.008) (Figure 2). In CVH and HCC patients, we could not find a significant rise in C-peptide level as compared to control values.

Analysis of serum level of IL-2 and TNF-α, in relation to IR in these cases could not show any significant change.
Figure 2. Serum level of c-peptide (in ng/ml) among different diseases groups. Data evaluated by Kruskal-wallis followed by Bon Feroni post test. Data expressed as mean ± SD.

CVH : Chronic Viral Hepatitis
CIR : Cirrhosis of liver
HCC : Hepatocellular carcinoma.

Table 2. Serum level of C-peptide and cytokines in different disease groups

<table>
<thead>
<tr>
<th>Disease groups</th>
<th>CVH</th>
<th>CIR</th>
<th>HCC</th>
<th>CONTROL</th>
<th>CUMULATIVE P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 Median (Range)</td>
<td>0</td>
<td>0</td>
<td>0 (0, 0)</td>
<td>0</td>
<td>Not significant</td>
</tr>
<tr>
<td>TNF-α Median (Range)</td>
<td>0</td>
<td>27.6</td>
<td>189.8 (0,1954.2)</td>
<td>163.1</td>
<td>Not significant</td>
</tr>
<tr>
<td>IL-6 Median (Range)</td>
<td>0 (0, 698.7)</td>
<td>7.85</td>
<td>54.55 (5.4,181.7)</td>
<td>0</td>
<td>0.0001</td>
</tr>
<tr>
<td>C-PEPTIDE Median (Range)</td>
<td>8.2 (0.9,14.5)</td>
<td>9.05</td>
<td>8.3</td>
<td>4.9</td>
<td>0.0607</td>
</tr>
</tbody>
</table>

p value was calculated for each parameters against the value of Control group.

Whereelse IL-2 levels was not detected in majority of patients in each group, TNF-α remained either undetectable or could be detected in normal range. This shows that disease conditions or IR level is not significantly affected by the serum level of IL-2 and TNF-α (Table 2).

The serum level of IL-6 was also tested in all the patients and related to IR. IL-6 level had a significant rise in all disease groups as compared to control (p<0.05) (Figure 3). When this level was further analysed in relation to HBV and HCV infection in HCC groups, we observed significant rise in IL-6 level in HCV patients as compared to that in HBV infected patients (p < 0.001) (Figure 4). All these values are a mean of triplicate values obtained for each parameter during analysis of blood samples. Comparison of IL-6 with IR in all the patients groups could not show any relation between IL-6 and IR (Table 3).
Figure 3. Serum level of Interleukin-6 (IL-6 in pg/mL) among different diseases groups. Data evaluated by Kruskal-wallis followed by Bon Feroni post test. Data expressed as mean ± SD.
CVH : Chronic Viral Hepatitis
CIR : Cirrhosis of liver
HCC : Hepatocellular carcinoma.

Figure 4. Serum level of Interleukin-6 (pg/mL) in patients infected with hepatitis B virus and hepatitis C virus among different liver diseases groups. Data evaluated by 2-way ANOVA with Bonferroni’s post test. Data represent mean ± SD, *** p< 0.001
CVH : Chronic Viral Hepatitis
CIR : Cirrhosis of liver
HCC : Hepatocellular carcinoma.
Table 3. Serum of IL-6 in relation to IR level in various disease groups with HOMA-IR index < 3 or > 3

<table>
<thead>
<tr>
<th>Disease groups</th>
<th>IL-6</th>
<th>HOMA-IR &lt; 3</th>
<th>HOMA-IR &gt; 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVH (35)</td>
<td></td>
<td>19.75 ± 42.25</td>
<td>14.24 ± 30.29</td>
</tr>
<tr>
<td>CIR (33)</td>
<td></td>
<td>24.43 ± 45.27</td>
<td>36.46 ± 37.78</td>
</tr>
<tr>
<td>HCC (11)</td>
<td></td>
<td>90.17 ± 79.5</td>
<td>66.56 ± 66.62</td>
</tr>
<tr>
<td>Control (20)</td>
<td></td>
<td>0.4429 ± 0.8715</td>
<td>NIL</td>
</tr>
</tbody>
</table>

Values in parenthesis indicate the no. Of patients studied in each group. The value of IL-6 were shown as mean ±SD. All calculations were done with reference to control values.

CVH: Chronic Viral Hepatitis
CIR: Cirrhosis of liver
HCC: Hepatocellular carcinoma.

DISCUSSION

Insulin resistance is assumed to be a major part of liver pathology during viral hepatitis (Davila et al., 2005; Sumie et al., 2007; Tazawa et al., 2002). Insulin resistance, steatosis and oxidative damage are interrelated and induce each other leading to cell damage in presence of some internal or external stimuli (Koike, 2005; Kawaguchi et al., 2002). Viral hepatitis is one important stimulant in causing IR and so initiates a damaging mechanism leading to liver cell necrosis synchronised with immune mediated cell lysis (Hui et al., 2003). Present study was designed to explore the impact of viral etiology and the level of blood cytokines on IR in liver disease caused by hepatitis viral infection.

In this study, a total number of 79 patients diagnosed with viral hepatitis were included as study subjects. Sera from all the patients were screened for hepatitis markers and those positive for hepatitis B and C markers were grouped separately for further investigations. These patients were diagnosed and categorised as those with CVH, Cirrhosis and hepatocellular carcinoma (HCC) on the basis of their investigation profile, clinical examination and histological results. Simultaneously, their blood were tested for insulin level, c-peptide, cytokines level and viral markers. All these parameters were analysed to find their status in relation to disease status.

On routine examination we observed that majority of patients had abnormal investigations depending on their disease condition. The IR level was related to various parameters including viral etiology and cytokines. Our results indicate that there is a significant change in the level of IR irrespective of etiology in all disease groups. This means that each hepatitis may initiate the disease and further complicate it synchronised with host-immunity against viral proteins to become responsible for raised IR. The nature of virus does not have a specific relation with rise of IR level.

Assuming that both hepatitis B and C have a significant effect on host immunity and inflammation during viral-host interaction, we also measured level of associated cytokines and investigate their relation with IR. To explore whether the effect of host response on IR is influenced by causative virus, IR level was analysed in relation to hepatitis B and C infection in each disease group. We observed no significant relation of IR with causative hepatitis virus. This supports the phenomenon that it is a disease condition having an overall impact and not the etiology on IR.

The previous studies show that insulin resistance is present in a large number of patients with cirrhosis: 60% to 80% are glucose intolerant and approximately 20% develop overt diabetes mellitus (Kruszynska et al., 1991; Petrides et al., 1998). Kruszynska et al., noted an elevated fasting insulin level and C-peptide concentration in most cirrhotic patients studied (Kruszynska et al., 1991). The levels of glucose and insulin measured in plasma were higher in cirrhotic patients than in the controls. Plasma insulin and C-peptide levels was 2-3 fold higher in cirrhotic patients than in non-cirrhotic controls (Kruszynska et al., 1991). The chronically elevated plasma insulin concentrations in cirrhotic patients result largely from decreased hepatic extraction due to shunting. In spite of all those informations available from previous studies, we could not see a rise in C-peptide level in our patients' population, though there was definitely a rise in IR. It may be possible that C-peptide is not released due to severe liver diseases in these patients.

The chronic elevation of different insulin antagonists such as free fatty acids, cytokines, adipokines, glucagons, growth hormone, catecholamines may contribute to insulin resistance in cirrhotic patients. Caronia et. al., showed that the prevalence of diabetes mellitus is higher in HCV-related cirrhosis than in hepatitis B virus (HBV)-related cirrhosis (23.6% vs. 9.6%) (Caronia et al., 1999). Mason et. al., demonstrated that HCV infection and age of the patient were independent predictors for diabetes, (Mason et al., 1999) while gender, race and cirrhosis did not constitute independent...
risk factors for diabetes in HCV-infected individuals. A wide array of inflammatory mediators participates in this phase including interferon, complement natural killer cells and macrophages (Nakamura and Imawari, 2000; Freeman et al., 2001). Our results do not support this observation of higher IR in HCV induced cases as compared to other cases.

It was demonstrated that fibrosis in the liver was more pronounced in patients with insulin resistance and correlated with enhanced insulin and/or glucose levels (Narita et al., 2004; Lecube et al., 2006). This presumably testifies for pro-inflammatory and fibrogenetic stimuli released by activated adipocytes. Some of them e.g. leptin and TGFβ trigger hepatic stellate cells to turn into myofibroblasts (Ikejima et al., 2001). Furthermore, they noted that the levels of tumour necrosis factor alpha (TNF-α) were more than two-fold higher in the HCV-core gene transgenic mice compared with control mice (Shintani et al., 2004). Obesity and inflammation lead to enhanced TNFα, but the exact mechanism by which HCV increases TNFα concentration in the hepatocyte remains unclear. Furthermore, an elevated TNFα concentration is also noted in other liver diseases, such as hepatitis B, yet these diseases are not associated with such high incidence of diabetes (Bozkaya et al., 2000). In our study population, we found only low level of IL-2 and TNF-α in blood. It means that there is only low level inflammation caused by HBV and HCV and it is not adequate to cause any change in glucose disposal or an inducement in IR. These data suggest that the connection between HBV and HCV and diabetes could be secondary to the ability of viruses to induce steatosis. Rise in IL-6 level in both HBV and HCV induced cases show that it is more influenced by underlying inflammation caused by hepatitis viral infection with little impact on IR or impact of IR on IL-6 level. There is a need of more extensive study on this aspect by including large population of patients to reach some logical conclusion.

REFERENCES


direct involvement of the virus in the development of insulin resistance. Gastroenterol. 126:840-848.