HLA-DRβ1, Circulating Th1/Th2 Cytokines and Immunological Homunculus in Coronary Atherosclerosis

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ABSTRACT

Coronary atherosclerotic disease is one of the most endangering health disorders worldwide. This study was designed to investigate the correlation between HLA-DRβ1 alleles and circulating Th1/Th2 type cytokines in coronary atherosclerosis. By Elisa, Th1/Th2 type cytokines were determined in serum samples of 31 subjects with unstable angina, 27 subjects with chronic stable angina and 24 individuals as normal control. By SSP-PCR, more than 100 alleles of HLA-DRB1 were typed in 24 subjects who had skewed serum levels of Th1/Th2 type cytokines. Lipid profiles were determined by the routine methods of clinical laboratory in all subjects.

The mean serum concentration of IL-10 in normal control subjects was higher in comparison to the patient groups, 0.33±0.59 pg/ml versus 0.064±0.3 pg/ml in unstable angina pectoris group (p<0.028) and 0.22±0.6 pg/ml in chronic stable subjects. There was no statistically significant difference among the groups in serum levels of other desired cytokines (IFN-γ, IL-4). 33.33% of normal control subjects were HLA-DR16 positive whereas none of the subjects with chronic stable angina or individuals with unstable angina pectoris was positive for this antigen. The mean concentration of serum LDL-cholesterol in normal control group was high 142.046±35.40 (pg/ml).

This preliminary study shows that the atherogenic effect of the LDL-cholesterol may be dampened by HDL-cholesterol through anti-inflammatory cytokine IL-10 and HLA-DR16, a phenomenon interpretable via immunological homunculus theory.

Key words: Cholesterol; Coronary Atherosclerosis; Cytokine; HLA-DRβ1 Antigen; Th1 Cells; Th2 Cells

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INTRODUCTION

Coronary atherosclerotic disease (CAD) is one of the most common autoimmune-inflammatory processes in which hypercholesterolemia as a primary trigger, and heat shock protein-60 (hsp-60) as a secondary trigger, are the key players in macrophage and T-lymphocyte activation, systemically or regionally. Indeed, inflammation and hypercholesterolemia are considered as “partners in crime” in the pathogenesis of this disease.1-3

There are accumulating evidences implying a prominent role for pro-inflammatory Th1 type cytokines, in particular IFN-γ, in initiation, progression and complications of coronary atherosclerosis.3,7 Through macrophage activation, these cytokines participate in the weakening of the atherosclerotic plaque’s fibrous cap and ultimately, plaque rupture. A phenomenon that causes stable angina to convert to unstable angina clinically. In contrary to Th1 type cytokines, Th2 type cytokines including IL-4, IL-10 act as macrophage deactivating factor and are considered as anti-inflammatory and anti-atherogenic cytokines. In other words the functions of Th2 type cytokines could lead to the prevention of atherosclerotic plaques instabilization and rupture and consequently keeping the plaque in a stable condition.1-8,9 Indeed, coronary atherosclerosis is accepted as a diseases exacerbated by dysregulation of T cell–originated cytokines within coronary artery wall.6

According to immunological homunculus theory explained for the first time by Ivan Cohen, in type 1 diabetes, protective Th2 type of natural auto-reactivity to some ubiquitous antigen like heat shock protein-60 (hsp-60) could prevent from aggressive auto immunity.10 This is a beneficial type of auto immunity and indeed this type of auto immunity is necessary for health.

Basically, it is well assumed that the affinity of interaction between T-cell receptor (TCR) and Human leukocyte Antigen- class II (HLA- class II) – antigenic peptide complex is one of the important determining factors in skewing naïve T-helper towards the effectors Th1or Th2 cells.11 There are some evidences indicating that the T-lymphocytes in patients with unstable angina pectoris express skewed families of T-cell receptor repertoire.4,12 Similar to other autoimmune diseases, the correlation between atherosclerosis and HLA-DR alleles has already been documented by several studies.12-14

With all the above mentioned evidences, however, there is no report on HLA- class II’s implication as a genetic risk factor through the skewing of naïve T-helper cells to anti-atherogenic Th2 or to atherogenic Th1 type cytokine producing cells, which is consequent to coronary atherosclerotic plaque stability or instability respectively. The present study is designed and conducted in order to investigate this possibility.

PATIENTS AND METHODS

Patients and Normal Control Groups

With a minor difference, the individuals were selected according to inclusion and exclusion criteria that were used in Caliguiri study.4 Subjects with infectious diseases, fever, immunologic disorders, known or suspected cancer, congestive heart failure, left bundle branch block, valvular heart disease, evidence of left ventricular aneurysm, recent (<3 months) major trauma, surgery, myocardial infarction, or coronary revascularization as well as individuals under immunosuppressive drug therapy were excluded from the study.

Thirty-one patients (male/female: 14/17, age average: 60.74 years, age range: 44-78 years) with clinical symptoms and ECG-documented new onset (<2days) unstable angina pectoris were recruited for the present study. All patients of this group had at least 1 coronary artery stenosis detected at angiography (>75% reduction of lumen diameter) and also had experienced at least 2 episodes of angina at rest or 1 episode lasting >20 minutes during the last 24 hours, accompanied by transient ischemic ST-segment changes and no detectable rise in creatine kinase-MB levels or troponin T levels.

Twenty-seven subjects (male/female: 11/16, age average: 60.92 years, age range 47-71 years) with effort-related angina stable for at least 6 months were selected as chronic stable angina pectoris group. All patients of this group had a positive exercise stress test or positive thallium -scan and at least 1 coronary stenosis detected at angiography (>75% reduction of lumen diameter) and also had experienced at least 2 episodes of angina at rest or 1 episode lasting >20 minutes during the last 24 hours, accompanied by transient ischemic ST-segment changes and no detectable rise in creatine kinase-MB levels or troponin T levels.

Twenty-four age and sex-matched people (male/female: 10/14, age average: 59 years, age range: 43-71 years) were recruited as the normal control. All the subjects of this group had normal ECG and
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Echocardiogram and no evidence of atherosclerosis by echography of carotid arteries or no evidences of ischemia in exercise test or thallium- scan. C - reactive protein, CRP- positive subjects were excluded from the study.

10 ml of venous blood were taken in fasting conditions, between 8:00 and 10:00 a.m and within 24 hours of hospital admission. The written informed consent to participate in the study was obtained from all patients. The research proposal was approved by the Ethics Committee of Babol University of Medical Sciences.

This study has been conducted in two steps. In the first step, IL-4, IL-10 as Th2 type cytokine, TNF-α, and IFN-γ as Th1 type cytokine were measured in serum samples from all individuals. In the second step, more than 100 alleles of HLA-DRB1 locus were typed in 24 subjects who had skewed serum levels of Th1/Th2 type cytokines.

**Cytokines Detection by Enzyme Linked Immunosorbent Assay (ELISA)**

The serum levels of Th1 type (IFN-γ, TNF-α) and Th2 type (IL-10, IL-4) cytokines were determined by using a kit based on sandwich ELISA (Tepnel, France). According to the kit instructions; 100 µL of capturing antibody for each related cytokines was dispensed onto each well of maxisorb- micro titration plate (Nunk, Denmark), after overnight incubation at 4 º C the plates were washed by washing buffer PBS 0.2 M that contained 0.05% tween-20 and then the uncoated site of wells were blocked by 1% skimmed milk (sigma, Germany) in PBS 0.2 M. Serum samples were diluted as 1/2 in diluent buffer (1% skim milk in PBS). In parallel, the known different concentrations of standard for each study cytokines were prepared and 100 µL of diluted samples and standards were dispensed to related wells. After 30 minute incubation at 37º C, the plates were washed 3 times by washing buffer. Then, 100 µL of second antibody conjugated with Biotin was added to each well and after 30 minutes incubation at 37 º C and 4 times washing, 100 µL of avidine protein, conjugated with horse radish peroxidase, was added to each well. The plates were incubated for another 30 minutes at 37 º C and finally, after thorough washing, the enzymatic reaction developed by adding tetramethyl benzidine as chromogen and H2O2 as substrate. The standard curve was drawn for each cytokine by ELISA reader (Rayto, China) according to the optical density of appropriated concentrations of the recombinant cytokine and the cytokine concentration values for each serum sample were given in pg/ml automatically if they were above zero.

**Lipid Profile and CRP Determinations**

High Density lipoprotein– cholesterol (HDL), Low Density lipoprotein (LDL), total cholesterol, Triglyceride and also C- Reactive protein (CRP) were determined by routine methods of medical laboratory.

**Determination of HLA-DRβ1 Alleles**

DR low resolution SSP kit (Olerup SSP AB, Sweden) was used for typing of the HLA-DRβ1 locus. According to the kit description all the HLA-DRβ1 alleles, i.e. HLA-DRβ1 *010101 to 10102 recognized by the HLA Nomenclature Committee in January 2006, are recognized by the kit.

DNA extraction was performed by Qiagen blood kit (Qiagen Germany) from EDTA anti coagulated blood of 6 subjects with unstable angina, 9 subjects with chronic stable angina and 9 normal control subjects.

DNA amplification was accomplished by adding 2 µl DNA (60 ng) and 3 µl PCR master mix (0.4 unit Taq DNA polymerase, 200µmol each dNTP, PCR buffer: 50 mM KCL, 1.5 mM MgCL2, 10mM Tris - HCL PH 8.3, 0.001% w/v gelatin, 5% glycerol and 100ug/ml cresol red) to each of the 23 PCR tubes containing 5 µl specific primer mix. PCR profile was: 2 minutes at 94º C for initial DNA denaturation, followed by 10 cycles at 94º C for 10 seconds for DNA denaturation and 1 minute at 65º C for annealing and extension, followed by 20 cycles at 94º C for 10 seconds, 50 seconds at 61º C and 30 seconds at 72º C. PCR products as well as a 100 base pair ladder were visualized on ultra- violet transilluminator after electrophoresis on a 2%(w/v) agarose gel containing 500ng/ml ethidium bromide in 0.5x TBE buffer, after 15-20 minutes migration at 8-10 volts/ cm. Each gel was documented by a UVi gel document instrument and finally the presence or absence of specific PCR products bands were recorded and interpreted by SCORE software.

**Statistical Analysis**

The normality of data’s distribution has been examined by Kolmogorov-Smirnov test. For the comparison of means of the data with Gaussian distribution (Lipid profiles) independent sample t-test...
and for comparing the cytokines levels Mann-Whitney U tests were carried out. Pearson test was used for testing of correlation between the cytokine levels and lipid profile. The similarity of demographic data between groups were analyzed by t-test and chi square test. In all tests \( p<0.05 \) was considered statistically significant. All statistical analyses were conducted by SPSS software version 17.

**RESULTS**

**Direct Correlation between Circulating Th2 Type (IL-10, IL-4) Cytokines and Serum HDL-cholesterol Levels**

The demographic data and the mean values of lipid profiles in different study groups are illustrated in table 1.

The serum levels of triglyceride, total cholesterol and LDL-cholesterol significantly increased and HDL-cholesterol levels obviously decreased in both unstable and stable angina patients groups when compared with the normal control group. \( p<0.001 \) However, according to the criteria of Rojas-Villarraga study, the normal control subjects had significant elevated levels of serum total cholesterol, triglyceride and specially LDL-cholesterol. We also considered dyslipidemia when the concentration of fasting serum total cholesterol was \( >200 \) mg/dl, high density lipoprotein cholesterol was \( <40 \) mg/dl, triglyceride was \( >150 \) mg/dl and LDL-cholesterol was \( >100 \) mg/dl. \(^{15}\)

There were no significant differences in age, gender and BMI among the two patients groups and normal control group.

The mean age in normal control group was 59 years, again according to Juan-Manual study, we assumed that the current age above 45 years for men and 55 years for women is a traditional risk factor for Cardio Vascular Disease (CVD), by considering both the presence of dyslipidemia and their old age, our normal subjects should be prone to CVD.

Interestingly, there was direct correlation between serum HDL-cholesterol levels and Th2 type cytokines. For IL 10, \( r = 0.3, p = 0.004 \) (Figure 1) and for IL-4 \( r = 0.187, p = 0.002 \) (Figure 2).

IL-10 serum levels were significantly higher in normal control groups in comparison to patients with unstable angina (Figure 3). However, there were no significant differences in serum levels of IL-4 and IFN-\( \gamma \).

The mean serum concentration of IL-10 in normal control group was 0.33\( \pm \)0.59 pg/ml versus 0.064 \( \pm \)0.3 pg/ml in unstable angina \( p<0.028 \) and 0.22\( \pm \)0.6 pg/ml in chronic stable subjects (Table 1). IL-4 serum levels in unstable angina, chronic stable angina and normal control groups were 0.26\( \pm \)0.3, 0.59\( \pm \)1.16, 0.38\( \pm \)0.36 pg/ml respectively. There is no statistically significant difference in IL-4 serum levels among the study groups. (Table 1)

The mean values for IFN-\( \gamma \) levels in serum samples of subjects with unstable angina was 2.26\( \pm \)3.36 pg/ml. This value was 2.11\( \pm \)2.93pg/ml for chronic stable subjects and 3.36\( \pm \)6.03pg/ml for normal control group (Table 1). Among the groups, there is no statistically significant difference in serum levels of IFN-\( \gamma \). In our study TNF-\( \alpha \) was detectable only in four serum samples. Two in stable angina group and two in normal controls.

**HLA-DR16 Antigen may Act as Resistance Factor in Coronary Atherosclerosis**

In order to investigate the correlation between circulating Th1/Th2 cytokine levels and HLA-DRB1 alleles and their corresponding serologic specificities, the subjects from each study groups who produced systemically higher levels of Th1 or Th2 cytokines and no or lower levels of respective antagonistic cytokines were selected to enter to the second step of our study.

![Figure 1. Correlation between serum IL-10 levels and HDL-cholesterol in whole individuals related to different study groups.](image)
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As illustrated in table 2, most of the selected subjects had high IFN-γ /IL-10 or IFN-γ /IL-4 ratios. There were statistically significant differences in mean concentrations of IFN-γ as Th1 cytokine and IL-10 (p<0.001) as well as IL-4 (p=0.001) as Th2 cytokines within this group. No significant differences in IL-4 and IL-10 levels were found within those subjects. As shown in table 2; because of the existence of the high degree of polymorphism in corresponding serologic specificities between the selected subjects, the application of statistical method of forward stepwise multiple logistic regressions for the test of correlation between HLA-DRβ1 alleles and serum levels of Th1 and Th2 type cytokines was not possible. Thus, the data were presented descriptively (Table 2). HLA-DR4 (8/24) and HLA-DR15 (8/24) were the most common HLA-DRβ1 specificities among the subjects who participated to our study. 3 out of the 9 individuals (33.33%) in normal control group were HLA-DR16 positive whereas none was positive for this antigen in chronic stable angina and unstable angina groups (Table 2).

Table 1. The demographic data and mean values of BMI, lipid profile, serum levels of Th1/Th2 type cytokines in different study groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unstable angina (n=31) Mean±SD</th>
<th>Chronic stable angina (n=27) Mean±SD</th>
<th>Normal control (n=24) Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI(kg/m²)</td>
<td>29.30±4.55</td>
<td>30.05±4.19</td>
<td>29.98±4.3</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>**195.81±60.47</td>
<td>**164.92±34.02</td>
<td>142.046±35.40</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>**30.1±4.48</td>
<td>**31.69±4.74</td>
<td>38.18±5.84</td>
</tr>
<tr>
<td>Total-cholesterol (mg/dl)</td>
<td>**281.13±58.87</td>
<td>**242.80±29.88</td>
<td>213.76±51.10</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>**265.97±89.06</td>
<td>**233.15±68.69</td>
<td>191.82±52.77</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>2.26±3.36</td>
<td>2.11±2.93</td>
<td>3.36±6.03</td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>0.26±0.34</td>
<td>0.59±1.16</td>
<td>0.38±0.36</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>*0.064±0.3</td>
<td>0.22±0.6</td>
<td>0.33±0.59</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.001 (in comparison to normal control group).
DISCUSSION

The interesting finding of our study is that the patients with unstable angina were found to have significant lower levels of serum IL-10 in comparison to normal control. Moreover, the mean concentration of serum LDL-cholesterol as well as their ages were high in these subjects when they were compared to the values of the related traditional risk factors reported by Rojas-Villarraga A. 15 This means that these people were at risk of coronary atherosclerotic disease. Furthermore, a substantial positive correlation between serum HDL-cholesterol levels and circulating levels of Th2 type cytokines, IL-10 and IL-4 were found in this study.

In contrary to a large number of evidence that imply an outstanding role for inflammatory Th1 derived cytokines in particular IFN-γ as well as IL-17 in different stages of atherosclerosis development, especially in plaque rupture, 5,6,7,13,16,17 The data generated in the present study show that in comparison with normal control group, the subjects with unstable angina have significantly lower serum levels of anti-inflammatory cytokine (IL-10). However, the serum levels of another Th2 type cytokine namely IL-4 and also serum IFN-γ levels as Th1 type cytokine did not show a significant difference among the three study groups. Another Th1 cytokine in this study, i.e. tumor necrosis factor – α (TNF-α) was detectable only in 4 cases in spite of obtaining a linear standard curve with recombinant human TNF-α protein and proper sensitivity of the kit. Moreover, in comparing to the normal individuals the serum levels of IL-10 decreased in subjects with chronic stable angina, but this decrement was not statistically significant. This group of patients showed higher level of IL-10 when
compared with the unstable patients group; however, this difference also was not statistically significant. Probably, similar to Kaski's study, it needed to collect larger samples to appear significant. Indeed, in this study a trend to decrease in circulating IL-10 levels was seen from normal control group to patient with unstable angina (Figure 1).

There is a general consensus about the role of activated macrophage in plaque instability and plaque rupture. Macrophage could be activated by the several substances including IFN-γ, a prototype Th1 cytokine called as macrophage activating factor, and self or microbial heat shock-60 molecule (hsp-60) through Toll like receptor-activation pathways. As a danger signal, hsp60 directly stimulates the macrophage to produce pro inflammatory cytokines like TNF-α, IL-1, IL-6 as well as nitric oxide (NO). Also it is well assumed that Th2 type cytokine especially IL-10 plays a pronounced role in anti inflammatory process. IL-10 as a macrophage inhibitory cytokine inhibits most aspects of macrophage activity including the secretion of matrix degrading enzymes such as matrix metalo- proteinase. These enzymes have pivotal role in digesting the fibrous cap of coronary atherosclerotic plaque and in consequence plaque instability and finally plaque rupture, a phenomenon that causes stable angina to convert to unstable angina clinically.

As expected the normal subjects showed a significant higher levels of HDL-cholesterol than patients group. We supposed that in these subjects, HDL-cholesterol in a dose dependent manner through HLADR molecule could modify the function of immune cells to produce higher levels of anti inflammatory IL-10 cytokine and in consequence the atherogenic effect of LDL- cholesterol could be dampened. In other words, according to immunological homunculus hypothesis, we consider a protective role for IL-10 in atherosclerosis induced by HDL-cholesterol, a similar status induced either by hsp60 molecule or its driven peptide called Diapep-277 in type 1 diabetes prevention. According to our supposition, if under some circumstances, i.e. in the presence of lower levels of HDL- cholesterol and higher levels of LDL-cholesterol or the presence or absence of some genetic factors, IL-10 concentration fall; then the initiation, progression and the complications of coronary atherosclerosis would be problematic.

We anticipated finding a correlation between HLA-DRβ1 molecules and circulating Th1/Th2 cytokine levels, in particular IL-10. Although 33.33% of individuals in normal control group were HLA-DR16 positive and none of the individuals was positive for this antigen in patient groups, due to the existence of indigenous high polymorphism in HLA-DRβ1antigen, the utilization of statistical test for investigating the correlation between HLA-DRβ1antigens and serum IL-10 levels or other desired cytokines was not possible. Dahleh et al. calculated for CAD a relative risk (RR) equal to 4 for HLA-DR17 (DR3). The association between artherosclerosis and 12 alleles of HLA- HLA-DRβ1 and 4 alleles of DQB1has been investigated by Jonasson L et al. Their findings indicated that atherosclerosis especially the early onset of coronary atherosclerosis, is not a disease associated with particular HLA alleles. Gonzalez et al. showed that increased risk of cardiovascular disease in patient with rheumatoid arthritis associated with HLA-DRβ1 *0404. Also Palikhe et al. have investigated the association of HLA-A, HLA-B and HLA-DRβ1 with Chlamydia pneumonia infection in patients with CAD. They have shown the association of HLA-B*35 with Chlamydia infection in this group of patients. However they could to show any association between HLADRβ1 and CAD in relevance to Chlamydia infection. Mas et al. showed that HLA-DRβ1 – TNF-α–TNF-β haplotype is strongly associated with severe aortoiliac occlusive disease (AOD), a clinical form of atherosclerosis. They suggested HLA-DR*0404 -TNF-α11-b4 haplotype carries a genetic factor that furnish susceptibility to AOD. Although the role of major histocompatibility complex in Th1/Th2 skewing is well underpinned in human and experimental animal model, we are the first group to investigate the association of a wide range of HLA-DRβ1 alleles (more than 100 alleles) with Th1/Th2 type cytokines in patients with stable / unstable angina as well as the normal control. The data generated in this study suggest a further study with a larger sample size for a definite determination of the correlation between HLA-DR16 as a possible resistance genetic marker, and IL-10 levels, as an anti-inflammatory cytokine in prevention of atherosclerosis. Nevertheless, according to the lesson learned from the positive correlation between HDL-cholesterol and IL-10 in normal control subjects who participated to this study, other antigen presenting molecules such as CD1 molecule and antigen specific CD1- restricted
regulatory T-Cell should be taken into account. This suggestion is supported by Schumann work. He showed that lipoprotein-mediated transport of immunological relevant lipids might have inhibitory effects on the lipid-specific immune response. 26

Taken together, this preliminary study shows that HDL-cholesterol as a beneficial lipid through HLA-DR16 and anti-inflammatory cytokine IL-10 may dampen the harmful atherogenic effect of LDL-cholesterol, a phenomenon interpretable via the immunological homunculus theory.

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REFERENCES

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