Influence of Diabetes on Circulating Apoptotic Microparticles in Patients with Chronic Hepatitis C

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Abstract. Background/Aim: Type 2 diabetes mellitus (DM) frequently occurs in patients with chronic hepatitis C (CHC) and is associated with atherosclerosis, in which circulating microparticles (MPs) play an important role. We asked whether the presence of DM affects endothelial-derived (EMPs) and platelet-derived microparticles (PMPs) levels; and whether MPs levels associate with biomarkers of inflammation and oxidative stress in patients with CHC. Materials and Methods: Overall, 136 patients were enrolled in the study, 86 CHC patients (41 with DM with moderate glycemic control), 20 outpatients with DM and 30 controls. Circulating MPs were phenotyped by flow cytometry. Results: When the MPs levels were analyzed individually in CHC patients, there was a positive association of plasma apoptotic MPs with oxidative stress markers. We report a hitherto undescribed relationship between diabetes prevalence and apoptotic MPs-associated inflammation in patients with CHC. Conclusion: The presence of apoptotic MPs in the plasma of CHC patients, with increased levels being found in patients with DM and moderate glycemic control was herein demonstrated. The simultaneous monitoring of plasma apoptotic MPs, oxidative stress markers and inflammatory biomarkers can be helpful for the cardiovascular disease control in diabetic patients with CHC.

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expression of adhesion molecules on endothelial cells and contribute functional effects in terms of conditions involved in atherosclerosis (namely, inflammation and coagulation) (13, 14). The number of circulating PMPs may vary in different inflammatory and cardiovascular diseases, reflecting the interaction of the pathological process with the endothelium. Indeed, it has been demonstrated that circulating PMPs are a marker of cardiovascular diseases (15, 16) and are elevated in patients with chronic hepatitis (17) and diabetes (18).

Recent studies have reported changes in some MPs levels in patients with chronic hepatitis (19, 20, 21). However, inconsistent conclusions, relating predominantly to methodological issues were reported in these studies. Moreover, none of these studies investigated possible associations between circulating EMPs and PMPs levels and both oxidative stress and inflammation in chronic hepatitis. The present study addressed the hypothesis that the presence of DM in patients with CHC may alter the circulating level of MPs including endothelial-derived and platelet-derived microparticles.

The aim of this study was to evaluate the associations between DM and circulating MPs including endothelial-derived and platelet-derived microparticles, plasma oxidative stress markers (CML-AGEs, oxidative stress index) and inflammatory biomarkers (hsCRP, IL-6, TNF-α) in CHC patients, comparing diabetic and non-diabetic patients with a control group with type 2 diabetes mellitus.

Patients and Methods

Patients and study design. This study was performed in 86 patients with chronic hepatitis C (CHC) infection admitted to the Clinic of Infectious Diseases, Liver Diseases and Acquired Immune Deficiency for evaluation. Patients were divided into two groups according to their HCV antibody status and the presence of type 2 diabetes mellitus: anti–HCV-positive diabetic patients (n=41) and anti–HCV-positive non-diabetic patients. (n=45). The control group with type 2 diabetes mellitus (DM controls) was comprised of diabetic outpatients (n=20, 12 males/8 females, mean age 59±10.4 years).

The exclusion criteria were as follows. 1) Liver cirrhosis. 2) Conditions other than diabetes and HCV infection that could influence either serum glucose levels: premenopausal women; alcohol consumption >40 g/day; treatment with steroid or nonsteroidal anti-inflammatory drugs, except aspirin; concomitant infection; and chronic diseases other than diabetes. 3) Hypertension, cardiovascular diseases, autoimmune or malignant diseases, anticoagulation or antplatelet therapy. 4) Type 1 diabetes (history of diabetic ketoacidosis or age <30 years with insulin requirement) and secondary diabetes due to chronic pancreatitis or pancreatic tumor. Liver cirrhosis was ruled out by liver biopsy performed within 18 months before inclusion (compensated patients) or by typical clinical features such as signs of portal hypertension (splenomegaly, ascites, and esophageal varices), hematologic evidence of hypersplenism, or biochemical evidence of hepatocellular failure.

The diagnosis of chronic hepatitis C infection was based on persistently increased alanine aminotransferase values, anti-HCV and HCV-RNA positivity and liver histology features. The HCV inflammation was confirmed by measurement of HCV Ab and HCV RNA in the serum, using the EIA methods and RT PCR – Cobas Amplicor Roche methods, respectively. For the anti–HCV positive patients with normal aminotransferase levels and no liver biopsy, we ensured that aminotransferase levels, and liver function tests results were persistently normal.

Participants were classified as having DM if they met the 2006 American Diabetes Association criteria: Fasting Blood Glucose (FBG) >126 mg/dl, a doctor’s diagnosis of diabetes and/or on medication for diabetes. No diabetes was defined as absence of a history of DM and FBG <100 mg/dl (1). All diabetic subjects in the present study had moderate hyperglycemia (HbA1c, 6-8%).

All laboratory measurements were performed on fasting blood samples. After 2 h of bed rest, blood pressure was determined with an automatic digital sphygmomanometer and blood samples were collected in ice-cooled, ethylenediaminetetraacetic acid (EDTA)-containing tubes for the determination of plasma CML, in tubes with no additive for routine biochemical study and cytokine concentrations. All samples were separated immediately by centrifugation at 4°C and stored at –80°C until further analysis.

Assessment of plasma circulating MPs. The circulating microparticles (MPs) were categorized into (1) platelet-derived activated MPs (PMPs-ac) (CD31 + CD42b+ AN-V−), 2) platelet derived apoptotic MPs (PMPs-ap) (CD31 + CD42b− AN-V+), 3) endothelial-derived activated MPs (EMPs-ac) (CD31 + CD42b− AN-V−), and 4) endothelial-derived apoptotic MPs (EMPs-ap) (CD31 + CD42b− AN-V+) based on a previous report (22) with some modifications. Blood collection was performed in the morning in overnight fasting patients and controls. Peripheral blood was collected in acid citrate dextrose vacutainer tubes, using a 21-gauge needle. The first 3 ml of blood were discarded to avoid contamination with EMPs due to vascular injury (23). The peripheral blood (1.5 ml) was centrifuged at 2,500 g for 15 min without acceleration or break to prepare platelet-rich plasma. The 250 μL plasma samples were thawed and centrifuged for 10 min at 19,700 g at 4°C and then collected for investigation of microparticles smaller than 1.0 μm. Size calibration was conducted with 1.0 μm beads. The MPs pellet was resuspended with 150 μl of Annexin-V binding buffer (BD Biosciences, San Jose, CA, USA). All buffers were sterile-filtered with a 0.2 μm filter. The 100μL MPs were then incubated in a TruCOUNT tube (BD Biosciences, San Jose, CA, USA) with fluorescent monoclonal antibodies: anti-CD31-PE, Anti-annexin-V-FITC, and anti-CD42b-PE (BD Biosciences, San Jose, CA, USA). The samples were incubated in the dark for 15 min at room temperature. The samples were then analyzed on a FAC500 flow cytometer (Beckman Coulter) after 400 μL Annexin-V binding buffer was added. The absolute count of MPs was measured setting the stop condition for TruCount beads at 100,000 events. Fluorescence minus controls and non-stained samples were used to discriminate true events from noise, and to increase the specificity for MPs detection. The contamination of CD31*CD42b− EMPs with CD45*CD31*CD42b− leukocyte MPs was tested using the pan-leukocyte marker CD45. The amount of CD45*CD31*CD42b− leukocyte MPs was low (4.6% in platelet-poor plasma). Values are reported as counts per microliter.
levels were measured using ion-exchange high-performance liquid chromatography (Bio-Rad Variant™ II Turbo HbA1c Kit). All other biochemical parameters were measured with routine laboratory methods.

**Ethical aspects.** The consent of the Bioethics Committee of the Wroclaw Medical University was obtained and all patients were informed about the analyses performed. Studies were conducted in compliance with the ethical standards formulated in the Helsinki Declaration of 1975 (revised in 1983).

**Statistical analysis.** Continuous variables are expressed as median (interquartile range IQR) and categorical variables as number (percentage). Unpaired data were analyzed by the independent samples t-test or the Mann-Whitney test. Paired data were analyzed by the paired Student t-test or Wilcoxon test. All reported p-values were two-tailed, and values <0.05 were considered significant. Correlation analysis between stochastically independent variables were performed by the Pearson correlation test. Multiple logistic
Table II. Clinical and biochemical variables associated with diabetes mellitus (DM).

|                         | CHC patients without diabetes | CHC patients with diabetes | DM controls |
|-------------------------|------------------------------|---------------------------|-------------|
| (n)                     | 45                           | 41                        | 20          |
| Male:Female ratio       | 26:19                        | 19:22                     | 12:8        |
| Age (years)             | 61±12                        | 64±11                     | 59±10.5     |
| Body mass index (kg/m²) | 22±2.3                       | 23.3±2.5                  | 29.5±2.6    |
| HbA1c (%)               | 5.2±0.55                     | 6.7±1.2**                 | 6.9±0.98**  |
| Alanine aminotransferase (IU/l)| 89 (33-150)       | 104 (37-190)              | 25 (20-27)*** |
| Aspartate aminotransferase (IU/l)| 76 (28-130)     | 84 (31-140)               | 28 (21-30)** |
| Albumin (g/dl)          | 4.35 (4.2-4.9)               | 4.4 (3.4-4.8)             | 5.0 (3.5-5.8) |
| Platelet count (x10⁹/l) | 216 (154-241)               | 214 (161-245)             | 200 (130-220) |
| WBC count (x10⁹/l)      | 4.9 (3.0-7.8)                | 6.4 (3.3-8.2)             | 4.7 (3.2-5.0) |

Oxidative stress markers

|                         | CHC patients without diabetes | CHC patients with diabetes | DM controls |
|-------------------------|------------------------------|---------------------------|-------------|
| CML-AGEs (ng/ml)        | 2.7 (1.7-3.6)                | 4.4 (2.5-8.2)**           | 4.3 (2.7-5.5)** |
| TAS (mmol/l)            | 1.1 (0.9-1.3)                | 0.68 (0.5-0.9)*           | 0.56 (0.4-0.81)** |
| OSI (arbitrary unit)    | 2.4 (1.9-2.8)                | 6.4 (5.1-9.1)**           | 5.7 (4.4-8.0)** |

Inflammatory markers

|                         | CHC patients without diabetes | CHC patients with diabetes | DM controls |
|-------------------------|------------------------------|---------------------------|-------------|
| hsCRP (mg/l)            | 1.74 (1.05-4.5)              | 3.9 (1.5-20.1)**          | 1.9 (0.58-2.5)* |
| IL-6 (pg/ml)            | 5.1 (2.8-9.8)                | 9.4 (4.8-12.1)*           | 6.5 (5.5-7.7) |
| TNF-α (pg/ml)           | 33.0 (29.0-36.0)             | 35.0 (32.0-42.3)          | 28.0 (22.0-35.0) |

PMPs-ac: platelet-derived activated MPs (CD31⁺ CD42b⁻ AN-V⁻); PMPs-ap: platelet derived apoptotic MPs (CD31⁺ CD42b⁻ AN-V⁺); TAS: total antioxidant status; TNF-α: tumor necrosis factor α; OSI: oxidative stress index; WBC: white blood cells.

Values are expressed as means±SD or as medians (interquartile range) for skewed data. Significance levels between groups: *p<0.05; **p<0.01, ***p<0.001 vs. CHC patients without diabetes; *p<0.01, **p<0.001 vs. CHC patients with diabetes. CML-AGEs: Ne-(carboxymethyl)lysine-advanced glycation end products; EMP-ac: endothelial-derived activated microparticles (CD31⁺ CD42b⁻ AN-V⁻); EMP-ap: endothelial-derived apoptotic microparticles (CD31⁺ CD42b⁺ AN-V⁺); FBG: fasting blood glucose; hsCRP: high sensitivity C-reactive protein; IL-6: interleukin-6; OSI: oxidative stress index; WBC: white blood cells.

regression analysis was used to identify factors related to high MPs level. Variables that achieved statistical significance in the univariate analysis (p-value<0.05) were subsequently included in a logistic regression analysis. Selection of variables was based on a stepwise regression analysis using a forward selection method.

Results

Clinical and biochemical characteristics of the study groups.

Clinical and biochemical characteristics of the study groups are reported in detail in Table I. In all subjects (45 males/41 females, mean age 62±14 years), diabetic outpatients belonging to the DM controls (12 males/8 females, mean age 59±10.5 years), and healthy controls (17 males/13 females, mean age 56±8.7 years), age, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP) were obtained together with measurements of albumin, various liver enzymes (ALT, AST and γ-GT), fasting blood glucose, total cholesterol, high density lipoprotein-cholesterol, low density lipoprotein-cholesterol and triglyceride concentrations (Table I). The levels of aminotransferases and γ-GT were significantly lower in DM controls compared to CHC patients (p<0.05). There were no significant differences among the three groups with respect to gender, systolic blood pressure, diastolic blood pressure, body mass index, serum albumin, serum total protein and lipids profile (Table I). The plasma levels of MPs in the group of patients with CHC and DM controls and healthy controls are presented in Table I. Plasma MPs were also checked for correlations with selected biochemical markers of liver function (albumin, prothrombin ratio, bilirubin concentration) and injury (aminotransferases). Comparable levels of EMPs-ac (CD31⁺ CD42b⁻ AN-V⁻) and PMPs-ac (CD31⁺ CD42b⁻ AN-V⁻) were found in all CHC patients and in healthy controls, but increased levels of EMPs-ap (CD31⁺ CD42b⁺ AN-V⁺) and PMPs-ap (CD31⁺ CD42b⁺ AN-V⁺) were observed in CHC patients and DM controls.
The plasma levels of circulating PMPs with immune phenotypes labeled as CD31+ CD42b− AN-V− (PMPs-ap) and CD31+ CD42b+ AN-V− (PMPs-ac) and EMPs-ap (CD31+ CD42b− AN-V+) and EMPs-ac (CD31+ CD42b− AN-V−) in group of patients with CHC and DM controls are presented in Table II. Distribution of sex was similar among groups. However, there was a difference in age among groups; subjects in CHC group with and without DM were older than subjects in DM controls (p>0.05). Additionally, the white blood cells and platelet counts did not differ between the three groups. HbA1c levels were higher in CHC patients with DM and DM controls than in CHC patients without DM (p<0.01, respectively), and aminotransferase levels were higher in CHC patients with and without DM than in DM controls (Table II). The circulating levels of EMPs-ac and PMPs-ac did not differ between the three groups, whereas the circulating levels of EMPs-ap and PMPs-ap were significantly higher in the diabetic CHC group and DM controls than in the non-diabetic CHC group (p<0.01, p<0.001, respectively) (Table II). Furthermore, plasma CML-AGEs concentrations were higher in CHC patients with DM (n=41, median 4.4 ng/mL, IQR 2.5-8.2 ng/mL) than in patients without DM, and this difference was statistically significant (p<0.01). Moreover, CML-AGEs levels in diabetic CHC patients were similar to those in the DM controls (Table II). Meanwhile, the TAS values were lower, while the OSI values were significantly higher (p<0.001) in the CHC patients with DM than in those with DM controls.
Correlations between high levels MPs-ap and low-grade inflammation, age and diabetes mellitus (DM) prevalence in patients with CHC. The correlations between high levels of MPs-ap and relevant baseline variables in the all CHC patients were investigated. The positive relationships were between DM and both CML-AGEs, as well as OSI (p<0.001, p<0.01; respectively). Moreover, significant relationships between diabetes and both hsCRP as well as IL-6 (p<0.01, respectively) were observed among the CHC patients. The univariate logistic-regression analyses showed that increased levels of FBG, HbA1c, hsCRP, IL-6, CML-AGEs, high OSI, and the presence of DM were significant predictors of high MPs-ap levels in patients with CHC (Table III). In contrast, there were no significant correlations between high MPs-ap levels and age, systolic or diastolic blood pressure, and body mass index. Based on stepwise multiple logistic regression analysis of factors (FBG, HbA1c, hsCRP, IL-6, CML-AGEs, OSI, and the presence of DM) the presence of DM was found to be an independent predictor of high levels of circulating EMPs-ap (OR=3.27, 95% CI =1.73-6.77, p<0.01) and PMPs-ap (OR=3.42, 95% CI =1.81-7.1, p<0.01) (Table IV).

Discussion

In our study, comparable levels of EMPs-ac (CD31+ CD42b– AN-V−) and PMPs-ac (CD31+ CD42b+ AN-V+) were found in all CHC patients, but increased levels of EMPs-ap (CD31+ CD42b– AN-V+) and PMPs-ap (CD31+ CD42b+ AN-V+) were observed in those with the DM, which may contribute to the increased atherogenic risk associated with the diabetes. Of particular interest was our finding that the presence of DM with moderate glycemic control was a significant risk factor for the presence of oxidative-driven generation of apoptotic MPs and MPs-associated inflammation in patients with CHC.

Several causes of liver disease trigger MPs production, in particular viral infection and DM (24, 25). Liver disease itself might also induce MPs release, as the main processes of MPs formation (namely, apoptosis and cell activation) are common in this context (26, 27). This study demonstrated that circulating levels of activated EMPs and PMPs did not differ between the two groups of CHC patients, whereas the circulating levels of apoptotic EMPs and -PMPs (called apoptotic MPs; MPs-ap) were significantly higher in those with diabetes. Moreover, DM was found to be a risk factor for high MPs-ap levels in all CHC patients examined. While there is ample in vitro evidence of the potential downstream biological effects of MPs (e.g., regulation of inflammation, promotion of coagulation, vascular dysfunction) (15, 9) many of which are known to be important in atherogenesis, in vivo data are few in patients with CHC at this juncture. Because the DM occurs in most patients with CHC, its presence likely accounts for most of the increased prevalence of atherosclerosis in chronic hepatitis C. Subjects with poor controlled hyperglycemia, hypertension and cardiovascular diseases were excluded from our study patients. Consequently, only uncomplicated DM has remained to be an atherogenic factor along with CHC and emerged to be related with high MPs-ap levels.

Many stimuli that promote MPs release, including oxidative stress and systemic inflammation are present in chronic liver diseases (7, 28-30). In the case of DM, it has been shown that inflammation, associated with oxidative stress, predicts atherosclerosis and cardiovascular disease risk (31). On the other hand, some studies suggest that oxidative stress and apoptosis might be key factors in promoting MPs production (32). Consistently, we found that plasma oxidative stress markers (CML-AGEs, OSI) were positively correlated with both DM as well as high levels of MPs-ap in CHC patients. The above findings suggest that oxidative stress in CHC patients with moderate glycemic control is associated with a process of MPs formation (namely apoptosis) involving platelets and endothelial cells, including MPs-ap which may account for the pro-coagulant potential of MPs (16, 33). It is also possible that the oxidative-driven generation of MPs may relate to low-grade inflammation in vasculature, which associates with overproduction of cytokines (34). Finally, our results show that circulating MPs-ap levels were associated with both markers of inflammation (i.e. IL-6 and hsCRP) as well as the presence of DM. The association of MPs-ap with the low-grade inflammation suggests that inflammatory alterations are important in relation to MPs-ap elevation in CHC patients with moderate glycemic control.

The platelet-derived MPs can inflict endothelial injury via induction of inflammation and impairment of endothelial-dependent vasodilation (35). The experiments in human umbilical vein endothelial cells showed that PMPs may also induce platelet/endothelial cell interaction (36), an important step in the initiation of atherosclerosis. In another report, Jansen et al. demonstrated that EMPs generated from high glucose treated cells, but not from healthy endothelial cells, induce vascular inflammation and promote atherosclerosis in vivo (37). Therefore, we suggest that an increased circulating level of apoptotic MPs in the setting of DM should be...
considered as an early indicator of atherosclerosis in CHC patients without a clinical history of cardiovascular disease. The exact nature of the significance of our findings, however, remains unclear.

This study is associated with a few limitations. Although the number of patients enrolled might seem small, it adequately represents the sample size estimated to provide the specific power. However, a primary limitation of the present study is its cross-sectional design and the inherent possibility that lifestyle factors may have influenced the results described here. In an effort to minimize confounding variables, we studied subjects of similar age and nonsmokers who were not currently taking medication that could influence inflammatory and oxidative markers. Being cross-sectional in its nature, our study allows commenting only on associations—pathomechanisms involved, causality or the direction of potential causal associations cannot be determined.

In conclusion, the simultaneous monitoring of plasma apoptotic MPs, oxidative stress markers and inflammatory biomarkers can be helpful for the cardiovascular disease control in CHC patients with moderate glycemic control. Larger prospective studies should investigate the practical clinical value of plasma apoptotic MPs measurements. As the burden of cardiovascular diseases increases in CHC patients, and as attempts focus on treating HCV infection worldwide, this problem requires urgent attention.

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