Level and Value of T Cell-derived Circulating Microparticles in Liver Cirrhosis Patients

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Abstract. Background/Aim: We examined the hypothesis that T cell-derived-circulating microparticles (MPs) are increased in liver-cirrhosis (LC) patients compared to normal subjects and are also increased in chronic hepatitis compared to acute-decompensated-liver cirrhosis (ADLC). Patients and Methods: A total of 66 LC patients, including 35 with ADLC and 31 with non-decompensated-LC (NDLC), were enrolled in the study. Ten volunteers served as controls. Results: Flow-cytometric analysis showed that circulating levels of T-cell derived MPs (i.e., total MPs and CD4+/CD8+/CD54+MPs) were higher in LC patients than in the controls (all p<0.003). Total MPs and CD8+MPs were higher in NDLC than in ADLC patients. There were good correlations between CD8+MPs and ADLC as well as between total MPs and chronic hepatitis. Multivariate-linear-regression analysis showed that NDLC was independently predictive of increased circulating CD8+MPs levels (p<0.05) and chronic hepatitis independently predictive of increased circulating total MPs levels (p<0.001)/CD4+MPs (p<0.05). Conclusion: Circulating levels of T-cell-derived MPs were increased in ADLC patients and were even more elevated in NDLC patients compared to healthy-control subjects.

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Liver cirrhosis (LC), a reversible wound-healing response to either acute or chronic cellular injury that reflects a balance between liver repair and scar formation (1), is the final pathological pathway of liver damage arising from a wide variety of chronic liver diseases (2-4). Clinical observational and epidemiologic studies have previously shown that LC is an increasing cause of morbidity and mortality in developed countries, being the 12th most
common cause of death worldwide but fourth in central Europe (5-7).

It is well known that liver fibrosis/LC is a common pathological consequence of a variety of chronic stimuli, including viral infection, autoimmune, drug-induced, cholestatic and metabolic diseases (8-11). Studies have further clarified that the etiology of cirrhosis varies geographically, with alcoholism, chronic hepatitis C virus infection, and nonalcoholic fatty liver disease being the most common causes in western countries (12-14), whereas chronic hepatitis B is the primary cause of LC in the Asia-Pacific region (15-17).

Although the causes of LC are multifactorial, there are some pathological characteristics that are common to all cases of liver cirrhosis, including degeneration and necrosis of hepatocytes, replacement of liver parenchyma by fibrotic tissues and regenerative nodules, and loss of liver function (18-22). Additionally, a key discovery in understanding LC is initiated by activation of hepatic stellate cells (HSC), which are the primary effector cells orchestrating the deposition of extracellular matrix (ECM) in the liver structure. Furthermore, progressive accumulation and decreased remodeling of the ECM, disrupts the normal architecture of the liver (10). If left untreated, fibrosis can progress to LC, ultimately leading to organ failure and death (23).

Limited studies have previously shown that lymphocytes have the capacity to modulate fibroblast fibrolytic effects through lymphocyte-derived microparticles (MPs) rather than cytokine mediated manner (24, 25). Interestingly, one of these studies has further exhibited that TCMPs circulated in blood plasma and were elevated in patients with active chronic hepatitis C (25). Moreover, MPs derived both from CD8+ and CD4+ T cells can induce a fibrolytic phenotype in HSCs (25). These findings raise the hypothesis that circulating T-cell derived MPs may be a novel diagnostic marker for inflammatory liver diseases/LC (25). However, the available data to support this hypothesis is still largely limited, suggesting more prospective clinical studies are required to strengthen this issue.

Clinical significance. This study tested the hypothesis that circulating T-cell derived microparticles (MPs) might be a novel diagnostic marker for inflammatory liver diseases/liver cirrhosis (LC). Consistent to the hypothesis, the results of our study showed that circulating levels of T-cell derived MPs, indicators of inflammatory reaction in chronic hepatitis, were significantly increased in acute decompensated liver cirrhosis patients and more significantly increased in non-decompensated LC patients than in healthy-control subjects, highlighting a persistent inflammation in LC patients.

Patients and Methods

Study design. This clinical study was approved by the Institutional Review Committee on Human Research in Chang Gung Memorial Hospital (100-3666A3) in 2013 and conducted at Kaohsiung Chang Gung Memorial Hospital. Informed consent was obtained from all study subjects.

Patient population, inclusion and exclusion criteria. Patients with age >18 years old who had a history of LC were enrolled into this prospective study. Patients with history of the followings were excluded from the study: intracranial hemorrhage, surgery or trauma within the preceding 3 months, hematology disorders, chronic renal disease in stage 5, malignancy other than hepatoma, febrile disorders, congestive heart failure, myeloproliferative disorder, without diagnosis of LC, acute or chronic inflammatory disease other than LC during the study period, history of autoimmune diseases with or without immunosuppressive therapy, and prior myocardial infarction with an onset of < 3 months.

Between October 2013 and November 2014, 66 patients who presented to the Department of Gastroenterology at Kaohsiung Chang Memorial Hospital with the diagnosis of LC were recruited. Patients were enrolled either from the outpatient department or during hospitalization for severe jaundice, ascites with hypoalbuminemia for plasma transfusion, acute aspartate aminotransferase (AST) and alanine aminotransferase (ALT) elevation, esophageal varices ligation or esophageal varices bleeding (Figure 1). All blood samples were collected prior to blood transfusion.

Ten age- and gender-matched healthy controls were also studied. Informed consent was obtained from all healthy subjects.

For analytical purposes, the patients were categorized into acute decompensated liver cirrhosis (ADLC) (defined as Child’s class B or C) and non-decompensated liver cirrhosis (NDLC) (defined as Child’s class A).

Ultrasoundographic examination and definitions. Ultrasonographic scans were performed by experienced hepatologists at our institute using an ultrasound system with a 3.5 MHz convex probe (SSA-340, SSA-370, Apionix-700 Toshiba, Tokyo, Japan; SSD 2000, Aloka, Tokyo, Japan; HDI 5000, ATL Ultrasound, Bothell, WA, USA; Preirus Hi-vision Hitachi, Tokyo, Japan). Chronic hepatitis C was defined as detectable serum antibody to hepatitis C virus (anti-HCV), and chronic hepatitis B was defined as detectable serum hepatitis B surface antigen (HBsAg). The definitions and diagnoses of hepatocellular carcinoma (HCC), LC, and the degree of decompensation were based on previous reports (26-30).

Circulating MPs were categorized into six groups. Circulating MPs were categorized into six types 1) Total MPs; 2) CD4+ apoptotic T cell (i.e., Annexin-V) derived MPs (i.e., CD4+ MPs); 3) CD8+ apoptotic T derived MPs (i.e., CD8+ MPs); 4) CD11a+ activated T cell (i.e., T-cell activating signal) (i.e., CD11a+ MPs); 5) CD54 (Cluster of differentiation 54), also known as ICAM-1 (intercellular adhesion molecule 1) is a member of the immunoglobulin superfamily (including antibodies and T-cell receptors) (i.e., CD54+ MPs); and 6) CD147 (also called basigin or EMMPRIN, is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily) (i.e., CD147+ MPs). The procedure and protocol for classification have been outlined in a previous report (25) and used with minimal modification.

Blood samples for biochemical analysis, blood cell count study, and flow cytometric analysis of plasma levels of MPs. Blood samples were obtained once at 9:00 am and analysed according to our recent
reports (31, 32). In detail, white blood cell (WBC) red blood cell (RB) and platelet counts, hemoglobin, biochemistry, and electrolyte levels were analyzed using standard laboratory methods in our hospital. Peripheral blood was collected from each study subject in acid citrate dextrose (ACD) vacutainer tubes. To prepare blood samples, peripheral blood (1.5 ml) was centrifuged at 2000 × g at 25°C for 20 min without acceleration or break. The 650 μl plasma samples were transferred to a new Eppendorf and centrifuged for 2 min at 13,000 × g at 25°C without break. The plasma samples were transferred to a new Eppendorf and centrifuged for 20 min at 13,000 × g at 25°C without break and then the pellet was collected for investigation of MPs smaller than 1.0 μm. Size calibration was conducted with 1.0 μm beads (Invitrogen, Carlsbad, CA, USA). The MP pellet was resuspended in 150 μl of Annexin-V binding buffer (BD Biosciences). All buffers were sterile-filtered through a 0.2 μm filter. The 100 μl MPs were then incubated in a TruCOUNT tube (BD Biosciences) with fluorescent monoclonal antibodies: 1) Annexin V-FITC; 2) CD4-PE; 3) CD8-PE; 4) CD11a-PE; 5) CD54-PE; and 6) CD147-PE (BD Biosciences). The samples were incubated in the dark for 15 min at room temperature. The samples were then analyzed on a FC500 flow cytometer (Beckman Coulter) after the addition of 400 μl Annexin-V binding buffer. The absolute count of MPs was measured setting the stop condition for TruCount beads at 10,000 events.

Statistical analysis. Continuous variables with normal distribution were expressed as mean±standard deviation, and the difference between the two groups was analyzed with independent t-test. We compared continuous variables among the three groups using one-way analysis of variance with post-hoc Bonferroni test. Discrete or categorical variables between different groups were reported as percentage and number, and then analyzed with Chi-square test or Fisher’s exact test as appropriate. Additionally, Pearson’s correlation analysis was adopted to assess the relation between the level of MPs and disease status. Furthermore, after logarithmization of MPs, the multivariate linear regression model was utilized to identify clinical or pathological predictors of circulating MPs change. Statistical analysis was performed using SPSS statistical software for Windows version 22 (SPSS for Windows, version 22; SPSS, IL, USA). p<0.05 was considered as statistically significant.

Results

Comparisons of baseline characteristics of study patients and control subjects (Table I). Table I shows the baseline characteristics of study patients and healthy volunteers. The results demonstrated that age, gender, body weight, body height, RBC count, WBC count, hemoglobin, circulating levels of AST, ALT, alkaline-phosphatase and creatinine did not differ between study patients and control subjects. However, the platelet count was significantly lower whereas the total number of the MPs were significantly higher in patients compared to control subjects. Additionally, circulating levels of MPs, including CD4+, CD8+ and CD54+ MPs were significantly increased in patients compared to control subjects.

Comparison of baseline characteristics between patients with acute decompensated liver cirrhosis (ADLC) and non-
decompensated liver cirrhosis (NDLC) (Table II). Age, gender, body weight, body height, WBC and platelet counts did not differ between the two groups of patients. However, the RBC count and hemoglobin levels were significantly lower in the ADLC group than those in the NDLC group.

The circulating levels of creatinine, AST, ALT, total bilirubin, α-fetal protein and ammonia were similar between these two groups of the patients. Additionally, the incidences of hepatitis B carrier, hepatitis C carrier and hepatocellular carcinoma did not differ between these two groups of patients. However, the incidences of alcoholic LC, splenomegaly and esophageal varices bleeding were significantly higher whereas acute hepatitis was significant lower in the ADLC group than in the NDLC group.

**Correlation between ADLC and microparticles (Table III).** The Pearson’s correlation analysis was utilized to examine the correlation between the ADCL and circulating level of MPs. The results showed that only CD8+ MPs were significantly correlated with the ADLC. On the other hand, the circulating levels of CD4+ MPs had a tendency of significant correlation with ADLC.

**Correlation between chronic hepatitis and microparticles (Table IV).** The Pearson’s correlation analysis was also utilized to examine the correlation between chronic hepatitis and circulating levels of MPs. The results showed that only total MPs had a strong significant correlation with chronic hepatitis.

**Multivariate linear regression analysis of the predictors of increased circulating microparticles (Table V).** The analytical results showed that the change in circulating CD8+ MPs was significantly predictive of NDLC. Additionally, circulating levels of total MPs and CD4+ MPs were significantly influenced by the existence of chronic hepatitis.

**Mean levels of total and different microparticles in control subjects, ADLC and NDLC (Figures 2 and 3).** Figures 2 and 3 show that the levels of total MPs and different kinds of MPs (i.e., CD4+, CD8+, CD11a+ MPs, CD54+ and CD147+) were notably higher in ADLC patients and more notably higher in NDLC patients than in control subjects.

**Discussion**

This study investigated the circulating levels of MPs in LC patients and yielded several striking clinical implications. First, as compared with healthy control subjects, the circulating levels of total MPs and different kinds of MPs (i.e., CD4+, CD8+, CD54+) were substantially higher in ADLC and more substantially higher in NDLC patients, highlighting that presence of persistent inflammation in LC patients. Second, the results of multivariate linear regression analysis showed that NDLC rather than ADLC was significantly predictive of increasing circulating levels of MPs (i.e., CD8+MPs), suggesting that the inflammatory response was vigorous in the former than in the later situation. Third, the results showed that chronic hepatitis was
the strongest independent predictor of increased circulating levels of total MPs and CD4+ MPs.

Currently, there are still limited data to address the correlation between hepatitis and increased levels of T cell derived circulating MPs. To the best of our knowledge, this is the second reported study to address human T cell derived MPs circulating in blood of hepatitis/LC patients. Accordingly, our findings would provide an additional information for the readers to understand the role of T cell derived MPs in the process of chronic hepatitis and LC.

One important finding of the present study was that, compared to healthy control subjects, the circulating levels

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Table II. Comparison of baseline characteristics between patients with acute decompensated liver cirrhosis (ADLC) and non-decompensated liver cirrhosis (NDLC).

| Variables                     | ADLC group (n=35) | NDLC group (n=31) | p-Value |
|-------------------------------|-------------------|-------------------|---------|
| Age (yrs)                     | 56.94±12.67       | 56.55±15.13       | 0.910   |
| Male gender                   | 68.6% (24)        | 61.3% (19)        | 0.536   |
| Body weight (kg)              | 63.30±13.72       | 64.44±11.48       | 0.769   |
| Body height (cm)              | 163.93±8.47       | 162.37±8.33       | 0.538   |
| WBC count (x10⁹/ml)           | 6.83±3.04         | 7.03±3.33         | 0.808   |
| RBC count (x10⁶/ml)           | 3.79±0.88         | 4.42±0.88         | 0.008   |
| Platelet count (x10⁹/ml)      | 145.38±81.51      | 157.38±57.86      | 0.526   |
| Hemoglobin (g/dl)             | 11.99±2.50        | 13.34±2.01        | 0.028   |
| AST (IU/l)                    | 151.63±231.0      | 394.38±749.23     | 0.122   |
| ALT (IU/l)                    | 128.12±314.18     | 522.62±1009.01    | 0.051   |
| Alkaline-phosphatase (IU/l)   | 151.87±133.49     | 98.68±55.45       | 0.086   |
| Creatinine level (mg/dl)      | 0.88±0.33         | 0.87±0.31         | 0.903   |
| Total bilirubin               | 3.21±4.70         | 2.67±5.22         | 0.676   |
| α-fetoprotein (μg/l)          | 3350.05±16326.43  | 67.31±273.19      | 0.335   |
| Ammonia                       | 95.59±58.73       | 77.43±30.72       | 0.287   |
| Splenomegaly                  | 58.8% (20)        | 23.1% (6)         | 0.006   |
| Hepatitis B carrier           | 17.1% (6)         | 22.6% (7)         | 0.579   |
| Hepatitis C carrier           | 22.9% (8)         | 22.6% (7)         | 0.979   |
| Acute hepatitis               | 2.9% (1)          | 25.8% (8)         | 0.110   |
| Alcoholic liver cirrhosis     | 37.1% (13)        | 0.0% (0)          | <0.001  |
| Hepatocellular carcinoma      | 11.4% (4)         | 9.7% (3)          | 1.000   |
| EV bleeding                   | 59.3% (16)        | 18.2% (2)         | 0.033   |

Independent t-test or Chi-Square test (+/– Fisher exact test) were utilized. WBC: White blood cell; RBC: red blood cell; AST: aspartate aminotransferase; ALT: alanine aminotransferase; EV: esophageal varices.

Table III. Correlation between the ADLC and microparticles.

| Variables                     | Total MPs | CD4+ MPs | CD8+ MPs | CD11a+ MPs | CD54+ MPs | CD147+ MPs |
|-------------------------------|-----------|----------|----------|------------|-----------|------------|
| r                             | 0.169     | 0.212    | 0.262    | 0.176      | 0.138     | 0.160      |
| p-Value                       | 0.144     | 0.066    | 0.022*   | 0.128      | 0.234     | 0.174      |

ADLC: Acute decompensated liver cirrhosis; MP: microparticles.

Table IV. Correlation between the chronic hepatitis* and microparticles.

| Variables                     | Total MPs | CD4+ MPs | CD8+ MPs | CD11a+ MPs | CD54+ MPs | CD147+ MPs |
|-------------------------------|-----------|----------|----------|------------|-----------|------------|
| r                             | 0.390     | 0.211    | 0.211    | 0.167      | 0.175     | 0.192      |
| p-Value                       | <0.001    | 0.068    | 0.423    | 0.150      | 0.131     | 0.102      |

MPs: Microparticles. *Chronic hepatitis: defined as hepatitis B and C carriers.
of MPs were remarkably increased in LC patients. A previous study (25) has shown that T cell derived MPs (i.e., CD4+, CD8+, CD54+, CD147) were strongly correlated with inflammatory liver diseases. Our findings, which are in agreement with this study (25), indicate that our LC patients were at a situation of inflammation that could result in chronic persistent hepatitis.

A previous study (25) has shown that MPs which circulate in blood could be a novel diagnostic marker for diagnosis of hepatic diseases and also represent a novel strategy to induce
The most important finding in the present study was that the circulating levels of total MPs and CD4+ MPs were significantly independently predictive of chronic hepatitis. In this way, our findings were consistent with the finding of this previous study (25).

An essential finding of the present study was that a significant correlation was identified between ADLC and circulating levels of CD8+ MPs. However, the multivariate linear regression analysis showed that only NDLC rather than ADLC was significantly independently predictive of increased circulating levels of MPs (i.e., CD8+MPs). Our finding indicated that a persistent and more rigorous inflammatory reaction might be activated in NDLC than in ADLC.

This study has limitations. First the sample size was relatively small and the standard deviation of circulating MPs was extremely high which, therefore, would distort statistical significance. Second, LC was resulted from different disease entities. This might have resulted in unreliable Pearson’s correlation coefficient “R” and coefficient values.

**Conclusion**

In conclusion, circulating levels of T cell-derived MPs may be a useful accessory biomarker for predicting active inflammation in chronic hepatitis and LC.

**Conflicts of Interest**

The Authors report no conflicts of interest regarding this study.

**Authors’ Contributions**

C.-H.C. and K.-H.C.: study design, data acquisition, analysis, drafting of manuscript; C.-H.C., and C.-L.C: laboratory assay and troubleshooting; B.-C.C., H.-H.C., and J.-Y.C: data acquisition, analysis, and interpretation; P.-H.S. and H.-K.Y.: study conception and design, coordination, drafting of manuscript.

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