Dynamics of Circulating Microparticles in Liver Transplant Patients

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Abstract

**Background & Aims.** Microparticles are small membrane vesicles released from the cell plasma membrane, particularly in cell stress, apoptosis and altered cellular viability. Hepatocellular carcinoma (HCC) is a hypervascular neoplasm with high levels of apoptosis and necrosis. We investigated the levels of circulating microparticles of both tumor and endothelial origins in liver transplant patients with hepatitis C (HepC) cirrhosis with and without HCC and compared them with healthy people and patients with partial hepatectomy. **Methods.** Using immunolabeling of microparticles of different origin and flow cytometry-based enumeration of microparticles, the levels of circulating microparticles were studied in 8 patients with HepC and 8 patients with both HepC and HCC before and within two weeks after the transplant. **Results.** The initial levels of circulating microparticles were increased in patients with HepC and HCC as compared to patients with HepC alone. They were also increased in liver transplant patients as compared to patients with partial hepatectomy or healthy people. Levels of circulating microparticles were dynamically changing after the transplant, showing an initial increase with a subsequent decrease by the end of the second week after surgery. In some patients with a complicated clinical outcome, the levels of microparticles were continuously increasing after the surgery. **Conclusion.** The levels of circulating microparticles of endothelial and hepatic origin in liver transplant patients dynamically change after surgery and correlate with the clinical outcome. Perspectives, the levels of circulating microparticles may be used in clinical practice as a marker of the functional status of the transplanted liver.

Keywords


Introduction

The ability of eukaryotic cells to shed components of their plasma membrane into the extracellular space has been previously demonstrated [1]. Such sealed fragments (known as microparticles) MPs typically range in size from 0.1 to 2 µm. Microparticles are small membrane vesicles released from the plasma membrane [2], that contain cell surface proteins and cytoplasmic components of the original cell [3]. Microparticle formation was first described for platelets following their activation by different stimuli, including thrombin, collagen and shear stress [4]. The release of MPs occurs both in vivo and in vitro in different cell types, including B and T lymphocytes [5], monocytes and endothelial cells [6]. Microparticles, shed by different cells, express a subset of cell surface proteins similar to the plasma membrane of the original cell, i.e. MPs shed by polymorphonuclear neutrophils express selectins and integrins, complement regulators, HLA-1 and other markers of neutrophils [7], while MPs derived from endothelial cells express CD31, CD54, CD62E, αβ3 integrins, etc [8, 9].

Increased numbers of circulating MPs have been observed during cardiopulmonary bypass, unstable angina, lacunar infarcts and diabetes mellitus [10, 11]. Formation of MPs seems to be a part of normal cell function [2], although it also accompanies cell stress, apoptosis and altered cellular viability [8].

We recently demonstrated that MPs of endothelial origin directly affect the endothelium, impairing endothelial functions [12]. In addition, endothelium-derived MPs impair angiogenesis in vitro, resulting in increased apoptosis and accelerated degradation of the capillary-like network formed by human umbilical endothelial cells on Matrigel [13].

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm worldwide and the third most common cause of cancer-related deaths. HCC is a hypervascular tumor.
with high levels of apoptosis and tumor necrosis [14, 15]. In contrast to most solid neoplasms, where prognosis and treatment are largely dictated by tumor stage at the time of diagnosis, there are no widely accepted criteria for predicting the prognosis in patients with HCC [16].

It has been recently demonstrated that the levels of circulating platelet MPs and vascular endothelial growth factor (VEGF) are possible markers for predicting gastric cancer metastasis [17]. In addition, an increased number of circulating platelet- and monocyte-derived MPs was identified in patients with lung cancer (reviewed in [18]).

The aim of the current study was to investigate the levels of circulating MPs of both hepatic and endothelial origin in patients with hepatitis C (HepC) cirrhosis with and without HCC, as well as to determine if the numbers of circulating MPs could be used as a novel marker for tumor growth and/or the clinical outcome in liver transplant patients.

**Patients and methods**

**Patients and blood sampling.** Patients with HCC, arising in the background of HepC cirrhosis, who received a total liver transplant at the Westchester Medical Center in 2006 and 2007, were selected for the study (HepC/HCC patients, n=8). Age- and sex- matched patients with HepC cirrhosis, but without HCC, who received a total liver transplant, were used as a comparison group (HepC patients, n=8). In addition, the number of circulating MPs in patients with HCC was compared with those in patients with a partial hepatectomy secondary to a metastatic cancer to the liver or trauma (n=5). A written informed consent was obtained from all the patients involved in the study.

Single or multiple nodules of HCC varying in size from 1 to 7 cm were identified in patients with both HepC and HCC. Histological size of the tumor was determined as the sum of all tumor nodules identified macro- and microscopically in the explanted liver. Histologically, tumor nodules were classified as moderately to well differentiated HCC, grade 2-3. Patients with viral HepC cirrhosis alone had grade 2-3 stage 3-4 lesions based on the Modified Histological Activity Index [19].

The numbers of MPs were evaluated in blood samples collected for a routine hematology analysis. Blood levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured on an automated lab analyzer Roche Hitachi Modular P800 (Diamond Diagnostics, Inc., Holliston, MA) utilizing the spectrophotometric method of detection.

Bleodo was collected in tubes containing 7.2 mg of potassium EDTA (Becton-Dickinson, Franklin Lakes, NJ) from a peripheral vein using a 21-gauge needle to minimize platelet activation and it was processed for assay within 4 hours. Blood samples were collected 1-3 hours before the surgery (designated as day 0) and on days 1, 7 and 14 following the transplant or partial hepatectomy. Blood was also obtained from a control group, which included age and sex-matched healthy individuals without known diseases (n=8). Platelet-poor plasma (PPP) was obtained by a two-step centrifugation as previously described [9, 10]. First, the blood was centrifuged at 160g for 10 minutes to obtain platelet-reach plasma (PRP). The PRP was centrifuged at 1800g for 20 minutes and the PPP was collected and stored at -80°C. To confirm that this protocol did not affect the number of circulating MPs, the number of MPs was measured in randomly selected samples before and after storage at -80°C.

**Antibodies and reagents.** The following antibodies and reagents were used: mouse anti-human CD144 antibodies; mouse anti-human CD31 PE-conjugated antibodies; mouse anti-human CD42a FITC-conjugated antibodies; goat anti-mouse IgG FITC-conjugated antibodies; goat anti-mouse IgG PE-conjugated antibodies; Annexin V PE-conjugated – all from BD Biosciences (San Jose, CA). Mouse anti-human hepatocyte (HepPar) antibodies were obtained from DAKO (Carpinteria, CA) and latex beads of 1 mcm of size - from Sigma (St. Louis, MO).

**Labeling the MPs and flow cytometry.** The number of circulating MPs of different origin was enumerated in the blood samples using flow cytometry as previously described [9, 12]. Briefly, a BD Bioscience (San Jose, CA) flow cytometer FACSCalibur equipped with CellQuest Pro software was used to enumerate circulating MPs. In order to determine a proper gating of the microparticles, non-fluorescent polystyrene latex beads of 1 µm size were used. Microparticles were gated on a forward scatter/side scatter plot in the 0.3-1.2 µm region. The concentration of circulating MPs was calculated as previously described [9]. The time to acquire the certain number of MPs (usually 5000) was measured, and the number of MPs was calculated according to the following formula: MPC=(1000 x Num x 60)/(V x t); where MPC = MP concentration, ml-1; Num = number of particles acquired by a flow cytometer; V = volume speed (60 µl/min); t – time in seconds.

Microparticles of different origins were identified by their immune properties, as previously described [9, 18]. Briefly, 200 µl of PPP was incubated with primary antibodies in the dark at room temperature for 1 hour in concentrations recommended by the manufacturer (usually in 1:50 dilution). The endothelium-derived MPs were identified as the CD144-positive MPs [20, 21], and the MPs of hepatic origin were identified as the HepPar-positive microparticles. Secondary FITC-conjugated antibodies were added to the samples stained with unconjugated primary antibodies (1:100 dilution) and the samples were incubated for additional 30 min in the dark at room temperature. In order to determine the proportion of apoptotic MPs in different subpopulations, a double-staining of MPs was performed with Annexin V, according to the manufacturer’s protocol. To confirm that CD144 adequately highlights all MPs of the endothelial origin, an additional staining with CD31 and CD42 antibodies was performed in the same blood samples, and the flow cytometry data were compared with CD144 staining alone. Endothelial MPs were determined as the CD31+/CD42- population [22].
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Statistical analysis. Results are presented as mean ± standard error if not otherwise specified. Differences between two groups were analyzed by two-paired t-test. Categorical variables were analyzed by the Fisher’s exact test. Data were analyzed using a Prizm statistical software (GraphPad Software, San Diego, CA).

Results

Patients involved in the study

The patient distribution between the two groups is reflected in Table I. There were no significant differences between HepC patients with and without HCC based on the age. A male prevalence was noted in HepC/HCC patients, whereas patients with HepC alone had an equal gender distribution, which correlates with epidemiologic data [23]. There were no significant differences in the native liver weight or liver enzymes data before the surgery. The blood pressure was similar in both groups of patients. The number of patients with history of diabetes mellitus, hypertension and cardiovascular diseases, which are known to affect the number of circulating MPs [1, 6, 10, 11], was similar in both groups of patients involved into the study.

Circulating MPs of different origin in liver transplant patients

Initially, HepC/HCC patients (n=8) had significantly increased total number of circulating MPs as compared to patients without HepC (n=8) (1.26*10⁶/ml±1.9*10⁵ and 2.6*10⁵/ml±4.9*10⁴ correspondingly, Fig. 1A). Patients with HepC alone had an increased number of circulating MPs as compared to controls without primary liver disease (8.7*10⁵/ml±5.9*10⁴), but in less extent than HepC/HCC patients. Detailed analysis of MP subpopulations revealed that the levels of circulating MPs of endothelial and hepatic origins were 3- to 4-folds higher in both HepC and HepC/HCC patients as compared to patients who underwent a partial hepatectomy for non-cirrhotic conditions or healthy individuals (6.6*10⁴/ml±7.9*10³ in HepC/HCC patients, 8.9*10⁴/ml±8.5*10³ in HepC alone and 1.8*10⁵/ml±4.2*10⁴ in patients without HepC for endothelial MP; and 1.9*10⁵/ml±1.3*10⁴ in HepC/HCC patients, 1.5*10⁵/ml±2.1*10⁴ in HepC alone and 2.0*10⁴/ml±880 in patients without HepC for hepatic MP). However, no significant differences were identified in the levels of endothelial and hepatic MPs between HepC and HepC/HCC patients (Fig 1 B, C). The initial levels of apoptotic MPs, which were determined as

Fig 1. Dynamics of the levels of circulating MPs in liver transplant patients within two weeks after the surgery. The numbers of MPs were analyzed in the circulation in patients with hepatitis C cirrhosis alone (●-Cirrhosis, n=8) or complicated by hepatocellular carcinoma (■-HCC, n=8) who underwent a liver transplantation. Control group included patients with a partial hepatectomy for non-cirrhotic conditions (▲- Lobectomy, n=5). The numbers of MPs were measured in blood samples obtained 1-3 hours before the surgery (day 0) and at days 1, 7 and 14 after the surgery. A – dynamics of total numbers of circulating MPs in patients after liver transplantation or partial hepatectomy; B – dynamics of circulating MPs of hepatic origin (HepPar-positive MP); C – dynamics of circulating endothelium-derived MPs (CD144-positive MP); D – dynamics of circulating microparticles derived from apoptotic cells (Annexin V – positive MP). * - MP concentration in healthy people (n=8). # - P<0.05 as compared to the partial hepatectomy group.
Annexin V-positive MP, were significantly elevated in HepC/HCC patients, as compared to patients with HepC alone or patients without HepC (1.7*10^5/ml ± 1.9*10^4, 8.4*10^4/ml ± 1.1*10^4 and 2.9*10^4/ml ± 6.9*10^3, correspondingly, Fig. 1D). Analysis of the dynamics of the circulating MPs revealed that levels of CD144-positive endothelium-derived MPs were elevated initially in both HepC and HepC/HCC patients and normalized to the levels observed in patients without primary liver disease within two weeks after the surgery (Fig. 1C). The same dynamics were observed for apoptotic and hepatic MPs in HepC patients (Fig. 1B, D).

In contrast, HepC/HCC patients had an initial decrease in the levels of hepatic and apoptotic MPs on the first day after the surgery with the subsequent changes similar to HepC patients (Fig. 1B, D). Remarkably, the number of HepPar-positive MPs of the hepatic origin was increased with time in all patients who received a liver transplant, while remained unchanged in patients with partial hepatectomy (Fig. 1B). Healthy control individuals without any surgery had a significantly lower proportion of MPs of hepatic origin as compared with both HepC and HepC/HCC patients (Fig. 1B).

**Correlation between the tumor size, liver function tests and the levels of circulating microparticles**

We analyzed the relationship between the histological tumor size in HepC/HCC patients and the levels of circulating MPs of different origin before the surgery. The levels of total circulating MPs did not correlate with the tumor size (Fig. 2A). However, the levels of hepatic and endothelial circulating MPs correlated directly with the tumor size in HepC/HCC patients (Fig. 2B, C). In contrast, the levels of apoptotic MPs were not linked to the tumor size (Fig. 2D).

The levels of circulating MPs in all patient groups did not correlate with liver enzymes (AST and ALT levels) (Fig. 3A, B). Detailed analysis of MPs of different origin

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**Table I. Demographic data of the patients with HepC cirrhosis involved into the study**

<table>
<thead>
<tr>
<th></th>
<th>HepC alone (n=8)</th>
<th>HepC + HCC (n=8)</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>Age (mean, range), years</td>
<td>55.5 (41-66)</td>
<td>61.6 (50-75)</td>
<td>0.1492</td>
</tr>
<tr>
<td>Female:male ratio</td>
<td>4:4</td>
<td>1:7</td>
<td>0.1410</td>
</tr>
<tr>
<td>Preoperative blood pressure (SBP/DBP, mm Hg, mean)</td>
<td>133/69</td>
<td>127/66</td>
<td>0.6872</td>
</tr>
<tr>
<td>History of diabetes and/or hypertension</td>
<td>2/8</td>
<td>2/8</td>
<td>1.000</td>
</tr>
<tr>
<td>Native liver weight, g, mean ± SER</td>
<td>1450 ± 163</td>
<td>1331 ± 167</td>
<td>0.6260</td>
</tr>
<tr>
<td>AST, U/L, mean ± SER</td>
<td>410 ± 336</td>
<td>210 ± 67</td>
<td>0.5681</td>
</tr>
<tr>
<td>ALT, U/L, mean ± SER</td>
<td>182 ± 147</td>
<td>83 ± 21</td>
<td>0.5408</td>
</tr>
</tbody>
</table>

SBP – systolic blood pressure; DBP – diastolic blood pressure; SER – standard error; AST - aspartate aminotransferase; ALT - alanine aminotransferase; U/L – units per liter.

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![Fig 2](image-url)  

**Fig 2.** Correlation between the tumor size and the numbers of circulating MPs. The size of the HCC in the native liver was correlated with the concentration of circulating MPs in patients with both HepC and HCC before the surgery (n=8). A – correlation between the total numbers of circulating MPs and the tumor size; B - correlation between the total numbers of MPs of hepatic origin and the tumor size; C - correlation between the total numbers of endothelium-derived MPs and the tumor size; D - correlation between the total numbers of MPs shed from apoptotic cells and the tumor size.
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Fig 3. Correlation between liver function tests and the numbers of circulating MPs in all patients and healthy individuals involved in the study (n=29). A – correlation between the total numbers of circulating MPs and AST level; B – correlation between the total numbers of circulating MPs and ALT level.

demonstrated that none of the MPs subpopulations correlated with the liver enzymes in any of the patient groups studied (data not shown).

Clinical outcome and proportions of MPs of different origin

Next, we analyzed the “differential count”, or the proportion of MPs of different origin, in the total pool of circulating MPs (based on the analogy with the blood differential count). Since only MPs of hepatic and endothelial origin were enumerated, all other sources MPs were designated as “others”. Using this approach, we were able to determine the fraction of MPs of the certain origin in the total MP population. In general, the dynamics of the proportional levels of circulating MPs were similar to the actual numbers of the MPs in all groups of the patients studied. However, analysis of individual data revealed several correlations, which were obscured when MP concentrations only were analyzed. Three out of 16 patients, who received a liver

Fig 4. Dynamics of proportional levels of circulating microparticles (MPs) was analyzed in individual patients having a complicated post-surgical course. Patient # 1 (○ - Pt 1) developed sepsis few days after the surgery. Patient # 2 (▲ - Pt 2) had a rejection of the transplant and deceased three weeks after the surgery. Patient # 3 (∇ - Pt 3) had an acute renal failure (ARF) after the surgery. Data from individual patients were compared with the pooled data from the patients who received a liver transplant, excluding the patients with complications, described above (n=13) (■). A – dynamics of the proportion of MPs of the hepatic origin; B - dynamics of the proportion of endothelium-derived MPs; C - dynamics of the proportion of MPs shed from apoptotic cells. * - proportion of MPs in healthy individuals.
transplant, had a complicated postoperative clinical course. These patients had dynamic changes in the MP proportions different from the patients with an uncomplicated clinical course. The dynamics of circulating MPs in patients with complications was compared with the similar data in patients with a favorable clinical outcome. For this analysis, all data from the transplant patients with an uncomplicated clinical course were pooled into one group. Hence, one patient was diagnosed with sepsis a few days after the surgery, and this patient had continuous increase in the proportions of hepatic, endothelial and apoptotic MPs within two weeks after the surgery (Fig. 4A-C). In contrast, patients with uncomplicated clinical course had the proportions of those MPs returned to the original levels, or the levels observed in healthy people, by the end of the second week after the surgery. Another patient had an acute transplant rejection and died three weeks after the surgery. This patient had significantly increased proportions of endothelial and apoptotic MPs even before the transplantation, and those proportions were not decreased within the time of the monitoring (Fig. 4B, C). In contrast, a patient with acute renal failure (ARF) had dynamical changes in MP proportions similar to those patients with an uncomplicated clinical course, namely a temporary increase in the proportion of hepatic and apoptotic MPs with a subsequent decrease in those proportions by the end of the second week after the surgery (Fig. 4A-C), which was correlated with the clinical progression of ARF.

Discussion

In the current study we investigated the dynamics of circulating MPs of different origins in liver transplant patients with HCC arising in the background of HepC and compared them with HepC patients without HCC. To our knowledge, this is the first detailed study of the dynamics and the origin of circulating cellular MPs in patients with an organ transplant.

Recently, increased levels of circulating MPs were identified in many pathological conditions, including cardiovascular diseases, sepsis, pre-eclampsia, diabetes mellitus and others [3, 6, 10, 24]. However, little is known about the role of MPs in the pathogenesis of malignant tumors. Kim et al (2003) proposed that elevated levels of platelet microparticles, along with blood levels of VEGF and interleukin-6 (IL-6), may predict clinical outcome of gastric cancer and serve as a marker of metastases [17]. Circulating levels of VEGF were proposed as a possible tumor marker for metastases in HCC [25]. Nevertheless, it is not clear if circulating MPs of the tumor origin may be used as markers of tumor growth and activity [26, 27].

In the current study, we demonstrated that levels of circulating MPs in HepC/HCC patients are not significantly elevated as compared to patients with HepC alone. However, we identified a direct correlation between the size of the tumor and the levels of hepatic and endothelial MPs in those patients (Fig. 2B, C). It had been demonstrated that HCC is a hypervascular tumor [28, 29], with increased angiogenesis and VEGF production [30], which correlates with an increased risk of vascular invasion and metastases [15]. Our data support these findings, since the levels of endothelium-derived MPs were directly correlated with the size of the tumor, suggesting an increase in the mass of endothelial cells in the tumor itself, which has been reported earlier [30, 31]. Interestingly, the levels of hepatic MPs were directly correlated with the size of the tumor, but there were no significant differences between the patients with HepC cirrhosis alone and patients with HCC, suggesting that an increased number of MPs is shed by hepatocytes in cirrhotic livers as well. Dynamical changes in the levels of circulating MPs after surgery were similar in all subsets of microparticles, namely an initial increase in the circulation with a subsequent decrease to the levels observed in healthy people. Interestingly, similar dynamics in the levels of circulating MPs was observed in all the patients after surgery, regardless of the underlying condition, suggesting that circulating microparticles, especially endothelium-derived microparticles, may reflect a general reaction on a stress. Indeed, the endothelium-derived MPs were significantly increased after the surgery with subsequent normalization by the end of the second week in all patients with uncomplicated clinical course.

Many published data depict the level of circulating MPs in patients with different diseases and compare it with patients without the disease. In the current study, we introduced the concept of “differential count” of circulating microparticles, calculating the proportion of MPs of different origin in the total MP pool, based on the well-accepted and widely used concept of the differential white cell blood count. We demonstrated that proportions of circulating hepatic, endothelial and apoptotic MPs correlated well with the clinical course of the patients. Thus, in patients with an uncomplicated clinical course, the proportion of MPs was decreased with time and reached levels similar to those of healthy individuals within two weeks after the surgery. In contrast, a patient who developed sepsis after surgery had an increased proportion of endothelial (and apoptotic) MPs for at least two weeks after surgery, which correlates with the previously published data [32]. The patient who died had an increased proportion of the endothelial and apoptotic MPs by the end of the second week after the surgery; we could not find literature data about this phenomenon. The third patient who developed ARF immediately after surgery, had a delayed dynamics of endothelial and apoptotic MPs. These data suggest that the dynamical changes in the proportion of endothelial and apoptotic MPs may be used as a marker of the liver functional status and, potentially, as a marker of the clinical outcome and the viability of the transplanted organ. However, this hypothesis needs to be confirmed by further broader investigations before it can be accepted in clinical practice.

Our study demonstrated that dynamics of circulating MPs is well correlated with the pathophysiological changes occurring after the liver transplantation. The levels of circulating MPs were significantly elevated in all patients.
on the first day after the surgery, including patients with a partial liver resection. After this temporal increase, the levels of MPs were decreased, reaching the levels observed in healthy people within two weeks after the surgery. The levels of endothelial and hepatic MPs were increased more substantially in patients who underwent liver transplantation as compared to the patients with a partial hepatectomy. Interestingly, in patients with both HepC and HCC, the levels of apoptotic MPs dropped on the first day after surgery with the subsequent changes similar to the patients with HepC alone, suggesting that the tumor was the major source of those microparticles. Indeed, these patients had elevated initial levels of hepatic MPs as compared to patients with HepC alone, but these differences were not statistically significant, based on the population studied. This may reflect the origin of apoptotic MPs from necrotic tissue that lost immunoreactivity to the HepPar antigen. Interestingly, no MP levels were correlated with liver enzymes (ALT, AST), suggesting that the generation of MPs is not directly dependent on the hepatocyte integrity.

There were many different attempts to correlate HCC progression and different tumor markers, including CEA, AFP etc [33]. However, no single marker has been proved to be useful as a diagnostic or prognostic marker for HCC. In our study we demonstrated that, even in a limited number of patients involved, circulating MPs dynamically changed after the surgery. The dynamics of the proportion of endothelial and hepatic MPs was different in patients with an adverse clinical outcome, suggesting that this may reflect functional changes in the transplanted liver and perspectively may be used as a prognostic or diagnostic marker of the clinical outcome. We correlated MP levels with liver enzymes and found no significant correlation, suggesting that MPs reflect different physiological processes in the liver and may be used as an additional source of information. Perspective, the levels of circulating MPs of different origin may be used in clinical practice as a diagnostic marker of a malignant tumor, as well as a marker of the functional status of the transplanted organ.

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Conflicts of interest

None to declare.

References


