Real-Time Monitoring of Rare Circulating Hepatocellular Carcinoma Cells in an Orthotopic Model by In Vivo Flow Cytometry Assesses Resection on Metastasis

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Abstract

The fate of circulating tumor cells (CTC) is an important determinant of metastasis and recurrence, which leads to most deaths in hepatocellular carcinoma (HCC). Therefore, quantification of CTCs proves to be an emerging tool for diagnosing, stratifying, and monitoring patients with metastatic diseases. In vivo flow cytometry has the capability to monitor the dynamics of fluorescently labeled CTCs continuously and noninvasively. Here, we combine in vivo flow cytometry technique and a GFP-transfected HCC orthotopic metastatic tumor model to monitor CTC dynamics. Our in vivo flow cytometry has approximately 1.8-fold higher sensitivity than whole blood analysis by conventional flow cytometry. We found a significant difference in CTC dynamics between orthotopic and subcutaneous tumor models. We also investigated whether liver resection promotes or restricts hematogenous metastasis in advanced HCC. Our results show that the number of CTCs and early metastases decreases significantly after the resection. The resection prominently restricts hematogenous metastasis and distant metastases.

Introduction

Hepatocellular carcinoma (HCC) is one of the prevalent human cancers worldwide ranking third for mortality and fifth for estimated new cases annually (1). The prognosis of HCC has been significantly improved in recent years due to earlier diagnosis and more effective treatments. However, tumor recurrence and metastasis are still the major obstacles for long-term survival (2, 3).

The metastasis is reported to correlate with the presence of circulating tumor cells (CTC) in the vasculature as a consequence of either advanced tumor growth and invasion or a therapeutic intervention (4, 5). To monitor CTCs, conventional methods usually isolate and count cells expressing epithelial markers from peripheral blood samples (4–10). However, these methods are restricted by invasiveness, lower sensitivity caused by small blood sample volumes, and difficulty to record the dynamics of CTCs, which in vivo flow cytometry could overcome (11–23). In vivo flow cytometry is optimized to quantify circulating fluorescently labeled cells in live animals, without the need to extract blood samples. In in vivo flow cytometry measurement, when the fluorescent cells in fast flowing blood pass through the laser slit across the artery, the fluorescence signal could be excited and be detected. The technique is capable of monitoring the varieties of target cells in circulation continuously, for example, cancer cells (15, 18), hematopoietic stem cells (19, 20), lymphocytes (14), and yields quantitative results without affecting the physiology of the subject.

Previously, the intravenous models have been commonly used in the in vivo flow cytometry measurement of CTCs studies (15, 18). In those studies, cancer cells are labeled ex vivo and injected into blood circulation of small animals. Although it reflects some circulating metastatic characteristics or therapy responses of cancer cells, the large number of injected CTCs does not exist in pathologic conditions (24, 25). Moreover, intravenous tumor models could only reflect the processes that CTCs leave the circulation. They do not provide the information of CTC forming. The subcutaneous metastatic tumor models have been also used in monitoring CTCs by...
**Materials and Methods**

**Cell culture and transgenic procedure**

A human HCC cell line HCCLM3 (32) with high metastatic potential was established at the Liver Cancer Institute, Zhongshan Hospital, Fudan University. The HCCLM3 cells were cultured at 37°C and 5% CO2 in high-glucose Dulbecco’s modified Eagle’s medium medium (Gibco) containing 10% FBS (HyClone). The HCCLM3 cells were transfected with linearized pEGFP-C1 (BD Clontech) using Lipofectin reagent (Invitrogen). Stably transfected populations were obtained by growing the cells in the same media supplemented with 600 μg/mL G418 (Invitrogen) at 37°C and 5% CO2. GFP+ cells were isolated by fluorescence-activated cell sorting (FACS) to establish stably transfected HCCLM3-GFP multi-clone cell line. HCCLM3-GFP cells were maintained in the same media supplemented with 600 μg/mL G418 (Invitrogen) at 37°C and 5% CO2.

**Mouse models and resection procedure**

Animal care and experimental protocols were in accordance with guidelines established by the Shanghai Medical Experimental Animal Care Commission. BALB/c nude mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd and raised under specific pathogen-free conditions. The study was approved by the Ethical Committee of Animal Experiments of Institutes of Biomedical Sciences, Fudan University.

For a subcutaneous metastatic tumor model, 5 × 106 HCCLM3-GFP cells of passage 0 or 1 (P0 or P1) were implanted subcutaneously on the back of the mice (male, 6-week-old, 20 ± 2 g). For an orthotopic metastatic tumor model, orthotopic tumor implantation with HCCLM3-GFP tumor was done under aseptic conditions. The tumor tissue from maternal tumor (subcutaneous tumor or orthotopic tumor) was cut into small cubes about 1 × 1 × 1 mm. The recipient mice (male, 6-week-old, 20 ± 2 g) were anesthetized with pentobarbital sodium salt (1%, 0.1 mL/g mouse weight). The upper abdomen was prepared with 70% alcohol and betadine scrub. A small left subcostal incision was made. The left lateral lobe of the liver was extracted. Following a small, superficial incision into the liver, the 1 × 1 × 1 mm cube of HCCLM3-GFP tumor was implanted. The incision in the liver was closed with a 7-0 suture to avoid possible early peritoneal seeding of tumor cells.

In the resection group, mice with orthotopic metastatic tumor underwent the resection on the day 30 after implantation. Through the left subcostal incision, the left lateral lobe where tumor had been implanted was excised. The tumor size was around 10 mm in diameter and the surgical margin was more than 5 mm.

**In vivo flow cytometry**

To monitoring GFP+ CTCs, we set up in vivo flow cytometer based on previous experience (11–13, 15, 17–19, 33). Briefly, we use transillumination with a 535 ± 15 nm light emitting diode (LED) to visualize the major veins and arteries of the ear microcirculation. An artery of 50 to 70 μm in diameter is selected for data acquisition. Light from the 488 nm laser was focused into a slit by a cylindrical lens and imaged across the selected ear artery. The size of the slit at the focal plane of the sample is approximately 5 × 72 μm. The depth of focus (i.e., the full width at half maximum of the light slit onto the sample in the axial direction) is approximately 50 μm, a value chosen to match the vessels of interest. The sample is positioned so that the long dimension of the slit traverses the width of the blood vessel; thus, fluorescently labeled cells traverse this slit and are excited one by one as they flow through the chosen artery, producing a burst of fluorescence for each cell. Fluorescence is detected with a photomultiplier tube placed directly behind the mechanical slit and the spectral filtering for GFP, sampled at a rate of 5 kHz with a data acquisition card, and displayed/stored on a computer.

To assess the depletion kinetics of CTCs, anesthetized mice were positioned onto the stage to detect GFP+ CTCs. The in vivo flow cytometry measurement was carried out for at least 1 hour once per week for each mouse starting from tumor implantation till its death. Each group included at least 6 mice.

To determine the sensitivity and specificity of in vivo flow cytometry, mice without tumor implantation are measured at least once every 2 hours as blank control. Mice with nonfluorescent tumor implantation are measured at least once every 2 hours as negative control. Each group includes at least 6 mice. Both control groups are used to optimize the analysis algorithm developed in house (34). In in vivo flow cytometry data, the width of signal peaks (with the unit "second") reflects the time during which the CTCs flow across the detecting laser slit, from the beginning of the cell encountering the slit to the whole cell leaving the slit. Thus, the flow rate can be estimated by time width of signal peak in in vivo flow cytometry results, with the formula: flow rate = (width of laser slit + diameter of the cell)/time width of signal peak. The time width values of more than 100 signal peaks from a number of representative data.
traces are used to estimate the average flow velocity in our experiments. Moreover, we estimate the flow volume of blood per hour in the arteries we detected with the formula: flow volume per unit time = (π × diameter of artery²/4) × flow velocity. Diameters of the arteries are obtained from at least 50 ear images by a charge coupled device during corresponding in vivo flow cytometry measurement.

**Flow cytometry**

To evaluate the sensitivity and specificity of the data acquired from in vivo flow cytometry, we used conventional flow cytometry analysis as a comparison. The blood samples from mice for conventional flow cytometry analysis were collected immediately after in vivo flow cytometry measurement. Whole blood sample was collected from anesthetized mice (at least 6 mice in each group for each time point) by heart extraction in heparin. Peripheral blood mononuclear cells (PBMC, including possible present CTCs) were enriched by Ficoll-Paque density centrifugation separation (16). PBMCs from nontumor mouse were used as negative control to exclude the influence of autofluorescence and select appropriate gate in conventional flow cytometry. GFP⁺ CTCs were then counted by using the selected gate.

**Tumor size measurement by ultrasound**

To assess tumor growth, in vivo ultrasound images were acquired by VisualSonics Vero 7700TM Imaging System (Visualsonics Inc.). Brieﬂy, anesthetized mice were ﬁxated, and ultrasound images were acquired following the standard protocols with the frequency of 17.5 MHz. The tumor size was calculated by the formula used commonly in clinics: Volume = (long diameter × short diameter²)/2.

**Pathology test**

To assess the metastasis in various organs, lungs, liver, spleen, kidneys, and a small number of lymph nodes were harvested from the mice and examined under pathology test. Tissue was ﬁxated by formalin and made to parafﬁn sections. Tissue sections were stained with hematoxylin and eosin (H&E) using standard protocols.

To evaluate whether GFP⁺ cells in conventional flow cytometry analysis of whole blood sample were indeed tumor cells, GFP⁺ cells were isolated and stained with H&E using standard protocols.

**Tissue metastasis imaging**

In addition to pathology test, we acquired in vitro images of tissue metastases by confocal ﬂuorescence microscope (Leica TCS SPE, Leica Microsystems CMS GmbH) to assess the level of metastasis. Lungs, liver, spleen, kidneys, and a small number of lymph nodes were harvested (at least 6 mice in each group for each time point) and examined under the microscope using a 10× water immersion objective (NA = 0.3) to visualize GFP-expressing tumor metastases. The tissue was immerged with saline in a 3.5-cm diameter dish, pressed, and ﬁxated for imaging.

Whole tissue images were acquired by in vivo optical imaging system (NigthOWL, II LB 983 NC100, Berthold Technologies GmbH & Co., KG). A 475 ± 20 nm excitation bandpass ﬁlter and a 520 ± 10 nm emission bandpass ﬁlter were used for GFP imaging. The images of tissues from nontumor mice were acquired as negative control.

**Results**

**In vivo CTCs counting by in vivo flow cytometry and validation**

Fast ﬂow rate in artery and sophisticated image processing algorithms restrict the use of confocal imaging in enumerating CTCs, whereas in vivo flow cytometry is a simple and potentially far more powerful method to enumerate circulating cells (11). CTCs in orthotopic GFP-labeled HCC tumor mice can be readily counted ﬂowing through the ear microcirculation by in vivo ﬂow cytometry (Fig. 1A). A typical data trace is shown in Fig. 1B, in which individual peaks correspond to single cells that are excited as they traverse the laser slit of the in vivo flow cytometry. The variations in the intensity of recorded peaks might be due to GFP expression level and deviations from focal plane of individual cells. The full width at half maximum is associated with the in vivo flow velocity of the corresponding cell because it indicates the amount of time required to pass through the excitation slit of light, which is approximately 5 μm across.

To assess the sensitivity, the control traces were acquired from similar arteries of the mice either without tumor implantation (Fig. 1C), or with nonﬂuorescent HCC tumor implantation (Supplementary Fig. S1). There was no noticeable signal peak present in both control groups. In addition, H&E analysis of GFP⁺ cells sorted by FACS in peripheral blood conﬁrmed that the cells we considered as CTCs were indeed tumor cells (Fig. 1D).

To assess the sensitivity, we used conventional flow cytometry to analyze GFP⁺ CTCs in whole blood at week 4, 6, and 8 after HCC tumor implantation, as a comparison. As Fig. 1E shows, when in vivo flow cytometry detects 2.95 ± 1.29, 13.50 ± 2.56, 35.02 ± 4.57 CTCs per hour at 4, 6, and 8 weeks, the corresponding CTC counts by conventional flow cytometry, which are 12.36 ± 5.70, 58.13 ± 15.11, 157.53 ± 26.92 per mL, respectively. We found that those 2 groups of data had good linear relationship. Therefore, if in vivo flow cytometry detects one signal peak per hour, it corresponds to 4.36 ± 0.66 CTCs per mL blood measured by conventional flow cytometry. Because blood flow velocity of the ear artery in our experiments is 11.9 ± 2.3 mm/s calculated by in vivo flow cytometry, the flow volume is 0.128 ± 0.021 mL/h, assuming that the cross-section area of the artery is 0.66 CTCs per mL blood are detected (1/0.128). Thus, our in vivo flow cytometry had approximately 1.8-fold (7.81/4.36) higher sensitivity than whole blood analysis by conventional flow cytometry.

**The difference of CTC dynamics in a subcutaneous versus an orthotopic metastatic tumor model**

Clinical observations have suggested that the local environment can inﬂuence the growth, metastasis, and therapy response of tumors. Therefore, orthotopic tumor models, which can reﬂect clinically relevant local environment, are better for studying tumor metastasis. To establish our orthotopic HCC
model, HCCLM3 tumor cells are labeled with GFP (97.5% positive in flow cytometry analysis, Supplementary Fig. S2) and implanted into the liver. Lung metastases are found at week 4 after implantation. Massive lung metastases were found at week 8 (Supplementary Fig. S3).

To investigate whether the subcutaneous and orthotopic metastatic HCC tumor models have different CTC dynamics, we carried out in vivo flow cytometry measurement on the 2 groups weekly after tumor implantation. Interestingly, there was a significant difference between the 2 curves of CTC dynamics (Fig. 2A). For our orthotopic tumor model, CTC dynamics showed similarity to an exponential curve. Therefore we made an exponential fit to the dynamic curve (\( N = \frac{0.0342}{C^3}e^{0.9185T} \); \( T \): time (weeks); \( N \): number of cell counts per hour; \( R^2 = 0.9679 \)). In contrast, the CTCs from our subcutaneous model fluctuate in a low level. In addition, we assessed the influence of tumor size on CTC dynamics. We used ultrasound imaging to measure tumor size accordingly (Fig. 2B). The dynamics of CTCs in our orthotopic tumor model correlated with the tumor size. In contrast, there was no correlation between CTC dynamics and tumor size in our subcutaneous tumor model. The tumor growth was similar between the 2 groups (Fig. 2C). Therefore, the difference of CTC dynamics between the 2 groups is not due to the tumor size. To investigate whether local environment contributes to the difference, we carried out pathology test of the tumor (Fig. 3). We found that the organ/tissue environments between the 2 groups, especially the blood supply, envelope, and boundary of the tumor have remarkable differences. The differences generally could influence the condition for tumor cell entrance into circulation. Furthermore, compared with subcutaneous tumor, the better blood supply condition in orthotopic tumor could cause the lower death rate of peripheral tumor cells. The death rate of peripheral tumor cells might be important to CTCs formed. Thus, the differences of CTC dynamics might be due to the organ/tissue environment, rather than tumor growth, emphasizing that orthotopic metastatic tumor models are better for metastasis study.

CTC dynamics change after liver resection

It remains unclear whether liver resection increases hematogenous metastasis in advanced HCC. Therefore, we assessed
the influence of liver resection to CTC dynamics by in vivo flow cytometry in our orthotopic tumor model. CTC dynamics of 2 mouse groups are obtained, one group being liver-resection group and the other being control group. As Fig. 4A shows, remarkable difference in CTC dynamics appears between 2 groups. In the control group, CTC dynamics shows similarity to an exponential increase \( N = 0.0285 e^{0.9375 T}, R^2 = 0.9375 \), consistent with what we have reported above. CTC dynamics of the resection group is similar to the control group before the resection. However upon the resection, CTC counts drop down to an undetectable level, followed by another exponential-like curve \( N = 0.0468 e^{1.0397 T}, R^2 = 0.9643 \) with a faster increase. Thus, although liver resection decreases the number of CTCs significantly, it might lead to higher hematogenous metastasis potential of residual tumor if the tumors are not removed completely.

To investigate the relationship between CTC dynamics and the tumor size, orthotopic tumor size measurements were done (Fig. 4B). In the resection group, tumor growth was similar to that of the control group before the resection. With tumors removed, tumor sizes dropped to an undetectable level and restart to increase subsequently. After comparing the data of CTC dynamics (Fig. 4A) and tumor growth (Fig. 4B), we found that CTCs increases as the tumor grows in our orthotopic metastatic tumor model, although without a linear relationship (data not shown). In addition, in contrast to CTC dynamics, orthotopic tumor growth does not show faster increase rate after the resection. The observed faster increase of CTC counts after the resection might be induced by advanced metastatic tumors in other organs, as well as by molecular changes. Therefore, in our tumor model, the number of CTCs mainly corresponds to the condition of tumor cell source, not only orthotopic tumor size but also advanced metastatic tumor.

To confirm results of CTC dynamics obtained by in vivo flow cytometry, conventional flow cytometry analyses was done on...
whole blood samples of the mice in both the resection and the control groups at weeks 4, 6, and 8, respectively. The results were similar to those obtained by \textit{in vivo} flow cytometry (Fig. 4C): although CTC counts increase continuously in the control group, the CTCs in the resection group decrease after liver resection and increase again subsequently.

The curative effect of the resection on metastasis assessed by \textit{ex vivo} imaging

To further assess the distant metastases in other organs after the resection, we conducted both traditional pathology test (Supplementary Fig. S3) and \textit{ex vivo} fluorescence imaging (Fig. 5A and B). Confocal fluorescence imaging has higher sensitivity than pathology test (20), maybe mainly because pathology test could only see one slice of tissue. Lung is the common site of metastasis in advanced HCC. Figure 5A shows fluorescent images of whole lung tissue. Although still going up after the resection, lung metastases increase remarkably slower in the resection group than those in the control. It confirms curative effect of liver resection on metastasis. Further details of metastases are shown by confocal microscope imaging (Fig. 5B). We found that the metastases can be classified into 2 groups according to the size. The metastases with diameter smaller than 100 µm (about 1 approximately 5 cells) are considered as early metastases (Fig. 5C), which could...
be rarely detected or distinguished by pathology test. The metastases with diameter larger than 100 μm are considered as advanced metastases (Fig. 5d). The metastases in control group are far more than those in the resection group with regard to both early and advanced metastases. Interestingly, the number of early metastases in control group is about 17-fold at week 8 than that in the resection group. In contrast, the number of advanced metastases in control group is about only 4-fold at week 8 than those in the resection group. Thus, the resection can inhibit early metastases more than advanced metastases. In addition, the number of early metastases decreases in the resection group at week 6, consistent with CTC dynamics, whereas such decrease is not present for advanced metastases. Therefore we speculated that early metastases might reflect hematogenous metastasis condition, whereas advanced metastases might reflect accumulative effects of hematogenous metastasis. As a consequence, liver resection might decrease the newly produced metastases by decreasing the CTCs.

Discussion

The major motivation for this work is to monitor the condition of hematogenous metastasis continuously in vivo in an orthotopic tumor model and determine whether the resection promotes or restricts hematogenous metastasis. This issue is important because there has been no research so far to our knowledge that has monitored CTC dynamics under clinically relevant physiology condition. Therefore, we take advantage of in vivo flow cytometry technique to monitor CTCs and assess its sensitivity and specificity of detection. Furthermore, we have discovered significant difference of hematogenous metastasis condition in subcutaneous and an orthotopic tumor model, which shows that orthotopic tumor models are better to study the metastasis. In addition, we show that the resection can restrict hematogenous metastasis by decreasing the number of CTCs and distant early metastases.

It has been disputed whether surgical therapy promotes hematogenous metastasis. Tumor resection is usually considered as the mainstay in solid tumor therapy; HCC is no exception for those patients without cirrhosis-induced limited liver function or extent of the tumor. However, previous studies have shown that surgical trauma, such as hepatectomy, may cause the variety of potential growth factors, which mediate liver regeneration after hepatectomy (35–37), enhance neo-vascularization, and tumor cell proliferation (38), and promote tumor metastasis (39, 40). Our study monitors the influence of the resection on hematogenous metastasis in real time. The in vivo flow cytometry results show that tumor resection can decrease CTCs, confirming positive effect of the resection. Imaging experiment also shows that the resection group has less distant metastases than the control group. Furthermore, the number of early distant metastases decreases after the resection. This indicates that forming of neometastasis is restricted after the resection, which might be due to CTC decrease. Therefore, our study shows that liver resection could decrease hematogenous metastasis in HCC. Nevertheless, we also observe the reappearance of CTCs after the resection. Compared with the control, the rate of CTC reincrease in the resection group is higher. Thus, if the tumors are not removed completely, the resection might lead to higher metastasis potential of residual tumor, consistent with previous studies. The addition of adjuvant therapy (e.g., chemotherapy or radiation therapy) may overcome this problem (34). Further studies are currently undergoing.

It has been more than a century since Paget proposed the seed and soil hypothesis to describe the mechanism by which cancer spreads or metastasizes throughout the body (41). CTCs might be considered as the seed in recent studies (4–10, 15–18). Nevertheless, there are few studies to monitor CTC dynamics under clinically relevant physiologic condition. We have observed some interesting phenomena that could explain the mechanism of CTC dynamics. First, we find out that CTC counts have relationship to the tumor size. Second, when the original tumor is removed, the number of CTCs drops to undetectable level (Fig. 4A, data in the resection group at week 5). These imply that CTCs could not maintain in circulation without the supply of solid tumor (also see Supplementary Fig. S4). Furthermore, all the mice with CTC recurrence after original tumor removed have metastases observed in both our imaging experiment and pathology test; all the mice without CTC recurrence after original tumor removed have no metastases observed. Therefore the recurrence of CTCs after resection indicates that the tumor still remained, in the form of either residual tumor or distant metastases. Putting together, we hypothesize that CTCs are in homeostasis as following. On the one hand, cells from original tumor enter circulation to form CTCs. In this stage, CTC increase depends on the size and the properties of original tumor and the microenvironment of host tissue. On the other hand, CTCs depletion might be caused by apoptosis, immune system killing, or metastasizing to target organs. Furthermore, in solid tumor cancer, CTCs could not stay long in circulation. Therefore, CTCs just reflect the combined result of these 2 processes—entrance and depletion. Further studies are needed to test our hypothesis. Research works for understanding molecular mechanism of cancer metastases and therapies by our methodology are promising to improve clinical treatment and are undergoing.

In conclusion, we have determined that our in vivo flow cytometry has higher sensitivity than the currently used whole blood analysis by conventional flow cytometry. Moreover, this study shows the significant difference of hematogenous metastasis between an orthotopic model and a subcutaneous model, and reveals the dynamics of CTC-dependent metastasis under clinically relevant oncology conditions. In addition, we have used CTC dynamics to show the significant effectiveness of surgical resection on cancer hematogenous metastasis, which is important to guide clinical therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Yan, J. Zhou, X.-B. Wei Development of methodology: Z.-C. Fan, J. Yan, G.-D. Liu, W.-Z. Wu, X.-B. Wei
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