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soluble vascular endothelial growth factor receptor-1; PIVKA-II, protein induced by vitamin K absence or antagonist II; TNM, Tumor, lymph Node and Metastasis; CT, computed tomography; MRI, magnetic resonance imaging; BCLC, Barcelona-Clinic Liver Cancer.

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Abstract

Angiogenesis is deemed to be a critical step in the development and progression of vascular-rich hepatocellular carcinoma (HCC). In this process, myeloid lineage cells, such as macrophages and monocytes, have been reported to act as vascular progenitor cells. TIE-2, a receptor of angiopoietins, conveys pro-angiogenic signals. We thus aimed to clarify the roles of TIE2-expressing monocytes (TEMs) in the clinical management of HCC patients. This study enrolled 168 HCV-infected patients including 89 with HCC and examined the frequency of TEMs, as defined as $CD14^{+}CD16^{+}TIE2^{+}$ cells, in the periphery and in the liver. The localization of TEMs in the liver was determined by immunofluorescence. Micro-vessel formation in the liver was quantified by counting $CD34^{+}$ cells. In HCC patients, the frequency of TEMs in the periphery was significantly higher than those in non-HCC groups, and also was higher in the liver than in the periphery. In patients who underwent local ablation or resection of HCC, the frequency of TEMs dynamically changed in parallel with the recurrence of HCC. Most TEMs were identified in the perivascular area of cancer tissues. A significant positive correlation was observed between micro-vessel density in HCC tissues and the peripheral or intra-tumor frequencies of TEMs, suggesting that TEMs are involved in angiogenesis in the liver. Receiver operating characteristic analyses revealed the superiority of TEM frequency to AFP, PIVKA-II and ANG-2 levels as a diagnostic for HCC. ***Conclusion:*** TEMs are increased in

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patients with HCC and change in parallel with the therapeutic response or recurrence. The frequency of TEMs can be used as a diagnostic marker for HCC, potentially reflecting angiogenesis in the liver.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies and the third leading cause of cancer-related deaths worldwide (1). Clinically, HCC frequently develops from liver cirrhosis, with most etiologies involving hepatitis B and C virus (HBV and HCV) infection (2, 3). Since the majority of HCC is characterized by a vascular-rich nature, vascular formation (i.e., angiogenesis) is deemed to be a critical step in the development and progression of HCC (4). Some clinical studies have demonstrated that the degree of vascularity in HCC tissues correlates well with the severity of the disease conditions (5), suggesting that the prevention of this process could have some beneficial impact on patient prognosis. However, the precise mechanisms of HCC-related angiogenesis in the liver remain obscure.

In general, two types of components are cooperatively involved in the progression of angiogenesis: humoral angiogenesis factors and vascular progenitor cells (4). Many studies have reported that angiogenesis factors produced from HCC drive vascularization that supports the development and progression of liver cancer, including invasion and metastasis (6). Among such factors, serum levels of angiopoietin-2 (ANG-2), macrophage migration inhibitory factor (MIF), vascular endothelial cell growth factor (VEGF) and soluble vascular endothelial cell growth factor receptor-1 (sVEGFR-1) have been reported to be higher in HCC patients than in those without HCC,

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being strongly correlated with poorer prognosis or survival (7-11). As for the diagnostic value for HCC, such angiogenesis molecules often fail to show any advantage over other clinically available markers (12).

To support the growth of cancer cells, vascular or endothelial progenitor cells are considered to accumulate in the vicinity of cancer cells. Progenitors, such as hematopoietic stem cells or myeloid lineage cells, are reported to be involved (4). Tyrosine kinase with Ig and EGF homology domains 2 (TIE2) is a receptor of angiopoietins (ANGs); it is primarily expressed on endothelial cells and is capable of binding with all the known ANGs (ANG-1, ANG-2 and ANG-3/ANG-4). The TIE2-expressing monocytes (TEMs) are a novel subpopulation of peripheral and tumor-infiltrating myeloid cells presumed to be equipped with profound pro-angiogenic activity, which is found in both humans and mice (13-15). In the clinical setting of human cancers, TEMs are reported to be found in tumors of the kidney, colon, pancreas and lung, as well as in soft tissue sarcoma (15), where angiogenesis is known to be important for tumor progression. However, it is uncertain whether TEMs are increased or not in HCC patients and what their clinical impact is on the pathophysiology of the disease.

In this study, we aimed at clarifying the roles of TEMs in the clinical management of HCC patients by investigating their frequency, localization and correlation with clinical parameters and microvessel formation in the liver. Our findings indicate that TEMs could serve as a diagnostic

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marker of HCC potentially reflecting angiogenesis in the liver.

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Materials and Methods

Subjects

Among chronically HCV-infected patients who had been followed at Osaka University Hospital, 168 patients were enrolled (Table 1) according to the inclusion and the exclusion criteria (Supplementary table 1). They were categorized into three groups according to the stage of the liver disease: chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC). The clinical stage of HCC was determined according to the TNM classification system of the International Union against Cancer (7th edition) or the BCLC staging classification system. The protocol of this study was approved by the ethical committee of Osaka University Hospital and Osaka University Graduate School of Medicine. At enrollment, written informed consent was obtained from all patients and volunteers. Some of the HCC patients in this study received radiofrequency ablation (RFA) therapy based on the therapeutic guidelines for HCC promoted by the Japan Society of Hepatology (16). After the RFA sessions, the efficacy of tumor ablation or HCC recurrence was evaluated by computed tomography (CT) or magnetic resonance imaging (MRI) scanning. With some of the HCC patients who underwent surgical resection, cancerous and adjacent non-cancerous tissues were obtained at operation for further analyses of TEMs. As controls, we examined healthy subjects (HS) without history of liver disease, HCC patients with HBV infection

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(HBV-HCC group) and those without HBV or HCV (non-B, non-C [NBNC]-HCC group). The clinical backgrounds of the subjects are shown in Table 1.

Reagents

The fluorescence-labeled mouse or rat monoclonal antibodies against relevant molecules used in this study were: CD14 (M5E2), CXCR4 (12G5), CD40 (5C3), CD16 (3G8), CD34 (563), CD11b (ICRF44), CD49d (9F10), CD80 (L307.4), CD86 (2331), CD33 (WM53), CCR4 (1G1), HLA-DR (L243) and CCR5 (2D7/CCR5), which were purchased from Becton Dickinson (BD) Biosciences, San Jose, CA. Anti-human VEGFR2 (89106) or TIE2 (83715) Abs were purchased from R&D SYSTEMS, Minneapolis, MN; anti-human CD45 (HI30) from BioLegend, San Diego, CA; anti-human CX3CR1 (2A9-1) was from Medical & Biological Laboratories (MBL), Nagoya, Japan, and anti-AC133 (AC133) was from Militenyi Biotec.

Phenotype and frequency analysis of peripheral and tumor-infiltrating TEMs

After peripheral blood mononuclear cells (PBMC) had been separated from heparinized venous blood by Ficoll-Hypaque (Nacalai tesque, Kyoto, Japan) density gradient centrifugation, they were stained with a combination of fluorescence-labeled anti-human mouse mAbs against CD14, CD16 and TIE2. For the analyses of liver-infiltrated cells, fresh liver specimens were washed twice with

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phosphate-buffered saline (PBS) and then diced into 5-mm pieces. After these pieces had been passed through a nylon mesh (BD Falcon, San Jose, CA), tumor-infiltrating and non-cancerous tissue-infiltrating leukocytes were isolated by density gradient centrifugation as described above. These cells were stained with fluorescence-labeled Abs (CD14, CD16 and TIE2) as done for PBMC. The stained cells were analyzed using FACS CantoII (BD) and FCS Express software (De Novo, Los Angeles, CA, USA).

Western-blot analysis

$CD16^+$ and $CD16^-$ monocytes were sorted using a FACS sorter. The sorted cells (10^5-5×10^5) were subjected to Western blot analysis for TIE2 expression as described elsewhere (15).

Immunofluorescence analysis

Tissue specimens were obtained from surgical resections of HCC from the patients. Five-micrometer sections were fixed in 4% paraformaldehyde (PFA) for 15 minutes and immunostained. Briefly, the sections were incubated with the following antibodies by detection with a polymeric labeling 2-step method as described (15): rabbit anti-human CD14 antibody (clone, HPA001887; Sigma), mouse anti-human CD16 (2H7; MBL) and mouse anti-human TIE2 (AB33; Upstate Biotechnology) antibodies and subsequently with secondary goat anti-rabbit Alexa

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Fluor®488 or goat anti-mouse Alexa Fluor®594 (Invitrogen, Molecular Probes) antibodies. Cell nuclei were counterstained with Dapi-Fluoromount-G™ (SouthernBiotech, Birmingham, AL). The stained tissues were analyzed by fluorescence microscopy (Model BZ-9000; Keyence, Osaka, Japan).

Immunohistochemical analysis and assessment of microvessel formation (Microvessel density)

To evaluate microvessel density (MVD), immunohistochemical analyses were performed with anti-CD34 antibody (1/50 dilution; QB-END/10, Novo-castra, Newcastle, UK) using the avidin-biotin complex (ABC) method (Vectastain) as described (17).

Single microvessels were detected as any brown CD34-immunostained endothelial cells. MVD was evaluated according to the method described by Poon et al. (17). Sections were read by two double-blinded pathologists according to staining intensity.

Statistical analysis

Differences between two groups were assessed by the Mann-Whitney nonparametric U test, and multiple comparisons between more than two groups by the Kruskal-Wallis nonparametric test. Paired t tests were used to compare differences in paired samples using GraphPad Prism software (GraphPad Prism, San Diego, CA, USA). To differentiate HCC and LC, receiver operating

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characteristics (ROC) analyses were done using JMP software (SAS, Cary, NC, USA). In order to identify optimal cut-off point of TEM frequency for balancing the sensitivity and specificity, we defined the optimal point as those yielding the minimal value for $(1-\text{sensitivity})^2 + (1-\text{specificity})^2$. The correlation between two groups was assessed by Pearson's analysis. The recurrence-free survival rate in patients with HCC who underwent the treatment was compared using the Kaplan-Meier method, with the log-rank test for comparison. Associations among the variables were determined by χ^2 test of Fisher exact test and Student's *t* test. All tests were two-tailed, and a P value of less than 0.05 was considered statistically significant.

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Results

TIE2 is selectively expressed on $CD14^+CD16^+$ monocytes

In order to examine which population of cells expresses TIE2, we stained PBMC obtained from HCC patients with the relevant Abs. Among them, $CD14^+$HLA-DR^+ monocytes express TIE2 (Fig. 1A), but the remaining $CD14^-$HLA-DR^- cells do not (Fig. 1A). More precisely, T cells, B cells, NK cells, NKT cells, dentritic cells did not express TIE2 (data not shown).

Monocytes are divided into two distinct subsets according to the expression of CD14 and CD16; they are $CD14^{++}CD16^-$ and $CD14^+CD16^+$, respectively (Fig. 1A). $CD14^+CD16^+$ monocytes express TIE2 to a degree higher than $CD14^{++}CD16^-$ monocytes (Fig. 1B). In Western blot, the band intensity of TIE2 in lysates from $CD14^+CD16^+$ cells was lesser than those of HUVEC as a positive control, whereas the intensity of $CD14^+CD16^+$ cells was much more than those in samples from $CD14^{++}CD16^-$ cells (Fig. 1C). Therefore, for the following sections of this paper, we define $CD14^+CD16^+TIE2^+$ cells as being TIE2-expressing monocytes (TEMs). As the control, $CD14^+CD16^-TIE2^-$ cells are TIE2-negative monocytes ($TIE2^-$ monocytes).

TEMs are phenotypically and functionally distinct from $TIE2^-$ monocytes or endothelial progenitor cells in myeloid lineage

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TEMs are positive for CD45, CD11b, CCR4, CCR5, CX3CR1, CD40 and CD86, the expressions of which are greater than those for TIE2^{-} monocytes (Fig. 1D, 1E). The expressions of CD33, HLA-DR, CD49d and CXCR4 are comparably high on monocytes regardless of CD16 or TIE2 expression. Since TEMs have been reported to be involved in the promotion of angiogenesis in some clinical settings (4, 13, 18). we next compared their phenotypes with those of endothelial progenitor cells (EPC) (19). We found that TEMs do not express AC133, VEGFR2 or CD34 (Fig. 1D). These results indicate that; TEMs are phenotypically and functionally distinct from TIE2^{-} monocytes or EPC in myeloid lineage.

TEMs are significantly increased in the peripheral blood of HCC patients and their increase is associated with cancer occurrence and recurrence

We compared the frequency of TEMs in PBMC among healthy subjects and chronically HCV-infected patients with various stages of liver disease. With respect to the demographics of the subjects, no difference was found in the clinical and pathological characteristics among patient groups (Table 1). In HCC patients, the frequency of TEMs in the periphery was significantly higher than those in all other groups (Fig. 2A). In addition, the frequency of TEMs did not differ between patients at advanced HCC stages (TNM stages III and IV) and those at early HCC stages (stages I and II) (Fig. 2B). Similar results were obtained with the classification according to the BCLC

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staging system. The frequency of TEMs did not differ between the patients with advanced HCC stages (BCLC, C and D) and those with early stages (A and B) (3.6 ± 2.2% vs. 3.3 ± 2.3%). These results show that the increase of TEMs is closely related to the presence of HCC, irrespective of the stage of cancer. Furthermore, in non-HCV-infected HCC patients (NBNC-, alcoholic- and HBV-HCC patients), the same increment of peripheral TEMs was observed (Supplementary figure 1), suggesting that the increase of TEMs is influenced by HCC, not by infection with hepatitis viruses.

We serially examined the frequency of TEMs in HCC patients who underwent RFA therapy or tumor resection. In clinical practice, we assessed the viability of HCC by CT or MRI scanning every 3 to 6 months after the treatment. In patients without HCC recurrence, the frequency of TEMs dramatically decreased after successful HCC ablation and resection (Fig. 2C). By contrast, in patients with subsequent HCC recurrence, TEMs increased again before the apparent radiological identification of HCC (Fig. 2C). Therefore, the TEM frequency dynamically changes in patients in correlation with the presence or absence of HCC.

In order to assess the clinical impact of TEMs, we compared various clinical parameters between patients with higher TEM frequency and those with lesser frequency. We categorized HCC patients according to their peripheral TEM frequency of above (TEM^{high}) or below (TEM^{low}) the median value (cut off value = 2.75). We found that the patients in the TEM^{high} group displayed a more

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advanced Child-Pugh grade (B) and higher the model for endstage liver disease (MELD) score, a lower prothrombin time and a lower albumin level (Table 2). These results imply that an increment of TEMs is associated with a deterioration of liver function in HCC patients. Furthermore, the TEM^{high} group as assessed before the RFA treatment or the resection of HCC shows significantly shorter recurrence-free survival rates than the TEM^{low} group, suggesting that the numerical assessment of TEMs holds some prognostic value (Fig. 2D). The overall survival of the patients with HCC after the treatments was not different between the TEM^{high} and TEM^{low} groups in this observation period (Supplementary figure 2).

TEMs are located in the perivascular areas of HCC

Most of the TEMs, identified by CD14^{+}TIE2^{+} cells in situ, are located in the perivascular area of cancer tissues determined under immunofluorescence staining (Fig. 3A-[A], [B], [D]). Most of the CD16^{+} signals are overlapped on CD14^{+}TIE2^{+} cells (Fig. 3A-[C]). Some of the TEMs are localized inside the lumen of vascular cells (CD14^{-}TIE2^{+} cells) in cancer lesions (Fig. 3A-[D]). However, they are scarce in adjacent non-cancerous tissues (not shown).

TEMs accumulate in HCC tissue

The frequency of TEMs in tumor-infiltrating leukocytes (TIL) was higher than that in cells

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infiltrating non-tumor tissue (NIL) and PBMC (Fig. 3B). Moreover, in patients with HCC, the frequencies of TEMs in PBMC and TIL are positively correlated (Fig. 3C). These results suggest that TEMs tend to accumulate into HCC tissue. Of particular importance is that the peripheral TEM frequency reflects the degree of their infiltration into HCC tissues.

The TEM frequency is well correlated with the degrees of microvessels in HCCs

To study whether the frequencies of peripheral or intra-hepatic TEMs are correlated with the degrees of vascular formation in liver cancer, we examined the presence of $CD34^+$ cells in liver tissues obtained from 12 HCC patients. The expression of CD34 was predominantly confined to the cytoplasm of vascular endothelial cells. In general, microvessels are represented by brownish yellow capillaries or small cell clusters. The $CD34^+$ cells were located mainly in tumor cell areas (Fig. 4A). The values of microvessel density (MVD) in HCC (67.0 ± 57.8) tended to be higher than those in non-cirrhotic (26.7 ± 7.5) or cirrhotic non-cancerous tissues (32.1 ± 11.6), respectively. Furthermore, the values of MVD in HCC tissues were positively correlated with the frequencies of peripheral and intra-hepatic TEMs in HCC patients (Fig. 4B). These results suggest that TEMs are involved in the promotion of neo-vascularization in liver cancer.

Peripheral TEM frequency is superior to AFP and PIVKA-II levels as a diagnostic marker for

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HCC

In order to evaluate the feasibility of TEM frequency as a diagnostic marker of HCC, we examined whether or not the frequency of TEMs is correlated with various clinical parameters of HCC patients. No correlation was found between TEM frequency and other HCC-specific markers such as α-fetoprotein (AFP) or protein induced by the absence of vitamin K or antagonist II (PIVKA-II) (Fig. 5A). In addition, peripheral TEM frequency was not correlated with any of the levels of several angiogenic factors, such as VEGF, ANG-2, sVEGFR-1 and MIF (Supplementary figure 3). As for the diagnostic value of TEM frequency for differentiating HCC from chronic liver disease (CLD; chronic hepatitis and liver cirrhosis patients) or liver cirrhosis, its sensitivity and specificity were 86.1 and 71% or 81.3 and 90%, respectively (Table 3). ROC analyses revealed that TEM frequency was superior to AFP, PIVKA-II and ANG-2 levels as a diagnostic marker for HCC (Fig. 5B and Table 3).

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Discussion

In this study, we defined TEMs as $CD14^+CD16^+TIE2^+$ and examined their frequency, localization and correlation with micro-vessel densities in the liver. We demonstrated that 1) the frequency of TEMs is significantly increased both in PBMC and in the liver of HCC patients and positively correlates with the degree of micro-vessels in the HCC tissue; 2) its frequency dramatically changes in parallel with complete ablation or recurrence of HCC; 3) the peripheral frequency of TEMs can serve as a better diagnostic marker of HCC than AFP, PIVKA-II and ANG-2 levels. These results show that certain HCC-derived factors are responsible for the generation of TEMs, the degree of which is correlated with the intensity of vascular formation.

According to the patterns of CD16 and CD14, it has been reported that monocytes can be categorized into distinct subsets, such as classical ($CD14^+CD16^-$) and non-classical ones ($CD14^+CD16^+$) (20). Such populations are regarded as functionally distinct, since the percentage of $CD14^+CD16^+$ cells predominantly increases under inflammatory conditions such as chronic hepatitis and inflammatory bowel disease (21, 22). We identified TEMs in $CD14^+CD16^+$ monocytes but not in $CD16^-$ monocytes, suggesting that TIE2 is predominantly induced in $CD16^+$ monocytes. However, the precise mechanisms of TIE2 induction on monocytes have been largely unknown. Furthermore,

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it is yet to be clarified whether CD16$^+$ monocytes are differentiated from CD16$^-$ monocytes or not. Multiple factors are reported to enhance CD16 expression on monocytes, such as macrophage-colony-stimulating factor (M-CSF), IL-10 and transforming growth factor (TGF)-β1 (23). In addition, some studies disclosed that hypoxia and MIF contributed to TIE2 expression on monocytes in vitro (14, 24). Cumulative data have been published showing that cancer cells, including HCC, are dichotomously capable of releasing various inflammatory (TNF-α, IL-1β) and anti-inflammatory (TGF-β and IL-10) cytokines, as well as hematopoietic factors (M-CSF and MIF) (7, 25-29). However, in this study, no correlation was found between the frequency of TEMs and any of serum angiogenesis factors (Supplementary figure 3). It is thus plausible that, not a sole but a mixture of such HCC-derived factors contributes to the generation of TEMs from CD16$^+$ monocytes with the aid of a hypoxic microenvironment. In our preliminary study, a combination of cytokines and growth factors were able to induce TIE2 on CD16$^+$ monocytes (manuscript in preparation).

Although the presence of TEMs has been reported in cancer tissues from patients with colorectal, pancreatic or renal cancer (15), the actual roles of TEMs in the angiogenesis process of HCC have yet to be elucidated. In this study, we showed that the peripheral and intrahepatic frequency of TEMs is positively correlated with the density of micro-vessels in the liver. Additionally, we found that TEMs accumulate in the liver and are located in the perivascular area of HCC tissues. In support for our observations, Venneri et al. reported that TEMs preferentially localize in the vicinity of tumor

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blood vessels and avascular viable areas in human cancer specimens but are not found in non-neoplastic tissues adjacent to tumors (15). These results suggest that TEMs may take some part in HCC-related vascular formation. Further investigation is still needed to disclose the molecular mechanisms of TEMs accumulating in the liver. In analogy of $CD14^{+}CD16^{+}$ monocytes, some interactions between chemokine and its receptors, such as CCR5 or CX3CR1, may be involved in such process (22), all of which are shown to be expressed in TEMs as well (Fig. 1D).

Thus far, many studies have reported that the histological degree of angiogenesis in the liver is closely correlated with the prognosis or survival of patients with HCC. In agreement with these results, several serum angiogenesis factors, such as VEGF, ANG-2, sVEGFR-1 and MIF, are reported to be feasible as markers of prognosis, invasiveness or post-therapeutic recurrence in HCC patients (7-11). Multivariate analysis disclosed that the degree of angiogenesis in the liver, as assessed by MVD, is an independent factor significantly involved in the disease-free survival rate in patients with resectable HCC (17) Therefore, the positive correlation between peripheral and intra-hepatic TEM frequency and MVD in the liver observed in this study offers the possibility of TEMs being as a prognostic marker of HCC patients. In support for this, the patients with higher TEM frequency were at a more advanced Child-Pugh stage, had poorer liver function and showed higher post-therapeutic HCC recurrence in this cohort.