

Retrospective Study

Macrophage colony-stimulating factor expressed in non-cancer tissues provides predictive powers for recurrence in hepatocellular carcinoma

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Abstract

AIM

To investigate the role of macrophage colony-stimulating factor (M-CSF) in patients with hepatocellular carcinoma (HCC) after surgery.

METHODS

Expression of M-CSF, distribution of M2 macrophages (M ϕ s), and angiogenesis were assessed in the liver, including tumors and peritumoral liver tissues. The prognostic power of these factors was assessed. Mouse isolated hepatic M ϕ s or monocytes were cultured with media containing M-CSF. The concentration of vascular endothelial growth factor (VEGF) in media was assessed. Furthermore, the role of the M-CSF-matured hepatic M ϕ s on proliferation of the vascular endothelial cell (VEC) was investigated.

RESULTS

A strong correlation between the expressions of M-CSF and CD163 was observed in the peritumoral area. Also, groups with high density of M-CSF, CD163 or CD31 showed a significantly shorter time to recurrence (TTR) than low density groups. Multivariate analysis revealed

the expression of M-CSF or hepatic M2M ϕ s in the peritumoral area as the most crucial factor responsible for shorter TTR. Moreover, the expression of M-CSF and hepatic M2M ϕ s in the peritumoral area had better predictable power of overall survival. Values of VEGF in culture media were significantly greater in the hepatic M ϕ s compared with the monocytes. Proliferation of the VEC was greatest in the cells co-cultured with hepatic M ϕ s when M-CSF was present in media.

CONCLUSION

M-CSF increases hepatocarcinogenesis, most likely by enhancing an angiogenic factor derived from hepatic M ϕ and could be a useful target for therapy against HCC.

Key words: M2 macrophage; Vascular endothelial growth factor; Vascular endothelial cell; Monocyte; Angiogenesis

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Core tip: This study was designed to investigate the role of macrophage colony-stimulating factor (M-CSF) in patients with hepatocellular carcinoma (HCC) after resection. Groups with high density of M-CSF, CD163 or CD31 showed a significantly shorter time to recurrence (TTR) than low density groups. Multivariate analysis revealed the expression of M-CSF or hepatic M2M ϕ s in the peritumoral area as the most crucial factor responsible for shorter TTR. The expression of M-CSF and hepatic M2M ϕ s in the peritumoral area had better power for the prediction of overall survival. Thus, M-CSF could be a useful target for therapy against HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a globally common cancer, resulting over one million deaths every year^[1]. The etiology of HCC is strongly associated with liver cirrhosis due to alcohol abuse and hepatitis viral infection, exposure to aflatoxin B1 and various metabolic liver diseases^[2]. The mechanisms of HCC are mainly still unknown.

Although liver resection improves overall survival in patients with HCC^[3], a high rate of postoperative recurrence is still one critical problem, including intrahepatic metastasis (IM) or multicentric (MC) recurrence. Biomarkers, which derived from carcinoma cells or tumor-associated fibrotic tissues, have

been studied^[4]; however, results have not been well elucidated. Previously, the oncogenesis of HCC has been predominantly investigated in terms of oncogenic factors above mentioned. Alternatively, chronic inflammation is also strongly linked to mechanisms of initiation or progression of carcinoma by increasing production of reactive oxygen species and inflammatory cytokines from inflammatory cells, such as hepatic macrophages (M ϕ), "Kupffer cells (KC)", and infiltrating neutrophils into the liver^[5,6]. Previously, we reported a relationship between chronic inflammation caused by hepatitis viral infection and DNA damage due to oxidative stress in patients with HCC. Furthermore, it was also reported that the period of post-operative recurrence was much shorter in patients with high oxidative stress compared with patients who had low oxidative stress in HCV-infected liver^[7]. In addition to the oncogenic factors, the microenvironment is also an important factor as the soil for initiation and progression of HCC. Budhu *et al*^[8] reported that metastasis of HCC was associated with an immune system in the liver, suggesting that liver tissues surrounding tumors have an effect on prognosis of HCC.

Macrophages, one of the predominant infiltrating cell types into the tumor^[9], are attracted by chemokines^[4]. When M ϕ s are activated, they can eliminate malignant cells or draw out reactions causing tissue destruction^[10]. Although the significant role of macrophage colony-stimulating factors (M-CSF) and tumor-infiltrating M ϕ s in IM of HCC was already reported by Budhu *et al*^[8], the postoperative prognostic significance has not been cleared. Results from the most recent report suggest expression of M-CSF was associated with poor survival after curative liver resection in patients with HCC, emphasizing the important role of the microenvironment in the intrahepatic recurrence of HCC^[11]. M-CSF can also induce production of Th2 cytokines^[12,13] and some growth factors by the monocyte, which are essential for cell growth and invasion of tumor^[14]. Indeed, M-CSF induces vascular endothelial growth factor (VEGF) production and angiogenesis in monocytes. Furthermore, suppression of M-CSF decreased the infiltration of M ϕ s and suppressed tumor progression. On the other hand, hyper expression of M-CSF or treatment of recombinant M-CSF enhanced infiltration of M ϕ s, which is associated with growth of tumor cells and angiogenesis^[15-17].

Based on these results, it was hypothesized that M-CSF expression in non-tumoral liver tissues may enhance recruitment of M2M ϕ and angiogenesis, and enhance growth of tumor cells. This may increase the rate of recurrence of HCC, leading to a poor overall survival. Furthermore, in this study, the role of M-CSF on production of VEGF by the hepatic M ϕ s and the proliferation of vascular endothelial cells (VEC) were investigated *in vitro*.

Table 1 Clinicopathological features of the patients

Clinicopathological features	
Patients	n = 77
Gender	male : female = 38 : 10
Age (yr)	mean = 66.6 (44-85)
Virus infection	HCV : HBV : NBNC = 72 : 4 : 1
UICC-TNM classification	I : II : IIIA : IIIB = 41 : 32 : 3 : 1
Tumor number	single : multiple : unknown = 48 : 28 : 1
Tumor size (cm)	mean = 3.0 (1.0-9.5)
Portal vein invasion	yes : no : unknown = 7 : 68 : 2
Tumor differentiation	well : mod : poor : unknown = 18 : 44 : 13 : 2
Number of platelet ($\times 10^4$)	mean = 13.4 (3.8-28.1)
Alanine aminotransferase (ALT) (U/L)	mean = 50.9 (16-207)
Total bilirubin (mg/dL)	mean = 0.9 (0.3-1.9)
Prothrombin activity (%)	mean = 77.1 (33.0-104.0)
Indocyanine green R15 (%)	mean = 19.1 (6.0-44.4)
Alpha-fetoprotein (ng/mL)	mean = 389.6 (1.8-602.6)

From July 2000 to June 2008, consecutive patients in the University of Yamanashi Hospital (Yamanashi, Japan) who (1) were diagnosed as having HCC and (2) underwent curative resection surgery, were enrolled in this study.

MATERIALS AND METHODS

Patients

This is a retrospective single-center, open-label study. From July 2000 to June 2008, consecutive patients in the University of Yamanashi Hospital (Yamanashi, Japan) who (1) were diagnosed as having HCC; and (2) underwent curative resection were enrolled in this study (Table 1).

Follow-up and postoperative treatment

All patients were observed until December 2013. The presence and identification of the hepatitis virus was determined by one or more of the following techniques: (1) the presence of anti-HCV and anti-HBV reactive serum proteins; (2) reverse transcription-PCR for serum HCV-RNA; or (3) the branched DNA-HCV probe assay. Following liver resection patients returned to the ambulatory care clinic for additional tests monthly. Serum α -fetoprotein levels were measured every month. In addition, ultrasonography and computed tomography (CT) of the liver were performed every 2 and 4 mo, respectively. Informed consent was obtained from all subjects who participated in the study, and the study was approved by the Institutional Board on Ethics for Human Science at the University of Yamanashi. All available clinical data for the patients enrolled in this study are summarized in Table 1. Overall survival (OS) was defined as the interval between the dates of surgery and death. Disease-free survival (DFS) was defined as the interval between the dates of surgery and recurrence; if recurrence was not diagnosed, patients were censored on the date of death or the last follow-up.

Animals

For the *in vitro* study, male C57BL/6 mice (8-10 wk of age, obtained from Jackson Laboratories, Bar Harbor,

ME), were housed in a clean, temperature-controlled environment with a 12-h light-dark cycle and were given free access to regular laboratory chow diet and water for several days. All animals received humane care, and the study protocols were approved by the Committee of Laboratory Animals at University of Yamanashi according to institutional guidelines.

Liver tissues

Sections of tumor and surrounding liver tissues were collected at the time of the operation. Then, sections were fixed in formalin (10%), dehydrated in absolute ethanol and embedded in paraffin. Serial sections (5 μ m thick) were prepared for further immunohistochemical analysis.

Immunohistochemical detection of M-CSF, CD68, CD163, and CD31

Paraffin-embedded serial sections of liver tissues were stained immunohistochemically with anti-M-CSF (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD68 (DAKO, Kyoto, Japan), anti-CD163 (Abcam, Cambridge, United Kingdom), or anti-CD31 (Abcam) antibodies. Briefly, after deparaffinization and rehydration, the antigen retrieval procedure was applied by heating the slides in 0.1 mol/L citrate buffer (pH 6.0) for 10 min. The sections were then incubated first with 0.3% H₂O₂ in distilled water for 5 min to block endogenous peroxidase and then incubated with one of the following antibodies: monoclonal mouse antihuman CD68 (diluted to 1:200), CD163 (diluted to 1:200), CD31 (diluted to 1:200), and M-CSF (diluted to 1:100) at 4 °C overnight. Biotinylated secondary antibody conjugated with avidin-biotin horseradish peroxidase (Dako Envision kit/HRP; Dako, Kyoto, Japan) and 3',3'-diaminobenzidine tetrahydrochloride were used for standard avidin biotinylated peroxidase detection. Quantitation of cells that stained positive for CD68, CD163, CD31 and M-CSF was performed using image analysis software (Scion Image; Scion Corp., Frederick, MD) by evaluating five different fields (magnification: $\times 400$) for areas positively stained and expressed as a percentage of the total area.

Effects of M-CSF on production of vascular endothelial growth factor by the isolated Kupffer cells and the monocytes from mice *in vitro*

Kupffer cells were isolated by counterflow centrifugal elution as described in our previous work with some modifications^[18]. Briefly, nonparenchymal cells were isolated by collagenase digestion and differential centrifugation using Nycodenz (Nycomed Pharma AS, Oslo, Norway) as described elsewhere^[19]. Furthermore, the peripheral blood monocytes were isolated by the centrifugation method using magnetic beads EasySep (Tokyo, Japan). These cells were incubated in media containing recombinant mouse M-CSF (100 ng/mL) (Sigma) for a designated time period (from 1 to 5 d). In another set of experiments, isolated KCs

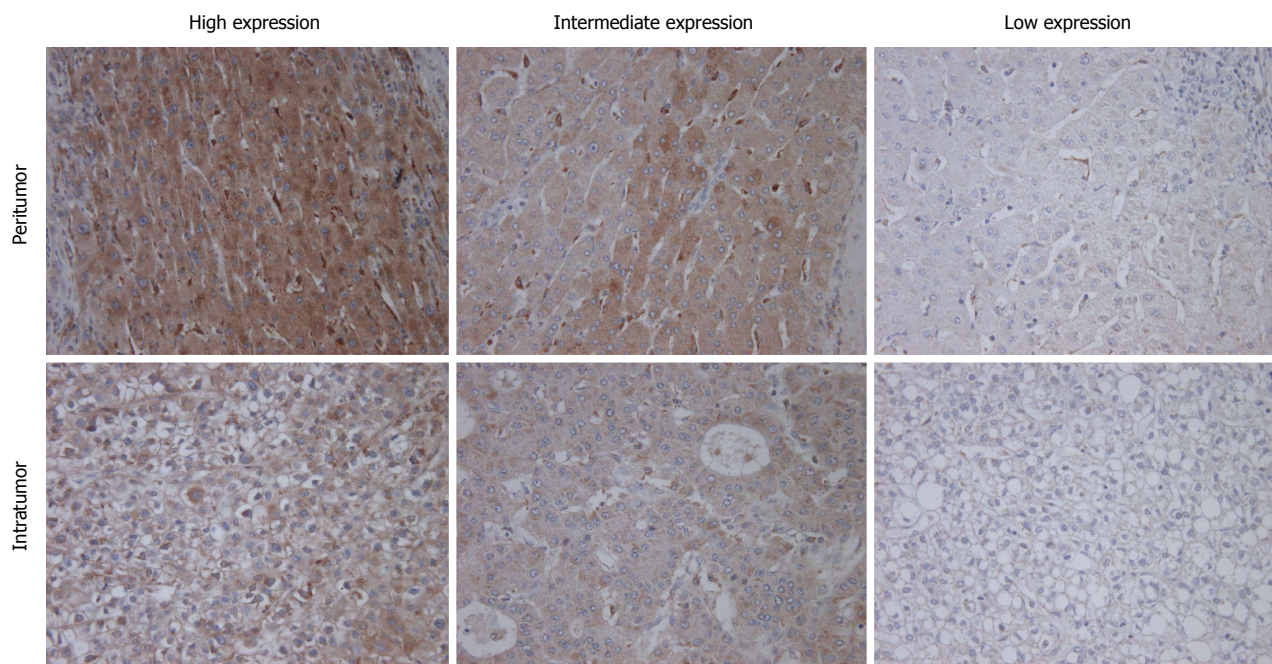


Figure 1 Immunohistochemical staining for macrophage colony-stimulating factor. Immunohistochemical staining for M-CSF in intratumoral and peritumoral tissues in the liver was performed as described in Patients and Methods. Representative photomicrographs are shown. Original magnification, $\times 400$.

were incubated with different doses of M-CSF (0, 1 ng, 10 ng, and 100 ng/mL) in media for 5 d. The concentration of VEGF in media was measured by an ELISA kit (R&D Systems, Minneapolis, MN).

Effects of hepatic macrophages and M-CSF on proliferation of the vascular endothelial cells

The effects of M-CSF and the KCs on the proliferation of the VECs were investigated. The KCs were isolated by collagenase digestion and the centrifugation method above mentioned, and the VECs were isolated from the aorta. The VECs were seeded on the bottom chamber of a trans-well chamber (Corning Inc., Corning, NY) and the hepatic macrophages were seeded on the upper chamber. These cells were incubated with the media containing M-CSF (mouse recombinant M-CSF; 100 ng/mL) (Sigma, St. Louis, MO) for 4 d and proliferation of the VECs was assessed.

Statistical analysis

Analysis was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago IL). The Pearson χ^2 test or Fisher's exact test was used to compare qualitative variables, and quantitative variables were analyzed by the *t* test or Pearson's correlation test. Kaplan-Meier analysis was used to determine the survival (time to recurrence and overall survival). Receiver operating characteristic (ROC) curve analysis was used to determine the predictive value of the parameters. For the univariate analysis, linear regression, or Log-rank test statistical procedures were used to assess which endpoints could be used for predicting the prognosis of HCC patients after surgery. For the multivariate analysis, the Cox

proportional hazard model was used to calculate hazard ratio and the *P* value of each parameter. *P* < 0.05 was considered statistically significant.

RESULTS

The clinical characteristics of HCC patients in this study are shown in Table 1. Experiments were performed using resected liver specimens including HCC and tumor free non-cancerous liver tissue by surgery.

Expression of M-CSF in intratumoral and peritumoral tissues in patients with HCC

Immunohistochemical staining for M-CSF was performed as described in the Patients and Methods section. Immunohistochemical staining for M-CSF was observed in the cytoplasm of both tumor cells and hepatocytes (Figure 1). On the other hand, most of the non-parenchymal cells were negatively stained.

The expression of M-CSF in intratumoral tissues was not correlated with DFS and overall survival rate (OS) (data not shown), consistent with the previous report^[11]. On the other hand, in peritumoral tissues, DFS and OS were significantly shorter in the high expression group than in the low expression group (Figures 2 and 3), consistent with the previous reports^[11].

Expression of CD68, CD163, and CD31 in liver tissues of patients with HCC

Immunohistochemical staining for CD68 (a marker of the M1M ϕ s and/or the monocytes), CD163 (a marker of the M2M ϕ s), and CD31 (a marker of the vascular

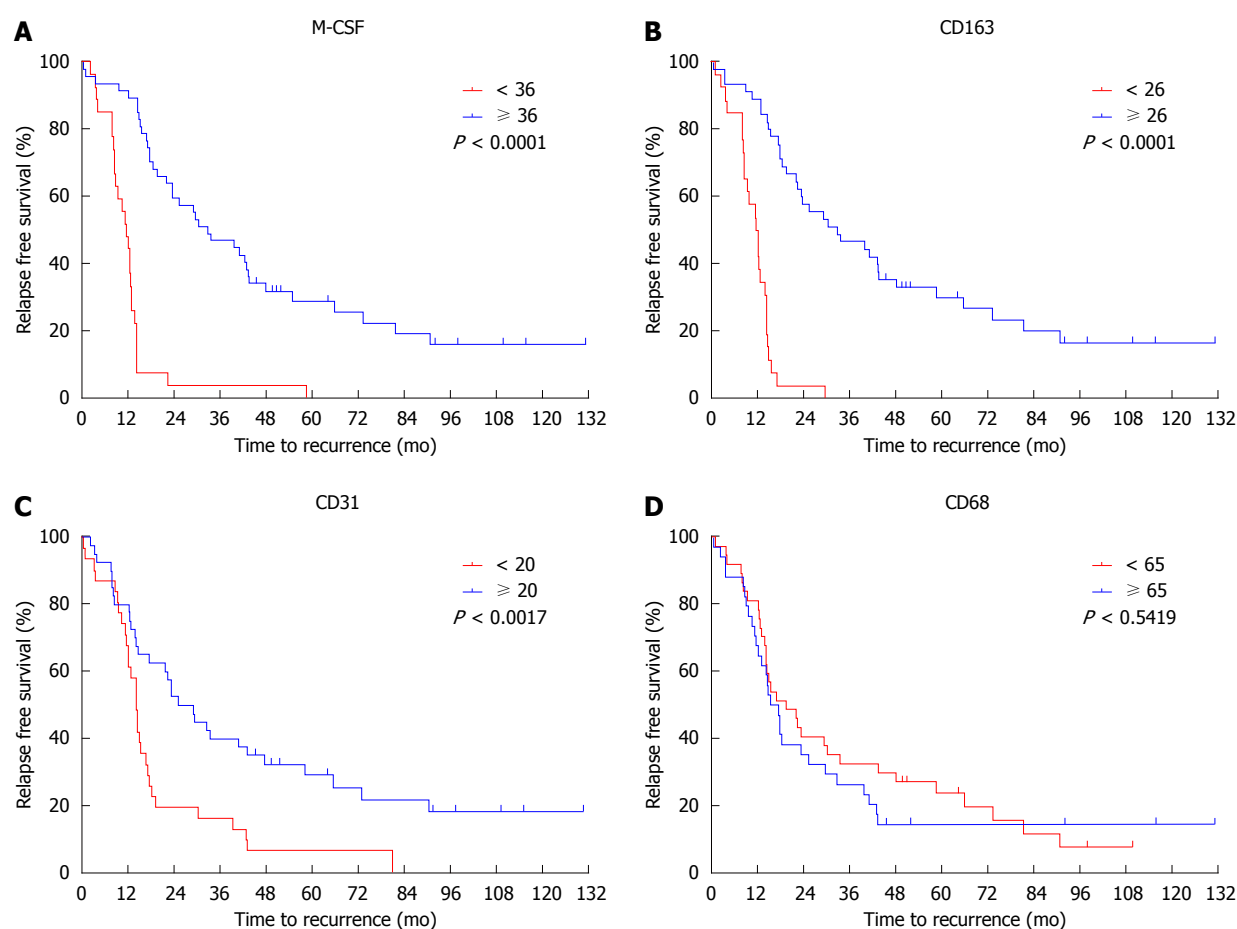


Figure 2 Cumulative disease-free survival curves of patients with high or low peritumoral features. Disease free survival curves of patients with high or low expression of CD68, CD163, CD31 and macrophage colony-stimulating factor (M-CSF) in peritumoral liver tissues are shown. A: M-CSF; B: CD163; C: CD31; D: CD68. The intratumoral features were not associated with disease-free survival.

endothelial cells) was performed as described in the Patients and Methods section. A positive correlation was observed between the expression of M-CSF and CD31 in the peritumoral liver tissues, but not between M-CSF and CD163 (Figure 4), or CD68 (data not shown).

To determine the cutoff value of the M-CSF positive area, an ROC curve was drawn using recurrence within one year. Patients with a high expression of CD31 or CD163 in peritumoral tissues showed significantly poorer DFS compared with patients with low expression of these markers (Figures 2 and 3). Furthermore, patients with high expression of CD163 in peritumoral tissues showed significantly poorer OS compared with patients with low expression of these markers. Although positive staining for CD68 was detected in peritumoral tissues, the number of CD68-positive cells was not correlated with DFS (Figure 2) or OS (data not shown).

Prognostic factors

According to the ROC curve, the ideal cutoff value of the densities of M-CSF, CD163, and CD31 were 36, 26, and 20, respectively. The median DFS was 12.3 mo for patients with high expression of M-CSF in peritumoral

tissues, which was significantly shorter compared with the median DFS for patients with low expression of M-CSF (43.3 mo). Furthermore, the median survival times for the high CD163 density and the low density group were 12.2 and 32.0 mo, respectively. The median survival times for the high CD31 density and the low density group were 13.6 and 37.3 mo, respectively.

M-CSF density, CD163 index, and CD31 index in peritumoral tissues are predictable factors for prognosis in patients with HCC

The univariate analysis of the prognostic value for various factors assessed in the present study is shown in Table 2.

In clinicopathologic factors, the size of the tumor, the clinical disease stage, serum ALT and AFP levels are useful to assess recurrence of HCC after surgery. In the present study, there were significant differences in the univariate analysis of DFS between the UICC-TNM Stages (Stage I vs Stages II and III) (Table 2). Furthermore, there were significant differences in the univariate analysis of OS between the tumor number (single vs multiple) and the fibrosis score (1-3 vs 4). In multivariate analysis of the prognostic power, the

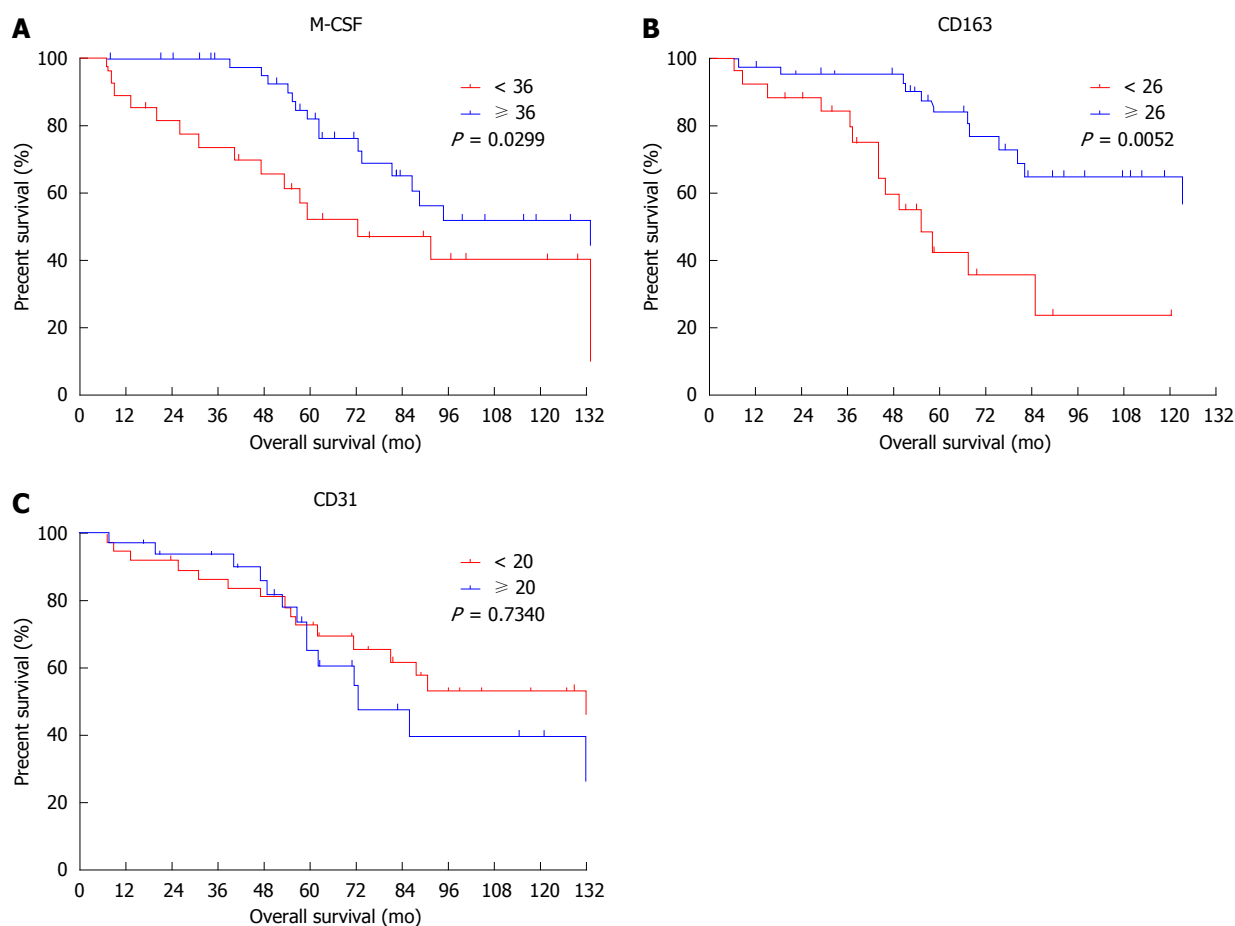


Figure 3 Cumulative overall survival curves of patients with high or low macrophage colony-stimulating factor expression in peritumoral features. Overall survival curves of patients with high or low expression of macrophage colony-stimulating factor (M-CSF) in peritumoral liver tissues are shown. A: M-CSF; B: CD163; C: CD31. The intratumoral features were not associated with overall survival.

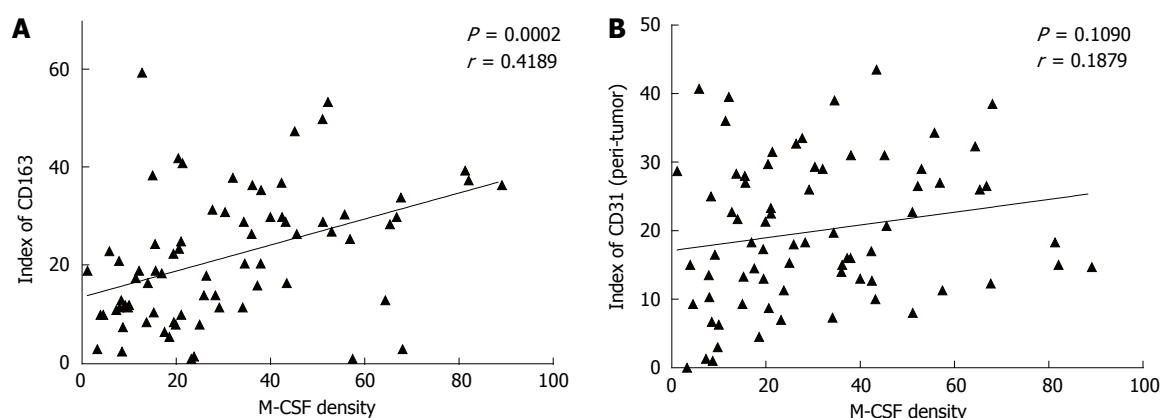


Figure 4 Correlation between the expression of macrophage colony-stimulating factor and CD163, or CD31 in the non-tumoral liver tissues. Immunohistochemical staining for macrophage colony-stimulating factor (M-CSF), CD163, and CD31 was performed in non-cancerous liver tissue collected from patients as described in detail in the Patients and Methods section. A (between M-CSF and CD163) and B (between M-CSF and CD31) show correlations between markers assessed in this study in each of the patients with HCC.

fibrosis score was a factor for predicting the length of overall survival in patients with HCC (Table 2).

M-CSF density, CD163 index, and CD31 index in peritumoral liver tissues appear to be risk factors for DFS by univariate analysis (Table 2). When each of these markers was included in the multivariate analysis, M-CSF density and CD163 index were

found to be the only significant predictors for DFS. Alternatively, peritumoral M-CSF density, CD163 index, and CD31 index appear to be risk factors for OS by univariate analysis. Furthermore, when each of these markers was included in the multivariate analysis, the CD163 index was found to be the only significant predictor for OS.

Table 2 Univariate and Multivariate analyses of factors associated with recurrence and survival

Factors	Time to recurrence				Overall survival			
	Univariate	Multivariate			Univariate	Multivariate		
	P value	Hazard ratio	95%CI	P value	P value	Hazard ratio	95%CI	P value
Age: ≥ 60 yr <i>vs</i> < 60 yr	0.240			NA	0.523			NA
Gender: female <i>vs</i> male	0.760			NA	0.111			NA
Indx of α -SMA: ≥ 12 <i>vs</i> < 12	0.751			NS	0.423			NS
Indx of CD163: ≥ 26 <i>vs</i> < 26	< 0.001	4.517	1.879-10.857	0.001	< 0.001	1.086	1.039-1.135	< 0.001
M-CSF density: ≥ 36 <i>vs</i> < 36	< 0.001	4.826	2.045-11.389	< 0.001	0.037			NS
Indx of CD31: ≥ 20 <i>vs</i> < 20	0.002			NS	0.020			NS
AFP: ≥ 100 <i>vs</i> < 100	0.232			NA	0.785			NA
AFP-L3%: ≥ 10 <i>vs</i> < 10	0.827			NA	0.900			NA
PIVKA-II: ≥ 100 <i>vs</i> < 100	0.841			NA	0.325			NA
Tumor size: ≥ 2 cm <i>vs</i> < 2 cm	0.185			NA	0.847			NA
Tumor number: multiple <i>vs</i> single	0.447			NA	0.038			NS
Tumor differentiation: well <i>vs</i> mod. <i>vs</i> poor	0.379			NA	0.745			NA
Fibrosis score: 1, 2, 3 <i>vs</i> 4	0.755			NA	0.015			0.002
TNM stage: I <i>vs</i> II <i>vs</i> IIIa	0.020			NS	0.173			NA

Tables are shown univariate and multivariate analysis of prognostic markers associated with the disease-free and the overall survival.

Production of VEGF by isolated Kupffer cells and monocytes from mice

The production of VEGF by isolated KCs increased in a dose- and time-dependent manner after incubation with M-CSF in media (Figure 5). Furthermore, the production by isolated monocytes also increased in a time dependent manner by stimulation with M-CSF (Figure 5). Although the production of VEGF increased in both isolated KCs and monocytes incubated with M-CSF, production was significantly greater in the KC compared with the monocytes (Figure 5).

Proliferation of isolated vascular endothelial cells isolated

Cell proliferation of isolated VECs markedly increased in cells treated with M-CSF compared with those that were not treated with M-CSF in media (Figure 6). Importantly, among the groups studied, the proliferation was greatest in the M-CSF-treated VECs co-cultured with the KCs.

DISCUSSION

M-CSF is involved in progression of hepatocellular carcinoma after curative resection

It was previously reported that overexpression of M-CSF is observed in tumor tissues in various human cancers and is related with poor survival^[20-25]. It was also reported that M-CSF is predominantly detected in peritumoral liver tissues^[8]. Furthermore, it was reported from this laboratory that the incidence of chemically-induced HCC was reduced in M-CSF deficient mice (KO) compared with their littermates (WT) by inhibiting the expression of M2 M ϕ and angiogenesis in peritumor liver tissues^[26]. In the present study, the high expression of M-CSF in peritumoral tissues was correlated with a high incidence of HCC, including MC recurrence and IM,

and poor prognosis after curative resection (Figures 2 and 4), consistent with the previous report^[11]. Thus, M-CSF is involved in hepatic carcinogenesis and its progression. Based on these reports, this study investigated whether M-CSF induces hepatic carcinogenesis, most likely by enhancing angiogenesis by M-CSF-induced hepatic macrophages.

In the present study, expression of M-CSF differed in intratumoral tissues and peritumoral liver tissues, suggesting that the expression level of M-CSF differs in each individual. Importantly, the level of M-CSF expression did not correlate with any pathophysiologic factors. Therefore, the expression of M-CSF may be associated with other genetic factors in each patient^[11]. Indeed, Okamoto *et al.*^[27] reported that a specific gene profile in non-tumoral tissue predicted MC recurrence or IM of HCC. Alternatively, placental^[28,29] and endothelial cells^[30] excreted M-CSF under hypoxic conditions. Based on this result, distribution of M-CSF may be caused by hypoxia in hepatocytes due to compression by the primary tumor. A tumor itself also enhances acute and chronic inflammation, causes cytokine production and attracts M ϕ s to peritumoral liver tissues. The previous study indicated that implanted or spontaneous tumors induced vascular endothelial growth factor in peritumoral tissue that was much greater than in the intratumoral tissue^[31]. Furthermore, angiogenesis was greater in peritumoral tissues than in the intratumoral tissues in chemically-induced HCC in mice^[26]. Taken together, the expression of M-CSF in peritumoral hepatocytes may be associated with the genetic heterogeneity in each individual or inflammatory factors in the liver.

Role of the hepatic microenvironment in initiation and progression of HCC

The high rate of IM and MC recurrence after complete resection indicated that the non-tumoral hepatic

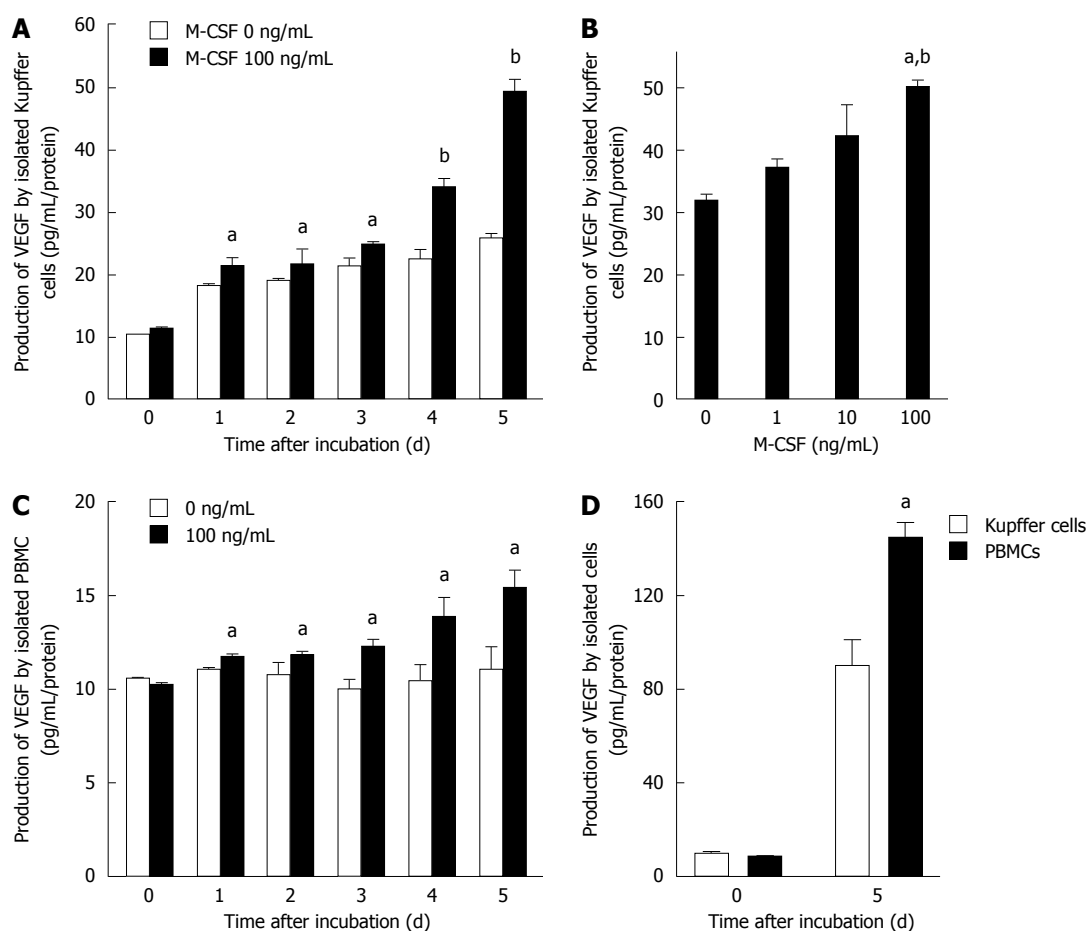


Figure 5 Production of vascular endothelial growth factor by isolated Kupffer cells and peripheral blood monocytes. Production of vascular endothelial growth factor (VEGF) by isolated Kupffer cells and peripheral blood monocytes was determined as described in the Methods. A: Kupffer cells were cultured with or without macrophage colony-stimulating factor (M-CSF) in media for designated experimental periods ($n = 6$). $^aP < 0.05$ vs with Kupffer cells without M-CSF stimulation; and $^bP < 0.01$ vs with Kupffer cells without M-CSF stimulation by ANOVA with Bonferroni's post-hoc test; B: Kupffer cells were treated with different doses of M-CSF in media, and cultured for 5 d ($n = 6$). $^aP < 0.05$ vs with Kupffer cells without M-CSF stimulation; and $^bP < 0.01$ vs with Kupffer cells with 10 ng/mL of M-CSF in media by ANOVA with Bonferroni's post-hoc test; C: Peripheral blood monocytes were isolated and were cultured for designated experimental periods. The concentration of VEGF in media was then determined as described in the Methods ($n = 6$). $^aP < 0.05$ vs with peripheral blood monocytes without M-CSF stimulation by ANOVA with Bonferroni's post-hoc test; D: Isolated Kupffer cells or PBMC were incubated with 100 ng/mL of M-CSF in media for 5 d. The concentration of VEGF was determined as described in Materials and Methods ($n = 6$). $^aP < 0.05$ vs with PBMC by ANOVA with Bonferroni's post-hoc test.

microenvironment plays a key role in tumor initiation and progression. Previous investigations of causes of HCC were mainly focused on factors of tumor initiation as "seeds." On the other hand, few studies that focus on hepatic microenvironmental factors as "soil" were reported. Indeed, Ezaki *et al.*^[32,33] reported that the hyper expression of thymidine phosphorylase in the peritumoral liver tissues was correlated with the higher incidence of postoperative recurrence of HCC. Furthermore, Yu *et al.*^[34] reported that vascular density was higher in peritumoral tissues compared with that in intratumoral tissue, leading to increased expression of VEGF and hypoxia inducible factor-1 in peritumoral liver tissues. Moreover, it was reported that immunological features in the peritumoral tissue predicted venous metastases in HCC^[35]. In this study, the high density of M-CSF in peritumoral liver tissues was correlated with a high incidence of HCC and prognosis, consistent with the previous report^[11] (Figures 2 and 3). Taken together, it is concluded

that the peritumoral microenvironment, as "the soil," is also important in understanding the mechanism of incidence of HCC. Furthermore, previous studies and this study suggest that postoperative adjuvant therapies could target not only the subclinical carcinoma cells, but also the microenvironment in the liver.

Role of M-CSF induced macrophage in the progression of HCC

The union of M-CSF expression, population of M2M ϕ s and angiogenesis in peritumoral tissues had a better predictable power for prognosis in HCC (Figures 2 and 3, and Table 2). The number of M ϕ s was greater in peritumoral tissue than in intratumoral tissue, consistent with results in previous reported studies^[35,36]. In the present study, expression of M-CSF, M2M ϕ s and angiogenesis in the peritumoral liver tissue was correlated with DFS after surgery (Figure 2). The role of microenvironments in intratumoral tissues and

Group	1	2	3	4
M-CSF (100 ng/mL)	-	+	-	+
KC (2×10^5 cell/well)	-	-	+	+
VEC (1.5×10^5 cell/well)	+	+	+	+

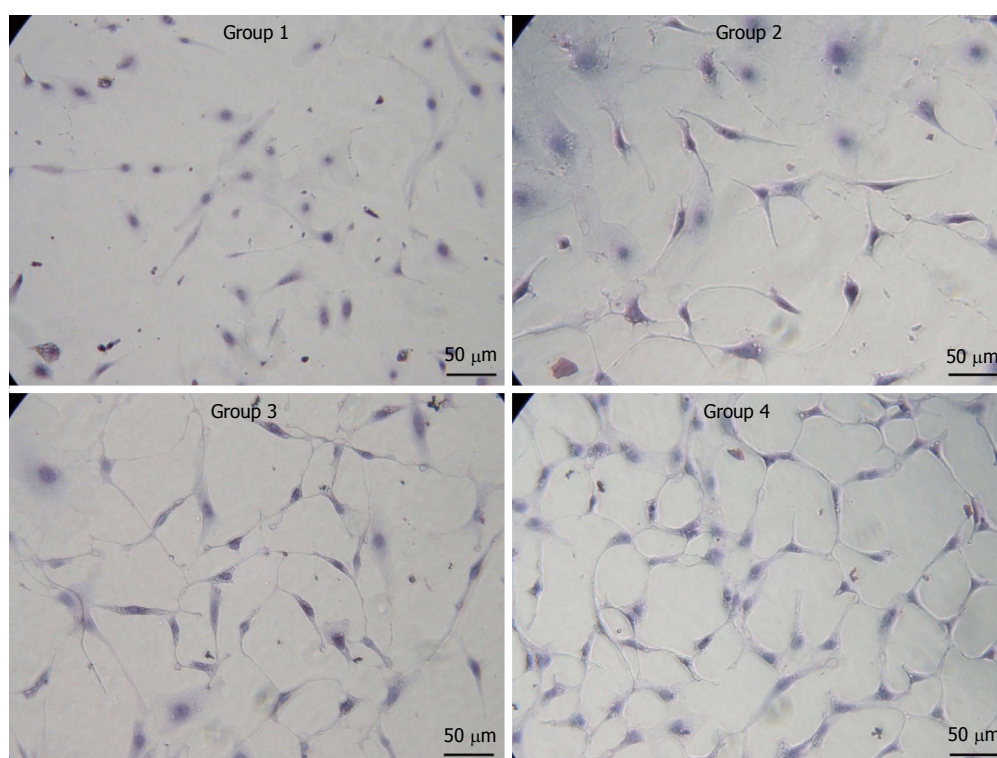


Figure 6 Proliferation of isolated vascular endothelial cells. Isolated vascular endothelial cells (VECs) were co-cultured with or without the isolated Kupffer cells in media containing of macrophage colony-stimulating factor (M-CSF). Cell proliferation of VECs was determined as described in the Patients and Methods ($n = 5$). Treatments of each group are shown in the table. Representative photomicrographs are shown. Original magnification, $\times 400$.

peritumoral tissues may be different in initiation and progression of HCC. Expression of M-CSF, CD31 and M2M ϕ s in intratumoral tissues may be involved in promoting the dissemination of the cancer cells^[37-39]. M-CSF and M2M ϕ s in the peritumoral tissues are involved in accelerating colonization and growth of disseminated tumor cells, leading to micrometastasis, since M-CSF and M2 M ϕ in the tumor is eliminated by operation. Therefore, as the defense to inhibit growth of the tumor, the peritumoral liver tissue, which supplies M-CSF to the tumor^[31], plays a pivotal role by providing a fruitful soil for micrometastasis of HCC as "seeds". The role of the M ϕ s in providing a fertile soil as the metastasis niche has also been reported^[40]. In this study, the number of M2M ϕ s in the peritumoral tissues was correlated with the expression of M-CSF in the peritumoral tissues and was linked with DFS and OS (Figures 2 and 3). Thus, M-CSF-induced M2M ϕ s may be involved in DFS. In addition to this result, the number of M2M ϕ s and the expression of angiogenic factors were also positively correlated. Importantly, VEGF production by isolated hepatic M ϕ was increased by M-CSF stimulation (Figure 5) and proliferation of isolated VEC was greatest in the cells incubated with M-CSF-stimulated hepatic M ϕ in the presence of M-CSF

in media (Figure 6). Thus, M-CSF-induced M2M ϕ s could be involved in progression of HCC by inducing angiogenesis.

Clinical applications and conclusion

Undoubtedly, M-CSF and M2M ϕ could be suitable targets for adjuvant therapy after surgery, since in the present study the expression of M-CSF and the number of M2M ϕ s increased in the peritumoral tissues, hepatic M ϕ s incubated with M-CSF in media produced VEGF, and the proliferation was greatest in the M-CSF-treated VECs co-cultured with the KCs among the groups studied. Thus, this study indicates that M-CSF expressed in the peritumoral liver tissue could predict the recurrence of patients with HCC after surgery and also indicates that the remnant liver may play an important role in recurrence and metastasis. Therefore, evaluation of M-CSF is a useful result that can be easily assessed in specimens collected during surgery.

COMMENTS

Background

Expression of macrophage colony-stimulating factor (M-CSF) was associated

with hepatocellular carcinoma (HCC) progression, disease recurrence, and poor survival after hepatectomy, highlighting the importance of the peritumoral tissue environment in the intrahepatic recurrence of HCC.

Research frontiers

In this study, the authors investigated the relationship between the M-CSF-matured hepatic macrophage and the prognosis of HCC after resection.

Innovations and breakthroughs

In this manuscript, the major point is that vascular endothelial growth factor derived from the M-CSF-matured hepatic macrophage plays a pivotal role in the prognosis of HCC.

Applications

M-CSF or its receptor could be a new molecular target for therapy against HCC.

Terminology

The present study indicates that M-CSF expressed in peritumoral liver tissues can predict recurrence in patients with HCC after surgery and also implies that the remnant liver may play a key role in recurrence and metastasis. Therefore, evaluation of M-CSF is a useful result that can be easily assessed in specimens collected during liver tumor resection.

Peer-review

In this paper, the authors aimed at investigating the role of M-CSF in patients with HCC after resection. They analyze the prognostic value of MCSF, CD163, CD31 and other clinicopathologic parameters for patients' disease-free survival (DFS) with HCC and find M-CFS in non-cancer tissues can act as a predictor for patients DFS after resection.

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