Basic Investigations

CO-EXPRESSION OF MACROPHAGE COLONY-STIMULATING FACTOR WITH ITS RECEPTOR IN HUMAN HEPATOMA CELLS AND ITS POTENTIAL ROLES

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ABSTRACT

Objective: To investigate the potential role of macrophage colony-stimulating factor (M-CSF) and macrophage colony-stimulating factor receptor (M-CSF-R) on the growth of human hepatoma cells. Methods: Specimens of different origin, including tissues of human hepatocellular carcinoma (HCC), human fetal liver (FL) and normal liver (NL), the hepatoma cell lines, as well as the peripheral blood mononuclear cells (PBMC) from patients with HCC or liver metastatic tumor (LMT), were used to detect the expression levels of M-CSF and M-CSF-R by ABC immunohistochemistry staining and reverse transcription polymerase chain reaction methods the expression levels of M-CSF and M-CSF-R. Influence of monoclonal antibody against M-CSF (B5) or M-CSF-R (RE2) on proliferation ability of hepatoma cell lines *in vitro* was also studied. Results: The results showed that hepatoma tissues produced elevated levels of both M-CSF and M-CSF-R compared with those of fetal liver ($P<0.001$). The M-CSF/M-CSF-R expression levels of PBMC from hepatoma patients were higher than those of LMT patients ($P<0.01$, $P<0.05$) and the normal people ($P<0.001$). The hepatoma cell lines showed strong positive for M-CSF and M-CSF-R production. Both B5 and RE2 displayed a dose-dependent inhibitory effect on the growth and proliferation of hepatoma cells. Conclusion: The study indicates a co-expression model for M-CSF-R in hepatoma cells, suggesting an involvement of M-CSF/M-CSF-R in growth signaling of those malignant cells. The M-CSF/M-CSF-R seems to function through an autonomy mechanism in human hepatoma.

Key words: Macrophage colony-stimulating factor (M-CSF), Macrophage colony-stimulating factor receptor (M-CSF-R), Hepatoma, Co-expression, Autocrine

Recently, the anti-cytokine therapy and anti-receptor therapy is being identified as an efficacious treatment in the field of biological therapy against cancer and related diseases.[1] Pathogenesis of hepatoma is quite complicate in which multi-factors, multi-genes and multi-processes are involved. Activation of certain oncogenes, inactivation of some tumor suppresser genes, or abnormal expression of cancer related genes might contribute to the development of the tumor. The *N-ras, c-myc, p53, c-fms* and IGF-2/IGF-2R are recognized as oncogenes or cancer related genes of primary liver neoplasms. Macrophage colony stimulating factor (M-CSF), a cytokine derived from monocyte/macrophage lineage of hematopoietic system and so nominated, has been proved recently to be associated with several nonhematopoietic disorders. The receptor of the

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cytokine (M-CSF-R) is encoded by the proto-oncogene *c-fms*, which contains a cytoplasmic protein tyrosine kinase domain. A few investigators confirmed the association between M-CSF/M-CSF-R and gynecologic malignancies. Yang, et al. detected a higher level of M-CSF-R production in human hepatoma tissue than in the normal liver or fetal liver tissue.[2] Inhibitory effect of an anti-serum against M-CSF-R on the growth of hepatoma cells has been documented by their colleagues thereafter.[3] We investigated previously that not only anti-M-CSF-R monoclonal antibody (MAb), but also anti-M-CSF MAb could inhibit the growth and proliferation of human hepatoma cells transplanted in nude mice,[4] suggesting an abnormal expression of M-CSF/M-CSF-R in those malignant cells. However, little is known about the expression status of M-CSF in hepatoma; the mechanism through which M-CSF and its receptor may confer their influence on the growth of those malignant cells is unclear. The present study was proposed to determine M-CSF/M-CSF-R expression level in hepatoma cells and tissue, and to analyzed the influence of antibodies against M-CSF and M-CSF-R on the proliferation of hepatoma cell lines, in hopes of elucidating the potential biological roles of these molecules in the pathogenesis of hepatoma.

MATERIALS AND METHODS

Clinical Samples and Cell Lines

All clinical specimens were obtained from Tangshan Worker's Hospital, Tangshan, Hebei, and the First Central Hospital, Tianjin. A total of 28 clinical specimens, include 8 tissues of hepatocellular carcinoma (HCC) tissue, 15 samples of peripheral blood mononuclear cells (PBMC) from HCC patients, and 5 PBMC samples from patients with liver metastatic tumor (LMT), 20 males and 8 females, ages from 24 to 74 years old. All patients were diagnosed and confirmed by routine histopathological examination. Twelve normal PBMC samples were collected from healthy donors in our institute, whereas 3 tissue specimens of normal human liver (NL) were collected from healthy individuals who underwent liver trauma resulted from accidents. Four samples of human fetal liver (FL) were obtained from normally induced abortions (20 ± 5 weeks old). Three human hepatoma cell lines, SMMC-7721, BEL-7402 and HOS-8603, were kindly provided by Professor Jun-Kui Zhang at our institute, and subcultured in our lab.

Tissue samples were collected after surgery and immediately frozen in liquid nitrogen. Prior to immunohistochemistry assay, frozen sections were prepared with a cryostat (Bright, England) at -20°C, dried at room temperature, and fixed with acetone. The PBMC were routinely isolated and the slides were prepared with a cytospinner (Shannon, England).

Immunohistochemical Staining

The ABC immunohistochemical assay was carried out according to the protocols we described before.[5] Anti-M-CSF MAb (B5)[6] and anti-M-CSF-R MAb (RE2)[7] were prepared in our lab. The second antibody, a goat anti-mouse IgG labeled with biotin, was purchased from Vector Co., USA. Two hundred cells were counted and the intensity of staining for each of those cells was adjusted. Five grades, –, +, ++, +++ and ++++ were employed to express the degrees of staining, which represent 5 reaction coefficient respectively, *i.e.* 0, 1, 2, 3 and 4. The 5 products of every coefficient and the corresponding cell number were added up, which resulted in the value of a positive score. All slides were measured in duplicate. Those samples with a positive score over 10 or frequency over 5% were considered as positive.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Two pairs of M-CSF primers, 5' GCGAATTCGGACCAGCTGCCCCGTATG 3'/5' GTGTGGACAAGATGCTGAGAGGAAG 3' and 5' AGAGCCCGCCAGACCCAGCAACTT 3'/5' CGG-GATCCCCCCAGGGCTCACAATAAA 3' (p1/p2, p3/p4), and one pair of M-CSF-R primers 5' ACTT-CCCCACCGAGGCCAATG 3'/5' CGGAAGAACA-TGGAGGT G GAG 3' (p5/p6), were designed according to literature,[8,9] and were synthesized in Sangon Co. (Shanghai). The RT-PCR was carried out as we described before[10] with slight modifications. For tissue samples , frozen sections of 10—15 μm thickness were prepared at -20°C. About 50 sections of each sample were transferred into a glass homogenizer, followed by homogenizing with an adequate volume of denaturing solution at 4°C. Total RNA was isolated using an acid guanidinium thiocyanate-phenol-chloroform extraction method (Gibco). The first strand of M-CSF/M-CSF-R cDNA was reversedly transcribed with its lower primer respectively. Two micrograms of RNA were reverse transcribed in a 25 μl-system containing 5 μl 5 × buffer (Gibco), 2 μl 0.1 M DTT (Gibco), 2 μl 10 mM dNTP (Tianxiangren Co.), 25U Rnasin (Sino-American Co.), 50 pmol primers and 200U MMLV reverse transcriptase (Gibco), incubated at 37°C for 2 hours. The PCR system included 10 × PCR buffer with 15 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 30 pmol of each primer of M-CSF or M-CSF-R, 5U Taq DNA polymerase (Gibco) and 1 μg of template cDNA. PCR was performed for 35 cycles, denatured

at 94°C for 30 sec., annealed at 58°C (M-CSF-R) or 60°C (M-CSF) for 40 sec., and followed by extending at 72°C for 90 sec. The amplified DNA products were analyzed on 1.5% agarose gel. For each PCR assay, over 0.05 g tissue or 5×10^6 cells were used.

Inhibition Test of B5 and RE2 on Hepatoma Cells

Hepatoma cells were grown in RPMI 1640, 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, in a humidified 5% CO_2 atmosphere at 37°C. Well growing cells were digested off with 0.02% EDTA and seeded into a 24-well plate (Nunc, Denmark) in 2×10^4/ml. B5 or RE2 in a serial titers, 1:16, 1:12, 1:8 and 1:4, was added to the culture after the cells adhered completely. The cells were incubated at 37°C for 96 hours thereafter. We then harvested the cells followed by counting the viable cells with trypan blue exclusion. Each titer of B5 and RE2 was determined duplicated. The blank control was performed with PBS instead of MAbs. Inhibition ratio of the antibodies was calculated as follows.

$$\text{Inhibition ratio (\%)} = \frac{\text{Viable cells incubated with PBS} - \text{Viable cells incubated withB5 or RE2}}{\text{Viable cells incubated with PBS}} \times 100\%$$

Statistics

M-CSF and M-CSF-R expression levels resulted from the immunohistochemistry assay were expressed as $\bar{x} \pm s$ and were subjected to an unpaired student's t test.

RESULTS

Elevated expression Levels of M-CSF/M-CSF-R in HCC Cells

Eight hepatoma tissues produced increased levels of M-CSF and M-CSF-R compared with those of HFL ($P<0.05$, $P<0.01$) or those of NL ($P<0.001$), with frequency and positive score as 52.2% and 63.4, and 47.7% and 65.8 respectively. The mRNA of M-CSF and M-CSF-R were detectable in 6/8 (75.0%) and 7/8 (87.5%) of the cases. For the fetal liver, positive reaction for both M-CSF and M-CSF-R expression were moderate, with mRNA frequency as 25.0% and with protein frequency as 26.3% and 19.9% respectively. Three normal liver tissues expressed both M-CSF and its receptor weakly, with protein frequency only as 13.8% and 8.4% respectively (Table 1). Strong positive reactions were observed in all hepatoma cell lines: The frequency of M-CSF and M-CSF-R expression exceeded 80% while mRNA frequency for these molecules reached 100%.

Table 1. M-CSF/M-CSF-R expression in cells or tissues of different origin ($\bar{x} \pm SD$)

		M-CSF		M-CSF-R	
	Cases	Frequency(%)	Score	Frequency(%)	Score
PBMC-HCC	15	44.6 ± 9.5**a	66.3 ± 11.0**a	52.4 ± 9.7*a	71.2 ± 14.5*a
PBMC-LMT	5	31.1 ± 7.0	49.9 ± 7.6	40.6 ± 8.2	55.7 ± 9.4
PBMC-Normal	12	<5	<10	<5	<10
HCC cell line	3	85.6 ± 15.6	95.6 ± 20.0	82.8 ± 7.6	103.1 ± 23.5
Tissue-HCC	8	52.2 ± 23.0*a	63.4 ± 25.0*a	47.7 ± 9.7**a	65.8 ± 16.7**a
Tissue-FL	4	26.3 ± 23.8	30.1 ± 25.6	19.9 ± 19.5	23.0 ± 21.9
Tissue-NL	3	13.8 ± 9.4	17.8 ± 9.2	8.4 ± 2.0	12.0 ± 3.2

* $P<0.05$, PBMC-HCC compared with PBMC-LMT; Tissue-HCC compared with Tissue-FL.

** $P<0.01$, PBMC-HCC compared with PBMC-LMT; Tissue-HCC compared with Tissue-FL;

a $P<0.001$, PBMC-HCC compared with PBMC-Normal; Tissue-HCC compared with Tissue-NL.

Expression Status for M-CSF/M-CSF-R in PBMCs of Different Origin

The M-CSF and M-CSF-R expression levels for PBMC from 15 hepatoma patients were higher than that of LMT patients ($P<0.01$, $P<0.05$), and were much higher than those of normal donors ($P<0.001$). Eight PBMC samples were randomly selected from those 15 hepatoma patients and subjected to RT-PCR, which resulted in mRNA frequency of M-CSF and M-CSF-R as 4/8 (50.0%) and 5/8 (62.5%) respectively. The results of immunohistochemistry assay are listed

in Table 1.

Localization of M-CSF and M-CSF-R on HCC Cells

The M-CSF located mainly on cell membrane or in cytoplasm, except for 2 HCC tissues and the hepatoma cell line SMMC 7721, in which nuclei-staining was dominant. However, M-CSF-R appeared dominantly on the membrane.

B5 and RE2 Effects on Proliferation of HCC Cells *in vitro*

As shown in Figure 1 and Figure 2, B5 and RE2 displayed an inhibitory effect on growth of the 3-hepatoma cell lines; the inhibition was of dose-dependence. Comparison between cell lines for its inhibition ratio: cell line 7721> cell line 7402> cell line 8603. After having been incubated for 48 hours with B5 or RE2 in 1:4 titer, 7721 and 7402 cells appeared less adhesive, with more non-adhered cells presented in the medium.

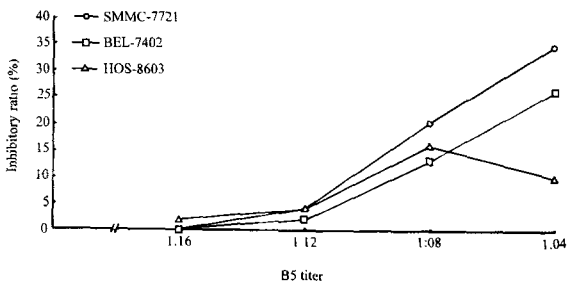

Fig. 1. Inhibitory effect of M-CSF MAb (B5) on proliferation of hepatoma cell lines

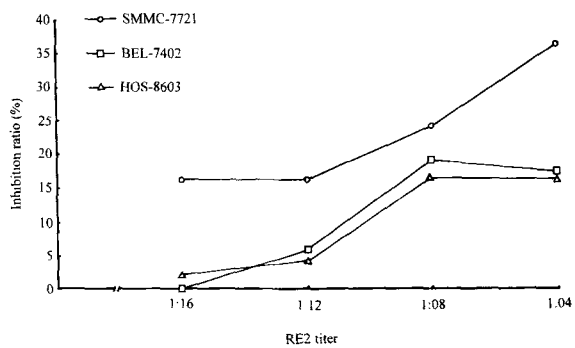

Fig. 2. Inhibitory effect of M-CSF-R MAb (RE2) on proliferation of hepatoma cell lines

DISCUSSION

Studies of the physiologic role of M-CSF demonstrated that, although M-CSF is required for survival, proliferation and differentiation of monocytic lineage, this cytokine is also associated with certain nonhematological diseases, such as gynecologic malignancies,[11-14] atherosclerosis and chronic renal failure.[15] M-CSF-R is the product of proto-oncogene *c-fms*, over activation of which results in accelerating the process of cell transformation and tumor growth through a tyrosine kinase autophosphorylation mechanism. An increased expression level of M-CSF-R was observed in human hepatoma tissues, with 1.68 and 6.09 folds higher than the para-hepatoma tissues and normal liver tissue respectively,[2] suggesting a close association between overproduction of the receptor and malignant transformation of the liver cells or growth of hepatoma cells. This paper showed that hepatoma tissues and cells produced higher levels of not only M-CSF-R but also M-CSF than human fetal liver tissues and normal liver tissues. To a great extent, activation of M-CSF-R depends on the specific binding of M-CSF. Simultaneous increase of M-CSF/M-CSF-R expression in hepatoma cells implies the two molecules may function in a synergetic way in hepatoma cells. Co-expression of M-CSF and *c-fms* in tumor cells of gynecologic malignancies has been frequently investigated.[11-13] Most tissues (70.4%) of primary gynecologic malignancies, but all of the metastatic tumors, were proved to express both M-CSF and *c-fms*, suggesting M-CSF and its receptor may participate in the development or progression of the tumors.[12] It is suggested therefor that, M-CSF and M-CSF-R may influence the growth of both hepatoma and gynecologic malignancies through the same mechanism. Interestingly, in this report, we found a predominant nuclear staining pattern for M-CSF in two hepatoma patients; these two patients showed an increased risk of metastasis and a poor survival (died with metastasis within 3 and 5 months respectively) compared with the others, as the follow-up indicated. This finding is exactly consistent to the literature,[13] in which an association between nuclear staining of M-CSF and poor prognosis of the breast cancer was described. The mechanism of this association remains to be clarified, however, we are undertaking further studies in characterizing the biological role of intracellular M-CSF in tumorigenesis.

M-CSF is physiologically produced by cells of different origin, including bone-marrow stroma cells, lymphocytes, endothelial cells, and activated monocytes/macrophages, etc. Human M-CSF has been recognized to influence immunological activities of mature macrophages, including tumor cytotoxicity, production of TNF-α and chemotactic activities for monocytes. Recently, Itoh et al. have observed a higher level of serum M-CSF in HCC patients than chronic hepatitis patients and normal people, but the

monocyte count of these patients did not increase,[16] which puzzled Itoh and colleagues. In an attempt to work out this divergence and explore the immunological function of M-CSF on hepatoma patients, we measured PBMC of HCC patients and LMT patients for M-CSF/M-CSF-R expression levels. The results showed that PBMC of HCC patients produced higher levels of M-CSF/M-CSF-R than that of LMT patients, while the expression levels of these patients were significantly higher than that of normal people. These data indicated that M-CSF/M-CSF-R expressed in PBMC of HCC patients might associate the immunological state of the patients, suggesting that over expressed M-CSF in PBMC of HCC patients contribute to the elevated serum M-CSF level.

It has been investigated that antiserum against two peptide fragments of M-CSF-R could inhibit growth of human hepatoma cells.[3] We have discovered recently that, when the nude mice transplanted with human hepatoma cells were injected with MAbs specific to M-CSF and M-CSF-R, growth of these tumor cells *in vivo* could be inhibited by 74.9% and 77.9% respectively.[4] This paper showed a dose-dependent inhibitory effect of those antibodies on the growth of hepatoma cells. When used in high titer (*i.e.* 1:4), this pair of MAbs may influence the adhesion ability of the cells. These data implied a crucial role in hepatoma growth or a possible role in maintaining the migratory behavior of the malignant cells played by M-CSF and its receptor, and a potential tumoricidal activity of the two antibodies in tumor biological therapy.

Autocrine and paracrine are considered as the dominant form of secretion by which the tumor cells are enabled to escape the immune attack of the host and grow in an autonomy way.[17] The IGF-2/IGF-2-R has been suggested to be the first pair of autocrine/paracrine system in hepatoma.[18] Based on the results of this study and our previous findings, conclusions or deductions may be drawn as following: a) There exist high levels of M-CSF/M-CSF-R in hepatoma cells; b) Antibodies for M-CSF/M-CSF-R display a inhibitory effect against hepatoma *in vivo*, and c) M-CSF/M-CSF-R may be considered as a novel autocrine/paracrine system in hepatoma cells. These data suggest that this pair of signal molecules may be involved in intercellular signal transduction pathways, through which proliferation and malignant phenotype transformation of the tumor cells were modulated. In earlier studies, we put forward a juxtacrine mechanism in leukemia cells, through which the membrane bound M-CSF (mM-CSF) specifically binded to its receptor and the behavior of the leukemic cells was under modulate.[19,20] Latterly, a receptor-like role of the mM-CSF had been demonstrated in our lab.[21] The present experiment shows that the majority of the M-CSF expressed in hepatoma cells were of membrane-bound isoform, implying an involvement of juxtacrine modulation mediated by M-CSF/M-CSF-R in hepatoma growth. The mechanism of M-CSF/M-CSF-R secretion in hepatoma cells remains to be clarified. Further studies on M-CSF/M-CSF-R secretion system will be helpful in elucidating the association between M-CSF/M-CSF-R and pathogenesis of hepatoma, and in exploring the feasibility of the two McAbs for their clinical applications in biological therapy against hepatoma.

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REFERENCES

[1] Wu KF. Treatment with anticytokines and soluble receptors. Chin J exp Hematol 1996; 4:1.

[2] Yang DH, Liu /ww, Gu JR, et al. Expression of gene products of IGF-II receptor and CSF-1 receptor in human primary hepatocellular carcinoma and non-cancerous liver tissues. Natl Med J China.

[3] Ma AQ, Xu XL, Qu SM, et al. Biological effects of antibodies against CSF-1 receptor (*fms*) on the growth of human hepatoma cells. Tumor 1994; 14:72.

[4] Qian LF, Wu KF, Geng YQ, et al. Biological effects of monoclonal antibodies against CSF-1 and CSF-1-R on human hepatic cancer cells transplanted in nude mice. Tumor 1997; 17: 207.

[5] Song YH, Qi SL, Li LQ, et al. Studies on micromegakaryocytes in myelodysplastic sydromes (MDS). Proc CAMS and PUMC 1988; 3:33.

[6] Geng YQ, Rao Q, Kong J, et al. Preparation and characterization of McAb against the leukemic cell J6-1 membrane associated factor (MAF-J6-1). Chin J Hematol 1994; 15: 630.

[7] Rao Q, Wu KF, Han JS, et al. Antigen association between J6-1 cell membrane associated factor receptor with macrophage colony stimulating factor receptor. Exp Hematol 1998; 26:709.

[8] Wong GG, Temple PA, Leary AC, et al. Human CSF-1: Molecular cloning and expression of 4-kb cDNA encoding the human urinary protein. Science 1987; 235: 1504.

[9] Coussens L, Van-Beveren C, Smith D, et al. Structural alteration of viral homologue of receptor proto-oncogene fms at carboxyl terminus. Nature 1986; 320:277.

[10] Yang WQ, Wu KF, Song YH, et al. Influence of human herpes virus 6 on expression levels of M-CSF in bone marrow mononucleate cells from patients with hematopoietic diseases. Chin J Hematol 1998; 19: 646.

[11] Kacinski BM, Dtanley ER, Carter D, et al. Circulating levels of CSF-1 (M-CSF) a lymphohematopoietic cytokine may be a useful marker of disease status in patients with malignant ovarian neoplasms. Intl J Radiat Oncol Biol Phys 1989; 17:159.

[12] Baiocchi G, kavanagh JJ, Talpaz M, et al. Expression of the macrophage colony-stimulating factor and its receptor in gynecologic malignancies. Cancer 1991; 67:990.

[13] Scholl SM, Pallud C, Beuvon F, et al. Anti-colony-stimulating factor-1 antibody staining in primary breast adenocarcinomas correlates with marked inflammatory cell infiltrated and prognosis. J Natl Cancer Inst 1994; 86: 120.

[14] Suzuki M, Sekiguchi I, Ohwada M, et al. Expression of *c-fms* proto-oncogene product by ovarian cancer cells lines with effects of macrophage colony-stimulating factor on proliferation. Oncology 1996; 53: 99.

[15] Yang WQ, Wu KF, Song NG. The role of macrophage colony-stimulating factor in development of atherosclerosis and chronic renal failure. Foreign Med Sci J Clin biochem Lab 1998; 19: 220.

[16] Itoh Y, Okanoue T, Enjyo F, et al. Serum level of macrophage colony stimulating factor (M-CSF) in liver disease. J Hepatol 1994; 21:527.

[17] Sporn MB, Todaro GJ. Autocrine secretion and malignant transformation of cells. New Eng J Med 1980; 303:878.

[18] Zhou XM, Gu JR, Cheng YQ, et al. Simultaneous over-expression of insulin-like growth factor-II(IGF-II) and IGF-II receptor (IGF-II-R) genes in human primary cancer: Implication of growth of hepatic cancer. Chin J Cancer Res 1992; 1: 10.

[19] Wu KF, Rao Q, Zheng GG, et al. Enhancement of J6-1 human leukemic cell proliferation by cell-cell contact: Role of an M-CSF-like membrane-associated growth factor MAF-J6-1. Leuk Res 1994; 18: 843.

[20] Wu KF, Rao Q, Zheng GG, et al. Enhancement of J6-1 human leukemic cell proliferation by MAF-J6-1 through a cell-cell contact mechanism II: Role of an M-CSF receptor-like membrane protein. Leuk Res 1998; 22:55.

[21] Wu KF, He ZH, Zheng GG, et al. Receptor role of membrane bound macrophage colony-stimulating factor. Chin Sci Bull 1998; 43:1034.