Membrane-Bound Macrophage Colony-Stimulating Factor Mediated Auto-Juxtacrine Downregulates Matrix Metalloproteinase-9 Release on J6-1 Leukemic Cell

QING RAO, GUO-GUANG ZHENG, GE LI, YONG-MIN LIN, AND KE-FU WU[1]

Institute of Hematology, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300020, China

Earlier studies indicate that J6-1 human leukemic cells proliferate and propagate via the membrane-bound macrophage colony-stimulating factor (M-CSF)-mediated auto-juxtacrine mechanism. Matrix metalloproteinases (MMPs) can modulate the activity of cell membrane molecules and influence many cellular behaviors. Therefore, we hypothesized that MMP may also be involved in the membrane-bound M-CSF-mediated juxtacrine mechanism. First, we investigated whether blocking of membrane-bound M-CSF by neutralizing antibody to M-CSF or M-CSF receptor and adding of exogenous M-CSF are able to influence MMP-9 release. Next, we determined whether MMP-9 participated in J6-1 cells proliferation and influence the shedding of membrane-bound M-CSF and its receptor. Current studies show that blockade of the interaction between membrane-bound M-CSF and M-CSF receptor by antibody to M-CSF or M-CSF receptor promotes MMP-9 release. Moreover, we demonstrated that because of M-CSF mediated juxtacrine, lack of MMP-9 promotes J6-1 cell proliferation, in which a decrease in the shedding of cell-surface M-CSFR is involved. Hence, we suggest that membrane-bound M-CSF inhibit MMP-9 release and down-regulated MMP-9 contribute to juxtacrine stimulating in leukemic cell growth. Exp Biol Med 229:946–953, 2004

Key Words: human leukemia; macrophage colony-stimulating factor; matrix metalloproteinase-9; juxtacrine; soluble receptor; shedding

This work was supported by The Climbing Program granted by The Ministry of Science and Technology of China (95-special-10).

[1] To whom requests for reprints should be addressed at Institute of Hematology, Chinese Academy of Medical Sciences & Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, China. E-mail: kfwu@public.tpt.tj.cn

Received February 5, 2004.
Accepted June 10, 2004.

1535-3702/04/2299-0946$15.00

Introduction

The cell surface is the location of many interactions, including those of cells with growth factors, the surface of neighboring cells, and extracellular matrix proteins (1). An increasing number of secreted proteins are recognized as being derived from integral plasma membrane proteins, and in this case, the secretory event involves the selective hydrolysis of the proteins from the cell surface (2). The cleavage generally occurs close to the extracellular face of the membrane, releasing physiological active protein. This secretion process also provides a mechanism for down-regulating the protein at the cell surface, which plays a regulatory role in physiological processes including hematopoietic cell growth and differentiation (3). A closely related family of metalloscretases controls the surface expression of multiple integral membrane proteins (2).

The matrix metalloproteinases (MMPs) are a family of extracellular matrix (ECM)-degrading enzymes that share common functional domains and activation mechanisms (4). It is now clear that MMPs not only remodel the ECM but also influence many cellular functions. MMPs activity may be required during development and normal physiology in several ways: to degrade ECM molecules and allow cell migration, to alter the ECM microenvironment and result in alteration in cellular behavior, and to modulate the activity of biologically active molecules by direct cleavage, release from bound stores, or the modulating of the activity of their inhibitors (5).

More evidence suggests that cell-cell interactions occurring at the surface membrane play a regulatory role in normal physiology (6). Lacraz's study has indicated a role for direct cell–cell contact in potentiating metalloproteinase expression in monocytes and T lymphocytes (7). Subsequently, Baram *et al.* demonstrated that mast cell-T cell contact up-regulated mast cell MMP-9 expression, which is essential for leukocyte extravasation(8).

Autonomy is a fundamental phenotype of all malignant

cells (9, 10). A number of membrane-bound growth factors have been found to play crucial roles in neoplastic processes through auto-juxtacrine mechanisms. The role of normal cell–cell contact in MMP expression has been elucidated. However, little is known about the response of malignant cell–cell contact in MMP production or about MMP's regulation role in autonomy growth of malignant cells.

In an early study, we showed that J6-1 leukemia cells coexpress macrophage colony-stimulating factor (M-CSF) receptor (M-CSFR) and membrane-bound M-CSF (mM-CSF), which can enhance the growth of J6-1 cells by stimulating a corresponding receptor coexpressed on the adjacent J6-1 cells (11). Subsequently, we reported that the growth and tumor development by J6-1 leukemic cells depend on mM-CSF-mediated cell–cell contact (12). The current study was aimed at gaining insight into the regulation of mM-CSF mediated J6-1 cell–cell contact on release of MMP and determining whether MMP inversely contribute to the leukemic cell's auto-stimulating growth and the release of the essential cytokine to J6-1 leukemic cells. We demonstrate that blockade of the binding of mM-CSF with M-CSFR by a neutralizing antibody to M-CSF or M-CSFR promotes MMP-9 release. Moreover, we demonstrate that a lack of MMP-9 caused by mM-CSF mediated juxtacrine leads to decrease in the shedding of cell-surface M-CSFR, which contributes to J6-1 cell proliferation.

Materials and Method

Cell Line and Reagents. The human leukemia cell line, J6-1 was established from a patient with acute monocytic myeloid leukemia in 1976 (13). The cells were maintained in culture using RPMI 1640 medium supplemented with 15% fetal calf serum (FCS), 2 m*M* L-glutamine, 10 m*M* HEPES, and antibiotics in 5% CO_2/ air-humidified atmosphere at 37°C.

Purified recombinant human M-CSF and MMP-9 were obtained from Peprotech EC LTD (Rocky Hill, NJ) and R&D Systems Inc. (Minneapolis, MN), respectively. Antihuman M-CSF neutralizing antibody and anti-MMP-9 polyclonal antibody were purchased from R&D System Inc. Anti-MMP-9 monoclonal neutralizing antibody was the product of NeoMarkers (Lab Vision Corp., Fremont, CA). Rabbit anti-M-CSFR polyclonal antibodies were prepared from recombinant soluble M-CSFR in our lab and have been previously shown to specifically block the binding of M-CSF to its receptor and neutralize M-CSFR activity. *o*-Phenanthroline, synthetic metalloprotease inhibitor, was the product of Sigma-Aldrich Chemical Co (St. Louis, MO). One millimole *o*-phenanthroline (ophen) was used in zymographic analysis, and 0.05 m*M* ophen was used in MMP-9 blocking experiment. All chemicals were obtained from Sigma-Aldrich Chemical Co.

Cell-Conditioned Media. After washed with serum-free RPMI-1640, J6-1 cells were incubated for 24 hours at a concentration of 2×10^6 cells/ml in the presence or absence of M-CSF and anti-M-CSF or anti-M-CSFR antibody. The supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) for MMP-9 and zymography. In zymographic analysis, the cell-conditioned media were concentrated by 5-fold with Microcon Centrifugal Filter Units (Millipore Corp., Bellerica, MA).

MMP-9 ELISA. MMP-9 antigen levels in the conditioned media were measured using specific ELISA kit (R&D Systems) as manufacturer's recommendation (sensitivity, 0.156 ng/ml). Recombinant MMP-9 from R&D Systems was used as a standard. The results are represented from at least triplicate experiments.

Zymographic Analysis and Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis. Gelatinolytic activities were analyzed by the zymography described earlier (14). This technique was performed on 8.5% polyacrylamide gel copolymerized with 2 mg/ml type I gelatin. Fifteen microliters of samples mixed with 5 μl of loading buffer were run under nonreducing conditions without prior boiling. After electrophoresis, gels were washed three times in 2.5% Triton X-100 to remove SDS allowing proteins to renature and then immersed in buffer contained 50 m*M* Tris pH7.5, 5 m*M* $CaCl_2$, 1 μ*M* $ZnCl_2$, and 0.01% NaN_3 for 24–48 hours at room temperature. The gels were stained with 0.4% (w/v) Coomassie Blue and destained in 35% ethanol/10% acetic acid. Clear zones of gelatin lysis against a blue background stain indicated enzyme activities. Enzyme activity determined by zymographic analysis actually represents the total amount of secreted gelatinolytic protein. A synthetic inhibitor of MMP, *o*-phenanthroline, treated the parallel gels to verify the zones of lysis were produced by MMPs. Protein markers were prestained to determine the molecular weights of gelatinases. Media conditioned by HT1080, known to secrete MMP-9 and MMP-2, was used as the standard (15). The intensity of the bands was quantified using Shimadzu CS-9000 Scanning Densitometer (Shimadzu Corporation, Kyoto, Japan). The results are represented from at least triplicate experiments.

RNA Extraction, cDNA Synthesis, and Semi-quantitative RT-PCR. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Complementary DNA (cDNA) was subsequently synthesized from 5 μg total RNA using M-MLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD).

PCR was performed using a Gene-Amp PCR System 2400 thermocycler (Perkin Elmer, Torrance, CA). The polymerase chain reaction (PCR) conditions and the numbers of PCR cycles were chosen according to preliminary experiments in which PCR product was detectable in an amount directly proportional to the quantity of starting cDNA. As an internal control, human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified using its specific primers. The PCR program used to amplify MMP-9 and GAPDH started with a predenatura-

tion at 94°C for 30 seconds, followed by 30 cycles (for MMP-9) or 28 cycles (for GAPDH) of denaturation at 94°C for 30 seconces, annealing at 65°C for 50 seconds, elongation at 72°C for 1 min, respectively, and final extension for 10 min at 72°C.

The primer sequences used for the specific amplification of MMP-9 and GAPDH were follows: 5′-CAACATCACCTATTGGATCC-3′, 5′-GGGTGTAGAGTCTCTCGCT-3′ for MMP-9 5′-TGAAGGTCGGAGTCAACGGATTTGG-3′, 5′-CATGTGGGCCATGAGGTCCACCAC-3′ for GAPDH. These primer pairs amplified PCR fragments for MMP-9 and GAPDH of 480 and 983 bp.

Ten microliters of the PCR products was electrophoresed on a 1% agarose gel containing ethidium bromide. The gels were then photographed under UV light using a Kodak digital camera (Rochester, NY). The intensity of each band was quantified by densitometry, using equipped Kodak image software. Relative expression of MMP-9 was calculated following the formula: R = Densitometrical Units of MMP-9/Densitometrical Units of GAPDH.

Colony Formation Assay. Colony formation by J6-1 cells was carried out in triplicate in 100μl essential medium (5×10^4 cells/ml) containing 1% methylcellulose, 10% FCS, 5×10^{-7} *M* 2-mercaptoethanol and 0.03% glutamine in 96-well culture plates. Cells were incubated for 5 days and colonies were counted with a reversed microscope. Cell aggregates having 40 or more cells were considered colonies.

Fluorescent Activated Cell Sorter (FACS) Analysis for Cell Surface M-CSF Receptor. For detection of M-CSF or M-CSFR on cell surface, 1×10^6 cells were prepared in cold PBS containing 0.01% sodium azide. Nonspecific binding was blocked by incubation with rabbit IgG. After blocking, the cells were incubated with anti-M-CSF or anti-M-CSFR mAb for 1 hour at 4°C. Cells were subsequently washed with PBS and incubated with fluoresecein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG for 30 minutes. Cells were then washed and resuspended in PBS and analyzed within 1 hour using flow cytometry analysis (FACScan, Becton Dickinson Immunocytometry Systems, San Jose, CA). At least 10,000 cells were analyzed per sample.

ELISA for Soluble M-CSFR. The soluble M-CSFR (sM-CSFR) was determined with monoclonal–polyclonal antibodies sandwich ELISA developed in our lab. The establishment and authentication of ELISA have been reported (16). Briefly, the capturing antibody was the mouse anti-M-CSFR monoclonal antibody and the detecting antibody was the rabbit anti-M-CSFR polyclonal antibody. Biotinylated anti-rabbit IgG and ABC complex were used as a signal detecting system. Recombinant human M-CSFR was used as the standard (17). The analytical detection limit was 0.1 ng/ml of sM-CSFR.

Statistical Analysis. All results of at least triplicates within one experiment or of at least three comparable experiments were expressed as the mean ± standard deviation (SD). Student's *t*-test was used to determine the significance ($P < 0.05$) of the differences between the mean values.

Results

M-CSF-Mediated Cell–Cell Contact Inhibits MMP-9 Production by J6-1 Cells. Previous studies have demonstrated that membrane-bound M-CSF (mM-CSF) mediated J6-1 leukemic cell proliferation in a juxtacrine manner. To determine the effects of M-CSF mediated cell–cell contact in MMP-9 release, we first measured the MMP-9 antigen level in media conditioned by J6-1 cells treated with anti-M-CSF monoclonal antibody (mAb to M-CSF) or exogenous M-CSF using ELISA. As shown in Figure 1, without treatment, J6-1 cells released low levels of MMP-9 into the cell culture supernatant. After the cell–cell contact was blocked with mAb to M-CSF, MMP-9 level was increased significantly in a dose-dependent manner. Furthermore, we measured MMP-9 level in the medium conditioned by the cells treated with anti-M-CSFR polyclonal antibody (pAb), and a similar effect was observed. Interestingly, addition of exogenous M-CSF to J6-1 cells caused a decrease in MMP-9 production in a dose-independent manner. These data show that M-CSF, and especially mM-CSF, down-regulates MMP-9 production by J6-1 leukemic cells.

To confirm the effect of mM-CSF on MMP-9 production, we next determined the gelatin activity in J6-1 cells conditioned media by zymography analysis. A weak band corresponding to the 92-kD MMP-9 was observed in

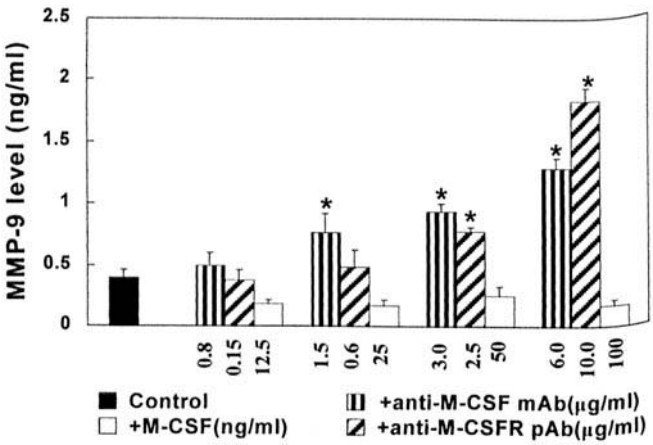

Figure 1. Blocking of macrophage colony-stimulating factor (M-CSF) binding to its receptor induces matrix metalloproteinase-9 (MMP-9) production by J6-1 cells. ELISA detected MMP-9 antigen level in J6-1 cell supernatants, with or without exogenous M-CSF and antibody to M-CSF or M-CSF receptor (M-CSFR) incubation for 24 hours. Blocking of J6-1 cells with anti-M-CSF monoclonal antibody (mAb) and anti-M-CSFR polyclonal antibodies (pAb) had a significant and dose-dependent enhancement on the level of MMP-9 production. Incubation of J6-1 cells with exogenous M-CSF inhibited MMP-9 production. The results shown are representative of three independent experiments. The asterisks indicate where statistical differences exist ($P < 0.05$) compared with control.

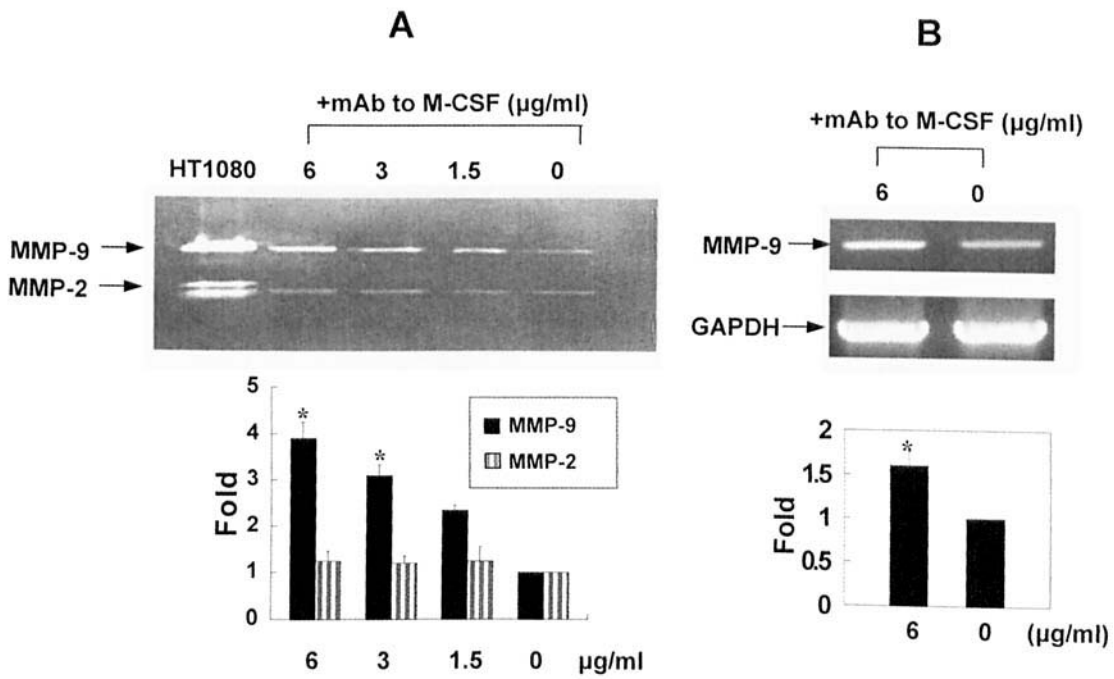

Figure 2. Blocking of macrophage colony-stimulating factor (M-CSF) binding to its receptor up-regulates matrix metalloproteinase-9 (MMP-9) gelatinolytic activity in J6-1 cell supernatant and MMP-9 mRNA expression in J6-1 cells. (A) Zymographic analysis of medium conditioned by J6-1 cells, with or without monoclonal antibody (mAb) to M-CSF for 24 hours. Gelatinolytic activities (MMP-9 and MMP-2) were analyzed by densiometry. The intensities of the bands were quantitated using a Shimadzu CS-9000 Scanning Densitometer. The quantitative results were expressed as the fold of relative intensity to the band of medium conditioned by J6-1 cells without treatment and expressed as fold ± standard deviations from three zymograms. The result shown is representative of three independent experiments, and the asterisks indicate where statistical differences exist ($P < 0.05$) in MMP-9 activities in response to anti-M-CSF mAb. Media conditioned by HT1080, known to secrete MMP-9 and MMP-2, was used as the standard. (B) Effect of anti-M-CSF mAb on MMP-9 mRNA expression in J6-1 cells. Total RNA was extracted from J6-1 cells incubated without or with mAbs to M-CSF and RT-PCR was performed as described in materials and methods. GAPDH served as an internal control. The intensities of the bands were quantitated. The results were expressed as the fold of relative intensity of MMP-9 compared to the control and are representative of three independent experiments. The asterisks indicate where statistical differences exist ($P < 0.05$) in relative expression of MMP-9 mRNA in response to anti-M-CSF mAb.

the media conditioned by J6-1 cells without treatment (Fig. 2A). Zymography consistently detected a higher gelatin activity corresponding to MMP-9 in the medium conditioned by J6-1 cells blocked with mAb to M-CSF. Quantification of antibody-induced increases in MMP-9 activity by densitometry indicated that this effect was significant and dose dependent. However, there was no influence on gelatin activity corresponding to MMP-2 when J6-1 cells were blocked with anti-M-CSF mAb.

Next, we further examined the effect of anti-M-CSF mAb on MMP-9 gene expression using the RT-PCR technique. Consistently, as shown in Figure 2B, after J6-1 cells were treated with anti-M-CSF antibody for 24 hours, the MMP-9 mRNA level was increased. Densitometry analysis of relative expression of MMP-9 revealed that the increase was significant.

These data showed that when J6-1 cell–cell contact mediated in mM-CSF was blocked by anti-M-CSF and anti-M-CSFR antibodies, up-regulation of MMP-9 was detected at mRNA, protein, and gelatin activity levels. It clearly demonstrates that membrane-bound M-CSF is capable of inhibiting the MMP-9 production in J6-1 leukemic cells.

Lack in MMP-9 Contributes to the Juxtacrine Mechanism and Promotes J6-1 Leukemic Cell Proliferation. Our data now have shown that membrane-bound M-CSF inhibited MMP-9 production by J6-1 cell. Considering that MMP-9 can regulate many cellular behaviors by modulating cell surface molecules, we then determined whether lack of MMP-9 was involved in J6-1 cells growth. We examined the effect of MMP-9 induced by anti-M-CSF mAb on the J6-1 cells proliferation, using colony formation assay. J6-1 cells grew vigorously in methylcellulose system in the absence of exogenous growth factors. The growth capacity of J6-1 cells was markedly inhibited when J6-1 cells were treated with mAb to M-CSF (6 μg/ml) (Table 1). Furthermore, we added anti-MMP-9 antibodies and MMP-9 synthetic inhibitor to block the activities of induced MMP-9.

Table 1 also showed that the growth-inhibiting effect caused by mAb to M-CSF was abolished by both anti-MMP-9 antibodies and MMP-9 inhibitor, indicating that anti-M-CSF mAb caused growth-inhibitory effect on J6-1 cells was partly owing to the induced MMP-9. Interestingly, direct addition of anti-MMP-9 antibodies to culture caused a weak enhancement of colony formation by J6-1 cells, which could be explained by the reason that J6-1 cells release low

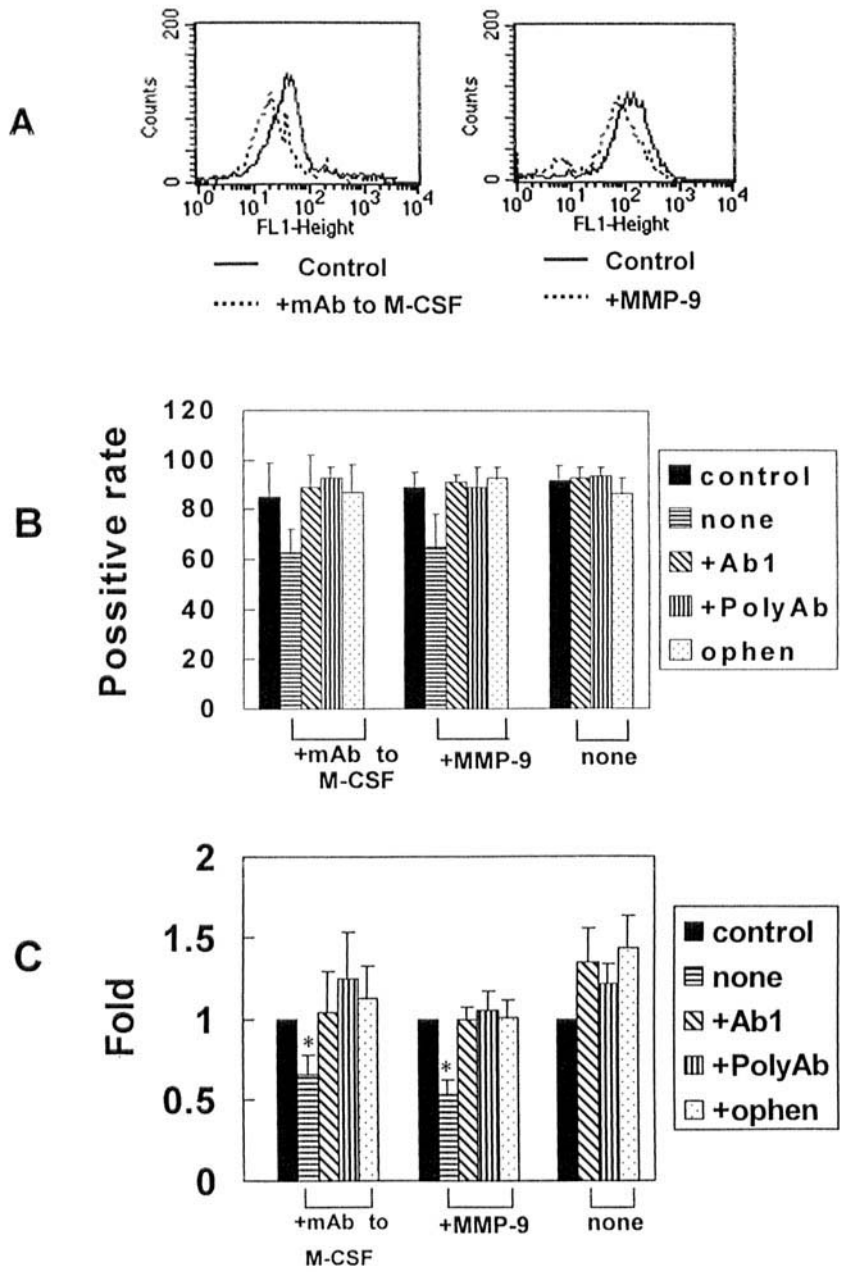

Figure 3. Effects of mAb to macrophage colony-stimulating factor (M-CSF) induced matrix metalloproteinase-9 (MMP-9) and recombinant MMP on cell surface expression of M-CSFR. After J6-1 cells were treated with anti-M-CSF monoclonal antibody (mAb) (6 μg/ml) or MMP-9 (1.2 ng/ml) for 24 hours, surface expression of M-CSFR was analyzed by flow cytometry as described in Materials and Methods. Surface M-CSFR levels were shown as the percentage of M-CSFR positive cell (positive rate in B) and the fluorescene intensity (fold of intensity relative to control in C). In the abolish experiment of anti-M-CSF mAb-induced MMP-9, J6-1 cells were pretreated with anti-M-CSF mAb for 2 hours and then treated with or without anti-MMP-9 antibodies and MMP inhibitor for 24 hours. In the abolish experiment of MMP-9, J6-1 cells were cultured in present of MMP-9 with anti-MMP-9 antibodies or MMP inhibitor for 24 hours. The results shown are representative of five separate experiments. The asterisks indicate where statistical differences exist ($P < 0.05$) compared with control. Ab1: monoclonal antibody to MMP-9. polyAb: polyclonal antibody to MMP-9. ophen: MMP synthetic inhibitor.

levels of MMP-9 without treatment and their inhibiting activity was neutralized. As a control, recombinant MMP-9 (1 ng/ml) was added to the colony formation system. As shown in Table 1, the growth capacity of J6-1 cells was consistently inhibited by MMP-9, and the growth-inhibiting activity was completely abolished by anti-MMP-9 antibodies and MMP-9 inhibitor. As shown here, MMP-9 could inhibit J6-1 leukemic cell proliferation. Thus, we concluded that in the absence of blocking mAb to M-CSF, MMP-9 production is down-regulated and the decrease in MMP-9 secretion contributes to the vigorous growth of J6-1 leukemic cells.

Influence of MMP-9 on the Shedding of Cell Surface M-CSFR. To further demonstrate the mechanism

Table 1. Effects of Anti-MMP-9 Antibodies and MMP Inhibitor on Anti-M-CSF mAb-Induced Inhibition on Proliferation of J6-1 cells

Treatment[a]	Colony number[b]	Inhibiting activity (%)
Control	49.2 ± 7.4	
mAb to M-CSF	27.7 ± 1.5	43.6[c]
mAb to M-CSF+Ab1	56.0 ± 8.0	−13.8
mAb to M-CSF+PolyAb	44.5 ± 3.5	9.5
mAb to M-CSF+ophen	48.7 ± 4.0	1.0
Control	52.3 ± 7.2	
MMP-9	30.0 ± 8.7	42.6[d]
MMP-9+Ab1	48.7 ± 5.5	6.9
MMP-9+PolyAb	41.6 ± 7.1	20.5
MMP-9+ophen	43.5 ± 6.8	16.8
Control	44.3 ± 9.7	
M-CSF	68.0 ± 5.6	−53.5[e]
Ab1	48.7 ± 13.1	−9.9
PolyAb	47.0 ± 5.6	−6.1
Ophen	41.3 ± 8.0	6.8

[a] Dosage: Monoclonal antibody (MAb) to macrophage colony-stimulating factor (M-CSF): 6 μg/ml, matrix metalloproteinase-9 (MMP-9): 1 ng/ml, M-CSF: 20 ng/ml, Ab1 (monoclonal antibody to MMP-9): 2 μg/ml. PolyAb (polyclonal antibody to MMP-9): 2.5 μg/ml. ophen (MMP synthetic inhibitor): 0.05 m*M*.
[b] The results shown are means ± S.D. from three separate experiments.
[c] $P = 0.03$
[d] $P = 0.02$
[e] $P = 0.01$

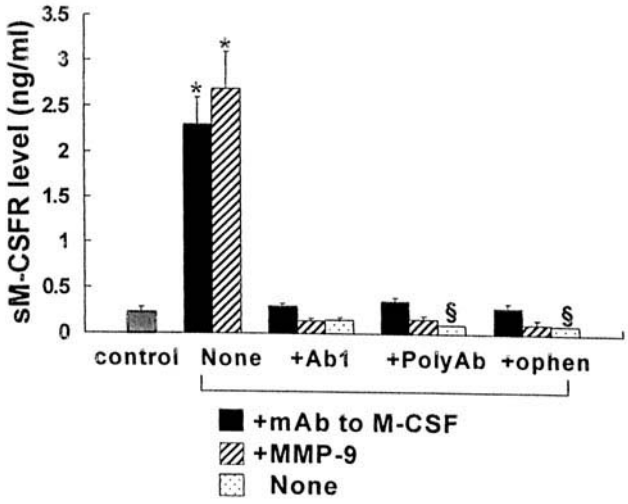

Figure 4. Effects of monoclonal antibody (mAb) to macrophage colony-stimulating factor (M-CSF) induced matrix metalloproteinase (MMP) and recombinant MMP on soluble M-CSFR level in J6-1 cell supernatants. After J6-1 cells were culture in serum free condition for 24 hours and followed by teatment with anti M-CSF mAb (6μg/ml) for 24 hours or MMP-9 (1.2ng/ml) for 8 hours, sM-CSFR level in J6-1 cell supernatants was detected using ELISA. In abolish experiment of anti-M-CSF mAb induced MMP-9, j6-1 cells were pretreated with anti-M-CSF mAb for 2 hours and then treated with or without anti-MMP-9 antibodies and for 24 hours. In abolish experiment of MMP-9, J6-1 cells were cultured in present of MMP-9 with anti-MMP-9 antibodies or MMP inhibitor for 8 hours. As a control, anti-MMP-9 antibodies and MMP inhibitor alone have a weak inhibition in sM-CSFR release. The results shown are representative of three separate experiments. *$P<0.05$, § The OD value was below the ELISA analytical detection limit (0.1ng/ml) and we assigned 0.1 ng/ml to any sample that was below detection limit. No SD existed. Ab1: monoclonal antibody to MMP-9. polyAb: polyclonal antibody to MMP-9. ophen: MMP synthetic inhibitor.

that lack of MMP-9 is involved in the M-CSF-mediated J6-1 cell growth, we determined the influence of MMP-9 on the shedding of the membrane-associated protein. Considering that membrane-bound M-CSF and cell surface M-CSFR are essential for J6-1 growth, we first investigated the effects of anti-M-CSF mAb induced MMP-9 on the level of membrane-bound M-CSF and on the cell surface M-CSFR by FACS analysis. Figure 3 illustrates the surface M-CSFR expression in J6-1 cells. FACS analysis revealed that the mean positive rate of cell with surface M-CSFR was 85 ± 14% without treatment. When cells were incubated in the presence of mAb to M-CSF, the positive rate decreased (63 ± 9%). This effect could be abolished by incubating cells with anti-MMP-9 antibodies and a MMP-9 inhibitor (89 ± 13%, 93 ± 3.5%, and 87 ± 11%, respectively) (Fig. 3A and B). Consistent results were demonstrated by the fluorescene intensity of cell surface M-CSFR (Fig. 3A and C). Figure 3C also showed that direct incubating the cells with anti-MMP-9 antibodies caused a modest increase in cell surface M-CSFR, illustrated by fluorescene intensity. This effect was possibly due to the result of the neutralizing function of anti-MMP-9 antibodies on MMP-9 produced by J6-1 cells.

There was no influence on the membrane-bound M-CSF level after the cells were treated with mAb to M-CSF.

We then measured the sM-CSFR levels in the supernatant of J6-1 cell treated with mAb to M-CSF. Interestingly, as shown in Figure 4, the sM-CSFR level was increased ninefold over that in the supernatant of J6-1 cells in absence of mAb to M-CSF (2.3 ng/ml vs. 0.23 ng/ml). This effect could also be abolished by both anti-MMP-9 antibodies and the MMP-9 inhibitor. Our results indicated that soluble M-CSFR could be generated from the cell surface M-CSFR in response to MMP-9. As a control, direct addition of anti-MMP-9 antibodies and MMP-9 inhibitor also can cause a modest inhibition in sM-CSF release.

To confirm that MMP-9 was responsible for the shedding of cell-surface M-CSFR, we further determined the direct effect of recombinant MMP-9 on the shedding of M-CSFR. As shown in Figure 3A and B, after J6-1 cells were treated with MMP-9, the positive rate of cells with surface M-CSFR was decreased (from 89 ± 6% to 65 ± 13%); that effect could be blocked by anti-MMP-9 antibodies and MMP-9 inhibitor. The mean value of fluorescene intensity of cell surface M-CSFR was also decreased significantly ($p = 0.01$) (Fig. 3 A and C). The direct effect of MMP-9 appears stronger than that of MMP-9, induced by anti-M-CSF mAb, even if the concentration of MMP-9 added to cell culture was equal to that in the supernatant induced by anti-M-CSF mAb.

We then measured the level of sM-CSFR released from J6-1 cell in response to MMP-9. In the presence of MMP-9,

sM-CSFR in the supernatant was increased 11-fold more than that in the supernatant of J6-1 cells without treatment (2.7 ng/ml vs 0.23 ng/ml), which was observed after only 8 hs of treatment. Anti-MMP-9 antibodies and MMP-9 inhibitor could also abolish this effect (Fig. 4).

Collectively, these results indicate that MMP-9 participates in the shedding of cell surface M-CSFR. However, because of the inhibiting of MMP-9 by membrane-bound M-CSF, the releasing of soluble M-CSFR was prevented.

Discussion

In a previous study, we reported on J6-1 leukemic cells proliferation and propagation by the juxtacrine mechanism, which was mediated by membrane-bound M-CSF and its receptor (11, 12, 18). In this report, we demonstrate that blockade of membrane M-CSF binding to its receptor expressed in adjacent cells by anti-M-CSF or anti-M-CSFR antibody caused an increase in both MMP-9 gene expression and MMP-9 release, indicating that MMP-9 production was being down-regulated because of the existence of membrane-bound M-CSF-mediated juxtacrine. Our result showed that the effect of anti-M-CSF mAb induced that MMP-9 release was dose dependent. Although addition of exogenous M-CSF also inhibited MMP-9 production, this effect was not dose dependent. That could be explained by the possibility that membrane-bound M-CSF expressed on J6-1 cell surface is so abundant that a limited amount of exogenous M-CSF could reach the platform of effective level to J6-1 cells. Now we concluded that J6-1 leukemic cells, because of overexpression of membrane bound M-CSF, have permanently down-regulated MMP-9 release.

Furthermore, we found that down-regulated MMP-9 contributed to the vigorous growth of J6-1 leukemic cells and influenced the production of sM-CSFR. In defining the effect of decreased MMP-9 on cell surface M-CSFR, which is essential for J6-1 cell growth, we found that after blocking membrane-bound M-CSF binding to its receptor, the cell surface M-CSFR level was decreased and the level of soluble M-CSFR in supernatant was elevated. These effects could be abolished by anti-MMP-9 antibodies and MMP-9 synthetic inhibitor. It indicates that MMP-9 participates in the shedding of cell surface M-CSFR, but because MMP-9 was down-regulated by membrane-bound M-CSF the shedding is inhibited. However, it cannot be excluded that the decreased cell surface M-CSFR caused by mAb to M-CSF was a possible result of a down-regulation of c-fms mRNA transcription. Actually, in our earlier study, we have demonstrated that anti-M-CSF mAb and M-CSF inhibitor caused c-fms mRNA expression to be up-regulated (12); these data raise the possibility that the decreased cell surface M-CSFR level was caused by the enhanced shedding of cell surface M-CSFR. This conclusion is also supported by the data of the direct effect of MMP-9 on the cell surface M-CSFR and the release of sM-CSFR by J6-1 cells. Now we conclude that because of the overexpression of membrane-bound M-CSF, MMP-9 production is down-regulated, leading to elevated cell surface M-CSFR, which further contributes to membrane-bound M-CSF-mediated juxtacrine stimulation in J6-1 leukemic cell growth.

It is now clear that MMPs are not only important for cell migration but also affect the biological activities of other molecules through proteolytic cleavage (19). MMP activity can lead to both gain and loss of function of biologically active molecules (20, 21). In the normal hematopoietic process, MMP-9-mediated release of sKitL enhances the motogenic potential of stem and progenitor cells, translocating them from their quiescent state into a permissive proliferative vascular niche (22). Of relevance to our study, Tanaka's study on the role of MMP-9 produced by mast cell precursors in their tissue invasion strongly indicates that MMP-9 production is down-regulated by c-kit receptor activation (23). In the studies on leukemic cells, more evidence focused on the role of MMP-9 in the leukemic cell's migration (15, 24, 25). This study provides the first demonstration that lack of MMP-9 promotes leukemic cell growth, in which MMP-9 mediated release of sM-CSFR is involved.

The shedding process provides a mechanism for down-regulating the protein at the cell surface under normal conditions. M-CSFR is one of numerous integral plasma-membrane proteins that could be released from lipid bilayer by proteolysis (26). In J6-1 leukemic cells, because of the lack of MMP-9, the sM-CSFR is down-regulated. In our earlier studies, sM-CSFR has been shown to be a potent inhibitor in J6-1 cell proliferation (27). It seems, therefore, that in response to the down-regulated sM-CSFR, its inhibiting effect may be prevented. In addition, elevated cell surface M-CSFR contributes to the M-CSF-mediated auto-juxtacrine. All of these events lead to J6-1 leukemic cell growth more vigorously.

In this work, we also detected MMP-9 production in response to blockade with anti-M-CSF mAb in other leukemic cell line and bone marrow cell samples of leukemia patients. A consistent effect was found in HL-60 leukemic cell, but not in K562 cells. Seven out of 13 leukemia patient samples exhibited elevated MMP-9 levels when the bone marrow mononucleus cells were treated with anti-M-CSF mAb. It should be noted that at least a few types of leukemia might use a similar MMP-9–involved mechanism in their proliferation.

It has been reported that c-fms and its ligand are coexpressed in some types of leukemia cell and in some malignancies (28, 29). Early study showed that coexpression of cellular M-CSF and its receptor is tumor associated (30). Our results indicate that binding of mM-CSF to its receptors lead to the lack of MMP-9, which contribute to the leukemic cell proliferation. Our data also demonstrate that in response to the lack of MMP-9, sM-CSFR is down-regulated. This phenomena has been consistently discovered by the earlier study on the serum soluble M-CSFR in

leukemia patients (31). It seems that MMP-9-involved juxtacrine mechanism may be shared by most malignant cells and play a potent role in the propagation of some tumor cells.

1. Nicholas D. Surface membrane-associated regulation of cell assembly, differentiation, and growth. Blood 78:264–276, 1991.
2. Hooper NM, Karran EH, Turner AJ. Membrane protein secretases. Biochem J 321:265–279, 1997.
3. Mackiewicz A, Wiznerowicz M, Roeb E, Karczewska A, Nowak J, Heinrich PC, Rose-John S. Soluble interleukin 6 receptor is biologically active in vivo. Cytokine 7(2):142–149, 1995.
4. Sternlicht M, Coussens LM, Vu TH, Werb Z. Biology and regulation of the matrix metalloproteinases. In: Clendeninn NJ, Appelt K, Eds. Cancer drug discovery and development: Matrix metalloproteinase inhibitors in cancer therapy. Totowa, NY: Humana Press Inc., p. 1–37, 2000.
5. Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. Genes & Development 14:2123–2133, 2000.
6. Liebermann DA, Hoffman-Liebermann B. Genetic programs of myeloid cell differentiation. Curr Opin Hematol 1(1):24–32, 1994.
7. Lacraz S, Isler P, Vey E, Welgus HG, Dayer JM. Direct contact between T lymphocytes and monocytes is a major pathway for induction of metalloproteinase expression. J Biol Chem 269:22027–22033, 1994.
8. Baram D, Vaday GG, Salamon P, Drucker I, Hershkoviz R, Mekori YA. Human mast cells release metalloproteinase-9 on contact with activated T cells: Juxtacrine regulation by TNF-α. J Immunol 167:4008–4016, 2001.
9. Kaiser HE. Neoplastic autonomy: a fundamental phenomenon. In Vivo 8(1):1–2, 1994.
10. Lynch RG. Differentiation and cancer: the conditional autonomy of phenotype. Proc Natl Acad Sci U S A. 92(3):647–648, 1995.
11. Wu KF, Rao Q, Zheng GG, Geng YQ, Li M, Kong J, Song YH, Ying HG, Chen BD. 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. 18:843–849, 1994.
12. Wu KF, Rao Q, Zheng GG, He ZH, Ying HG, Song YH, Chen BD. Enhancement of J6-1 human leukemic cell proliferation by membrane-bound M-CSF through a cell-cell contact mechanism II: role of an M-CSF receptor like membrane protein. Leuk Res. 22:55–60, 1998.
13. Wu KF, Zhang YQ, Qi SL. Establishment of myeloid monocytic leukemic cell lines and studies of their cell biological properties. Acta Genetica Sinica 7:136–143, 1980.
14. Masure S, Billiau A, Van Damme J, Opdenakker G. Human hepatoma cells produce an 85 kDa gelatinase regulated by phorbol 12-myristate 13-acetate. Biochim Biophys Acta 1054:317–325, 1990.
15. Janowska-Wieczorek A, Marquez LA, Matsuzaki A, Hashmi HR, Larratt LM, Boshkov LM, Turner AR, Zhang MC, Edwards DR, Kossakowska AE. Expression of marix metalloproteinases (MMP-2 and –9) and tissue inhibitors of metalloproteinases (TIMP-1 and –2) in acute myelogenous leukaemia blasts: comparison with normal bone marrow cells. Br J Haematol 105:402–411,1999.
16. Rao Q, Han JS, Sha XJ, Yang RC, Geng YQ, Zheng GG, Wu KF. Determination of serum soluble macrophage colony-stimulating factor receptor levels in patients with hematological diseases. Chinese J Cancer Res 13:185–189, 2001.
17. Luo SQ, Zheng DX, Liu YX, Rao Q, Wu KF. Analysis of the ligand-binding domain of macrophage colony-stimulating factor receptor. Chinese Sci Bull 45:1191–1194, 2000.
18. Li G, Song YH, Wu KF, Lin YM, Cao ZY, Zheng GG. Clone and expression of mutant M-CSF and its receptor from human leukemic cell line J6-1. Leuk Res 26:377–382,2002.
19. Schonbeck U, Mach F, Libby P. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. J Immunol 161:3340–3346, 1998.
20. Imai K, Hiramatsu A, Fukushima D, Pierschbacher MD, Okada Y. Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth fctor-beta 1 release. Biochem J 322:809–814, 1997.
21. Whitelock JM, Murdoch AD, Iozzo RV, Underwood PA. The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin and heparanases. J Biol Chem 271:10079–10086, 1996.
22. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of Kit-ligand. Cell 109:625–637, 2002.
23. Tanaka A, Arai K, Kitamura Y, Matsuda H. Matrix metalloproteinase-9 production, a newly identified function of mast cell progenitors, is downregulated by c-kit receptor activation. Blood 94:2390–2395, 1999.
24. Hayashibara T, Yamada Y, Onimaru Y, Tsutsumi C, Nakayama S, Mori N, Miyanishi T, Kamihira S, Tomonaga M, Maita T. Matrix metalloproteinase-9 and vascular endothelial growth factor: a possible link in adult T-cell leukaemia cell invasion. Br J Haematol 116(1):94–102, 2002.
25. Dias S, Hattori K, Zhu Z, Heissig B, Choy M, Lane W, Wu Y, Chadburn A, Hyjek E, Gill M, Hicklin DJ, Witte L, Moore MA, Rafii S. Autocrine stimulation of VEFGR-2 activates human leukemic cell growth and migration. J Clin Invest 106:511–521, 2000.
26. Downing JR, Roussel MF, Sherr CJ. Ligand and protein kinase C downmodulate the colony-stimulating factor 1 receptor by independent mechanisms. Mol Cell Biol 9:2890–2896, 1989.
27. Zheng G, Rao Q, Wu K, He Z, Geng Y. Membrane-bound macrophage colony-stimulating factor and it receptor play adhesion molecule-like roles in leukemic cells. Leuk Res 24:375–383, 2000.
28. Parwaresch MR, Kreipe H, Felgner J, Heidorn K, Jaquet K, Bodewadt-Radzun S, Radzun HJ. M-CSF and M-CSF-receptor gene expression in acute myelomonocytic leukemias. Leuk Res 14:27–37, 1990.
29. Baiocchi G, Kavanagh JJ, Talpaz M, Wharton JT, Gutterman JU, Kurzrock R. Expression of the macrophage colony-stimulating factor and its receptor in gynecologic malignancies. Cancer 67:990–996, 1991.
30. 30.Wu KF, Zheng GG, Rao Q Geng YQ, Yang WQ, Song YH. Cellular macrophage colony-stimulating factor and its role. Haematoloica 84:951–952, 1999.
31. Rao Q, Han JS, Geng YQ, Zheng GG, Qian LS, Wu KF. Decreased serum soluble macrophage colony stimulating factor receptor level in leukemia patients. Hematologica 86:989–990, 2001.