Basic Investigations

ANTIGEN ASSOCIATION OF J6-1 CELL MEMBRANE ASSOCIATED FACTOR RECEPTOR WITH MACROPHAGE COLONY STIMULATING FACTOR RECEPTOR

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ABSTRACT

Objective: To verify the antigen association of MAF-J6-1 receptor with M-CSFR and to further study the role of M-CSF and its receptor mediated juxtacrine in promoting leukemic cell proliferation. Methods: Monoclonal antibody (McAb) of MAF-J6-1R RE2 and polyclonal antibody (PolyAb) of rhM-CSFR were prepared. The specificity of McAb RE2 to M-CSFR was confirmed by indirect ELISA, cross-neutralizing assay with J6-1 cell colony formation and neutralization test by ELISA. Results: The reactive activity of purified RE2 to M-CSFR was over 1: 16000. The inhibitory activity of M-CSFR and MAF-J6-1R could be blocked by RE2 and anti-M-CSFR antibody. The reactivity of RE2 to M-CSFR could be reduced by M-CSFR. Conclusion: The specificity of RE2 to M-CSFR was confirmed and the antigen association of MAF-J6-1R with M-CSFR was proved. It suggests that M-CSF and its receptor mediated auto-juxtacrine stimulation could be an operative mechanism in either leukemia or non-hematological malignancies.

Key works: Macrophage colony stimulating factor, Receptor, Monoclonal antibody, ELISA

Macrophage colony stimulating factor (M-CSF) or colony stimulating factor-1(CSF-1) is a lineage specific hematopoietic regulator which can stimulate the proliferation and support the survival of mononuclear phagocyte series.[1] Although originally defined through its activities on hematopoietic cells, M-CSF may have important functions outside the context of hematopoiesis, such as breast cancers and female reproductive tract tumors formation etc.[2]

In the hematopoietic system, M-CSF exerts its pleiotropic effects by binding to a single class of high-affinity receptor[3] expressed predominantly on monocytes, macrophages and their committed bone marrow precursors. M-CSF receptor (M-CSFR) is encoded by c-fms protooncogene and is a member of a family of growth factor receptors that exhibit ligand-induced tyrosine-specific protein kinase activity.[4]

Despite the finding of the physiological actions of M-CSF and its receptor as an important receptor/ligant pair, little is known of their interaction mechanism in regulating hematopoiesis. In our previous work, we have shown that human leukemic cell line J6-1 expressed M-CSF like factor (MAF-J6-1) and the receptor of MAF-J6-1(MAF-J6-1R), which mediated auto-juxtacrine between J6-1 cells.[5,6] The juxtacrine-stimulating mechanism, which has been suggested to be operative between several types of tissue cells in development biology,[7] is now proved functionally related to neoplastic proliferation in myeloid leukemia and non-hematological malignancies. To further understand the role of M-CSF-like growth factor (MAF-J6-1) and its receptor in juxtacrine mechanism, which is operative in promoting leukemic cell proliferation, it should prove that MAF-J6-1R is related to M-CSFR. The number of the receptors of MAF-J6-1R was so low that it is difficult to verify it directly. In this study, several studies to examine the antigen association of MAF-J6-1R with M-CSFR were carried out. The polyclonal antibody of rhM-CSFR and monoclonal antibody of MAF-J6-1R were prepared in our lab. By means of demonstrating the cross blocking activity of M-CSFR's antibody to MAF-J6-1R and the specificity of

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MAF-J6-1R's McAb to M-CSFR, the antigen association of MAF-J6-1R with M-CSFR could be proved indirectly.

MATERIALS AND METHODS

Reagents

Recombinant Hu M-CSF was purchased from Cetus Corporation (Emeyville, CA, USA). Rat anti-M-CSFR McAb (Ab1) was purchased from Oncogene Research product (Cambridge MA, USA). The Mouse Monoclonal antibody Isotyping Kit was purchased from Sigma. Recombinant of human soluble M-CSF receptor (rhM-CSFR) was cloned from human placenta. Three Ig-like domains of extracellular part of c-fms were expressed in E-coli.[8]

Colony Formation Assay

Activity of MAF-J6-1R or M-CSFR was determined by their ability to inhibit the colony formation of J6-1 cells. Colony formation by J6-1 cells (5000 cell/well) was carried out in triplicate in 100 μl of medium containing 1% methylcellulose, 10% fetal calf serum, 5×10^{-7} mol/l 2-mercaptoethanol and 0.03% glutamine in 96-well culture plates (Nunc). Cells were incubated at 37°C with 5% CO_2 for 5 days. Cell aggregates having 40 or more cells were considered colonies. The growth inhibiting activity was determined according to the following formula:

$$\left[1-\frac{\text{Colony number (experimental)}}{\text{Colony number (control)}}\right]\times 100\%$$

Preparation and Purification of Monoclonal Antibody against Receptor of MAF-J6-1 and Polyclonal Antibody against M-CSFR

The monoclonal anti-MAF-J6-1R antibodies were prepared according to the procedure described by Bartal.[9] Briefly, BALB/C spleen cells were immunized in vitro with approximately 100 μg of partial purified MAF-J6-1R. Four days later, the spleen cells were fused with SP2/0 cells according to the procedure reported previously.[5] After fusion, the hybrids were selected against J6-1 cell membrane antigens by avivid-biotin complex peroxide (ABC) immunoassay using Vectastain ABC Kit's.[5] After three consequent selections by limiting dilution, positive clones that produced anti-MAF-J6-1R antibodies were verified by neutralizing activity determined by colony formation assay. Briefly, before the colony formation assay was carried, 10 μl of serially diluted MAF-J6-1R was mixed with the same volume of hybridomas supernatant and incubated for one hour at 37°C. The mixture was then subjected to colony formation system using J6-1 cells. The hybridoma clones whose supernatant blocked the biologic action of MAF-J6-1R were considered anti-MAF-J6-1R antibodies. Subclass of the most positive clone's antibody was determined with The mouse monoclonal antibody Isotyping Kit. The ascites fluid was prepared by intra-abdominal injection with 5×10^6 hybridoma cells seven days after injection of pristane. Ten days later the ascites fluid was taken.

The IgM monoclonal antibody of MAF-J6-1R was purified on hydroxyapatite column.

To examine the specificity of McAb of MAF-J6-1R to M-CSF, as control, the poly-valent antisera against M-CSFR was prepared in rabbits. The rabbits were immunized with a subcutaneous injection of 2 mg of the pure rhM-CSFR emulsified in an equal volume of Freund's complete adjuvant. Four further immunizations were administered at 2-week intervals. Blood was collected seven days after the last immunization. Indirect ELISA as described below determined the titer of the polyvalent antisera. Immunogloblin (Ig) fraction from antiserum to rhM-CSFR was purified by ion-exchange column (DEAE-cellulose) chromatographic technique following precipitation by ammonium sulfate at 33% saturation. Each fraction was estimated for anti-M-CSFR activity by means of ELISA.

Indirect Enzyme Linked Immunosorbent Assay (ELISA)

To determine the reactive activity of PolyAb and McAb to M-CSFR, indirect ELISA was performed. Each well of a flat-bottomed 96-well microtiter plate (Linbro) was coated with 100 μl of coating solution containing 0.1 μg of rhM-CSFR. As for the non-specificity negative control, each well was coated with 0.1 μg/100μl M-CSF. Then, the plate was sealed and allowed to stand for 24 hours at 4°C. After the incubation, each well was washed three times with 0.05% Tween-20 in PBS and filled with 150 μl of blocking solution (1% BSA, 0.2% gelatin in PBS) for 2 hours at 37°C. Prepared 2 fold serial dilutions of PolyAb or McAb were added separately and incubated for two hours at 37°C. After the solution was discarded and washing was done, 100 μl of diluted HRP-conjugated anti-rabbit IgG or anti-mouse IgG was added and incubated for two hours at 37°C. After four washings was taken, 100 μl of enzyme substrate was added. The reaction was stopped by adding 50 μl of 1N H_2SO_4 per well and the color was read on the microtiter plate reader at 492nm (Vamed Engineering, Austria).

Cross-neutralizing Assay of the Blocking Activity of Anti-MAF-J6-1R McAb and Anti-M-CSFR PolyAb on MAF-J6-1R or rhM-CSFR with Proliferation of J6-1 Cells

J6-1 cell colony formation assay was performed. In preliminary assays, concentrations of antibodies to block the activity of MAF-J6-1R and M-CSFR were determined by serial dilution analysis. The cross neutralization experiment of anti-MAF-J6-1R and anti-M-CSFR on inhibitory activity of MAF-J6-1R and rhM-CSFR with J6-1 cell colony formation was performed. 10 μl of MAF-J6-1R (28 μg/ml) and rhM-CSFR (10μg/ml) was treated with an equal volume of anti-M-CSFR Ab1 (50 μg/ml) and RE2 supernatant at 37°C for 1h. The mixture was then subjected to colony formation assays using J6-1 cells.

Neutralization test of Anti-MAF-J6-1R McAb and Anti-M-CSFR PolyAb on rhM-CSF and M-CSFR by Indirect ELISA

To determine the specificity of PolyAb and McAb to M-CSF-R, neutralization experiment was carried out. With indirect ELISA, before the antibody was added to the wells coated by rhM-CSFR, PolyAb and McAb were mixed with various concentrations of M-CSF or M-CSFR and incubated for one hour at 37°C. Then the indirect ELISA was performed as described above.

RESULTS

Preparation and Purification of Anti-MAF-J6-1R Monoclonal Antibody and Anti-M-CSFR Polyclonal Antibody

After screening with ABC enzyme immunoassay, 24 clones of hybridoma cells were detected against J6-1 cell membrane antigens. To assess whether these antibodies blocked the biologic action of MAF-J6-1R, supernatants from primary isolating were tested by J6-1 colony formation assay. Anti-MAF-J6-1R activity was detected in supernatants from 4 of 24 primary selection. The most active isolating was cloned via limiting dilution technique. The stable one was designated RE2, which could neutralize 90-100% inhibitory activity of MAF-J6-1R on J6-1 colony formation. RE2 was selected producing McAb against MAF-J6-1R in this study.

The RE2 antibody was of the IgM subclass as judged with Mouse Monoclonal Antibody Isotyping Kit. Ascetics fluid from BALB/C mice was harvested on the 10th day after hybridoma injection. Then the McAb anti- MAF-J6-1R was purified on the hydroxyapatite column. To assess whether anti-MAF-J6-1R monoclonal antibody exhibits anti-M-CSFR activity, the IgM fractions from hydroxyapatite column were estimated for specific activity to M-CSFR by ELISA. The fractions No. 30–38 appeared to be considerably specific to M-CSFR. (Figure 1B)

Sera from the rabbits which immunized by rhM-CSFR exhibited specific anti-M-CSFR activity. The positive titer of serum to M-CSFR determined by indirect ELISA was over 1: 2000. Normal rabbit serum had no effect in this assay. After being purified with DEAE-cellulose, each fraction was estimated for anti-M-CSFR activity by ELISA. Corresponding to the IgG peak, which eluted by 0.01 mol/L PBS, pH 7.4, fraction 10—15 exhibited specificity to M-CSFR. (Figure 1A)

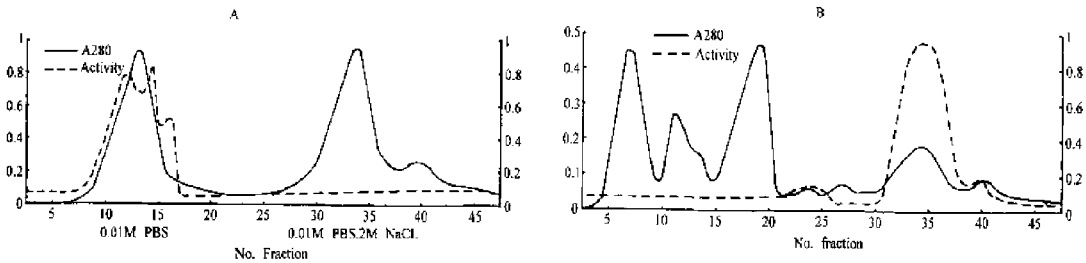

Fig 1. A. Purification of PolyAb of M-CSFR on DEAE-Cellulose; B. Purification of McAb RE2 ascetic fluid on hydroxyapatite column. The specific activity to M-CSFR of each fraction (---) was determined by ELISA

Determination of Specificity of Anti-MAF-J6-1R and Anti-M-CSFR Antibodies to M-CSFR by indirect ELISA

The specificity of monoclonal antibody RE2 and polyclonal antibody of M-CSFR was assessed by indirect ELISA. The serial dilutions of both

antibodies were tested. The titer of PolyAb was over 1: 2000, its non-specificity to rhM-CSF was less than 1: 8. (Figure 2A). Compared with PolyAb, the monoclonal antibody RE2 exhibited identical specificity to M-CSFR. Dilutions through 1: 16000 exhibited marked reactive activity to rhM-CSFR, However, there was no positive effect in dilution over 1: 16 in response to M-CSF. (Figure 2B). The reactivity of McAb RE2 with M-CSFR showed that RE2 had corresponding specificity to M-CSFR. As monoclonal antibody RE2 was produced from MAF-J6-1R, it suggests that MAF-J6-1R is an antigen associated with M-CSFR.

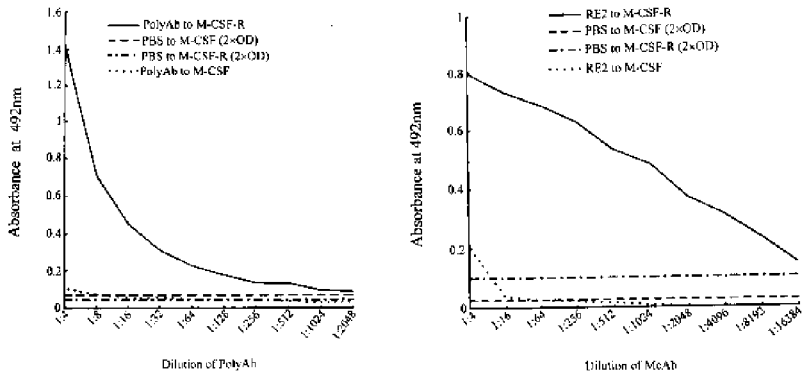

Fig. 2. Determination of specificity of purified PolyAb (A) and McAb RE2 (B) to M-CSFR and M-CSF by indirect ELISA. The values less than twice of OD value of PBS to M-CSFR or M-CSF were regarded as negative.

Cross Effect of Anti-MAF-J6-1R Antibody and Anti-M-CSFR Antibody on Proliferation of J6-1 Cells

The inhibitory activity of MAF-J6-1R on J6-1 cell colony formation has been demonstrated in our previous work.[6] The anti-MAF-J6-1R McAb could block the inhibitory activity of MAF-J6-1R. To determine whether MAF-J6-1R is related to M-CSFR or not, cross effects of anti-MAF-J6-1R McAb and anti-M-CSFR McAb on MAF-J6-1R and rhM-CSR's inhibitory activity on J6-1 cells colony growth were examined. The anti-M-CSFR antibody employed was obtained from commercial source (Ab1). Similar to MAF-J6-1R, rhM-CSFR exhibited inhibitory activity on J6-1 cell colony formation. Figure 3 shows that MAF-J6-1R caused reduction on proliferation of J6-1 cells. Although anti-MAF-J6-1R McAb RE2 and anti-M-CSFR Ab1 exhibited inhibitory activity on J6-1 cells, they could block the inhibitory action of MAF-J6-1R consistently. Figure 3 shows that both RE2 and Ab1 could neutralize the growth inhibitory activity induced by rhM-CSFR. This result proved that the specificity of anti-MAF-J6-1R antibody was consistent with that of anti-M-CSFR antibody. It proved that MAF-J6-1R is related to M-CSFR.

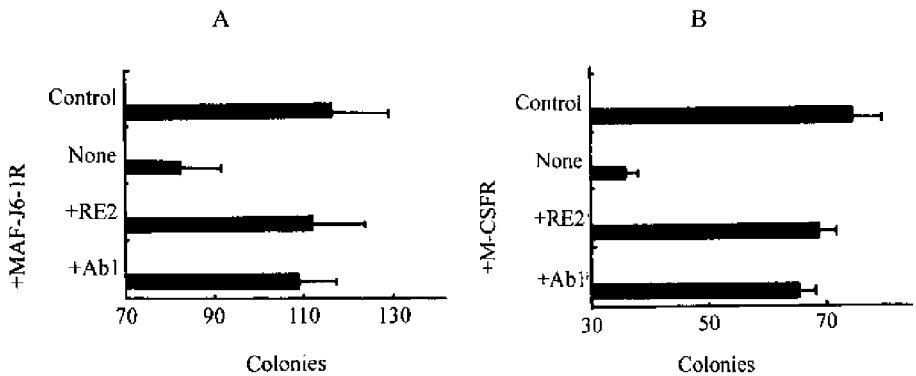

Fig 3. Cross neutralization action of RE2 and Ab1 on inhibition activity of MAF-J6-1R and M-CSFR by J6-1 colony formation assay. Data represent $\bar{x} \pm s$ from three identical experiments. Control=no receptor or antibody; None=receptor only; +RE2=receptor and RE2; +Ab1=receptor and Ab1.

Neutralization Test for PolyAb of rhM-CSFR and McAb RE2 by ELISA

The specificity of PolyAb and McAb RE2 was tested by neutralization test with M-CSF's soluble receptor and M-CSF. In the course of ELISA, before antibodies were added, the PolyAb and McAb were mixed with various concentrations of M-CSFR or M-CSF. As shown in Figure 4, after being neutralized by rhM-CSFR, the antibody reaction level with rhM-CSFR was dramatically decreased, but was not influenced by M-CSF. The reduction responding to rhM-CSFR was dependent on the dosage of M-CSFR. The effect that the interaction of RE2 with M-CSFR was reduced by M-CSFR but not by M-CSF confirmed the specificity of McAb RE2 to M-CSFR and antigen association of MAF-J6-1R with M-CSFR.

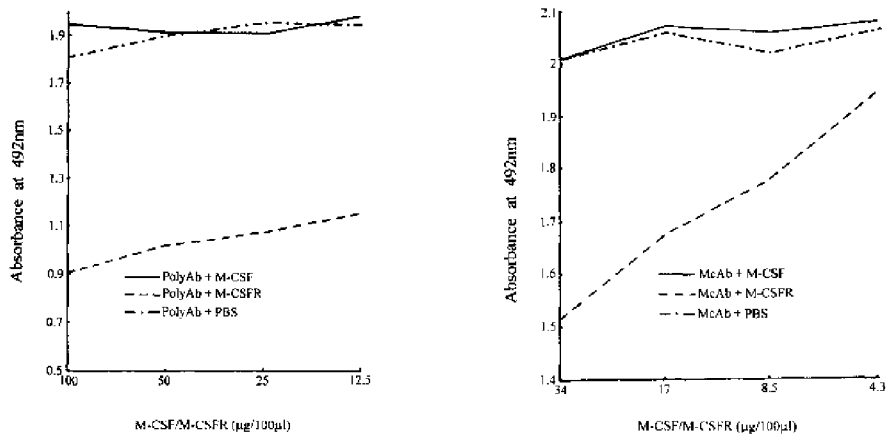

Fig. 4. Determination of the specificity of PolyAb (A) and McAb RE2 (B) to M-CSFR by neutralization-ELISA experiment.

DISCUSSION

J6-1 is a leukemic cell line that was established in our laboratory in 1976.[10] This cell line exhibits strong cluster forming and density-dependent growth characteristics *in vitro*. Our previous studies[5,6] suggested that "juxtacrine" is an important mechanism in promoting J6-1 leukemic cells proliferation. MAF-J6-1 and its receptor have been isolated and thought to be responsible to auto-juxtacrine in J6-1 cells. MAF-J6-1 has been characterized as being related to M-CSF.[5] The amount of the receptor of MAF-J6-1 was extremely low, preventing us from further characterization on this receptor directly.

In this study, several ways to verify MAF-J6-1R were taken. Indirect evidence suggests that this receptor is an antigen related to M-CSFR. (1) The inhibitory activity of MAF-J6-1R on J6-1 cell colony formation can be neutralized by anti-M-CSFR antibody obtained from a commercial source. RhM-CSFR exhibits consistent inhibitory activity with MAF-J6-1R and this activity can be blocked by anti-MAF-J6-1R monoclonal antibody prepared in this study; (2) Similar to the anti-M-CSFR PolyAb prepared from rhM-CSFR in this study, anti-MAF-J6-1R antibody exhibits strong positive titer to M-CSFR and specificity to M-CSFR; and (3) Neutralization test of anti-MAF-J6-1R McAb and anti-M-CSFR PolyAb with M-CSF and its receptor by indirect ELISA further confirmed the specificity of McAb of MAF-J6-1R to M-CSFR. The specificity of anti-MAF-J6-1R McAb to M-CSFR confirmed the antigen association of MAF-J6-1R with M-CSFR.

Normal monocytes produce M-CSF in response to certain physiologic inducers.[11] Assay of M-CSFR and M-CSF expression level in human myeloid leukemic cells has indicated that this receptor/ligand pair may be simultaneously and stably expressed in some cases,[12,13] and suggested that the growth of such cells might be enhanced through an autocrine mechanism. Activating mutations within M-CSF, such as the membrane-bound form in J6-1 cells and the responsible receptor, might therefore facilitate disease progression.[14] Using a nude mice model, we found that treatment of J6-1 cells with MAF-J6-1R before inoculation led to a complete inhibition of tumor formation in the animals.[6] The association of MAF-J6-1R with M-CSFR provides us with the model to study the M-CSFR's essential action for the growth and development of leukemia.

The antibodies described herein that are directed

against human M-CSF receptor should be useful for monitoring the serum M-CSFR level in normal adults and patients by sandwich ELISA which is being established in our Lab. It should be useful to clarify the role of hM-CSF and its receptor in normal and abnormal stages of hematopoiesis. Our finding that specific antibodies against the receptor diminish growth of J6-1 cells is of clinical significance in treating leukemia by interferring with ligand-receptor interactions.

FMS is a member of the type III receptor tyrosine kinase family that includes KIT (steel factor receptor), STK-1 (stem cell tyrosine kinase 1) and platelet-derived growth factor receptor. STK-1 expression is restricted to $CD34^+$ cell.[15] J6-1 is an EBV and HHV-6 double infected CD34 negative cell line.[16] Recently, Strockbine, et al. reported that the EBV BARF 1 gene encoded a novel, soluble colony-stimulating factor-1 receptor.[17] In this study, very close antigen association is revealed between MAF-J6-1R and M-CSFR. The cDNA cloning and sequence of MAF-J6-1R is being done in our lab to search the genetic relationship between MAF-J6-1R and M-CSFR (c-fms).

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