Isolation and Characterization of Two Monoclonal Antibodies that Recognize Different Epitopes of the Human *c-kit* Receptor

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MORITA, S., TSUCHIYA, S., FUJIE, H., ITANO, M., OHASHI, Y., MINEGISHI, M. and KONNO, T. *Isolation and Characterization of Two Monoclonal Antibodies that Recognize Different Epitopes of the Human c-kit Receptor.* Tohoku J. Exp. Med., 1996, 178 (2), 187-198 — After immunizing mice with a human megakaryoblastic leukemia cell line, M-MOK, we obtained two monoclonal antibodies which recognize the human *c-kit* receptor. The monoclonal antibodies, designated MTK1 and MTK2, were found to specifically recognize Balb/3T3 cells transfected with human *c-kit* cDNA and not parent Balb/3T3 cells while showing different immunological, biochemical and biological behaviors. Both allowed visualization of the 140 kDa *c-kit* protein by Western blot analysis, but MTK1 detected only positive band with non-reducing conditions for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. MTK1 partially inhibited the stem cell factor (SCF) induced proliferation of M-MOK cells, whereas, MTK2 was without effect. MTK1 also inhibited the bone marrow derived colony forming unit granulocyte/macrophage (CFU-GM) formed by granulocyte-macrophage colony stimulating factor (GM-CSF) and SCF. Not only anti-CD34 antibodies (HPCA-1) but also MTK1 could be shown to concentrate bone marrow CFU-GM and burst forming unit erythroid (BFU-E) effectively. The presently described monoclonal antibodies may therefore be useful for functional analysis of the ligand binding domain of the human *c-kit* receptor, as well as for further classification of hematopoietic stem cells in addition to the CD34 positive cells. — human *c-kit* receptor; monoclonal antibodies; biological activities; MTK1; MTK2

We have established a feeder cell- and cytokine-dependent human megakaryoblastic leukemia cell line, M-MOK (Itano et al. 1995). The cell line is positive for CD34, CD41, CD42b and CD61, and negative for HLA-DR and glycophorin. In addition, it expresses the transcription factor GATA2 but is negative for GATA1 as with KG1a cells representing a stage of immature myeloid cells (Civin et al. 1984; Nagai et al. 1994). In order to isolate specific monoclonal antibodies

against immature myeloid cells we immunized Balb/c mice with M-MOK cells and picked up two hybridoma clones, MTK1 and MTK2, which recognize the human *c-kit* receptor. There have been several reports on hybridomas producing monoclonal antibodies with specificity for the human *c-kit* protein (Bühning et al. 1991; Lerner et al. 1991; Broudy et al. 1992; Yamaguchi et al. 1993). YB5.B8 was established by immunizing with myeloid leukemia cells of FAB-M1 phenotype (Lerner et al. 1991), SR-1 by immunizing with an erythroleukemia cell line, OCIM1 (Broudy et al. 1992), 17F11 by immunizing with myeloid leukemia cells (Bühning et al. 1991) and NUc-kit by immunizing with L-cells transfected with human *c-kit* cDNA (Yamaguchi et al. 1993). Since the human *c-kit* receptor has been reported to be expressed on erythroleukemia cell lines, megakaryoblastic leukemia cell lines including M-MOK, and fresh myeloid leukemia cells (Hu et al. 1994; Itano et al. 1995; Morita et al. 1996), it was likely that immunizing with M-MOK cells would give rise to anti-*c-kit* receptor antibody producing hybridomas with high frequency. In the present study we report characteristics of MTK1 and MTK2 monoclonal antibodies, emphasizing their value for functional analysis of ligand binding domain of the *c-kit* receptor.

MATERIALS AND METHODS

Cell lines

M-MOK cells and lymphoblastoid cell lines LCL-MOK, immortalized by Epstein-Barr virus, were established from the same patient. M-MOK cells are routinely maintained in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT, USA) and 5 ng/ml of GM-CSF (Kirin Brewery, Tokyo). LCL-MOK cells were cultured in RPMI-1640 with 10% FCS. Balb/3T3 and a transfectant bearing human *c-kit* cDNA named Balb/3T3hc-kit (provided by Dr. Toshio Suda, Kumamoto University) (Yamaguchi et al. 1993) were maintained in DMEM (Gibco) with 10% FCS. KG1a (provided by Dr. Masayuki Yamamoto, Tohoku University) and THP-1 (Tsuchiya et al. 1980) were maintained in RPMI-1640 supplemented by 5% FCS.

Bone marrow and peripheral blood mononuclear cells

After obtaining informed consent, bone marrow and peripheral blood were drawn from healthy volunteers and leukemic patients in complete remission. Whole blood or mononuclear cells separated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation were used for surface immunostaining. Bone marrow mononuclear cells were also processed to purify CD34 positive cells.

Production of the monoclonal antibodies

A 4-week-old female Balb/c mouse received three intraperitoneal injections of 1×10^7 M-MOK cells at 2-week intervals. The methods of cell fusion and growth of hybridomas were described elsewhere (Aarden et al. 1985; Ohashi et al. 1992).

The solid phase enzyme-linked immunosorbent assay (ELISA) method was used to screen hybridoma-supernatants recognizing immunized M-MOK cells, but not LCL-MOK (Engvall and Perlmann 1972). After the second limiting dilution cloning we established two positive clones, designated as MTK1 and MTK2, which reacted with M-MOK but did not recognize LCL-MOK or peripheral white blood cells. MTK1 and MTK2 ascites were harvested according to Goding (1980). Monoclonal antibodies were purified using an Affi-gel protein A MAPSII kit (Bio-Rad, Richmond, CA, USA). Monoclonal antibody subclass was determined with an Isotype Ab-STATM-I kit (Sang Stat Medical Co., Menlo Park, CA, USA).

Flow cytometric analysis

The monoclonal antibodies were labelled with fluorescent isothiocyanate (FITC) and biotin (NHS-biotin, Pierce, Rockford, IL, USA) according to Ishii's method (Ishii et al. 1994). Reactivity of MTK1 and MTK2 to human bone marrow and peripheral blood leukocytes was examined with a flow cytometer according to the method described previously (Tsuchiya et al. 1991). Balb/3T3 and Balb/3T3hc-kit cells were also stained with FITC-MTK1 and- MTK2. To determine epitope specificities of the *c-kit* protein, cross-blocking experiments were performed. Briefly, Balb/3T3hc-kit cells were incubated with an excess amount of MTK2 ascites for 30 min at 4°C and then stained with FITC-MTK1, -MTK2 or commercially available FITC-anti-*c-kit* monoclonal antibody (NUc-kit, Nichirei, Tokyo) for 30 min at 4°C before further routine processing. For double staining experiments, purified CD34 positive cells were mixed with FITC-HPCA2 (CD34 antibody, Becton Dickinson, San Jose, CA, USA) and biotin-MTK1 simultaneously and incubation continued for 30 min at 4°C. After washing three times PE streptavidin (Becton Dickinson) was added and incubated for 30 min at 4°C.

Purification of CD34 or c-kit positive cells

CD34 positive cells in bone marrow mononuclear cells were concentrated by means of the immunomagnetic beads procedure with CD34 antibody, HPCA1 (Becton Dickinson) (Cairo et al. 1992). *C-kit* positive cells were concentrated in the same manner as the CD34 positive cells, but MTK1 was used as the first antibody instead of HPCA1. When purified CD34 positive cells were double stained with FITC-HPCA2 and biotin-MTK1 for flow cytometric analysis, they were detached from immunomagnetic bead-HPCA1-cell complexes by treatment with chymopapaine (200 μ /ml, Baxter, Deerfield, IL, USA). Purified CD34 and *c-kit* positive cells were subjected to CFU-GM and BFU-E assays according to the methods described previously (Minegishi et al. 1989). Human recombinant IL-3 (100 μ /ml, Kirin Brewery) and human recombinant erythropoietin (Epo, 2 U/ml, Chugai, Tokyo) were added as colony stimulating factors. Effects of MTK1 and MTK2 on the CFU-GM and BFU-E in bone marrow mononuclear cells were also

examined. Stem cell factor (SCF, 50 ng/ml, Kirin Brewery) was supplemented in addition to IL-3 and Epo in the presence or absence of 10 μ g/ml of MTK1 or MTK2.

Growth inhibition study

Effects of MTK1 and MTK2 on the proliferation of M-MOK cells were quantitated by a ^3H -thymidine (^3H -TdR) uptake assay (Itano et al. 1995). M-MOK cells were starved for 24 hr in RPMI medium supplemented with 10% FCS and then seeded in a 96-well microplate at the density of 3×10^3 cells/0.1 ml/well in the presence of 50 ng/ml of SCF. After adding graded amounts of MTK1 or MTK2 antibodies from 1 ng/ml to 10 μ g/ml in a 0.1 ml volume the cells were cultured for 5 days at 37°C in 5% CO_2 in air. As a negative control, an unrelated monoclonal antibody of the same subclass (PAR3, anti-parvovirus monoclonal antibody, Yaegashi et al. 1989) was used. The cells were pulsed for the last 4 hr with ^3H -TdR (74 KBq/ml, 1.0 TBq/mmol, Amersham, Buckinghamshire, England), harvested on a glass filter using an automatic cell harvester (Belco, Vineland, NJ, USA) and counted using a liquid scintillation counter (LS6000SC, Beckman, Fullerton, CA, USA).

Preparation of cell membranes and Western blot analysis

One ml of lysis buffer (0.02 M Tris-HCl, pH 7.3, 0.145 M NaCl, 1.5 mM MgCl_2 , 2.5% Tween 40, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM N-p-tosyl-L-arginyl methylester hydrochloride, 0.2 mM aprotinin and 3% sucrose) was added to 1×10^7 cells and then was homogenized using a Dounce homogenizer. After removing the nuclear fraction the membrane fraction was pelleted by ultracentrifugation for 1 hr at 15,000 rpm. Pellets were solubilized by 1% sodium dodecyl sulfate (SDS) and SDS-polyacrylamide gel electrophoresis (PAGE) and the Western blot analysis were carried out (Laemmli et al. 1970; Ohsako et al. 1994). The positive bands were detected with an ECL Western blotting detection kit (Amersham).

RESULTS

Antibody screening

Supernatants from 831 hybridoma wells were screened by solid phase ELISA assay and 6 supernatants which bound to M-MOK cells, but not to LCL-MOK cells, were found. Among these six supernatants two did not demonstrate any reaction to peripheral lymphocytes, monocytes or granulocytes. By the limiting dilution method, two clones, MTK1 and MTK2, were obtained, both being of the IgG1 subclass.

Specificity of the monoclonal antibodies

We hypothesized that the two monoclonal antibodies were directed to the *c-kit* receptor-protein on the basis of the following observations: 1) MTK1 and

Fig. 1. Binding of MTK1 and MTK2 by purified CD34 positive cells from bone marrow. CD34 positive cells were purified from bone marrow mononuclear cells of healthy volunteers by an immunomagnetic bead-procedure and then stained with FITC-HPCA2 and biotin-MTK1 or MTK2 and streptavidin PE. Two examples are shown; (a) 40% of CD34 positive cells are MTK1 positive and (b) 80% of CD34 positive cells are MTK2 positive.

Fig. 2. Specific binding of MTK1 and MTK2 by the Balb/3T3hc-kit transfectant. Balb/3T3hc-kit was stained with FITC-MTK1 (b) and FITC-MTK2 (d). As negative controls Balb/3T3 cells were also stained with FITC-MTK1 (a) and FITC-MTK2 (c).

MTK2 recognized approximately 3% of bone marrow mononuclear cells (data not shown), 2) MTK1 and MTK2 did not react to KG1 a cells which strongly express CD34 (data not shown), and 3) approximately 40-80% of purified CD34 positive cells were positive for MTK1 and MTK2 (Fig. 1a and 1b). In order to prove this hypothesis Balb/3T3hc-kit cells were used. After confirming that only Balb/3T3hc-kit and not the parent Balb/3T3 produced *c-kit* mRNA by Northern blot analysis (data not shown), those cells were immunostained with FITC-MTK1 and -MTK2 and analyzed by a flow cytometer. As shown in Fig. 2 Balb/3T3hc-kit but not parent Balb/3T3 were recognized by MTK1 and MTK2. These data clearly indicated both MTK1 and MTK2 to be anti-*c-kit* receptor antibodies.

Western blot analysis of MTK1 and MTK2 recognition molecules

Balb/3T3hc-kit and M-MOK cells were MTK1 and MTK2 positive, and Balb/3T3 and THP-1 were negative. Membrane fractions were prepared from these 4 cell lines, and SDS-PAGE and subsequent Western blot analysis were performed. The results are shown in Fig. 3a and 3b. Both MTK1 and MTK2 detected positive bands of 140 kDa and 112 kDa only in the lanes for Balb/3T3hc-kit and M-MOK. The molecular weight of the detected proteins coincided with that of the *c-kit* protein (Lerner et al. 1991). However, MTK1 recognized the *c-kit* protein only under non-reducing conditions and the presence of 2-ME in the

Fig. 3. Western blot analysis of molecules recognized by MTK1 and MTK2. After lysing the cells SDS-PAGE electrophoresis and Western blot analysis were performed according to the Materials and Methods. Lane 1, Balb/3T3hc-kit; lane 2, Balb/3T3; lane 3, M-MOK; and lane 4, THP-1. 1) MTK1, SDS-PAGE was carried out under non-reducing conditions and b) MTK2, SDS-PAGE under reducing conditions. The arrows indicate the molecular weights of the MTK1- and MTK2-reactive proteins.

sample buffer completely abrogated the positive bands. In the MTK2 case, *c-kit* positive bands were observed under both reducing and non-reducing conditions. Taking these results into consideration we concluded that MTK1 and MTK2 are true anti-*c-kit* receptor antibodies.

Effects of MTK1 and MTK2 on SCF-induced proliferation of M-MOK cells

Since excess amounts of unlabelled MTK1 did not inhibit the binding of FITC labelled MTK2 to M-MOK cells (Fig. 4) and also excess amounts of unlabelled MTK2 did not inhibit the binding of FITC labelled MTK1 (data not shown), MTK1 and MTK2 were concluded to recognize different epitopes on the *c-kit* receptor.

SCF can induce transient proliferation of M-MOK cells (Itano et al. 1995). Effects of MTK1 and MTK2 on this SCF-induced proliferation were examined in order to ascertain whether MTK1 and MTK2 show the different modes of inhibitory function (Fig. 5). MTK1 inhibited the proliferation of M-MOK cells in a dose dependent manner and as little as $0.1 \mu\text{g/ml}$ of MTK1 inhibited $^3\text{H-TdR}$ incorporation by approximately 70%. In contrast, MTK2 did not inhibit the

Fig. 4. A cross-blocking experiment between MTK1 and MTK2. The *c-kit* receptors on M-MOK cells were blocked with an excess amount of MTK1 ascites and then stained with b) unrelated ascites (PAR3) and FITC-MTK1, c) MTK1 ascites and FITC-MTK1, d) MTK2 ascites and FITC-MTK1 and e) $10 \mu\text{g}$ of NUC-kit monoclonal antibody and FITC-MTK1. a) a negative control histogram showing the pattern of M-MOK cells incubated only with PAR3.

Fig. 5. Effects of MTK1 and MTK2 on the SCF induced M-MOK cell proliferation. Graded amounts of MTK1 (▨) and MTK2 (▩) from 1 ng/ml to 10 μ g/ml were added separately or simultaneously (▧) to M-MOK cells seeded at the concentration of 3×10^3 /well in a 96 well microplate in the presence of 50 ng/ml of SCF. The Y-axis indicates cpm of ^3H -TdR uptake and the X-axis indicates the amounts of monoclonal antibodies. Purified PAR3 (□) with an unrelated specificity (10 μ g/ml) was used for the negative control wells.

Fig. 6. Effects of MTK1 and MTK2 on CFU-GM formation. 5×10^4 bone marrow mononuclear cells/dish were cultured according to the methylcellulose method in the presence of 20 ng/ml of GM-CSF, 50 ng/ml of SCF, and monoclonal antibodies. MTK1, (▨), MTK2 (▩) and PAR3 (□) antibodies were used at the concentration of 10 μ g/ml. Control cultures without antibodies were also carried out (▧). CFU-GM was counted after 10 days and BFU-E after 14 days of culture.

TABLE 1. *Separation of CFU-GM and BFU-E from bone marrow mononuclear cells by HPCA1 and MTK1*

Fraction	CFU-GM	BFU-E
Experiment 1		
Unfractionated bone marrow cells	18 ± 8	15 ± 6
Bone marrow cells fractionated by HPCA1	100 ± 60	990 ± 435
Bone marrow cells fractionated by MTK1	235 ± 155	850 ± 180
Experiment 2		
Unfractionated bone marrow cells	40 ± 4	39 ± 2
Bone marrow cells fractionated by HPCA1	265 ± 75	1,755 ± 75
Bone marrow cells fractionated by MTK1	435 ± 55	595 ± 55

The numbers in the table indicate formation of CFU-GM and BFU-E from 5×10^4 bone marrow mononuclear cells. Each data represents mean ± S.D. of triplicate assay.

growth of M-MOK at all. No additive inhibition was evident with both MTK1 and MTK2. M-MOK cells are able to form colonies in the presence of SCF in methylcellulose medium. In this system MTK1 inhibited the M-MOK colony formation by 85%, whereas, MTK2 was without effect (data not shown). SCF itself does not induce colony formation by bone marrow mononuclear cells, but in combination with GM-CSF increases CFU-GM synergistically. Effects of MTK1 and MTK2 on the CFU-GM induced by this synergistic system of GM-CSF and SCF were examined (Fig. 6). Forty percent reduction of CFU-GM was observed with 10 µg/ml of MTK1 as compared with isotype matched unrelated mouse ascites. MTK2 did not influence CFU-GM caused by GM-CSF and SCF. The presence of MTK1 and MTK2 did not exert any effects on CFU-GM induced by GM-CSF only.

Concentration of CFU-GM and BFU-E by MTK1

Expression of CD34 or the *c-kit* receptor is a hematopoietic stem cell marker (Fig. 1) (Civin et al. 1984; Catlett et al. 1991). It has been reported that CD34 monoclonal antibodies are able to concentrate functional bone marrow colony forming cells in combination with anti-mouse immunoglobulin-antibody conjugated immunomagnetic beads (Cairo et al. 1992). With the same immunomagnetic beads-separation method, MTK1 was examined for whether it could selectively fractionate CFU-GM and BFU-E like HPCA1. As shown in Table 1, CFU-GM was concentrated approximately 10-fold and BFU-E 15-50-fold from bone marrow mononuclear cells after immunomagnetic bead-separation by MTK1. MTK1 was also shown to concentrate approximately double the CFU-GM and one third to two thirds of BFU-E as compared with the HPCA1 case.

DISCUSSION

After immunizing Balb/c mice with M-MOK cells, we raised two hybridoma-clones producing monoclonal antibodies named MTK1 and MTK2. These monoclonal antibodies were confirmed to be directed to the *c-kit* receptor by demonstration of specific reactivity to the *c-kit* cDNA transfectant, Balb/3T3hc-kit (Yamaguchi et al. 1993). We consider MTK1 and MTK2 to recognize different epitopes of the *c-kit* receptor because of the following observations: 1) an excess amount of either one does not inhibit the binding of the other to M-MOK cells, 2) MTK1 inhibits the SCF-induced proliferation of M-MOK, and SCF and GM-CSF induced CFU-GM formation of bone marrow mononuclear cells, but MTK2 does not show such inhibitory effects, and 3) Western blot analysis revealed positive bands for the *c-kit* receptor only under non-reducing condition for MTK1, but under both reducing and non-reducing conditions for MTK2. The open reading frame of the *c-kit* gene consists of 976 codons, from which the extracellular domain is formed by 520 amino acids (Yarden et al. 1987). This extracellular domain has 13 cysteine residues, of which 10 are completely conserved among such receptor tyrosine kinase family members as *c-kit*, *v-kit*, *c-fms* and PDGF receptors. Since cysteine residues play an important role in the tertiary structure of the *c-kit* receptor through the generation of intramolecular SH bonds, the fact that MTK1 binds only under non-reducing condition would indicate recognition of the functionally important three dimensional structure of the SCF binding site, which has been said to reside in the first and second Ig-like domains of the *c-kit* receptor (Blechman et al. 1993). To our knowledge there have been no earlier reports of anti-*c-kit* monoclonal antibodies which act only under non-reducing conditions like MTK1 and this antibody might therefore be useful for studies of the functional binding site based on the tertiary structure of the molecule.

In the present study, MTK1 and MTK2 showed different behavior in terms of SCF-induced proliferation of M-MOK cells, only one being inhibitory. It has been reported that there were three kinds of anti-*c-kit* antibodies regarding such inhibitory action. SR-1 is a representative antibody which completely inhibits the SCF-induced proliferation of a factor dependent leukemic cell line, MO7e (Avanzi et al. 1988), at a 1/5,000 final dilution of ascites. YB5.B8 is the opposite which does not exert any influence, and 17F11 is an intermediate which partially inhibits growth (Ashman et al. 1994). Both of the antibodies were used at 5 μ g/ml final concentration. MTK2 is thus functionally similar to the YB5.B8 antibody. These monoclonal antibodies are biologically significant because they can select hematopoietic progenitor cells without the necessity of activation through the *c-kit* tyrosine kinase (Okayama et al. 1994).

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