Journal of Pharmacological and Toxicological Methods 63 (2011) 250–257

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Original article

Generation of a panel of monoclonal antibodies against atypical chemokine receptor CCX-CKR by DNA immunization

Shogo Takatsuka, Aya Sekiguchi, Masayuki Tokunaga, Akira Fujimoto, Joe Chiba *

Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278-8510, Japan

ARTICLE INFO

Article history:
Received 31 October 2010
Accepted 16 December 2010

Keywords:
Monoclonal antibody panel
Atypical chemokine receptor
CCX-CKR
DNA immunization
Hepatic cancer
Ligand-like antibody

ABSTRACT

Introduction: Chemokines are regulated by a family of 'atypical' chemokine receptors, D6, DARC and CCX-CKR, each of which efficiently internalizes its cognate chemokine ligands. Development of monoclonal antibodies (MAbs) that would recognize CCX-CKR on the cell surface will be helpful to identify primary CCX-CKR-expressing cell types and analyze the fate of CCX-CKR after ligand binding to the receptor. **Methods:** We generated IgG MAbs recognizing the cell-surface CCX-CKR by DNA immunization using a molecular adjuvant, and analyzed the epitope recognized by the MAbs. Then, the reactivities of the MAbs with CCX-CKR-transfected cells, and also hepatocytes and hepatic tumor lines were evaluated. Finally, we also tested the ligand-like activities of the MAbs, namely, induction of internalization of CCX-CKR by the MAbs. **Results:** A panel of MAbs reacting with CCX-CKR expressed on the cell surface was prepared. The panel was a small one, consisting of only ten MAbs, but was rich in terms of diversity of the Ig isotypes and of the epitopes. Epitope analyses revealed that all the 10 MAbs recognized at least three different, although very close, peptide structures of the N-terminal domain. Three MAbs, namely, 2F11, 13E11 and 14F10, were selected to represent the panel. All of the MAbs were applicable for flow cytometry and immunoflurescent assays and immunoprecipitation. The reactivity of the 2F11 MAb was also confirmed by western blotting. Endogenous expression of CCX-CKR on human hepatocytes and hepatic tumor cell lines was demonstrated using the 13E11 MAb. Interestingly, binding of the 13E11 MAb with B300-19 cells expressing CCX-CKR resulted in induction of CCX-CKR internalization. **Discussion:** This panel of MAbs may be expected to prove valuable for further study of the functions of this silent chemokine receptor, including those related to the homeostasis of lymphoid cells, and to the growth and metastasis of hepatic cancer.

1. Introduction

ChemoCentryx chemokine receptor (CCX-CKR) or CCRL1 is the third atypical chemokine binder or decoy chemokine receptor to be discovered after the Duffy antigen receptor for chemokine (DARC) and D6. They do not induce signaling along classic G-protein-mediated pathways; instead, they efficiently internalize their cognate chemokine ligands and act as scavengers (Mantovani, Bonecchi, & Locati, 2006; Wang et al., 2006; Wu et al., 2008). CCX-CKR binds to and scavenges chemokines of both the CC and CXC subfamilies, especially the "homeostatic" members, CCL19, CCL21, CCL25 and CXCL13 *in vitro* (Comerford, Milasta, Morrow, Milligan, & Nibbs, 2006; Feng, Ou, Wu, Shen, & Shao, 2009; Gosling et al., 2000; Hansell, Simpson, & Nibbs, 2006; Haraldsen & Rot, 2006; Mantovani et al., 2006; Townson & Nibbs, 2002). Regulation of the availability of these chemokines may modulate the axis role of CCR7/CCL19 or CCL21, CCR9/CCL25 and CXCR5/CXCL13 in both the processes of leukocyte migration and cancer cell migration. However, these ideas are based on work on *in vitro* systems that may not adequately model *in vivo* functions. Recently, two *in vivo* studies demonstrated that CCX-CKR modulates the physiological homing of dendritic cells to the skin-draining lymph nodes and the immigration of thymic precursors to the embryonic thymic anlage (Heinzel, Benz, & Bleul, 2007), and that it influences the localization, kinetics and nature of the adaptive immune response (Comerford et al., 2010). The antitumor activity of DARC and D6 (Feng et al., 2009; Wang et al., 2006; Wu et al., 2008) as also that of CCX-CKR against human breast cancer has been reported. Although the involvement of CCX-CKR has been reported in the migration process of both leukocytes and cancer cells, the precise mechanism of modulation of the availability of these chemokines by this decoy receptor still remains to be investigated.

In order to analyze the mechanism and more precisely understand this enigmatic molecule, it would be essential to develop methods to determine internalization of CCX-CKR and analyze the fate of CCX-CKR after ligand binding to the receptor. The development of

Abbreviations: CDC, Complement-dependent cytotoxicity; CTM, Cytotoxicity medium; DARC, Duffy antigen receptor for chemokine; *E. coli*, *Escherichia coli*; FBS, Fetal bovine serum; FCM, Flow cytometry; FITC, Fluorescein isothiocyanate; IF, Immunofluorescence; IP, Immunoprecipitation; MAb, Monoclonal antibody; MFI, Mean fluorescent intensity; RT, Room temperature; PBS, Phosphate-buffered saline; PMSF, Phenylmethylsulfonyl fluoride; PI, Propidium iodide; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WB, Western blotting.

* Corresponding author. Tel./fax: +81 4 7122 9695.
E-mail address: chibaj@rs.noda.tus.ac.jp (J. Chiba).

1056-8719/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.vascn.2010.12.003

monoclonal antibodies (MAbs) that would recognize CCX-CKR on the cell surface and in the cytoplasm will be helpful for such studies.

In this paper, we report on our attempt to produce anti-CCX-CKR MAbs that would react with the cell-surface CCX-CKR by DNA immunization using *Escherichia coli* chaperon GroEL as a molecular adjuvant (Fujimoto, Takatsuka, Ishida, & Chiba, 2009). A panel of MAbs reacting with the CCX-CKR expressed on the CCX-CKR gene-transfected cells and hepatic cancer cells was produced. We discuss the potential usefulness of this panel of MAbs, especially of the 13E11 MAb, which also showed ligand-like activity, in future analysis of the functions of this decoy or atypical chemokine receptor.

2. Material and methods

2.1. Animals, immunization and hybridomas

Female BALB/c mice (Sankyo Lab Service, Tokyo) (about 6 weeks old) were used for the immunization. Plasmid injection via the intramuscular route followed by *in vivo* electroporation was performed according to a previously described method (Aihara & Miyazaki, 1998; Fujimoto et al., 2009), with minor modifications. In brief, 30 μl of pCADEST1 encoding the CCX-CKR gene and the same amount of pCADEST1 encoding the GroEL gene were injected together into the quadriceps muscle using a syringe fitted with a two-stage needle (26-gauge), and needle electrodes (26-gauge) spaced at 5 mm were placed in each of the muscles. Then, 6 pulses (100 V, 50 ms, polarity reversal per 3 pulses) were delivered to the injection site. Muscles were also pre-treated about 10 min before the gene transfer by injection of bovine hyaluronidase instead of bupivacaine, to decrease the viscosity of the extracellular matrix and facilitate DNA diffusion (McMahon, Signori, Wells, Fazio, & Wells, 2001). Two and three weeks after the primary immunization, the same DNA immunization protocol was repeated in the BALB/c mice. Additional immunizations were also repeated at 5, 7 and 9 weeks after the primary immunization in the BALB/c mice. Blood samples were collected weekly from the mice; the sera were separated and stored at 4 °C for subsequent assay. After the immunization at 9 weeks after the primary immunization, the antibody titers in each animal were monitored until (several weeks) they declined to level that made it difficult to detect the cell-surface expression of CCX-CKR. Then, selected mice (No. 3 and 5) which showed the highest Ab titer at 9 weeks after the primary immunization, received a booster immunization by hydrodynamic delivery of 5 μg of pCADEST1 that encodes CCX-CKR via the tail vein (Herweijer & Wolff, 2007). Five days after the booster immunization, hybridomas were prepared by conventional methods using SP2/0 myeloma as the fusion partner. The mice were maintained under specific-pathogen-free conditions in the animal facility at the Tokyo University of Science. The care and handling of the animals conformed to the NIH guidelines for animal research. The Institution Animal Care and Use Committee approved the experimental protocols.

2.2. Construction of the expression vectors and purification of plasmid DNA

Expression plasmids for immunization were constructed using routine molecular-biologic techniques. The expression vector pCADEST1 was constructed based on pCADEST2.2 (Ainai et al., 2006). Human CCX-CKR gene and the GroEL gene were sub-cloned into the expression vector pCADEST1 using the LR-recombination reaction and Gateway LR Clonase II enzyme mix (Invitrogen), in accordance with the manufacturer's instructions. Expression plasmids for the MAb epitope mapping were constructed as follows: 1) CXCR4 chimera receptors in which each extracellular domain was switched to the corresponding domain of CCX-CKR were constructed by overlapping PCR. Human CXCR4 gene was sub-cloned from pCAGGS-CXCR4, which was received as a kind gift from Dr. Ishida, Kyowa-Kirin Co. Expression vectors containing the cDNA encoding the N terminus of CCX-CKR (1–10, 1–12, 1–14 residues) were constructed using the pDisplay vector system (Invitrogen). Plasmid DNAs isolated from *E. coli* DH5α cells were purified by cesium chloride density gradient centrifugation.

2.3. Cell culture and transfection

COS7 and HeLa cells, immortalized hepatocyte cell line Fa2N-4, and hepatic tumor cell lines, HLE, HLF, Hep3B, HepG2, HuH-7, PLC/PRF/5, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). B300-19 cells and their derivatives were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 50 μM 2-mercaptoethanol. Plat-E cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, blasticidin (10 μg/mL) and puromycin (2 μg/mL). For transient expression of the N-terminal epitopes of CCX-CKR, COS7 cells or HeLa cells were transfected with plasmids encoding CXCR4 chimeras or cDNA encoding whole or parts of the N-terminal domain of CCX-CKR cloned in the pDisplay vector by Fugene HD (Roche).

2.4. Establishment of B300-19 cells stably expressing chemokine receptors

The CCX-CKR gene was sub-cloned into the pMRX-IRES-Puro vector and B300-19 cells stably expressing CCX-CKR cells (CCX-CKR/B300) were established by retroviral infection using Plat-E cells, as previously described (Morita, Kojima, & Kitamura, 2000). Cells were infected and selected for 3 days using puromycin (SIGMA, 1 μg/mL).

2.5. Flow-cytometric analysis

The antibody (Ab) response was assessed by flow-cytometric (FCM) analysis as previously described (Fujimoto et al., 2009), using FITC-conjugated goat anti-mouse IgG (H + L) antibodies (Jackson ImmunoResearch, no. 115-095-062) as the secondary Abs.

2.6. Complement-dependent cytotoxicity

CCX-CKR/B300 cells (2.5×10^4 cells/well) were incubated with the anti-CCX-CKR 2F11 MAb or isotype-matched MAb in triplicate in 96-well plates, in 50 μl cytotoxicity medium (CTM; RPMI medium containing 25 mM HEPES, 0.14% $NaHCO_3$ and 0.3% bovine serum albumin). After incubation for one hour at 4 °C, 50 μl of CTM with 10% rabbit complement (PEL-FREEZ) was added. After 30 min of incubation at 37 °C, PI was added and the numbers of PI-positive dead cells were analyzed by FCM assay. Cytotoxicity was calculated as the percentage of dead cells out of 2.5×10^4 total events.

2.7. Immunoprecipitation, SDS-PAGE and western blot analysis

10×10^6 of CCX-CKR/B300 cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.5 containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodiumdeoxycholate, and 0.1% sodium dodecyl sulfate together with 1 mM PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin and 1 μM pepstatin) for 30 min on ice with continuous rocking, then centrifuged (15,000 ×*g*, 15 min). Protein extracts pre-cleared by incubation with protein G Sepharose for 1 h at 4 °C were centrifuged at 15,000 ×*g* for 1 min. The pre-cleared extracts were immunoprecipitated by the anti-CCX-CKR 2F11 MAb or isotype-matched MAb (5 μg/sample) for 1 h at 4 °C. The precipitates were collected by incubation with protein G Sepharose for 1 h at 4 °C. The immunoprecipitates or protein extracts were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon-P, Millipore, Tokyo). Membranes were blocked for 1 h at 37 °C with Block-Ace (Snow Brand Milk Products, UK-B80, Tokyo), washed with 0.1% Tween 20 in PBS for 3 min, then probed with 2F11 MAb (500 ng/mL) for 1 h at